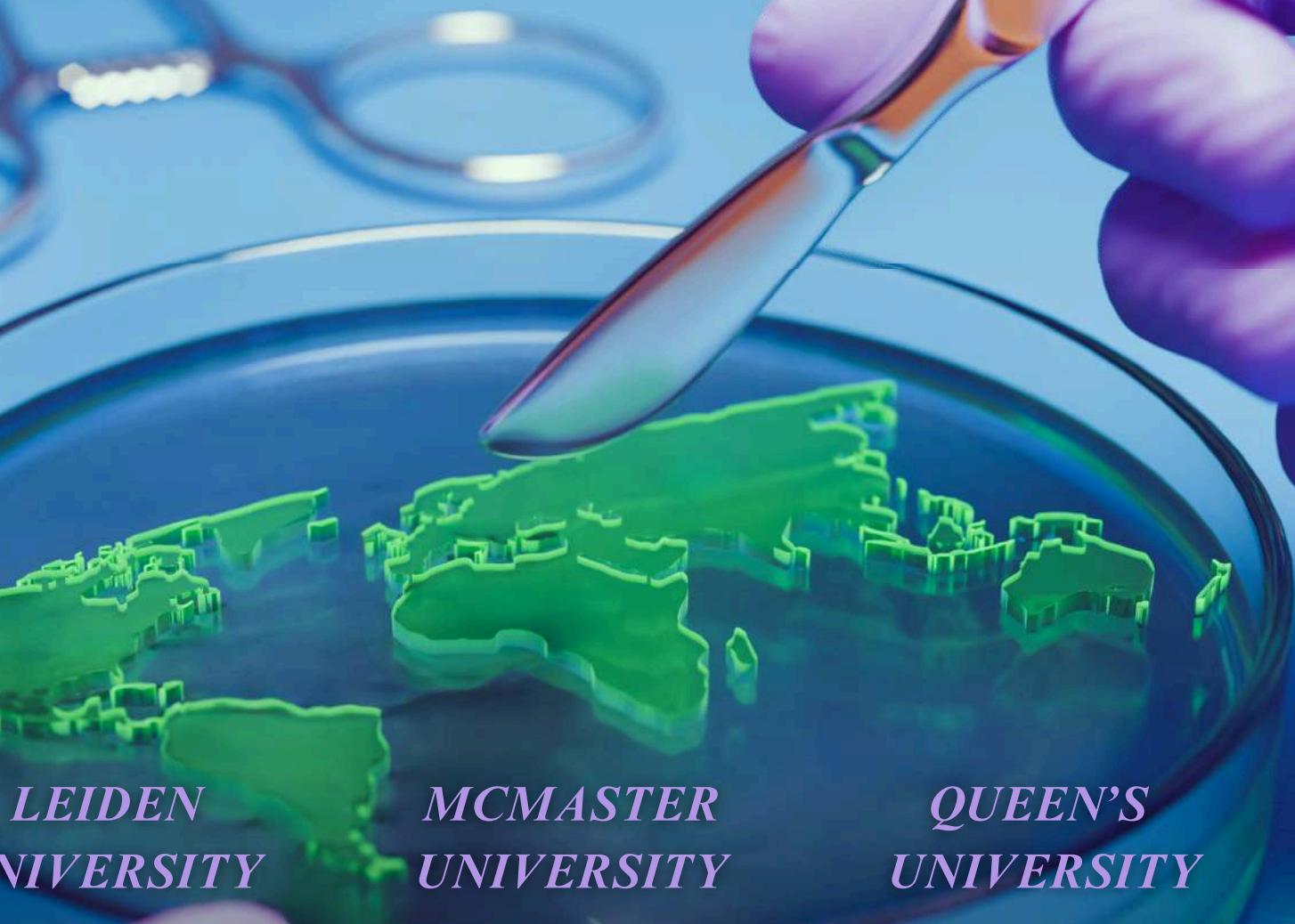


The Unofficial iGEM Proceedings Journal 2025:

VECTOR

by the 2025 MSP-Maastricht iGEM Team

October 2025, Vol. 4



**LEIDEN
UNIVERSITY**

Novel Method for Protein Engineering: Post-Translational Modifications Using Self Integrating Scaffold

Pages: 6 - 10

**MCMASTER
UNIVERSITY**

A Review on Acute Graft Rejection Detection: Current Methods and Emerging Solutions

Pages: 11 - 16

**QUEEN'S
UNIVERSITY**

Foundations for an AHL-Responsive Genetic Circuit in Eukaryotic Cells: A Biofilm-Disrupting Therapeutic for Cystic Fibrosis

Pages: 17 - 21



Cover by **Hassan Tahini at ScienceBrush Design**

The *Vector* Journey

2020



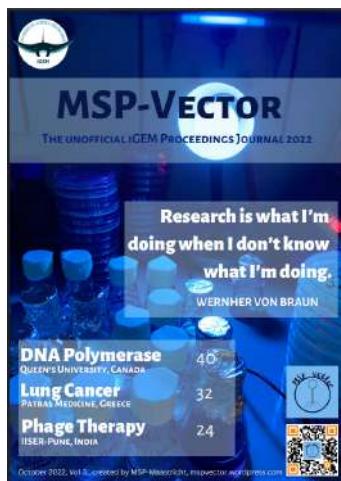
The 2020 MSP-iGEM team, Oakshield, founded and created **the first edition of *Vector***, at that point in time called *iGEM Vector*. The journal was based on a simple idea - to provide iGEM teams with the opportunity to develop their academic skills, such as scientific writing, and in turn, provide an educational platform where they could learn about new discoveries and advances in the field. The first edition, consisting of 34 submissions, was **just the beginning!**

2021



The peer review form developed for the second edition of the journal was further improved upon in the third edition (2022), based on the input and feedback received from several members of the **MSP teaching staff** specialising in and teaching courses on academic skills and writing. This change went hand-in-hand with the most notable improvement - the **addition of expert reviewers**.

2022



After two long years, the journal was **restarted** by the 2025 MSP-iGEM team for its fourth edition. This year's edition, comprised of 19 submissions from teams in 10 different countries, was renamed to ***Vector***. The name change, from *MSP-Vector*, reflects our mission - creating an educational platform for **all** iGEM teams, allowing them to improve their scientific and non-scientific skills.

It is our hope that this initiative will be continued by future MSP-iGEM teams and transform into a tradition, and in turn, continue to provide a platform for generations of young researchers where they can hone their scientific skills and **grow, not only as researchers, but as people**.

The 2021 edition introduced several changes. In addition to the name change, from *iGEM Vector* to *MSP-Vector*, the major improvement of note was the development and implementation of a **peer review form** for the participating teams which ensured that all papers would be reviewed in a thorough and **standardized** fashion.

2025



THE
FUTURE...

2025 MSP-

Maastricht

Team:

CoreSpin

Copper Recombinant Spidroin Interface

The Team

This year's MSP-Maastricht iGEM team consists of 14 undergraduate students from 17 different countries who can speak a total of 14 languages. Twelve of us are studying at the interdisciplinary Maastricht Science Programme focusing on various fields ranging from biology to physics; one is a biomedical sciences student and one a computer science student. In our challenging efforts to push the boundaries of knowledge, we are led (and sometimes dragged kicking and screaming against our will) by our supervisors: Dr. Erik Steen Redeker and Dr. David Cortens.

The Project

This year, the Maastricht team has decided to tackle the issue of **heat exchanger biofouling**. While at first seemingly a niche problem, energy used for cooling accounts for **10 % of the total global energy usage**. Biofouling of such cooling systems, all containing heat exchangers, leads to increased energy consumption and green house gas emissions on an alarming scale.

Our plan involves the development of a novel protective coating made from recombinant **spider silk proteins** that will prevent the formation of biofilms on heat exchangers in cooling systems, such as those found in HVAC units and data centres, while at the same time maintaining their thermal conductivity. This solution addresses energy losses and biofilm formation, offering a sustainable alternative that supports global efforts to reduce environmental impact and enhance industrial performance.

Powering a Greener Future — Together!



Meet the *Vector* Team

Denise Lemanczyk

Denise is a 2nd year bachelor student from the Czech Republic and Germany, who grew up in Belgium. Her favourite organism is the willow tree, *Salix babylonica* and her favourite book is the one she read last. She can be found formatting the depths of this journal, and has memorised all of your titles by heart.



Lovro Hranilović Petričić

Lovro is a 3rd year bachelor student from Croatia. His favourite organism is LUCA and his favourite book is *The Stranger* by Albert Camus. He can be found scouring the internet to find publicly available emails of iGEM teams to ask them to join Vector.



Jonas Hermans

Jonas is a 3rd year bachelor student from Canada and Belgium, who grew up in Germany. His favourite organism is the giant redwood, *Sequoiaadendron giganteum*, and his favourite book is *The Overstory* by Richard Powers. He can be found philosophising about syn-bio and writing his thoughts down for the foreword.

Sri Katyayani (Katya) Mannar

Katya is a 2nd year bachelor student from India and Germany. Her favourite organism is the Mallard duck, *Anas platyrhynchos* and her favourite book is *Villette* by Charlotte Brontë. She can be found looking over the peer review submissions, nervous about whether everyone will submit on time.

Foreword

Welcome to the 2025 edition of *Vector*, the unofficial journal for the iGEM competition, the largest synthetic biology competition in the world! iGEM encourages young aspiring scientists to push the boundaries of knowledge in an already pioneering field and to ask fundamental questions about the nature of our creations. Despite this, it can be easy to fall into the trap of becoming stagnant in our thinking, only navigating within our academic bubble, and forget the truly amazing nature of the work we do. Synthetic biology in and of itself is a subversion of natural processes; we are literally changing life. Humanity has progressed from the generational modification of organisms through agricultural selective breeding, to working with the smallest units of life with staggering accuracy and modifying the genetic makeup of organisms in real time, all in an evolutionary instant. We are working in a field still in its infancy, but which has the potential to drastically change our way of life and whose applications are practically limitless.

It is therefore imperative, especially in times of social uncertainty, that we as scientists consider the wider impact of our findings. We are not only biological engineers, but case studies in philosophy. The work we do challenges conventional definitions of life, is at odds with the direction of information flow in and between biological systems, and blurs the lines between living and machine. Can a synthetic construct still be classed as natural or biological if it did not arise out of biological processes? Does my creation “deserve” to exist? Do I “deserve” to have created it? These questions, in peaceful moments between juggling labwork, wiki coding and human practices, do and should keep us up at night.

Because synthetic biology at its core aims to use organisms for novel purposes, it directly interacts with many aspects of society, from healthcare and agriculture to industrial production and ecological conservation, which the research presented in this journal broadly reflects. Given this intimate connection, we as synthetic biologists should be hyper aware of the people whose lives our inventions have the possibility to impact, and how they can do so, both in a positive and negative light. The technology we currently use for genetic engineering, as well as its successors, will no doubt play an immense role in the future and survival of humanity. We would therefore like to encourage our readers to remain vigilant and critical of new technology, closely follow trends in the development of biotechnology and be amazed at the impressive collection of iGEM research we are proud to present to you in this year's edition of *Vector*.



Our Reviewers

Dr. Yevgeniya Nusinovich

Yevgeniya Nusinovich, M.D., Ph.D., is a Senior Editor at *Science* handling research papers on cardiology, metabolism, reproduction, medical genomics, and a selection of other biomedical topics. Before coming to *Science*, Yevgeniya was an editor at *Science's* sister journal *Science Translational Medicine*, where she also handled a variety of subject areas. Her Ph.D. was in molecular medicine, but she also trained in clinical pediatrics and worked with patients at Children's National Hospital in Washington, DC before fully switching to an editorial career.



Dr. Michael Funk

Dr. Michael Funk joined AAAS in 2017 and is now a Senior Editor handling papers for *Science* in the fields of biochemistry, structural biology, chemical biology, and environmental microbiology. His responsibilities include selecting articles for review, choosing appropriate reviewers, ensuring data availability and integrity, participating in scientific meetings, and coordinating with others at AAAS/*Science* to promote and communicate research to the scientific community and public. He has also judged annual prizes administered by AAAS/*Science*, including the inaugural BioInnovation Institute Prize in 2022. His background is in enzymology and crystallography, and he obtained his Ph.D. in Biological Chemistry from MIT.



*Art by Hassan Tahini from
ScienceBrush Design

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A Novel Method for Protein Engineering: Post-Translational Modifications Using Self Integrating Scaffolds

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ABSTRACT

The lack of precise control over glycosylation is a major challenge in the production of biopharmaceuticals. In order to overcome this hurdle, we aim to develop glycosylated Self Integrating Scaffold (SIS) fragments that can be inserted in proteins of interest, including immunoglobulin backbones. We will verify the ability of two split intein pairs to insert an extrinsic group into a protein using the MBP-HA-GFP assay. Next, we will assess the suitability of using different split intein pairs together by performing a FRET assay. Finally, we will create SIS fragments by inserting our plasmid containing split inteins surrounding a glycosylation site, which will induce the production of glycan trees on the flanked sites. Together, the results will provide SIS fragments that can be utilized to insert glycan groups in a wide variety of proteins as well as identify the most efficient split intein pairs to perform this insertion. This modular system could be used to precisely control the constitution of glycoproteins, promoting the production of optimized biotherapeutics and the execution of fundamental research. Future studies will focus on expanding this glycosylated SIS library to a “plug-and-play” repository, which facilitates the precise addition of a multitude of post-translational modifications in different protein backbones.

Index Terms - Post-Translational Modifications (PTMs), Glycosylation, Split Inteins, Immunoglobulin G (IgG), Self Integrating Scaffold (SIS), Protein Engineering

I. INTRODUCTION

Proteins are the cornerstone of all life. To fold and function correctly, most proteins undergo a process called post-translational modification (PTM), in which groups can be added. Examples include methylation, phosphorylation, and acetylation. Another PTM involves the addition of different glycan trees also known as glycosylation. Glycans can have different linking sites and compositions which all have effects on the function and therapeutic characteristics of a protein¹.

Antibodies are used in both therapy and research due to their ability to bind to specific motifs, making them useful for targeting and labelling². Immunoglobulin G (IgG) is the main antibody isotype in the blood circulation, and it consists of two light chains and two heavy chains. The conserved region of the heavy chains feature an N-linked glycosylation site at asparagine 297³. The type of glycan tree present on the heavy chain of IgG affects the affinity to certain receptors, making it important for the effectiveness of therapy⁴. Another key aspect for the regulation of synthetic medicinal antibodies is the homogeneity of these glycans⁵, or in other words, whether the glycans on the two chains are identical. Glycan heterogeneity can cause a severe immune response, such as serum sickness⁶. Heterogeneous antibodies can complicate regulation and clear structure-activity relationship analysis⁷. It is crucial for biotherapeutics to have homogenous glycoforms of antibodies⁶.

Glycosylation is one of the biggest hurdles in producing these antibodies synthetically. Even Chinese hamster ovary (CHO) cells, the industry standard, lack natural enzymes for producing certain human glycans³. Bacteria, yeast, and plant cells are also not an option without humanisation, as their glycans differ from human ones making them either inactive or harmful for humans³. To solve the problems with the current glycoengineered antibody production, we propose a new system using split inteins.

Intervening proteins, or inteins, function like protein introns. They are amino acid sequences within the translated peptide chain that excise themselves in order to fuse the remaining parts⁸. This process is called protein splicing and it is responsible for the formation of mature proteins⁹. Inteins can also be fragmented into two smaller halves, after which they are referred to as split inteins⁹. Those are particularly interesting, as they can fold independently during protein trans-splicing and can allow for the introduction of external sequences into a protein of interest. As ligation tools, inteins and their split variants have been used for labeling, cyclisation of proteins, and some PTMs such as acetylation and methylation^{9,10}. Another potential application involves the use

of two pairs of split inteins flanking an insert, which allows for the insertion in a peptide chain (Fig. 1).

In this work we will explore the production of Self Integrating Scaffold (SIS) fragments that will be composed of a glycosylation site flanked by two pairs of split inteins. By building this platform, we focus on modular N-glycan engineering of IgG, and aim to develop a solid basis for developing different intein-based tools for PTMs. This modular system could be used to precisely control the constitution of glycoproteins, promoting the production of optimized biotherapeutics and the execution of fundamental research.

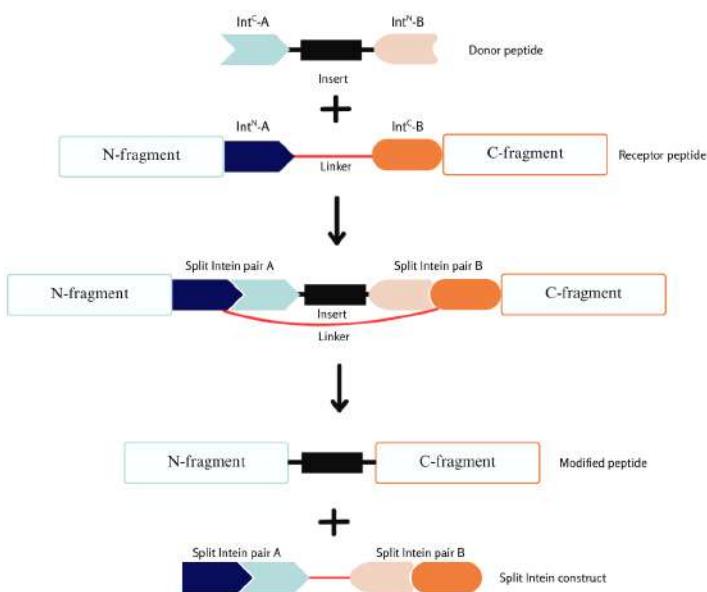


Figure 1. Schematic diagram of the split intein reaction. The inteins (A and B) are both split, and the fragments with the amino (N) and carboxyl (C) terminals are represented for both. When the donor and acceptor are added together, the N- and C-terminals of the same inteins will bind to each other, fold and splice themselves out. This happens while taking the linker with them and leaving the insert behind as a scar.

II. MATERIALS AND METHODS

Plasmid Construction

The gBlocks and the pET-16b backbone were amplified by PCR, with flanking regions added for Gibson Assembly using Q5 High-Fidelity 2× Master Mix (NEB M0492S). PCR products were verified by electrophoresis on a 1% agarose gel stained with Midori Green Advance. Correct products were purified using the NucleoSpin® Gel and PCR Clean-up kit, according to the manufacturer's instructions. Plasmids were assembled using the Gibson Assembly® protocol (E5510). The assembly mixtures were transformed into chemically competent *E. coli* TOP10 cells by incubating on ice for 30 min, heat-shocking at 42 °C for 1 min, and returning to ice for 2 min. Subsequently, 500 µL of LB medium was added, and cultures were incubated at 37 °C for

1 h. Cells were plated on LB agar containing ampicillin and incubated overnight at 37 °C. Colonies were screened by colony PCR using GoTaq® Green Master Mix. Positive colonies were inoculated into LB medium supplemented with 100 µg/mL ampicillin and grown overnight at 37 °C. Plasmids were purified using the NucleoSpin® Plasmid EasyPure kit and verified by Sanger sequencing (Macrogen). The MBP-HA-GFP assay requires two plasmids, the FRET assay requires six plasmids and the SIS fragment is synthesized in one plasmid. When these plasmids were confirmed, they were transformed into *E. coli* BL21 ai cells for protein production following the same transformation protocol described above.

Protein Expression and Isolation

E. coli BL21 ai cells containing the verified plasmids were inoculated into 20 mL LB medium with ampicillin and grown overnight at 37 °C at 160 rpm. This pre-culture was used to inoculate the expression culture at a 1:100 ratio in fresh LB medium with 100 µg/mL ampicillin. Protein expression was induced at an OD₆₀₀ of 0.6 by adding IPTG and arabinose to final concentrations of 0.5 mM and 0.2%, respectively. Cultures were incubated for 18 h at 18 °C.

Cells were harvested by centrifugation at 6000 g for 15 min and resuspended in a lysis buffer (150 mM NaCl, 100 mM phosphate 20 mM imidazole, pH = 7.2). Cells were lysed by sonication on ice using the KE76 tip with a 1 second ON/OFF pulse for 1 minute. This was repeated 5 times. The lysate was separated by centrifugation at 15.000 g, and the supernatant was purified using HisPur™ Ni-NTA Spin Columns, according to the manufacturer's instructions.

Protein trans-splicing

The following protein trans-splicing protocol was applied throughout our research lines. The acceptor and donor proteins were individually incubated in a reaction buffer (100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 1 mM TCEP; pH 7.2, unless otherwise specified) on ice for 15~ min. The reaction was initiated by mixing equal amounts of the donor and acceptor proteins. At the indicated time points, aliquots were withdrawn and quenched by adding a SDS sample buffer. Samples were analyzed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue.

MBP-HA-GFP Assay

The trans-splicing mechanism test replicates experiments from a recent paper⁶. The model proteins eGFP (enhanced Green Fluorescent Protein) and MBP (Maltose Binding Protein) were used, as well as an HA tag, all for easy validation. Upon mixing the proteins, the reaction setup follows the protein trans-splicing protocol. This will validate the possibility to insert a peptide in an unnatural place in an unnatural protein construct. This lays the foundation for future bioengineering of any imaginable protein.

FRET Protocol

Proteins will be diluted in buffer (PBS or HPLC-grade water) to a final concentration of 0.1 - 1 μ M and transferred into a black 96-well microplate. Fluorescence measurements will be performed using a Tecan plate reader, with excitation set to 435–455 nm (CyPet), and emission spectra collected at 477–504 nm (CyPet) and at 530 nm (YPet). FRET efficiency is then determined by calculating the ratio of YPet to CyPet emission intensities.

Control samples include CyPet-only, YPet-only, and CyPet+YPet mixtures to account for bleed-through and direct acceptor excitation. To further validate interactions, five frames are going to be recorded, followed by selective photobleaching of YPet. A constant CyPet signal indicates no interaction, whereas an increased CyPet signal after YPet bleaching indicates a FRET interaction. For the final product containing both CyPet and YPet, stronger FRET signals corresponded to closer proximity and tighter interaction between the fluorophores.

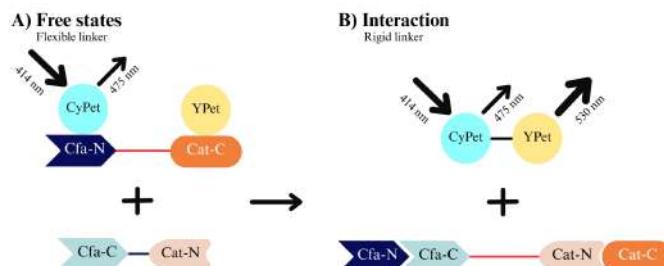


Figure 2. FRET Linker Swap Assay. A) The starting proteins are the unspliced donor and acceptor pair. The fluorophores are in a free state. B) After the intein reaction the flexible linker between the fluorophores is swapped out for the rigid linker and FRET will be detected.

Glycan Sorting and Characterization

The obtained isolated protein contains tags and a glycosylation signal peptide, which are going to be separated from the SIS fragments using a TEV protease. Next lectin chromatography will be performed to isolate the SIS fragments by glycan tree type. The SIS fragment solutions will be checked for purity and for exact glycan tree composition after lectin chromatography. The solutions will be analysed on intact protein mass, using first RP-HPLC and next ESI-MS/MS.

Mass Spectrometry of SIS Fragments

The intact protein mass, which will be measured via RP-HPLC, of the SIS fragments without glycans is going to be 79.7 kDa. The N-linked glycans are expected to add 1.3-2.8% of weight, meaning there are going to be fragments of 80.7-81.9 kDa.

III. RESULTS

Construct Verification

The donor and acceptor plasmids of the MBP-HA-GFP assay and FRET linker swap assay are constructed (Fig. 3). The FRET assay's positive control, the CyPet and YPet plasmids have been constructed too (Fig. 3). The negative control plasmid and the SIS fragment plasmid are still under construction. Colony PCR was performed in triplicate.

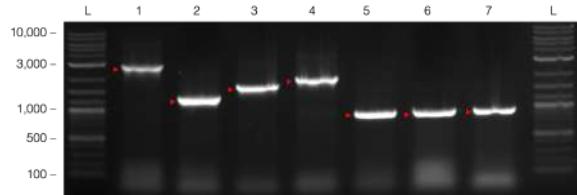


Figure 3. Gel Electrophoresis of Colony PCR Products. A colony PCR was performed on the *E. coli* BL21 cells with the transformed plasmids on a pET16b backbone. Correct bands are indicated by a red arrow head. L refers to the Quick-Load Purple 1 kb Plus DNA Ladder (NEB N0550S). (1) MBP-CfaN-linker-CatC-GFP acceptor (2867 bp); (2) CfaC-HA-CatN donor (1355 bp); (3) FRET positive control (1732 bp); (4) FRET acceptor (2020 bp); (5) FRET donor (904 bp); (6) CyPet (943 bp); 7: YPet (953 bp).

Protein Expression

The MBP-HA-GFP assay's donor has been expressed in both LB (Fig. 4) and TB. It shows a molecular weight of around 94.5 kDa. Expression in LB is higher than in TB. The pellet contains higher levels of protein.

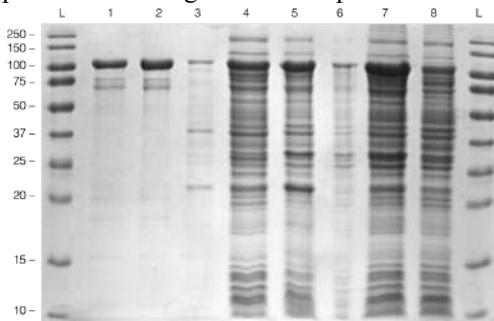


Figure 4. SDS-PAGE Showing the MBP-Cfa-linker-Cat-GFP Construct. (1) second elution of purified protein; (2) first elution; (3) first wash of lightly bound protein in HisPur® column; (4) flowthrough of unbound proteins; (5) supernatant after ultracentrifugation of sonicated pellet; (6) sonicated pellet; (7) induced cells; (8) uninduced cells.

IV. DISCUSSION

Our results will provide a readout of how efficient the selected split inteins are. The FRET assay offers insights into the specificity and splicing activity of the Cat and mut-Cfa pair and can serve as a comparative tool for evaluating other intein combinations. However, the current setup is restricted to qualitative data, as the assay only shows whether splicing

occurred. To determine the efficiency, rate and completeness of the splicing we will need to measure reaction kinetics.

Future research lines in our project will employ mass spectrometry to quantify splicing yield and product formation. In addition, alternative reporter systems, such as MBP-HA-GFP constructs, could be tested in humanised yeast strains or mammalian cells to evaluate intein folding and activity in more physiologically relevant environments. Together, these approaches will extend our current qualitative findings into a quantitative framework for assessing split intein performance.

The construction of the first required proteins has been successful (Fig.4). However, when taking a closer look at the isolation, we notice the presence of two additional proteins. Possible, these are constructs where only one split intein have reacted, where the MBP-HA-Cat construct would be 72.7 kDa and Cfa-HA-GFP would be 63.4 kDa.

A key advantage of the SIS architecture is its modularity, which enables split production strategies. In such a workflow, the protein backbone can be expressed in low-cost, high-yield organisms, while the glycan-bearing SIS modules are produced separately and subsequently ligated. This approach has the potential to increase overall yields and reduce manufacturing costs for complex glycoproteins. Furthermore, the modular design allows rapid adaptation of glycan profiles: exchanging a SIS fragment permits modification of the glycan component without re-engineering the entire expression strain, thereby streamlining both exploratory plug and play research, and process optimisation. This would allow us to create antibodies with homogenous glycan composition and to explore the role of glycan function. In turn, this knowledge enables the generation of biotherapeutics with optimised effector properties or pharmacokinetic behaviour.

Nonetheless, limitations are anticipated. Highly folded proteins with buried or sterically restricted insertion sites may pose accessibility challenges, reducing splicing efficiency or preventing effective integration¹¹. Additionally, the use of split inteins introduces a predictable “scar” of three amino acids, at the ligation site, which could locally alter structure or function¹². Furthermore, recent studies have demonstrated “scarless” intein variants that remove or minimise junction residues altogether¹³. Minimising the structural impact of these residues, either by rational design of the insertion site or by engineering scar-reduced inteins, will be important for expanding the applicability of this approach.

Even small scars left by inteins can affect protein folding and function. For example, Tasfaout et al. showed that inserting six-residue “footprints” into GFP reduced its fluorescence by ~50%, highlighting how additional residues can significantly impair protein activity¹⁴. The severity of scarring, however, is protein-specific and largely depends on the location of the insertion. To minimize this problem, inteins have been engineered with reduced extein requirements. For instance, Ciragan et al. demonstrated that introducing a serine at the +1 position enabled scarless ligation, provided that the target protein naturally contained serine at the splice

junction¹⁵. Looking ahead, more advanced systems are being developed, such as intracellular protein editing platforms that incorporate chemically unique residues to install labels into proteins with minimal disruption¹⁶. By combining these advancements with SIS fragments researchers will gain powerful tools not only to investigate glycan function, but also to broaden the application to a wide variety of PTMs and the generation of optimised and novel biotherapeutics.

V. CONCLUSION

Efficient synthetic production of proteins with PTMs, including glycosylated therapeutics, remains complicated. Creating a library containing glycans surrounded by efficient split intein pairs allows the precise insertion of known glycan trees on chosen locations in proteins, which provides new opportunities to make the synthetic production of glycosylated therapeutics.

For this research seven out of nine plasmids are constructed correctly, followed by one successful protein isolation. This lays the foundation for future assessment of the generated biobricks. While our system demonstrates promising modular engineering of glycoproteins, the main limitation is scarring, which may restrict broader applications in some proteins. Overcoming these constraints will be essential for enabling the full potential of SIS fragments.

As a result, the SIS fragments will be a modular approach that does not only regard glycosylation of therapeutics, but has the potential to be applied for a wide variety of proteins and PTMs. Therefore, a plug-and-play research style can pave the way towards improved characteristics of therapeutics with PTMs such as increasing efficiency and prolonged shelf-life, aid the elucidation of the effect of different PTMs on different locations within proteins, and simplify the synthetic engineering and production of proteins with PTMs.

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A Review on Acute Graft Rejection Detection: Current Methods and Emerging Solutions

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ABSTRACT

Acute graft rejection, or the immune activation against transplanted tissue, remains a significant concern for graft recipients within days to months following transplantation. Early detection of acute graft rejection enables timely immunosuppressive treatment, preventing irreversible loss of donor tissue. Current diagnostic tools and biomarkers often fall short in accuracy, invasiveness, cost, and accessibility. This review discusses current and emerging methods for detecting acute graft rejection, highlighting the pressing need for more rapid, accessible, and affordable tools to rule out early acute rejection in transplant patients. Although addressed by McMaster iGEM 2025, this problem presents a new frontier for synthetic biology innovations inspired by recent advancements in engineered systems.

Index Terms - Acute rejection, transplant, biomarker, detection, monitoring

I. INTRODUCTION

Dialysis and transplantation are the only courses of treatment available to patients undergoing kidney failure (Eflein, 2025); although studies demonstrate that renal transplant recipients survive longer than patients receiving dialysis (National Kidney Foundation, 2024). Over 150,000 solid organ transplants are performed annually worldwide (World Health Organization, 2024), with a total of 111,135 kidney transplants in 2023 (Eflein, 2025). Of the 105,000 people in the U.S. awaiting organ transplantation in 2023,

90,465 of these patients require a kidney transplantation, and only approximately 43,090 people received kidney transplants (Eflein, 2025). Despite matching patients to donor graft tissue of compatible blood group and tissue type, approximately 15% of transplants still face some degree of rejection (Kidney Research UK, 2025). Allograft rejection describes the recognition of donor antigen in the allograft as

foreign by the recipient's immune system, which activates an immunological response resulting in inflammation (Kidney Research UK, 2025; Naik & Shawar, 2023). Acute rejection (AR) is the rapid onset of rejection within days to months following the transplant and often occurs in the absence of any symptoms (Kidney Research UK, 2025; Naik & Shawar, 2023). There are two types of AR: antibody-mediated rejection (ABMR) and Tcell-mediated rejection (TBMR), both of which require immunosuppressant drugs to regulate the body's immune responses before the graft is destroyed (Kidney Research UK, 2025; Naik & Shawar).

During the post-transplant period, routine blood tests measure serum creatinine (sCr) levels, enabling physicians to suspect rejection when sCr levels rise over 25% above baseline (Naik & Shawar, 2023). However, changes in creatinine may be indicative of other conditions, and physicians often must rule out these possibilities before taking action (Kidney Research UK, 2025). Furthermore, conventional noninvasive methods lack specificity and sensitivity for detecting early graft injury or subclinical rejection —injuries that occur without apparent clinical symptoms (Lee et al., 2023). With organ failure patients experiencing prolonged wait times and some even passing before they can receive a suitable transplant (Canadian Institute for Health Information, 2023), clinicians and organ recipients require adequate tools to detect signs of inflammation and ensure longterm graft survival. This review defines the existing and emerging landscape for future research, providing potential avenues of integration and innovation to improve the accuracy, affordability, accessibility, and timeliness of AR detection.

II. RESEARCH ELABORATIONS

The literature search was conducted primarily on Google Scholar. We selected articles published or last updated between 2020 and 2025 to focus on recent developments. Other criteria include a minimum of 20 references pertaining to acute rejection, transplant, detection, and diagnosis. We also complemented literature findings with real-world insights

from interviews with expert stakeholders, utilizing a creative research method inspired by iGEM values of responsibility, responsiveness, and reflection. Comprehensive interview notes can be found on the McMaster iGEM 2025 Wiki.

III. RESULTS

A. Current Methods

The current post-transplant AR diagnostic process requires patients to present clinical features involving the crafted organ (Justiz Vaillant et al., 2024). As outlined in the Clinical Guidelines for Kidney Transplantation, patients undergo routine bloodwork, urine testing, and imaging, overseen by a multidisciplinary team of physicians, surgeons, clinical coordinator nurses, and a social worker (BC Transplant, 2021). Based on this assessment, both invasive and noninvasive methods are considered. AR remains a high risk after surgery, especially for kidney transplant recipients, with episodes occurring in less than 20% of low-risk graft recipients within 26 weeks post-transplant (BC Transplant, 2021). Estimated glomerular filtration rate (eGFR) is often measured as a functional marker (Oellerich et al., 2022). More commonly, AR is detected based on an increase in serum creatinine sCr (BC Transplant, 2021; Parajuli et al., 2023). If abnormalities in creatinine or changes in urine appear, the diagnostic process can escalate; imaging tests can be done, such as a Doppler ultrasound, followed by a renal biopsy for confirmation (Varkey, 2021). A kidney biopsy further confirms the diagnosis of AR, ruling out other factors such as volume and surgical factors. (BC Transplant, 2021).

Subclinical AR does not often present with a noticeable decline in organ function, requiring routine tests for early detection. Following a transplant, physicians routinely order a protocol biopsy at three and twelve months, along with regular blood tests to test for creatinine (Cieślik et al., 2023). However, the invasive nature of biopsy, its emotional and physical repercussions on patients, and demands for technical expertise hinder the frequent use of this procedure in clinical settings (BC Transplant, 2021; Justiz Vaillant et al., 2024). Geddes et al. (2025) found that minor complications occurred in 20% of biopsy cases. In comparison, major complications occurred in nearly 2% of cases (Geddes et al., 2025), restricting access to consistent and safe graft monitoring. Furthermore, conventional biomarkers like eGFR and sCr lack specificity and sensitivity, with levels often remaining within normal ranges for 24–36 hours after injury, delaying detection until substantial damage has already occurred (Naik & Shawar, 2023; Parajuli et al., 2023; Ollerich et al., 2022). In one study of transplant recipients with stable sCr, biopsies revealed that 29% of patients had AR (Parajuli, 2025). Therefore, a non-invasive test must ensure the selected biomarkers are capable

of detecting subclinical allograft injury before irreversible damage occurs.

A non-invasive blood test detecting donor-derived cell-free DNA (dd-cfDNA), DNA fragments shed from injured donor cells, has recently been clinically adopted to detect allograft rejection. (Oellerich et al., 2022; Varkey, 2021;

Westphal & Mannon, 2025). Rising dd-cfDNA levels result in earlier detection of graft injury with greater specificity than creatinine, especially ABMR (Parajuli et al., 2023; Oellerich et al., 2022). Donor-derived cellfree DNA is a promising emerging biomarker due to its high Negative Predictive Value (NPV), ranging between 87% and 95% (Han & Lubetzky, 2023). However, the test's Positive Predictive Value (PPV) falls between 18% to 40% (Han & Lubetzky, 2023); Hence, elevated levels often result in false positives, which can lead to unnecessary biopsies. Cost represents another significant barrier to accessing accurate and advanced diagnostics. The ddcfDNA assay is reimbursed by MediCare at approximately \$2,841 USD per test for patients who meet eligibility criteria (Securities and Exchange Commission, 2021), while renal biopsies can cost patients more than \$4,000 USD per visit (Florea et al., 2024). Although these tools provide an invaluable window into graft health, their high costs limit access to consistent, accurate monitoring.

Dr. Christine Ribic, Director of the Division of Nephrology at McMaster University, provides a valuable clinical perspective. Her statement, “Time is kidney,” underscores the highly timesensitive nature of graft rejection, with tissue scarring appearing if the organ is left untreated for as little as three weeks (C. Ribic, personal communication, June 11, 2025). She lends a clinical perspective on the value of the life of the graft, sharing that timely clinical intervention may recover some kidney function and prevent irreversible loss of graft function. Whereas the chance of finding another donor is slim, Dr. Ribic explains, most rejections can be treated with immunosuppressants, emphasizing the importance of early AR detection. While current methods combine biomarkers, imaging, and biopsy with promising molecular tools, patients face trade-offs between invasiveness, accuracy, affordability, and timeliness. Recognizing this lived experience provides context for evaluating current methods and highlights the gaps yet to be addressed in patient care.

B. Emerging Solutions

Non-invasive biomarkers display potential in AR detection. CXCL10, an urinary chemokine, has emerged as a novel marker of local inflammation in transplanted kidneys, rising in both AR and BK polyomavirus infection (Janfeshan et al., 2024; Loon et al., 2023; Oktay et al., 2021; Tinel et al., 2024). Persistently high CXCL10 levels indicate ongoing immune injury, while low levels effectively exclude AR and viral replication. CXCL10 alone shows ~78% sensitivity and ~82% specificity, while combining it with CXCL9 improves

accuracy to ~88% sensitivity and ~95% specificity. Advances in clinical implementation, such as rapid and affordable immunoassays like the Ella® platform, have further improved feasibility (Tinel et al., 2024). This technology demonstrates strong diagnostic performance with an area under the receiver operating characteristic curve (AUC) of ~0.84, reflecting good overall accuracy and supporting broader adoption. Urinary Neutrophil Gelatinase-Associated Lipocalin (uNGAL) is another emerging biomarker, rising quickly after kidney injury and providing an earlier warning signal than creatinine. One review study confirms uNGAL has a sensitivity of 83% and specificity of 81% for predicting acute kidney injury (Zou et al., 2023). Combining uNGAL with immunological markers such as donor-specific antibodies further improves diagnostic precision, reducing false positives and supporting better clinical decisionmaking (Sharaby et al., 2023).

Dr. Julie Ho, a clinician-scientist with the Transplant Manitoba Adult Kidney Program, is currently conducting clinical trials in Canada and Australia for a urine test which proactively identifies patients at risk of AR, allowing physicians to tailor treatment plans to prolong the life of the transplant (J. Ho, personal communication, June 16, 2025). CXCL10 is a significant improvement over sCr, she points out, and no point-of-care (POC) tests currently exist for the chemokine, suggesting a potential area of innovation for synthetic biology. Similarly, Dr. Tom BlydtHansen, a clinical pediatric nephrologist at the BC Children's Hospital, is translating the success of his CXCL10 biomarker detection test in the lab to clinical trials. Since physicians can only diagnose AR once the graft is already failing, he explains, highvolume, inexpensive biomarker tests present opportunities to monitor and intervene in a timely manner (T. Blydt-Hansen, personal communication, June 4, 2025).

Urie et al. (2024) developed a small implantable microporous scaffold that serves as a remote immune sensor in transplant recipients. By attracting immune cells that can be sampled with a minimally invasive biopsy, the scaffold enables gene activity analysis to detect early signs of AR before organ damage occurs. This approach offers a less invasive alternative to direct organ biopsies, allowing more frequent and precise monitoring. Furthermore, Madhvapathy et al. (2023) developed an implantable bioelectronic system to monitor the temperature of the graft, ultradian rhythms, and disruption of the circadian cycle in real time. A soft electronics interface detects abnormal changes across these measures before changes in sCr occur, enabling earlier AR detection. Such “smart kidney” systems could eventually integrate into bioengineered organs for continuous, non-invasive graft surveillance. Artificial Molecular Probes (AMPs) are another emerging solution for the real-time AR detection of the renal allograft (ARAR) (Cheng et al., 2023). Following intravenous administration, AMPs selectively accumulate in the kidneys and can be tailored to react with prodromal immune biomarkers, generating signals detectable through

near-infrared fluorescence imaging. Due to its high kidney specificity, this noninvasive optical urinalysis identifies rejection earlier than traditional methods to prevent graft loss and distinguishes allograft rejection from other kidney diseases. Artificial intelligence (AI) enhances biomarker interpretation and introduces novel imaging-based approaches (Ningappa et al., 2022). Machine learning models that integrate CXCL10, NGAL, and routine clinical data outperform individual markers, providing patient-specific dynamic predictions. Similarly, deep learning analysis of diffusion-weighted MRI achieves >90% accuracy for early AR detection. AI-driven decision support could therefore reduce unnecessary biopsies and enable earlier therapeutic intervention.

C. Future Research Considerations

The future of AR management relies on advancements in both diagnostic and therapeutic approaches that are not only more accurate but also predictive, standardized, and accessible across diverse healthcare settings. Advanced research into comprehensive molecular analyses using biomarkers such as ddcfDNA, transcriptomic signatures, microRNAs, and proteomic panels attempts to address these limitations. While such methods show promise for improved sensitivity compared with conventional measures, their high cost, lack of assay standardization, and limited large-scale validation continue to prevent widespread clinical implementation (Song et al., 2025; Lafont et al., 2025).

Expanding the role of biomarkers beyond diagnosis to include prognostic and predictive applications represents an important future direction. Multi-biomarker panels combined with computational approaches, such as machine learning algorithms, may allow early identification of patients at higher risk of rejection and enable individualized tailoring of immunosuppression (Lafont et al., 2025; Pan et al., 2025). Achieving this will require standardized assay protocols, prospective multicenter trials, and the integration of these tests into real-world clinical practice (Lafont et al., 2025). Emerging technologies also offer promise as complementary tools alongside standard care. For example, infrared thermal monitoring has demonstrated the ability to detect localized vascularized allograft rejection up to two days before histological confirmation, raising the possibility of wearable or portable surveillance devices for continuous, noninvasive monitoring (Filz von Reiterdank et al., 2025). These approaches could allow more proactive treatment planning.

At the same time, POC molecular assays and enhanced clinician education are essential to support wide-scale use, particularly in settings with limited medical resources (Naik & Shawar, 2023). Dr. Joseph Macri, a clinical biochemist experienced with a variety of hospital laboratory tests, affirms an emerging push towards more accessible, convenient tests efficiently conducted outside of hospital laboratory standards (J. Macri, personal communication, June 16, 2025). POC tests

should be highly sensitive, he explains, so that negative results can effectively rule out the condition, while positive results can warrant further follow-up for early AR detection.

Finally, advances in diagnostics must be matched by parallel progress in therapeutics. ABMR remains a major cause of late graft failure (Gupta et al., 2025). The development of ABMR-specific diagnostics, targeted immunotherapies, and patient-centered adherence strategies is critical to improving long-term outcomes (Böhmig et al., 2025). In addition, adopting standardized pathology reporting systems and incorporating insights from failed graft analyses can strengthen post-transplant management and inform preventive strategies (Böhmig et al., 2025). Together, these efforts point toward a future in which AR is anticipated, detected, and mitigated through alternative interventions in a timely manner. Future research must prioritize affordability, standardization, and personalization to ensure that advances in monitoring and treatment translate into meaningful improvements in graft survival (Lafont et al., 2025; Pan et al., 2025; Song et al., 2025).

IV. CONCLUSION

Early AR detection remains challenging with conventional methods. Researchers express concerns over the delayed detection, invasiveness, inaccuracy, or lack of accessibility of such methods. Urinary biomarkers such as CXCL10 and NGAL may improve screening but require further research and design prior to clinical adoption. Machine learning and other computational methods can contribute to more accurate predictive models. Researchers suggest combining multi-biomarker panels with computational approaches, imaging, and clinical data to predict AR with greater precision. Studies also demonstrate that implantable or portable devices can continuously monitor physiological changes, representing a methodological shift from traditional, episodic diagnostics to advanced, real-time graft surveillance. Developments in AR immunotherapy in parallel with diagnostic improvements highlight opportunities for closed-loop therapeutic systems able to detect AR and deliver localized immunoregulation. Altogether, these innovations point towards an evolving future of early AR detection, where diagnostics and therapeutics integrate seamlessly to improve the accessibility, accuracy, affordability, and timeliness of current methods. With the foremost goal of improving long-term graft survival, this review provides researchers with the information necessary to help bridge the gap between unmet needs and innovations in AR detection.

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Foundations for an AHL-Responsive Genetic Circuit in Eukaryotic Cells: A Biofilm-Disrupting Therapeutic for Cystic Fibrosis

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ABSTRACT

Pseudomonas aeruginosa biofilms are a hallmark of chronic lung infections in cystic fibrosis (CF), conferring high antibiotic resistance and contributing to patient morbidity. Quorum sensing (QS) via acyl-homoserine lactones (AHLs) drives biofilm maturation and virulence, presenting a therapeutic target. Previous work has focused on engineering AHL detection systems into prokaryotes, however, there has been limited investigation into expressing these systems in eukaryotic cells (Gupta et al., 2017). Here, we describe the design and early-stage characterization of eukaryotic genetic circuits that couple AHL detection to gene expression through engineered chimeric receptors derived from LasR and RhlR. In silico docking (HADDOCK) was used to assess AHL binding and dimerization stability across receptor variants, guiding construct selection. This work establishes the foundational engineering steps for AHL-responsive circuits in mammalian cells. This platform may ultimately enable ex-vivo cell therapies to sense *P. aeruginosa* quorum signals and disrupt biofilm integrity in CF lungs.

Index Terms – Quorum Sensing, Biofilm, *P. aeruginosa*

I. INTRODUCTION

Cystic fibrosis (CF) is a life-limiting genetic disorder affecting over 180,000 people worldwide (Guo et al., 2024). A major complication of CF is chronic lung infection, where thickened mucus facilitates bacterial colonization (Mall et al., 2024). *Pseudomonas aeruginosa* is the most prevalent pathogen in this setting, and its ability to form biofilms has been shown to be closely linked to morbidity and mortality (Hoiby et al., 2010; Yoon et al., 2002). Biofilms consist of bacterial aggregates encased in an extracellular matrix (ECM) that provides mechanical stability, protects against immune responses, and decreases antibiotic susceptibility by several orders of magnitude compared to planktonic cells (Hoiby, 1993). Once established, *P. aeruginosa* biofilms are extremely

difficult to eradicate, and treatment failures contribute to progressive lung damage in CF patients.

A key driver of *P. aeruginosa* biofilm formation and virulence is quorum sensing (QS), a bacterial communication mechanism that regulates gene expression in response to population density. In this system, acyl-homoserine lactones (AHLs) such as 3OC12-HSL and C4-HSL activate the transcriptional regulators LasR and RhlR, which in turn induce expression of virulence factors and biofilm matrix components. Targeting QS has long been proposed as a therapeutic strategy for disrupting pathogenic biofilms (Høiby, 2002). Synthetic biology provides a promising approach by engineering cells to detect bacterial QS signals and trigger therapeutic responses. Previous work has demonstrated the feasibility of engineered QS-based detection in prokaryotes (Balagaddé et al., 2005; Balagaddé et al., 2008; Gupta et al., 2017) but these systems are limited due to inherent immunogenicity (Gamage et al., 2003). Adapting this system to eukaryotes poses challenges due to differences in transcriptional machinery, protein folding, and localization. To address this, chimeric LasR and RhlR proteins with mammalian-compatible domains were designed to couple AHL detection with reporter expression. We propose a design that may be adapted to produce enzymes that degrade biofilm, with future applications for targeting *P. aeruginosa* QS in the CF lung.

II. MATERIALS AND METHODS

In silico circuit design

LuxR homologs were surveyed from literature as candidate QS regulators for use in the genetic circuit. LasR and RhlR were selected based on their well-characterized AHL ligands, 3OC12-HSL and C4-HSL, respectively (Keegan et al., 2023; Rust et al., 1996).

To adapt these QS proteins for mammalian use, several modifications were necessary to ensure proper localization, expression, and functionality within eukaryotic cells. A nuclear localization signal (NLS) was incorporated to direct the receptor-ligand complex into the nucleus, where

transcriptional regulation occurs. This step is critical as LuxR-type receptors natively function in bacterial cytoplasm but, in mammalian cells, they must access chromatin to regulate gene expression (Shiner et al., 2004). A transcriptional activation domain (TAD), derived from iGEM part *BBA_K1150001*, was fused to the receptor to recruit mammalian transcriptional machinery. To optimize translation, a Kozak consensus sequence was placed upstream of the coding region, and a flexible linker was introduced between the receptor and TAD to reduce steric hindrance, allowing each domain to fold and function independently while maintaining conformational flexibility (Shiner et al., 2004). The sequences for these two chimeric receptors are depicted in Supplementary Figure 1a and b. Coding sequences in the prokaryotic constructs were codon-optimized for *E. coli* BL21(DE3), and eukaryotic constructs optimized for *Homo sapiens*.

The genetic circuit uses a modular design, which allows the chimeric receptor to detect and respond to AHLs. A constitutive promoter is needed to express the chimeric receptor, and a strong terminator is needed to stop transcription. These components are grouped together in “*Module 1 (M1)*”, which acts as the AHL sensing component of the circuit. The chimeric receptor expressed in *M1* binds and activates the promoter of *Module 2 (M2)* which acts as the responding component of the circuit. *GFP* allows *M2* activation to be quantifiable. *M2* finishes with a strong terminator. These two modules are depicted in **Supplementary Figure 2a and b**.

In silico circuit validation

Structural validation of the engineered chimeric receptors was conducted using HADDOCK. This was done to ensure the chimeric receptor found in *M1* was still able to bind to AHLs and the *M2* promoter, despite the modifications made (NLS, TAD, Linker). Docking simulations compared wild-type and chimeric receptors to assess (i) ligand binding to the cognate AHLs, (ii) dimerization of receptor monomers, and (iii) binding of receptor dimers to AHL-dependent promoters. Active residues were defined based on structural data from crystallography, mutagenesis, and computational modelling performed by Zhang et al. (2002).

Candidate promoter sequences were obtained from the *lasB* and *phzA2* genes, selected for their strong activation by lasR and RhlR respectively (Keegan et al., 2023; Rust et al., 1996). Docking scores were recorded for all conditions.

Assembly of genetic circuits

Prokaryotic and eukaryotic genetic circuits containing the chimeric receptors along with regulatory elements (promoter, RBS, terminator, etc.) were ordered as gene blocks (gBlocks). The prokaryotic gBlocks are to be inserted into pUC19 using HiFi DNA assembly. Recombinant pUC19 is to be transformed into protein expressing BL21(DE3) *E. coli*. Eukaryotic gBlocks will be inserted into a 3rd generation lentiviral transfer plasmid (pLJM-eGFP or pLenti-EF1a-

IRES-Puro) using HiFi cloning. *E. coli* Stable NEB is to be transformed with the recombinant plasmids.

To introduce the two-module circuit into HEK293T (eukaryotic) cells, a 3rd generation lentiviral system will be used. Each circuit is to be HiFi cloned into the lentiviral transfer plasmid pLenti-EF1α (Addgene #85132) or pLJM-eGFP (Hayer et al., 2016). Successfully integrated clones are to be transformed into recombination-stable *E. coli*. Sequence validation will be conducted prior to transfection into HEK293T. Lentiviral packaging plasmids include pHMD-HgpM2, pHDM-tat1b, pRC-CMV-rev1b, and the VSV-G envelope plasmid. Post plasmid purification, co-transfection of the lentiviral plasmids into ~70% confluent HEK293T cells should enable the production of pseudoparticles. High virulence of the pseudoparticles will allow transduction of fresh HEK293T cells. GFP quantification of *M2* expression is to be evaluated by a plate reader.

Enzyme expression & purification

In parallel with circuit design, enzymes targeting biofilm ECM components have been selected as proof-of-concept therapeutic effectors. AiiA lactonase, alginate lyase, and α-amylase were pursued. Enzyme coding sequences were ordered as gBlocks and assembled into pET28a vectors. Recombinant sequence verified pET28a (**Supplementary Figure 3a, b, and c**) were transformed into BL21(DE3) *E. coli* and trialed for ideal expression conditions (**Supplementary Tables 1, 2, and 3**). Ideal conditions were then utilized for large scale enzyme production. Enzymes will be purified under native conditions by Ni-NTA IMAC resin, and eluates will be desalting to remove imidazole prior to downstream assays.

Biofilm Degradation Assay

To evaluate the extent of *P. aeruginosa* (PAO1 or PA14) biofilm reduction by addition of the isolated enzymes, a microtiter spectrophotometric biofilm production assay (Erriu et al., 2012) will be utilized. For each assay plate, wells will be allocated as follows: enzyme-treated (five concentrations: 5, 10, 25, 50, 100 mg mL⁻¹; n=3 technical replicates per concentration), untreated biofilm controls (n=3), and blanks (n=3, no cells; used for background subtraction). Enzyme incubation with biofilm was done for 60, 90, 120, 240, or 360 minutes. Crystal violet will be used for staining, and absorbance will be read at 570 nm. This allows quantification of the relative biomass of enzyme treated samples, when compared to untreated samples. All *P. aeruginosa* work is conducted under Biosafety Level 2 protocols in a CL-2 facility.

For dose-response analyses, % biomass versus log₁₀ (concentration) will be fit to a 4-parameter logistic model to estimate BR₅₀ (concentration yielding 50% biomass reduction), Top/Bottom plateaus, and Hill slope.

Statistical analysis of GFP output from construct iterations

For each receptor (LasR and RhlR, analyzed separately), a library of constructs will be tested across a range of AHL concentrations, with *M2* output being quantified by GFP. Raw signals will be corrected by subtracting media-only, plate matched controls, and when indicated, background-normalized to a co-reporter. Dose-response data will then be fit to a 4-parameter Hill model using nonlinear least squares.

I. RESULTS

Chimeric RhlR and LasR constructs maintain ligand binding in silico

Molecular docking simulations confirmed both chimeric LasR and RhlR variants formed stable complexes with 3OC12-HSL and C4-HSL respectively. The simulation results for both the LasR design when bound to C12-HSL and the RhlR design bound to C4-HSL are shown in **Table 1**. Key binding residues for both LasR and RhlR are also shown in Supplementary Tables 4 and 5.

Table 1. HADDOCK simulation results for LasR bound to C12-HSL and RhlR bound to C4-HSL.

| | Chimeric LasR | Chimeric RhlR |
|-----------------------------|-------------------|-------------------|
| Parameter | Value (\pm SD) | Value (\pm SD) |
| HADDOCK Score | -38.9 \pm 1.8 | -35.9 \pm 1.2 |
| Van der Waals Energy | -19.1 \pm 1.0 | -18.4 \pm 0.5 |
| Electrostatic Energy | -57.7 \pm 15.8 | -57.7 \pm 9.9 |
| Restraints Violation Energy | 17.3 \pm 11.2 | 0.9 \pm 0.2 |

Chimeric RhlR and LasR dimerize in silico

Following ligand docking, dimerization simulations were conducted to evaluate whether the chimeric receptors retained DNA binding ability. LasR and RhlR dimerization scores are displayed in **Supplementary Table 6** respectively, and key residues found in this dimerization for both chain A and chain B are shown in **Supplementary Tables 7 and 8** respectively. Residue-level mapping of the RhlR dimer interface revealed a clear network of stabilizing contacts within 4 Angstroms (\AA) across chains as shown in **Figure 1**.

α -amylase and AiiA lactonase successfully isolated, alginate lyase not detected in BL21(DE3) lysate

The open reading frames of the biofilm-modulating enzymes (Alginate lyase, AiiA lactonase and α -amylase) were successfully assembled into pET28a by HiFi DNA assembly and transformed into *E. coli* NEB5a. Agarose gel electrophoresis following restriction enzyme digestion confirmed plasmid sizes consistent with the designs for AiiA lactonase, α -amylase, and alginate lyase (Supplementary Figure 4, and 5). Whole plasmid sequencing verified construct

integrity (Supplementary Figure 3a-c), with alginate lyase showing 100 percent nucleotide identity to the design. The α -amylase constructs contained only synonymous substitutions relative to the design, and AiiA was entirely synonymous except for one amino acid substitution from a proline residue to a glycine. α -amylase and AiiA lactonase enzymes was visualized on SDS-Page, however alginate lyase was not visualized. α -amylase and AiiA lactonase was isolated using Ni-NTA IMAC. Biofilm assays are currently in progress, and results will determine the optimal concentration range for maximal ECM degradation.

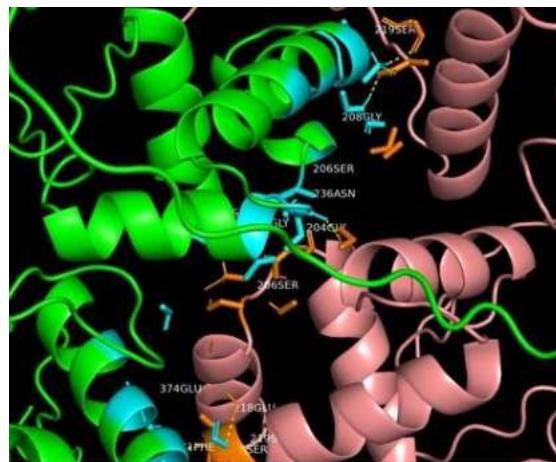


Figure 1. Residue-level mapping of the RhlR dimer interface

Prokaryotic and eukaryotic circuits incorporating *M1* and *M2* for both LasR and RhlR have been assembled and validated in silico; wet-lab characterization is ongoing. The system will be used to quantify GFP expression in response to C4-HSL, 3OC12-HSL, both AHLs together, and no ligand. Dose-response curves will be generated to compare basal and induced activation levels, as well as cross-reactivity between autoinducers.

III. DISCUSSION

This work establishes a foundation for engineering mammalian cells that are capable of sensing bacterial QS signals and responding with programmable gene expression. By adapting the canonical QS regulators, LasR and RhlR, for mammalian expression, we demonstrated through in silico modeling that engineered receptors preserve the capacity for ligand binding, dimerization, and promoter recognition. These results suggest that essential features for mammalian expression, such as nuclear localization and transcriptional activation domains, can be successfully engineered into bacterial QS modules while also retaining wild type function. In future work, expression in mammalian systems may be possible.

Section A - Chimeric receptor in silico validation

Docking simulations confirmed that the engineered LasR and RhlR receptors retained functional binding to their cognate ligands. Both proteins showed HADDOCK scores and interaction energies supporting stable ligand orientation. These findings suggest that the modifications did not disrupt stable ligand orientation.

Dimerization analysis further showed that both receptors maintained stable cooperative interfaces. LasR dimers relied on the residues seen in **Supplementary Table 7**, while RhlR dimers involved a broader set of residues (**Supplementary Table 8**). These interactions produced strongly negative electrostatic energies and large buried surface areas, suggesting robust interface stability.

While promoter-binding simulations are ongoing, preliminary alignment with the TraR-DNA complex (member of LuxR receptor family identified in Protein Data Bank) identified candidate promoter-binding residues in LasR (193THR, 204SER, 216/217ARG, 218LYS, 223SER,) and RhlR (192THR, 206SER, 210SER, 221LYS) that are positioned similarly to DNA-contacting residues in TraR: 190THR, 204SER, 219ARG, 220SER, 221LYS (Zhang et al., 2002). These alignments were validated in PyMOL by mapping residues within 4 Å of DNA bases. This confirmed that several LasR and RhlR side chains are positioned analogously to the DNA-contacting residues of TraR. This suggests plausible DNA binding interfaces that will be directly tested in upcoming docking studies with promoters (lasB, lasA, PhzA1, PhzA2, PhzH).

Section B – Prospectives, future directions, and limitations

This work establishes a framework for engineering mammalian cells to sense bacterial QS signals and presents a potential application as an autologous ex vivo therapeutic for chronic infections such as those caused by *P. aeruginosa* in CF. Biofilms underpin persistent infection and antibiotic resistance, while current therapies demand strict adherence and frequent hospitalization. An AHL-responsive system in patient-derived eukaryotic cells could provide long-term, adherence-independent therapeutic delivery, overcoming limitations of bacterial and phage-based approaches that are rapidly cleared by the immune system.

Translation of this concept will require validation of the eukaryotic AHL-detection system in immune cells and animal models, alongside optimization of enzymatic payloads for efficacy against mature biofilms. The modular promoter library enables tuning of expression levels to suit therapeutic needs, and ongoing wet-lab experiments will evaluate promoter performance in mammalian cells as well as the ability of α -amylase and related enzymes to disrupt biofilms of varying maturity.

While promising, this strategy remains at an early proof-of-concept stage. The behavior of bacterial QS regulators in mammalian cells requires extensive characterization to ensure reliable function and avoid off-target effects. Some additional challenges include confirming sustained biofilm degradation *in vivo*, ensuring safety and stability of engineered circuits,

and addressing the cost and complexity of autologous cell therapy.

IV. CONCLUSION

α -amylase and AiiA lactonase was successfully produced in BL21(DE3) *E. coli*, as well as isolated and purified to support downstream biofilm-degradation assays, which are ongoing. In parallel, *in silico* designs of the chimeric receptor–promoter circuits were finalized; modeling indicates retained ligand binding, and appropriate receptor dimerization. These computational results provide a basis for engineering gene circuits in mammalian cells toward an *ex vivo* biofilm therapeutic. By measuring output variation of the construct iterations, this allows for determination of an *M2* promoter that correlates to effective biofilm degradation. This, being determined by the threshold to which α -amylase is seen to successfully reduce biofilm biomass. Further, using this data, a finalized translational design, and a clinically effective dosing window may be determined. Validation by additional wet-lab data is needed to draw further conclusions. In combination with future wet-lab data, our results should provide a strong foundation for a biofilm-disrupting therapeutic for CF.

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Supporting information: [Vector 2025 Supplementary Information](#)

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The history of biosynthesis of vanillin from ferulic acid and its key events

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ABSTRACT

Vanillin is the second most expensive flavour compound after saffron and one of the most commercially relevant aroma molecules, widely used in the food, fragrance, pharmaceutical, and materials sectors. With the rising global population, the demand for vanillin continues to increase across all industries. Emerging biotechnologies aim to lower costs, improve access and provide new sustainable production methods, with ferulic acid as an extensively studied biotransformation candidate for the biotechnological production of vanillin. While several previous reviews have focused on the different methods of vanillin bioproduction, there are still few reviews which take into account the vast research boost of the 2020s. In an attempt to comprise the research of ferulic acid biotransformation to vanillin up to this date and its relevance for a sustainable flavour and fragrance industry, this review will highlight the history of bioproduction of vanillin and the role of ferulic acid in the vanillin pathway, its efficiency in different microorganisms as well as production bottlenecks which highlight the significant potential of vanillin bioproduction through ferulic acid for future research.

Index Terms – Vanillin, Ferulic acid, Biotechnology, Environment, Bioproduction

I. INTRODUCTION

Vanillin, also known as 4-hydroxy-3-methoxybenzaldehyde, is a molecule widely used in various industries, particularly in the food and beverage industry for flavouring, the fragrance industry, and the pharmaceutical industry. Vanillin, being the second most expensive flavouring compound after saffron (Olatunde et al., 2022), holds a great demand in global consumption. In 2022, the global vanillin market size was estimated at USD 627.1 million and is projected to reach USD 1119.3 million by 2030 (Grand View Research, Inc., 2023).

The pharmaceutical sector holds the largest share of the global vanillin market, followed by food and beverages, and then fragrances (Fig. 1, Grand View Research, Inc., 2023).

Vanillin may be naturally sourced from the pods of *Vanilla planifolia*, *Vanilla tahitiensis* or *Vanilla pompona* (Ranadive, 1992). Only vanillin extracted from those plants and

bioproduced vanillin can be called natural, as defined by the Regulation (EC) No 1334/2008 for the EU and the 21 CFR § 101.22 from the U.S. Food and Drug Administration for the U.S.

Currently, guaiacol is the primary raw material for the chemical synthesis of vanillin, accounting for more than 75% of all vanillin production. The reason for this is the cost-effectiveness (Kumar et al., 2025; Nayak et al., 2023), despite the environmental pollution caused by the formation of hazardous waste, as well as high water and energy consumption (Kumar et al., 2025; Martäu et al., 2021; Zhao et al., 2021) Another method is the biotechnological production of vanillin from ferulic acid, which this review will focus on. The β -oxidation pathway for ferulic acid, with the key enzymes Vanillin dehydrogenase and HMPHP-CoA dehydrogenase, converts ferulic acid to vanillin and exhibits a 90.5% conversion efficiency at 30°C and pH 7, with high selectivity, despite limited enzyme stability. The non- β -oxidation pathway for ferulic acid, with the key enzymes Feruloyl-CoA hydratase/lyase, converts trans-Feruloyl-CoA to vanillin with an even higher conversion efficiency of 96.7% at 37°C and under aerobic conditions, offering the advantageous direct conversion but with a dependency on CoA (Kumar et al., 2025, Table 4). Since this method, from all biotechnological production ways of vanillin, is widely used today as a cheaper alternative to natural vanillin and as a more sustainable alternative to synthetic, chemically synthesised vanillin, there are reviews on various aspects such as methodology, yield, and sustainability, but few on the historical development of this essential method and its key events that have shaped it. Therefore, in this review, the history of this method will be summarized, its pivotal events will be analysed, and an outlook on its future potential will be provided.

II. HISTORY

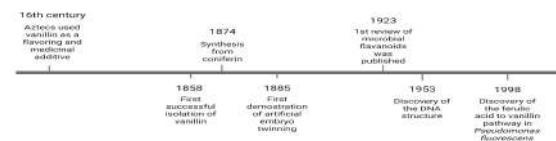


Figure 1. Schematic timeline of key events in the history of Vanillin synthesis. Created in <https://BioRender.com>

The key events of vanillin research are highlighted in Fig. 1.

The first recorded human employment of vanilla dates back to the 16th century when it was used as a flavoring, medicinal and ceremonial additive by the Aztecs (Fig. 1; (Bythrow, 2005; Rain, 2004). Scientific investigation into its main flavoring compound, vanillin, only commenced with its first isolation in 1858 (Fig. 1; (Goble, 1858) marking the onset of academic studies on the compound. This milestone enabled the structural characterization and first synthesis of vanillin from coniferin (Fig. 1; Reimer & Tiemann, 1876; Tiemann & Haarmann, 1874), which signified the beginnings of the modern flavouring industry (Krings & Berger, 1998 p. 1). Research on microbiological aroma compound production began in 1923 (Fig. 1; Omelianski, 1923). The necessary tools for genetic engineering were not available yet. This began to change in the early 20th century with the first observation of transformation in bacteria by Griffith (Griffith, 1928) and the discovery that genetic characteristics are determined by DNA (Watson & Crick, 1953). The uncovering of the DNA double-helix structure by Watson and Crick, facilitated through Franklin's X-ray diffraction data (Watson and Crick, 1953), led to the onset of the field of molecular genetics (Priefert et al., 2001) As a result of the emergence of gas chromatography in the 1950s, it was soon possible to separate and structurally elucidate volatile compounds (James & Martin, 1952), which enabled a multitude of research on flavour and fragrance compounds produced by microorganisms (Krings & Berger, 1998, p. 1). The first genetically modified organism in 1973 (Cohen et al., 1973) marked the beginning of modern synthetic biotechnology. Genetic modifications could now be used to induce the production of non-native pathways in organisms. The benefits of heterologous microbial production compared to deriving the compound from its original source may include a higher production yield and a smaller environmental footprint (e.g. Di Gioia et al., 2011; Gallage & Møller, 2015)

Since its discovery, the demand for vanillin as a flavouring and fragrance compound has continued to grow steadily and has long surpassed the vanillin derived from harvested vanilla pods (Grand View Research, Inc., 2023). To meet production demands, chemical synthesis of vanillin from lignin or guaiacol was introduced (Hocking, 1997). Both compounds are byproducts of the wood pulp and paper industry and are thus readily available in large quantities at a cost-effective price (Nayak et al., 2023). While being cost-effective, such synthetic vanillin is reliant on petrochemical resources, resulting in hazardous byproducts and environmental pollution (Zhao et al., 2021).

Using renewable feedstocks to derive vanillin through biological processes has emerged as an environmentally friendly alternative, inspiring various microbiological approaches to producing bio-vanillin. However, major challenges of cultivating microorganisms for bioproduction include the cytotoxicity of vanillin, high yields of unwanted byproducts, costly downstream isolation methods and further metabolism of the desired product by the selected

microorganisms. Such drawbacks have been addressed through different biotechnological approaches. (Gallage & Møller, 2015)

One of the most promising approaches is the microbial production of vanillin through ferulic acid or eugenol via bacteria or yeast, a pathway discovered in 1998 (Ciriminna et al., 2019; Gasson et al., 1998). Ferulic acid, or 3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid, is one of the plant secondary metabolites that confers rigidity in cell walls and is amenable to bioconversion into vanillin (Di Gioia et al., 2011; Ou & Kwok, 2004) that is abundant in agro wastes (Kumar et al., 2025). It is one of the most commonly used precursors due to its low toxicity to microorganisms and minimal by-product formation (Qiu et al., 2022).

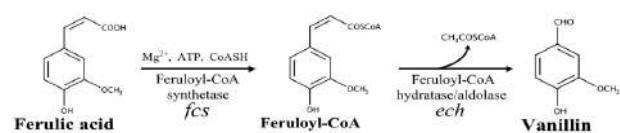


Figure 2. Schematic representation of the non- β -oxidative pathway for conversion of ferulic acid into vanillin. (Luziatelli et al., 2019)

Fig. 2 illustrates the pathway from ferulic acid to vanillin in an abbreviated form. Ferulic acid is first activated to feruloyl-CoA by a feruloyl-CoA synthetase, also known as the fcs gene. Then, the CoA-thioester is subsequently hydrated and cleaved to vanillin and acetyl-CoA by an enoyl-CoA hydratase/aldolase, otherwise known as ech genes. (Yoon et al., 2005) Vanillin, although produced from ferulic acid by microorganisms, is quickly oxidized or reduced to vanillic acid and vanillyl alcohol after its formation due to its high toxicity levels for most microorganisms. (Luziatelli et al., 2019; Priefert et al., 2001) In the absence of a vanillin degradation pathway, Escherichia coli was investigated as a potential vanillin producer by introducing genes related to the bioconversion of ferulic acid to vanillin. (Yoon et al., 2005)

Escherichia coli strains are now the most efficient vanillin producers (Di Gioia et al., 2011) with a promising conversion rate of up to 90.5%, while maintaining cost competitiveness with synthetic alternatives. (Kumar et al., 2025) The price of vanillin obtained through the fermentation of ferulic acid is approximately USD 700/kg (Ciriminna et al., 2019).

III. Future Outlook

Growing trends of health consciousness and a focus on wellness can drive the demand for organic foods perceived as healthier and free from synthetic chemicals (Rahman et al., 2024) thus creating a rising demand for naturally synthesised vanillin. With high costs and scarce availability of natural vanilla pods, there is significant potential for bioproduced vanillin from agricultural byproducts to gradually meet the

demand for global bio-vanillin consumption and potentially replace its cheaper counterpart, synthetic vanillin. (Luziatelli et. al, 2019) Its relatively low environmental and economic impact attracts various scientists and stakeholders and is “well regarded” by consumers (Wang et al., 2021). However, several bottlenecks, such as enzyme instability, product toxicity for the producing organisms, and downstream processing, still hinder biosynthetic vanillin from becoming the industry standard. Many research articles and journals have discussed various strategic approaches to optimise bio-vanillin production, such as stabilising recombinant *E. coli* strains, introducing different buffers or low-cost nitrogen sources and building effective biosynthetic pathways (Luziatelli et. al, 2019; Liu et al., 2023; Taiwo et al., 2024). Another often overlooked aspect is the potential for vanillin in the pharmaceutical industry. Several studies have reported the pharmacological activities of vanillin, including anticancer, antidiabetic, antioxidant, antisickling, antimicrobial, anti-inflammatory, aphrodisiac, cardioprotective, and diuretic effects (Martău et al., 2021; Olatunde et. al, 2022; Iannuzzi et al., 2023), which could be of interest to consumers in the future.

Based on previous literature, it is reasonable to assume that consumers who strongly value the environment will request health benefits, thereby increasing demand for natural vanillin, which can be provided mainly through biosynthetic means (Lai & Chen, 2020; Sigurdsson et al., 2023).

The impact of a more privileged society in Western countries may not be underestimated. Awareness is growing about the importance of good health, and in view of the threat of the climate crisis, a polluting method for producing vanillin is a serious concern. As a result, products with labels like “sustainable” or “healthy” may be preferred (Sigurdsson et al., 2023).

IV. DISCUSSION

Recent advances in the bioproduction of vanillin through ferulic acid as a sustainable and industrially viable alternative to synthetic vanillin have been collected in the scope of this review. Particular attention was paid to its historic relevance and its development towards . It has been established that vanillin has long been a topic of particular interest to humans. Arguably, the key event marking the onset of industrial vanillin production was the discovery of lignin and guaiacol biotransformation into vanillin, particularly due to their abundance and affordability as byproducts of wood pulp production for paper.

The discovery of the vanillin pathway based on ferulic acid was a crucial step towards that. While the field has progressed rapidly, several important challenges remain. To reach the full potential of biobased vanillin, it is essential to overcome the following main limitations: (1) Enzyme stability, (2) vanillin

toxicity for producer microorganisms. (3) downstream processing.

V. CONCLUSION

In summary, the increasing demand for vanillin in general, and for sustainably produced vanillin in particular, as a flavouring compound, fragrance and health supplement has been demonstrated. Further research into vanillin's reported antineoplastic, antidiabetic, antioxidant, antisickling, antimicrobial, anti-inflammatory, and cardioprotective properties is warranted to fully exploit its therapeutic potential.

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CD38 and Tumor Immunosuppression: Mechanisms and Therapeutic Insights

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ABSTRACT

CD38 is a multifunctional ectoenzyme that plays an important role in manipulating the tumor microenvironment (TME) by influencing local immune cell metabolic pathways and promoting immune suppression. It is mostly expressed on tumor cells directly and affects proximal immune cells, including regulatory T cells, myeloid-derived suppressor cells, and exhausted CD8+ T cells. CD38 causes immune inactivation in the TME through two key mechanisms: NAD+ depletion and adenosine accumulation. The enzyme degrades NAD+, causing T cells to shift to abnormal cellular pathways to produce energy, affecting their survival and effector functions. Furthermore, it works with other ectoenzymes like CD203a and CD73 to convert NAD+ to adenosine, which further reduces anti-tumor immune function through A2A receptor signalling. The enzyme is also often upregulated in response to interferon- γ and hypoxia, making it a major factor in resistance against PD-1/PD-L1 inhibitors. This literature review examines the molecular mechanisms CD38 utilizes to create an immunosuppressive TME, highlights its role as an immune exhaustion biomarker, and discusses the problems associated with targeting this enzyme, namely, off-target toxicity and the use of secondary metabolic pathways. A comprehensive understanding of these factors could positively influence the creation and implementation of future immunotherapeutic methods of combating immune cold tumors.

I. INTRODUCTION

The ability of cancer cells to evade and suppress the immune system is a defining hallmark of tumor progression and a major barrier to effective therapy. Recent strides have been made by the approval and subsequent therapeutic adoption of immune checkpoint inhibitors (CTLA-4 and Anti PD-1/PD-L1). However, despite their unprecedented success, a large group of patients exhibits either primary resistance or acquires resistance over time, highlighting the complexity and effectiveness of tumor immune evasion mechanisms beyond these checkpoints. These processes are primarily driven by the immunosuppressive tumor microenvironment (TME), a

dynamic system consisting of tumor cells, immune cells, and metabolic factors that inhibit effective anti-tumor immunity. Researchers have identified multiple possible causes for this evasive nature of tumor cells, one of which is Cluster of Differentiation 38, also known as CD38 for short. This ectoenzyme uses NAD+ as a substrate and produces ADPR (Adenosine Diphosphate Ribose) or cADPR (Cyclic ADPR), which can further be broken down into adenosine. Working alongside CD39 and CD73, two enzymes with similar functions, they reduce extracellular NAD+, rework calcium signalling cascades, and produce extracellular immunosuppressive adenosine.

Beyond its metabolic functions, elevated CD38 expression has been linked to increased infiltration of immunosuppressive cells, the exhaustion of cytotoxic T lymphocytes, and the development of resistance to immune checkpoint blockade. For example, CD38 is shown to have a direct link to the activation of the FOXP3, a forkhead transcription factor which plays an essential role in suppressing the function of NFAT and NF- κ B cellular pathways. (Pérez-Lara et al., 2021) This causes suppression of many genes, including IL-2 and effector T-cell cytokines, causing an overall reduction in local immune response. These effects contribute significantly to the creation of a highly tolerogenic tumor microenvironment (TME), allowing for malignant cells to evade the immune system and thrive despite immunotherapeutic interventions. (Konen et al., 2019).

In this review, we will characterize the molecular mechanisms by which CD38 reforms the TME, explore its interactions with different immune cell types as well as checkpoint resistance, and finally discuss the current challenges and future prospects for therapeutically targeting CD38 in cancer immunotherapy.

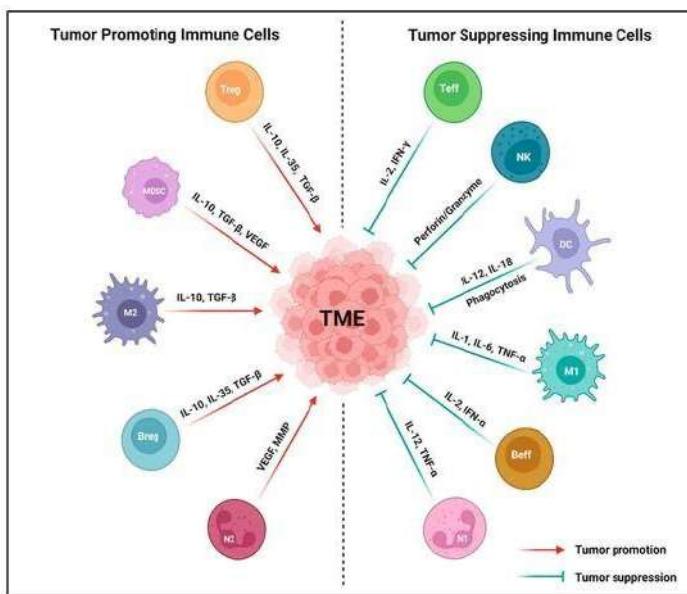


Figure 2. Tumor-promoting and tumor-suppressing immune cells in the tumor microenvironment. (Adapted from Giri et al., 2025)

II. MOLECULAR MECHANISMS OF CD-38 MEDIATED IMMUNOSUPPRESSION

NAD⁺ Depletion

CD38 functions primarily as an ectoenzyme catalyzing the degradation of extracellular Nicotinamide Adenine Dinucleotide (NAD⁺), a coenzyme involved in cellular metabolism and immune cell functioning. Recent studies reiterate the importance of NAD⁺, an important metabolite required for many pathways, ranging from the generation of second messengers to serving as a substrate and regulator of NAD-dependent deacetylases, commonly known as sirtuins. (Chini, 2009) Sirtuins are a group of enzymes that are indispensable in regulating cellular metabolism, stress responses, and gene expression. Sirtuins adjust chromatin structure and influence transcriptional programs that control processes like DNA repair, inflammation, and mitochondrial function. (Wu et al., 2022) They work by cleaving acetyl groups from histones and various proteins. From an immunological perspective, we can concentrate on its contributions to functions such as T cell activation, differentiation, and survival. Regarding T cells, regulatory T cells (Tregs) are a key subset of immune cells within the tumor microenvironment that play a crucial role in maintaining immune tolerance and suppressing excessive immune responses. SIRT1 modifies Treg function using the NF-κB pathway and its influence on the aforementioned FOXP3 transcription factor. However, when CD38 depletes extracellular NAD⁺, it affects sirtuin activity by impairing their regulatory control over Tregs and leading to enhanced

Tregs-mediated immunosuppression in the tumor microenvironment. Coupled with the effects of a lack of available second messengers, cell signalling becomes slow and inefficient. This results in the immune system's ability to detect pathogens, activate immune cells, and coordinate an effective defense being severely compromised. (Newton, Bootman, & Scott, 2016)

Adenosine Accumulation

CD38 also drives a second immunosuppressive pathway through the accumulation of extracellular adenosine, a potent modulator of immune cell activity within the tumor microenvironment. It is an extremely relevant physiological compound as it is one of the building blocks for nucleic acids, a key part of ATP, works as a neurotransmitter, and plays a role in cellular signalling. However, despite the essentiality of this compound in the regular functioning of a cell, in the TME, the role it occupies is far more counterintuitive. Studies show, the binding of excess adenosine to its respective receptors causes a wide range of suppressed effects, including reduced tumor antigen presentation, lowered anti-tumor cytokine secretion, a marked decrease in chemokine secretion and KCa3.1 channel activity, all of which impede effector T cell trafficking and infiltration into the tumor site. (Wang et al., 2024) The build-up arises when CD38 enzymatically converts NAD⁺ into ADPR and cADPR, which are subsequently processed by the ectoenzymes CD203a and CD73 into adenosine, leading to persistently elevated concentrations within the TME. Additionally, the excess adenosine signals predominantly travelling through the A_{2A} receptor on T cells, NK cells, and other immune effectors, activate the cAMP–PKA pathway, further inhibiting TCR signalling. (Chen et al., 2024) This in turn reduces the production of cytokines such as IFN-γ and IL-2, and impairs cytotoxic function. Through these combined effects, adenosine accumulation orchestrates a broad suppression of both innate and adaptive immunity.

Together, NAD⁺ depletion and adenosine accumulation represent both complementary arms of CD38-triggered immunosuppression, creating a metabolically and signal restricted environment that strongly favors tumor persistence despite immune surveillance and immunotherapeutic methods.

III. CD38 EXPRESSION PATTERNS AND IMPACT ON IMMUNE SUBSETS

CD38 is frequently detected on tumor cells in a variety of cancers, but it is also expressed by several types of immune cells within the tumor microenvironment. Here, its presence plays a pivotal role in shaping immune responses and facilitating tumor immune evasion.

Understanding these patterns of CD38 expression among both tumor cells and a diverse group of immune cell subsets is crucial to outlining its role in shaping the tumor immune landscape.

Tumor cells and Regulatory immune populations

As previously mentioned, CD38 affects a plethora of signalling pathways that make up the backbone for extremely necessary cellular processes such as cell growth, proliferation, survival, and regulation of metastasis. (Wang et al., 2025) CD38 is upregulated on tumor cells across a range of solid tumors and hematologic malignancies, including multiple myeloma, melanoma, lung, ovarian, and colorectal cancers. (Angelicala et al., 2021) Often, it is prompted by conditions present in the tumor microenvironment, like interferon- γ and hypoxia, and has been linked to aggressive disease phenotype and poor prognosis. (Angelicala et al., 2021) Within regulatory immune cell populations, Tregs and myeloid-derived suppressor cells (MDSCs) both express high levels of CD38. This is strongly linked to their enhanced immunosuppressive functions. CD38 $^{+}$ Tregs show increased FOXP3 activity and produce higher amounts of immunosuppressive cytokines. (Pérez-Lara et al., 2021) The increased expression by MDSCs and certain macrophages causes suppressed anti-tumor immune responses, partly through mechanisms like generating reactive oxygen species, depleting key nutrients, and altering metabolic pathways. Clinically, upregulated CD38 expression in these regulatory populations has been correlated with poorer patient outcomes, underscoring its potential as both a prognostic marker and a therapeutic target in cancer immunotherapy.

Effector immune cells and Myeloid populations

Besides regulatory populations, CD38 expression is becoming increasingly more recognized on key effector immune cells within the tumor microenvironment, such as exhausted CD8 $^{+}$ T cells and natural killer (NK) cells. (Marie Otsuka et al., 2023) Elevated CD38 levels on CD8 $^{+}$ T cells coincide with markers of immune exhaustion such as PD-1 and TIM-3, reflecting an immunologically dysfunctional state. Reduced cell division, cytokine production, and cytotoxic activity are some of the hallmark features presented by this abnormal state. Additionally, CD38 $^{+}$ NK cells have impaired cytotoxicity and diminished secretion of interferon- γ , limiting innate anti-tumor immune responses. (Ma et al., 2019)

In other myeloid populations, CD38 is found on various macrophage subsets and dendritic cells, where it controls their function and polarization. Specifically, its expression on tumor-associated macrophages promotes an M2-like immunosuppressive phenotype, which secretes anti-inflammatory cytokines. This contributes to supported tumor

growth, increased metastasis, and remodelling of the extracellular matrix. Dendritic cells expressing CD38 show altered antigen presentation capacity, which hampers the activation of adaptive immune responses. (Huang, Kang, & Chen, 2024)

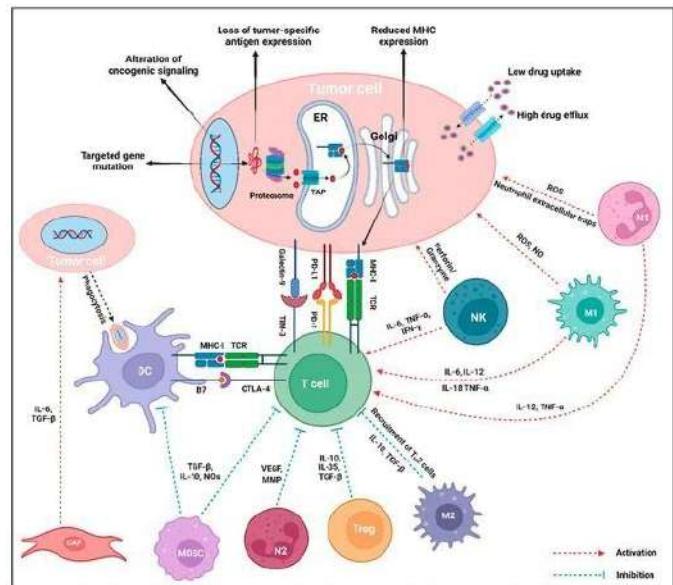


Figure 1. Immune evasion mechanisms in the tumor microenvironment. (Adapted from Giri et al., 2025)

While anti-CD38 therapies have made a substantial impact in hematologic cancers, tailored combination approaches taken together with enhanced delivery and specificity seem to be the path forward to effective treatment of solid tumors.

IV. THERAPEUTIC TARGETING OF CD38 IN CANCER IMMUNOTHERAPY

Through researchers attempting to characterize the enzyme and all its effects, CD38 has quickly attracted significant interest to fulfill a role as a therapeutic target in cancer treatment. The clinical success of anti-CD38 monoclonal antibodies such as daratumumab and isatuximab in multiple myeloma, for example, demonstrate the potential of using a CD38 blockade to reverse immune evasion and induce tumor regression. (Horenstein et al., 2025) (Valentina et al., 2024) These antibodies function by mechanisms including antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and depletion of immunosuppressive regulatory cells. However, its application in tackling solid tumors still remains riddled with complications. Factors such as tumor heterogeneity, physical barriers within the stroma, and the coexistence of multiple concurrent immunosuppressive pathways limit the efficacy of

monotherapies such as this. (Smolarska et al., 2025) (Zhang et al., 2022) Furthermore, interfering with CD38 expression on normal immune cells raises concerns of off-target effects and autoimmunity. (Piedra-Quintero et al., 2020) (Giri et al., 2025) The approach to tackling these issues currently is through combination therapy. Integrating CD38 blockade with immune checkpoint inhibitors such as PD-1/PD-L1 antibodies shows promise in overcoming resistance mechanisms mediated by CD38 upregulation. (Chen et al., 2017) Instead of solely using a CD38 blockade, emphasis is being placed on using it alongside simultaneous inhibition of adenosine-producing enzymes CD73 and CD39, bispecific antibodies, antibody-drug conjugates, and nanobody-based therapeutics. This approach allows for much more precise targeting and reduced systemic side effects. Metabolic modulation through NAD⁺ precursors is another possible approach. This presents a complementary mode of reinvigorating exhausted effector cells suppressed by CD38-driven NAD⁺ depletion. (Wan et al., 2023) Biomarker-guided patient selection and comprehensive profiling of tumor metabolic states are essential to optimize this therapeutic strategy.

While anti-CD38 therapies have made a substantial impact in hematologic cancers, tailored combination approaches taken together with enhanced delivery and specificity seem to be the path forward to effective treatment of solid tumors.

V. DISCUSSION

This literature review discusses CD38's complex relation to the immunosuppressive nature of the TME. Its increased upregulation over various different populations of cells, ranging from tumor cells to Tregs and exhausted CD8⁺ T cells, heavily implies CD38's integral function in helping tumors evade immune action and build resistance to checkpoint inhibitors. (Konen et al., 2019) (Pérez-Lara et al., 2021) (Marie Otsuka et al., 2023) While this approach has made great strides in efficacy for hematological malignancies, it remains in its infancy for solid tumors due to challenges such as metabolic complexity, local immune cell variety, and overall tumor diversity. The most pressing issues of which are: off target toxicity, risk of autoimmunity, and tackling multiple similar parallel pathways. (Smolarska et al., 2025) (Zhang et al., 2022) (Piedra-Quintero et al., 2020) (Giri et al., 2025).

The ReSET platform offers a promising solution that addresses several of these current gaps simultaneously. We have created a logic-gated probiotic that can detect

hallmark features of the tumor microenvironment, such as increased presence of lactate and hypoxia. In response, it selectively releases an anti-CD38 nanobody, allowing for precise targeting while minimizing systemic exposure and off-target effects. The simultaneous production of salicylate as a noninvasive biomarker for real-time monitoring of therapeutic activity, enabling personalised treatment adjustments. Furthermore, the use of nanobody technology enhances specificity and tissue penetration, potentially overcoming stromal barriers typical of solid tumors. (Valentina et al., 2024).

VI. CONCLUSION

CD38's large influence on immune regulation within the tumor microenvironment has established it as a critical therapeutic target, especially for cancers characterized by immune evasion and resistance to checkpoint blockade. Considerable progress has been achieved in treating hematologic malignancies, yet solid tumors present their own unique and complex challenges that demand more precise, tailored therapeutic approaches. (Konen et al., 2019) (Angelicola et al., 2021)

Emerging approaches such as ReSET, which combine logic-gated and targeted delivery with integrated diagnostic reporting, represent a promising direction for CD38 inhibition. By customizing immune modulation to the specific environment within the tumor microenvironment and offering real-time feedback, these platforms open the door to safer, more effective, and flexible cancer immunotherapies that could be applied to a wide range of tumor types in the future. (Valentina et al., 2024) (Giri et al., 2025) (Piedra-Quintero et al., 2020).

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Synergistic Roles of Biopolymers and Antimicrobial Agents in Burn Wound Therapy: A Synthetic Biology-Driven Review

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ABSTRACT

Burn wounds present a major therapeutic challenge due to delayed healing and high susceptibility to infections, particularly from antimicrobial-resistant pathogens. This review explores a dual-layer wound treatment concept comprising natural biopolymers and bioactive antimicrobial components. Electrospun casein-based scaffolds emulate dermal structure and function, while oxidized hyaluronic acid (O-HA) hydrogels provide moisture retention and crosslinking functionality. Propolis adds broad-spectrum antimicrobial properties, and bacteriophages offer targeted elimination of resistant pathogens. Throughout the review, we highlight how synthetic biology tools—including protein engineering, recombinant gene expression, and phage design—can enhance the functionality and specificity of these materials. This integrative approach exemplifies how bio-inspired systems, informed by synthetic biology, could advance next-generation wound therapy.

Index Terms – burn wounds, biopolymers, casein, oxidized hyaluronic acid, electrospinning, propolis, bacteriophages, synthetic biology

I. INTRODUCTION

Burn injuries affect millions worldwide and frequently result in severe tissue damage, infection, and prolonged rehabilitation (Greenhalgh, 2019). Traditional wound dressings offer basic protection but often lack the structural or biological complexity required for optimal healing (Esteban-Vives et al., 2016). In recent years, multifunctional biopolymer systems have gained interest as advanced materials capable of mimicking skin architecture and supporting regeneration. Simultaneously, synthetic biology offers new methods for designing bioactive molecules and tailored delivery platforms (Martinez et al., 2022). This review focuses on a layered strategy that integrates biopolymers with antimicrobial agents, while exploring how synthetic biology can optimize each

material component through gene design, recombinant expression, and modular biosystems.

1. Casein as a Biomaterial

Casein, the predominant protein in milk, forms stable micelles composed of α -, β -, and κ -caseins, lending it unique structural and rheological properties (Zhu et al., 2023). Its biodegradability and compatibility with cellular systems make it suitable for scaffolds and coatings in tissue engineering. While traditionally sourced from bovine milk, recent efforts in synthetic biology explore human-derived casein genes for enhanced biocompatibility, expressed in microbial systems like *E. coli* or yeast (Chatterjee et al., 2020). Recombinant casein enables more controlled material properties and reduces immunogenicity risks. Furthermore, electrospinning casein into nanofibers results in ECM-like topography, promoting fibroblast adhesion and guiding tissue repair (Memic et al., 2019).

2. Hyaluronic Acid and Oxidized HA

Hyaluronic acid (HA) is a linear glycosaminoglycan with water-retaining and cell-interactive properties, commonly used in wound care. Oxidized HA (O-HA), produced via periodate-mediated cleavage, introduces reactive aldehyde groups that allow Schiff base crosslinking with amine-containing polymers like casein (Choi et al., 2015). This crosslinking can be fine-tuned for degradation rate and stiffness. Synthetic biology tools allow tailoring of HA-modifying enzymes and offer recombinant production of HA with precise molecular weights (Lee et al., 2023), optimizing the physicochemical properties of hydrogels. Casein–O-HA hydrogels thus represent a promising platform for moisture-retentive, cell-friendly wound matrices.

3. Propolis as Antimicrobial Component

Propolis, a resinous bee product rich in flavonoids and phenolic compounds, demonstrates significant antimicrobial and anti-inflammatory activity. It has shown efficacy against key wound pathogens including *Staphylococcus aureus* and *Streptococcus mutans* (Asawahame et al., 2014). However, its

variable chemical composition poses challenges for standardization. Recent studies explore propolis nanoparticle formulations and their integration into polymeric scaffolds for controlled release (Hashemi et al., 2023). Synthetic biology may help improve standardization by producing propolis-inspired antimicrobial peptides or by engineering microbial systems to biosynthesize consistent propolis analogues with defined bioactive profiles (Chen et al., 2022).

4. Bacteriophages in Burn Infection Control

Bacteriophages (phages) selectively infect and lyse bacteria, offering an alternative to antibiotics, particularly against multidrug-resistant strains like *Pseudomonas aeruginosa* (Piranagh et al., 2023). Phages can be embedded into hydrogels for sustained release while preserving infectivity (Wang et al., 2022). Synthetic biology plays a critical role in phage engineering—enhancing host range, improving lytic potency, and reducing immune recognition (Roach & Debarbieux, 2017). Engineered phages with modified tail fibers or lysins can overcome limitations of native phages and provide precise, programmable antibacterial activity tailored to wound microbiomes.

5. Electrospun Fiber Systems

Electrospinning enables the production of nanofibrous scaffolds with ECM-mimicking architecture and tunable porosity (MacEwan et al., 2022). Casein electrospun fibers can support cell migration and nutrient exchange while gradually degrading. Synthetic biology adds another dimension—engineered casein variants or peptide sequences can be designed for specific biofunctions, such as promoting angiogenesis or modulating inflammation (Pang et al., 2021). Combining biopolymer processing with rational peptide design facilitates smart scaffold development that responds to the biological context of the wound.

6. Dual-Layer Architecture and Functional Synergy

The design mimics native skin architecture: the fibrous casein scaffold represents the dermis, providing structural support and bioactivity, while the O-HA hydrogel imitates the epidermis, ensuring hydration and cell recruitment. Phages offer pathogen-specific lysis, while propolis provides broad-spectrum protection and anti-inflammatory effects. Such synergy enhances tissue integration and may reduce the need for systemic antibiotics, aligning with global efforts to mitigate antimicrobial resistance.

7. Limitations and Challenges

Despite promising results, key challenges remain. Phage formulations must preserve infectivity during storage and avoid triggering immune responses (Rolain et al., 2016). Propolis standardization and stability require further optimization. Casein, though biocompatible, can cause allergic responses in sensitized individuals (Kuitunen et al., 2012). From a synthetic biology perspective, safe implementation of engineered components—such as phages or recombinant

proteins—requires robust biosafety measures and clear regulatory pathways, especially in clinical applications. Clear documentation of biocontainment strategies and safety validations is essential.

8. Biosafety and Biosecurity Consideration

While all materials used in the system (casein, O-HA, propolis) are biocompatible and GRAS-listed, allergic reactions to casein must be considered, particularly in pediatric patients (Kuitunen et al., 2012). Regarding phage therapy, strict biosafety level 1–2 protocols must be followed, and endotoxin testing, genome sequencing, and host range profiling are essential before clinical use (Rolain et al., 2016). Moreover, ethical and regulatory aspects of engineered or synthetic phages fall under the Cartagena Protocol on Biosafety and should be addressed in translational research.

9. Future Perspectives

Emerging strategies include bioresponsive materials that release antimicrobials in response to infection cues, integration of growth factors for enhanced healing, and the use of 3D bioprinting to create patient-specific constructs (Kim et al., 2023). Synthetic biology is poised to play a pivotal role in these developments, enabling precise control over material properties, therapeutic payloads, and biological interactions. As biosensors, gene circuits, and engineered phages become more sophisticated, they may be incorporated into dressings that adapt dynamically to the wound environment—ushering in a new era of intelligent wound care.

Conclusions

Burn treatment remains a complex therapeutic field due to the dual challenges of regeneration and infection. Integrating electrospun casein scaffolds with O-HA hydrogels, propolis, and phages presents a promising multifunctional strategy. This review highlights how synthetic biology can enhance each component—from engineered caseins and phages to programmable materials—contributing to personalized, antibiotic-sparing therapies. While challenges of standardization and biosafety remain, the convergence of materials science and synthetic biology offers unprecedented opportunities for next-generation burn care.

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Biosensing and Biodegradation: Engineering *Pseudomonas putida* for Smart Environmental Remediation and Value Recovery

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ABSTRACT

*Environmental pollution, especially from petroleum hydrocarbons, has severe consequences on marine ecosystems and human health. Synthetic biology offers new opportunities to engineer *Pseudomonas putida* KT2440 as a chassis for bioremediation, enhancing its ability to sense and degrade pollutants while resisting environmental stress. Beyond depollution, this approach also enables the production of valuable industrial intermediates, linking pollution control with circular bioeconomy solutions. Recent advances demonstrated that engineering *P. putida* with catabolic modules such as the NAH7 plasmid can significantly improve naphthalene degradation in polluted environments. Also, it is possible to enable the conversion of degradation intermediates like cis,cis-muconic acid into precursors for industrial products such as nylon. This year, the Sorbonne University iGEM team is engineering *P. putida* to not only sense hydrocarbons and degrade naphthalene more efficiently but also to use the sensing output to dynamically regulate metabolic processes for sustainable remediation. This review synthesizes recent advances in engineering *P. putida* for pollutant detection, degradation, and conversion into industrially relevant intermediates, while highlighting the challenges of scalability, biosafety, and regulatory acceptance.*

Index Terms – Environmental pollution, *Pseudomonas putida*, bioremediation, synthetic biology, naphthalene, circular bioeconomy, metabolic engineering, marine pollution, iGEM

I. INTRODUCTION

Environmental pollution has become one of the most important global challenges with a great impact on ecosystems

and human health. Most pollutants are heavy metals and organic compounds such as mineral oil hydrocarbons, polyaromatic hydrocarbons, benzene derivatives and halogenated hydrocarbons [1]. Oceans are particularly affected by pollution. Annually, about one million tonnes of petroleum hydrocarbons enter the marine environment, and nearly 90% of this input is attributable to human activities such as industrial discharges, routine shipping operations, and oil exploitation. This pollution has dramatic consequences for marine ecosystems: it destroys habitats, poisons wildlife and disrupts the food chain. Seabirds, fish and marine mammals are particularly vulnerable, often victims of poisoning or loss of their natural environment. Human populations living on the coast are also affected, through the degradation of fishing resources, beach pollution and health risks. Among these pollutants, aromatic hydrocarbons like naphthalene are particularly concerning due to their high thermodynamic stability, which makes them persistent in soils and aquatic systems [2].

Over the past decades, bioremediation has emerged as a promising alternative to physicochemical treatments. It relies on the natural capacity of microorganisms to degrade or transform environmental contaminants into less harmful products. Microbial degradation of aromatic compounds has been studied for more than 40 years. However, due to the continuous generation of novel pollutants due to human activities, it is difficult for microorganisms to acquire novel degradation pathways through natural evolution. Synthetic biology comes as an innovative approach to overcome the limitations of natural evolution [1].

Within this context, *Pseudomonas putida* has emerged as one of the most promising chassis for bioremediation due to its metabolic versatility, stress resistance and ease of genetic manipulation [3]. Beyond simply removing pollutants, researchers are now exploring how this organism can convert

breakdown products into compounds of value, such as biosurfactants or chemical precursors.

This year, Sorbonne University iGEM team decided to focus on engineering *Pseudomonas putida* to sense hydrocarbons, to break down naphthalene efficiently, and survive in a polluted environment in order to reduce pollution in a sustainable way. This review summarizes recent advances in engineering *Pseudomonas putida* as a smart bioremediation platform that combines pollutant sensing, efficient degradation, and value-added production. It also addresses key challenges such as scalability, biosafety, and regulatory constraints, emphasizing the potential of synthetic biology to link pollution control with circular bioeconomy solutions.

Pseudomonas putida as a synthetic biology chassis

Pseudomonas putida is a gram-negative bacterium found in diverse ecological habitats such as soil, water or plant rhizosphere. It is a non-pathogenic bacterium commonly used in research. The strain KT2440 is widely studied because of its versatile metabolisms, its genetic traceability and capacity to tolerate physicochemical stress [3]. It is also a non-pathogenic strain classified as BSL-1 (Biosafety Level 1). Recently, its potential in industrial biotechnology has been underlined with many applications whether in depollution or production of chemicals and biomaterials [4]. This strain can naturally degrade a wide range of aromatic and aliphatic hydrocarbons such as toluene, xylene, naphthalene, phenol or benzoate [3].

Pseudomonas putida KT2440 is considered a reference strain. Its genome was fully sequenced in 2002, and it was among the first Gram-negative soil bacteria officially certified as safe for laboratory use. Also, numerous genetic engineering tools (including CRISPR systems, transposons, and stable integration platforms) are already available for KT2440, making it easy to manipulate. These features make it a well-characterized and reliable chassis for metabolic engineering. Although KT2440 is the most studied chassis, other *Pseudomonas* strains, such as *Pseudomonas taeanensis* isolated from oil-contaminated seawater, could also be considered. They are naturally adapted to marine conditions but lack the genetic background for naphthalene degradation, making KT2440 still the best starting point [5].

Biosensing: Engineering pollutant detection circuits

Ocean pollution from hydrocarbons has been steadily increasing for years. In fact, the oceans are polluted by a large number of substances such as plastic, heavy metals, hydrocarbons, microorganisms, pesticides and others. All these pollutants are harmful and have a devastating effect on the marine environment, affecting both fauna and flora, as well as human health [6][10]. Environmental monitoring and quantification of these pollutants is an important issue for the environment, human health and the socio-economic development of our societies [9]. This is why research into biosensors has only increased and advanced in recent years.

Biosensors offer a fast and low-cost alternative to other ways of monitoring marine pollution, such as manual sampling, laboratory testing, and physical-chemical sensors, which are more expensive, slower, and often provide indirect indicators [7].

The Sorbonne University iGEM team decided to use this method of detecting hydrocarbons by integrating it into the bacterial strain, *Pseudomonas putida*. They drew on the work carried out in 2020 by the iGEM DeNovocastrian team. For their bioremediation project, they used a biosensor employing transcription factors involved in benzene degradation to transform these into a fluorescent signal by linking the transcription factor genes with fluorescent protein genes such as mCherry and GFP [8].

The Sorbonne University iGEM team wants to use a similar approach. To do this, with *P. putida* bacterial strain, They will use the transcription factors of the genes involved in the degradation pathway of naphthalene to salicylate and then salicylate to catechol, hybridising them with GFP to detect and quantify the naphthalene present and degraded.

Biosensors play an important role in this project because they enable the presence of hydrocarbons to be detected in situ and in real time, which can guide decontamination efforts and accelerate the response in the presence of hydrocarbons. In addition, the specificity of biosensors enables reliable identification.

Engineering Biodegradation Pathways

Degradation of aromatic hydrocarbons like naphthalene by microbial strains such as *P. putida* is well known. But, enhancing this degradation and giving the strain strategies to better resist and survive in various environments is important [11].

In KT2240, multiple strategies have been explored to expand its natural ability to degrade naphthalene. For example, salt tolerance can be improved by introducing heterologous genes such as *nhaA* (Na^+/H^+ antiporter) and *betA* (betaine-aldehyde dehydrogenase) from *E. coli*. This strategy allows the strain to remain functional up to 6% NaCl , which is above seawater salinity. This is interesting if we are to use it for marine depollution [12].

In order to enhance hydrocarbon degradation such as naphthalene, strategies focus on boosting catabolic genes such as *nahA* and *nahB* (naphthalene dioxygenase system), increasing biosurfactant production (rhamnolipids) to improve substrate availability or adding molecules like *alkB* or *cyp153* for alkane degradation. The plasmid NAH7, originally from *Pseudomonas putida* G7, remains one of the most efficient ways to improve naphthalene degradation in KT2440. Fernández et al. showed that transferring the NAH7 plasmid into *P. putida* KT2440 increased tolerance to naphthalene and

doubled rhizoremediation efficiency compared to the wild-type strain [13].

Value Recovery: Toward a circular bioeconomy

Value recovery links pollutant degradation by *Pseudomonas putida* to the production of valuable industrial compounds, supporting a circular bioeconomy. The degradation of aromatic hydrocarbons such as naphthalene generates compounds that can serve as industrial platform chemicals. In this way, we could at the same time diminish pollution but also create value while increasing the potential interest from industry and highlighting the broader impact of the project [14].

One of the compounds of interest is salicylate, an important precursor for salicylic acid widely used in pharmaceuticals such as aspirin. Catechol can also be produced. It is a central intermediate formed from the conversion of salicylate and many other aromatic compounds. Catechol is commonly used for the synthesis of polymers, dyes, and fine chemicals. By blocking catechol dioxygenases, engineered strains can slow down its conversion and favor catechol accumulation. Another interesting molecule is cis,cis-muconic acid which is derived from catechol cleavage. This molecule can then be converted into adipic acid, a key monomer for the production of nylon-6,6 which is one of the most widely used nylons in the textile industry. Kohlstedt et al. showed that engineered *P. putida* can produce cis,cis-muconic acid from lignin-derived catechol, reaching up to 74 g/L with the optimized MA-10 strain, which makes the process relevant for nylon production [15].

Altogether, this shows how we can combine depollution with circular bioeconomy, turning pollutants into resources instead of waste.

II. CONCLUSION

Engineering *Pseudomonas putida* as a smart environmental remediation platform seems a promising way of reducing pollution. Its natural capacity to degrade aromatic cycles can be enhanced while redirecting pathways toward the production of valuable compounds for the economy.

However, important challenges remain before such approaches can be applied outside the lab. Scalability is a key issue. Indeed, engineered strains must remain stable and functional in complex environments like seawater or soil. Biosafety is another concern. We must add robust containment systems, such as kill-switches to prevent unintended release and ecological risks (Ivanov et al., 2019). Regulatory aspects and public acceptance also represent major hurdles. Since *Pseudomonas* species are sometimes linked to opportunistic infections, communicating clearly about the safety of *P. putida* KT2440 and ensuring strict biosafety measures will be essential for societal trust. [16]

Altogether, while challenges remain, this illustrates the potential of synthetic biology to combine environmental protection and sustainable innovation.

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The Biotechnological synthesis of RNA circles

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ABSTRACT

In the last five years there has been a sudden burst of new research on circular RNAs. These products of alternative RNA splicing were soon to be associated with higher stability in cells, due to their lack of free 3' and 5' ends. Their various emerging roles in cell signaling and molecular biology, as well as their exhibited stability, has ignited the interest in their artificial production for biotechnological applications. In this review we focus on how their biogenesis led to multiple techniques of their artificial production and try to summarize their potential applications in synthetic biology.

Index Terms - Circular RNA, Circular RNA Biosynthesis, Ribozymes, Alternative splicing, RNA biotechnology

I. INTRODUCTION

Long after the initial discovery of circular RNAs as an alternative form of viral genomes, it was discovered that they are also produced by a “faulty” side mechanism of RNA splicing called ‘back splicing’ where the donor site of the splicing reaction is located downstream of the acceptor site (thus the reaction goes from front to back)(Li Yang et al, 2022). The resulting alternative transcript lacks free 3' and 5' ends as they are ligated together at the end of the splicing reaction. For this reason, circular RNA exhibit resistance to some of the main RNA degrading enzymes of living cells, the exonucleases and end up accumulating in cells, sometimes even exceeding the levels of their linear counterparts (Li Yang et al, 2022). While for a long time these circular transcripts were mostly ignored as a simple side product of normal RNA splicing, in the last years there has been a sudden bloom of new insights on their biological function. They were soon to be discovered as stable scaffolds of RNA binding proteins and non coding RNAs(Li Yang, 2022), indirect regulators of gene expression(Xiaoyu Sun et al,2024), molecular scaffolds for cell components and even protein coding platforms(Ling-Ling Chen et al, 2022). Following these discoveries, many

attempts started being made primarily in medicine, of synthetic circular RNAs acting as stable forms of vaccines(Zhaohui Gong et al., 2025) or medicines acting on the post transcriptional regulation of cellular processes of many diseases(Xiaofei Cao et al.2025). This interest in both basic and applied research regarding circular RNAs is what drove the effort behind developing a number of different methods for producing them biotechnologically, in order to study their function or produce them in large scale for commercial applications.

Their natural biogenesis can be summarized in two wide categories of different mechanisms: they are either produced during the function of the spliceosome in the process of the alternative splicing of mRNA, or by the activity of ligation enzymes found in unique RNA maturation pathways which recognise specific chemical modifications or RNA structures (Prisca Obi et al, 2021). In the spliceosome dependent pathways, cis and trans-acting elements work synergistically to bring the two ends of the spliced RNA together in order to facilitate the back splicing activity of the spliceosome, with many mechanistic details not yet known. In the spliceosome independent pathways, specific ligation enzymes found either in viral genomes or non-canonical splicing pathways of eukaryotes act on RNA substrates with specific modifications. As such, we decided to follow that categorisation while presenting the current RNA circularization methods, depending on whether their mechanism uses the cells' splicing machinery.

II. MATERIALS AND METHODS

The relevant publications of the literature were searched in PubMed using the key words: “circular RNA synthesis”, “artificial circRNA” and “circRNA biogenesis”. The articles were read thoroughly, and we referred to their references for the finding of additional literature. When the downloaded articles started repeatedly referencing each other we decided we had covered a large enough part of the existing literature.

III. RESULTS

Splicesome dependent methods

Inverted repeats and intron complementary sequences
 Observing the natural back splicing events in human cells revealed that back splicing was significantly promoted for sequences found between Alu repetitive elements. Inverted Alu repetitive sequences were found to promote the back splicing of intermediate RNA sequences, as after transcription, their hybridization forms a closed lariat structure that brings together the two ends of the splicing donor and acceptor site, implying that this lariat structure is important for the back splicing activity of the spliceosome.(Li Yang et al.,2022) Indeed, genetic constructs containing inserts between inverted Alu sequences proved to be sufficient in circularizing an artificial microRNA sponge in lung cancer cell lines(Ana R. Rama et al.,2022) Attempts in other organisms however revealed that these repetitive elements need to be organism specific: Inverted Alu repeats fail to promote back splicing in drosophila and vice versa.(Prisca Obi, 2021)

The complementarity of the repetitive sequences does raise the question of how much these sequences need to be repetitive. Interestingly, it was observed that more than 90% of the human sex determining gene SRY transcripts are circular but the gene is not located between inverted repeats. Instead, it was found that non repetitive intron sequences were complementary and led to a lariat structure containing the circularized exon. These intron complementary sequences (ICS) as they were called, were proved with genetic constructs to be sufficient in circularizing inserts, with the ICS being reduced to as much as 50 nucleotides.(Prisca Obi, 2021)

Even though these two methods faithfully copy the most abundant mechanism of circRNA biogenesis, with no apparent restrictions for the sequence to be circularized, the sideproducts can be quite unpredictable. Alternative backsplicing is known to happen with natural circular transcripts and ICS are even harder to predict in their alternative structures than inverted repeats. Yet, there have been successful constructs using these techniques. (Y. Wang et al. 2015)

RNA binding proteins

The lariat structure of the spliceosome can also be achieved with RNA binding proteins that bind each end of the target sequence and dimerize in order to bring the two ends together. Two RNA binding proteins in drosophila, Quaking and Musclebind, were found to be in a feedback loop with the circularization of their own transcripts, by binding in their respective binding sites on RNA. Homologs of these proteins are found in other organisms including humans. As such, a sequence can be circularize by being inserted in a construct

with binding sites of these proteins on each end. (Xiaofei Cao et al, 2025)

The binding sites of these proteins are not particularly long which raises the chances of off targets. For that reason, there have been multiple attempts to engineer these proteins in order to recognise longer binding sites mostly by constructing larger dimers of the binding complexes (Xiaofei Cao,et al 2025). That way, it becomes easier to regulate the activity of the circularization and the specificity of their substrates. It is a method that has been established as a dependable *in vivo* circularization method for eukaryotes and it is capable of being combined with inverted sequences as described above, as a means of synergistic circularization of RNA targets. Still, there is room for the optimization of constructs using these proteins, regarding not only their specificity but also the regulation of their expression

Spliceosome independent methods

The PIE constructs

The biochemistry of ribozymes governs the field of self-circularizing constructs, which this time, are able to produce circular RNA not only *in vivo* but also in *in vitro* reactions. Among ribozymes, there is a special family of RNA introns which are able to splice themselves out of the premature RNA and ligate the remaining RNA into a final product.(Annica Hedberg et al, 2013) Based on the enzymology of these self-splicing introns, they are categorized into Group I and Group II introns, and as such, their respective constructs. (Kyung Hyung Lee et al, 2024)

Normally, the self-splicing intron is located between two exons, recognises the boundaries of the exonic sequences and splices itself out, cutting itself on one end and ligating on the other. The logic behind the ‘permuted intron-exon’ constructs, lies on the rearrangement of the intron and exons, in order to promote the back splicing of an insert.(Xiaofei Cao, 2025) The intron is cut in two halves pointing outward, while the exonic boundaries point inward, towards the inserted sequence. The part of the intron that cuts itself becomes the donor site while the other half functions as the acceptor, and the splicing happens from front to back, leading to a circular insert. Because of their particular enzymology, Group I introns leave an exonic scar sequence in the circular RNA product, while Group II introns leave the product scarless. (Kyung Hyung Lee et al., 2024) The capacity of these constructs to circularize inserts does not exceed 5kb length.(Prisca Obi et al., 2021)

The PIE Group I introns have been used in this method for much longer than the GroupII the use of which came much more recently. The existence of the scar sequence has been the main problem that had to be addressed in the use of the PIE GroupI constructs(Lei Wang et al, 2024). There have

been many alterations in the architecture of these constructs in order to minimize the length of the remaining scar, as the structures of these scars are often immunogenic in host cells (Lei Wang et al 2024, Kyung Hyung Lee et al, 2023, Shaojun Qi et al 2024, Yifei Du et al 2024). For protein coding sequences, there is also a silent mutation method in order to incorporate the scar into the inserted open reading frame (Zhonghao Qiu et al 2022).

Interestingly, while libraries with Group I introns have been explored, only Leukine tRNA intron of Anabeena and the thymidine kinase gene from T4 bacteriophage have exhibited decent efficiency(Kuo-Chieh Liao, 2025). As for the recently used Group II introns there are still studies on how to optimally distribute the various domains of the enzyme to the two halves.(Xiayang Zhao et al 2024, Huanhuan Wei et al 2025,Xiang Gao et al 2025). Group I PIE is by far the most standard method for producing circular RNA in vitro and in vivo with most work done with this construct, while the Group II PIE is still emerging as an alternative. Notably, two iGEM projects, one by GIFU 2014 and one by SYSU China 2021 have used the Group I PIE construct as the best strategy for their approach.

The tRNA inspired systems

It was discovered that tRNA introns are spliced with a unique mechanism different from the transterification reaction of usual RNA splicing. The introns of tRNA genes are cut by a special protein complex that “measures” the amount of intron to be cut from the premature tRNA transcript, leaving it with non-canonical ends: The 5’ end becomes a hydroxyl group while the 3’ group a cyclic phosphate. A completely independent ligation reaction joins the two halves of the tRNA exons into a mature tRNA, while the intron is ligated in its two ends and becomes a circular RNA (a tricRNA, as it was called). The ligation reaction is performed by a single enzyme in bacteria and animals, the RtcB ligase, while in fungi and plants it is performed by a separate protein complex. (John J. Noto et al, 2017)

The first system that was inspired by this unique splicing pathway, was the tricY system (C.A. Schmidt et al 2016). The sequence to be circularized is inserted in the middle of a natural tRNA gene at the site of the tRNA intron and is under the transcriptional control of an RNAPol III promoter and terminator. Inside a host cell, the resulting tRNA is spliced and the insert takes the form of circular RNA. Depending on the organism, the tricY method can circularize inserts of up to 800 nt(Prisca Obi et al, 2021). There is still a scar from the tRNA intron, but it is not significantly immunogenic. Indeed, a proof-of-concept gene therapy for glioblastoma chose this system for their experiments. (Hadi Bayat et al, 2023)

A relatively new system that built on the tRNA splicing pathway is the tornado method. In this technique, the restriction of the inserted RNA is catalyzed by self-splicing ribozyme sequences and there is no need for a natural tRNA gene (Jacob L. Litke et al 2019). The insert is put between two ribozyme sequences of the twister and twister sister families which cleave themselves out of the produced transcript leaving the insert with a 5’ hydroxyl and a 3’ cyclic phosphate group. The resulting RNA is ready to be ligated by the RtcB ligase which recognizes the chemical groups and the scar sequences left by the ribozymes, designed to resemble tRNA introns. It is a method that can work both in vitro and in vivo. The tornado method has a notably high yield compared to other circularization methods, and has even been developed to ligate different methods and into a single circular RNA through trans ligation(Jacob L. Litke et al, 2021). That is the method that was chosen by this year’s iGEM Thessaly team for our project design. Even though a thorough characterization of the insert length capacity is not yet available, it has been proven to circularize efficiently open reading frames of about 1000 nt in length (Samie R. Jaffrey et al, 2024).

A note on in vitro ligation reactions

A more expensive but straightforward method that has been extensively used for the dependable circularization of pure RNA mixtures is the use of special RNA ligases often found in viral genomes (Xiaofei Cao et al 2025). Enzymes like the T4 RNA ligase, can be guided by a DNA oligonucleotide that hybridizes both the two ends of the RNA substrate and ligate the two ends of the RNA. Other RNA ligases can also connect the 5’ phosphate and 3’ hydroxyl ends given that they form a dsRNA helix (Eoghan O’Leary et al 2025). It should be noted however, that further enzymatic manipulation of the substrates is often needed, like removing the triphosphate group of the 5’ end. iGEM NHTU Taiwan 2023 however did use this method to produce circRNA for their colorectal cancer diagnosis kit.

Applications in synthetic biology

Medicine has been the primary field of research for circular RNA and thus received a big portion of their potential applications. Because of their prolonged life in cells, circular RNA has been a promising candidate for the improvement of mRNA vaccines (Zhaohui Gong et al., 2025). Transfection of cells with constructs expressing circular RNAs or the transcripts themselves has been an emerging strategy of providing gene therapies or RNA medicines, as their linear forms often face the challenge of fast degradation(Xiaofei Cao et al 2025).

Biomanufacturing has also been in contact with the recent advances in circular RNA technologies. Protein coding sequences can be circularized for the prolonged and stable expression of proteins either in in vitro translation systems or in specialized chassis organisms(Samie R. Jaffrey 2024).

Another promising application is the use of their ability to act as protein scaffolds, assembling as such entire consortia of enzymes fused with RNA binding proteins, in order to produce platforms for efficient catalysis of reactions needing multiple enzymes (Eogan O' Leary et al 2025).

Basic molecular biology methods at last also have a lot to take from the application of circular RNAs. The circular form of the Broccoli RNA aptamer exhibits much higher fluorescence and half-life than its linear form, enabling the study of cellular functions with a stable reporter on the level of RNA (Jacob L. Litke et al 2019). Also, circular guide RNAs for the CRISPR/Cas systems have been developed as more stable forms for the Cas-RNA complex. For the same reason, circular forms of dsRNA and siRNA are explored as more stable molecules for RNAi technologies (Eogan O' Leary et al 2025).

IV. DISCUSSION

Circular RNA technology has recently been centered in the limelight of current molecular biology research, and the plethora of biosynthetic methods proves the amount of attention that has been allocated to developing their applications. Despite their recent upgrade to the new "hot topic" of molecular biology research, by reading some of our references one can notice that their study and development has its roots far back into the last two decades. It is truly fascinating how originally niche topics as the biosynthesis of RNA circles can suddenly explode with potential once the scientific community realizes their roles in RNA biology. Hence, it will not be surprising if we see in the future even more methods for their synthesis, drawing on the knowledge gained by basic research in RNA splicing and the cellular pathways involving circular RNAs. By any means however, the different methods should not be viewed antagonistically as there is no single method that can cater to the needs of every biological application. For this reason, we summarize the information presented in (Table 1) as a beginner's guide in choosing the best method that suits their project's special needs.

Synthetic biology as we should not forget is not just a synonym for molecular biology, but it is built around the principles of engineering. We greatly anticipate the use of engineering principles to optimize the circularization methods, like it has been done for the PIE and Tornado methods. On the other hand, we wonder how the various components of the genetic constructs for circRNA production will be reimagined as modules of synthetic biology, like logical gates and circuits of information. Only experimentation and trial and error will uncover the full potential of these methods to extend the capabilities of synthetic biology in handling RNA. Finally, we eagerly wait for these methods to be extended to applications other than medicine, like environmental science and agriculture, either by novel applications or by facilitating existing technologies like CRISPR and RNAi.

Table 1. A small recap of the main points of this paper about the different circRNA production methods

| Circularization Method | Main Advantage | Main consideration |
|------------------------|--|--|
| Inverted Repeats | Natural biosynthetic method | The repeats should match the chassis organism +needs spliceosome |
| ICS | Also natural and added sequences can be smaller | Sideproducts can be unpredictable +needs spliceosome |
| RBP | Proteins can be regulated and they can work synergistically with other methods | Need careful engineering to avoid the off targets of the proteins +needs spliceosome |
| PIE Group I | Simple and classic method | It leaves highly immunogenic scar sequences in the product+ it works up until 5kb |
| PIE Group II | Like Group I without the scar | Very new, it is still under development |
| Tornado | Rapid and efficient | No RtcB in fungi and plants, different splicing pathway |

V. CONCLUSION

In this review we went over the current methods of circular RNA biosynthesis and tried to scratch the surface of their potential applications. The logic behind the different methods was explained and important considerations when choosing each method were outlined. We hope that by this review, we will inspire future iGEM teams and synthetic biologists to incorporate circRNA technology into their projects.

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Aligning Human Practices in the International Genetic Engineered Machine Competition with best practices from Innovation Science

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ABSTRACT

The International Genetic Engineered Machine (iGEM) competition is at the heart of the synthetic biology community, driving advances, providing new ideas and sparking debates. The main focus of teams in this competition is the engineering of an organism, peptide or other biological component to perform functions that it did not initially have, but which would be beneficial to certain stakeholders. Over time, iGEM has increasingly advocated for teams to be more involved in stakeholder interaction, through the Human Practices (HP) aspect of the project. This has led to teams enhancing their products to suit their stakeholders or niche better through practical improvements. We were interested in using theoretical frameworks from Innovation Science to identify and prioritise what aspects should be improved, as well as to identify other factors that influence adaptation of controversial techniques. In this article we describe multiple frameworks that can help iGEM teams establish courses of action for the implementation of their ideas, investigate whether or not iGEM teams have a history of incorporating such frameworks, and see how successfully they have been adopted by iGEM teams so far. By investigating these questions, we hope to highlight foundational frameworks for the HP aspect of iGEM teams and promote their incorporation into the iGEM competition.

Index Terms - iGEM, Human Practices, Innovation Science, Sustainable Transition Science

I. INTRODUCTION

The International Genetically Engineered Machine (iGEM) competition is a cornerstone of the synthetic biology community, bringing together interdisciplinary teams from around the world to tackle pressing global challenges through innovative biological engineering. In order to design solutions with valuable real-life applications, teams need a fundamental

understanding of the science involved, as well as a deep understanding of the current solutions available to stakeholders, their wants and needs and where such practices can be improved. iGEM encourages teams to develop such an understanding of stakeholder needs through the mandatory Human Practices (HP) aspect. Yet, while most of the lab work performed by teams is based on literature from molecular and synthetic biology, organic and inorganic chemistry, ecology, and many other fields, we wonder if the approach of iGEM teams to HP is given a similar fundamental theoretical basis.

One aspect of HP is reflecting on the crosstalk between your project and society. Working with synthetic biology causes many ethical, moral and regulatory tensions, as it brings about shifts in the mindset and methods used by industry, agriculture and government, as well as consumers (Kurtoğlu et al., 2024, Robinson & Nadal, 2025). Such tensions lead to barriers in implementation, such as a lack of technical expertise or infrastructure required to execute on the solution, risktolerance or funds available for investments in new methods of operation, or a welcoming attitude to solutions that rely on genetic modification (Adhikari et al., 2020; Ribeiro & Shapira, 2019). Therefore, successful implementation of a synthetic biological solution can only be achieved through detailed analysis of such factors, in order to mitigate potential drawbacks and further build on advantages compared to current and potential future alternatives. Sustainability transition models can be used to have a structured approach to such analyses (Zolfagharian et al., 2019). In this article, we use five of the most commonly used frameworks (Atkins et al., 2017; Zolfagharian et al., 2019).

Two of these frameworks employ a systems approach. Transition management (TM) aims to map and direct complex societal changes through participatory processes (Richter et al., 2014; Kumar, 2021). Technological innovation systems (TIS) structures actors, networks and institutions surrounding a certain development and details 8 specific criteria (detailed

in table 1) to analyse a system's ability to develop new innovations (Bergek, 2019). Two other frameworks focus on the current practices, also known as regime, and designing niches that fix issues in the regime. Multi-level perspective (MLP) focuses on defining and overcoming barriers in the regime that prevent it from attaining a desired goal (Geels, 2019). Similarly, strategic niche management (SNM) aims to improve the regime, but has a larger focus on continual

improvement of the niches (Raven, 2024). Lastly, theoretical domains of behaviour change (TDF) frames implementation through the lens of behavioural change, mapping barriers and facilitators on the way towards the desired change, and making efforts to design interventions to this behaviour that makes change possible (Atkins et al., 2017; Mather et al., 2022).

This article aims to investigate to what extent such theoretical frameworks are already incorporated into HP by analysing whether or not teams formulate their approach to HP based on a theoretical framework, whether their HP work

Table 1. Five theoretical frameworks and the criteria used to judge their usage in iGEM HP projects.

| Theoretical framework | Criterium 1 | Criterium 2 | Criterium 3 |
|---|--|---|--|
| Transition management (TM) | Identify transitions that would facilitate movement towards desired societal outcomes. | Map the complex socio-technical systems that are currently in place, and understand their interdependencies and potential lock-ins. | Steer the project to act in such a way that it directs changes within the system to the desired outcome. |
| Technological innovation systems (TIS) | Describe and analyse the structure of the TIS, in terms of its network of actors and institutions. Also identify its current state of development and formulate a development goal. | Analyse the performance of the TIS based on 8 functions: Knowledge development, Knowledge dissemination, Entrepreneurial experimentation, Resource mobilization, Development of social capital, Legitimation, Guidance of the search, and Market formation. | For the functions the TIS does not adequately perform, provide suggestions to the actors and institutions. |
| Multi-Level Perspective (MLP) | Define the system context by outlining the regime, relevant niches and the broader sociotechnical landscape. | Identify the factors, practices and rules that lock-in the regime in its current state. | Identify and evaluate niche innovations that could facilitate incremental changes in the regime. |
| Strategic niche management (SNM) | Define your niche and map the current regime in terms of laws, regulations, and institutional structures. | Engage a network of key actors within your niche, involve them in your design process. | Plan for developments in the niche to interact with and influence the greater regime and landscape over time. |
| Theoretical domains of behaviour change (TDF) | Identify a modifiable behaviour that is central to bringing about a certain desired change for the implementation of their product, with the understanding of positive or negative effects on other behaviours | Design an appropriate study using the TDF framework to systematically explore barriers and facilitators | Identify relevant theoretical domains for implementation, such as the Capacity, Opportunity and Motivation to execute the behavioural change of the actors involved. |

reflects the methodology of theoretical frameworks from transition science, and whether teams that do incorporate these theoretical frameworks are judged more favourably by iGEM.

II. MATERIALS AND METHODS

To get insight in the usage of theoretical frameworks by iGEM teams, a selection of teams was made to investigate. The integrated human practices special prize winners and two of the runner ups were selected for the high school, undergrad, as well as overgrad categories for the past 4 years, giving us 36 teams of which iGEM has judged their human practices approach favourably. To compare, we randomly selected 3 non-winners in each category per year. For both the group of winners and the non-winners, we compared the human practices approach as described on the team's wiki to the following characteristics of five theoretical frameworks (table 1). The total sum of met criteria was taken as that team's "score". These scores were then analysed using a student's T test.

III. RESULTS

Though none of the iGEM teams investigated in this paper explicitly named a theoretical framework on their HP wiki page at all, we were able to recognise some of the criteria described in table 1 in the approach of the teams. The total scores of nonwinners and winners in the high school, undergrad and overgrad categories are detailed in figure 1.

Despite only the undergrad category showing a significant difference in score between winners and non-winners ($n=12$, $p=0.0029$), all categories appear to be following the same trend. axis.

III. CONCLUSION & DISCUSSION

With this paper we hoped to investigate the importance of theoretical frameworks to the HP approach of iGEM teams. We argue for the importance of HP for the translation of solutions provided by HP to real world application, and for the benefits of using established theoretical frameworks from Innovation Science. In addition, we show that past iGEM teams have not utilised theoretical frameworks for HP, but that there is a trend that teams whose work more closely aligns with frameworks from literature tend to perform better.

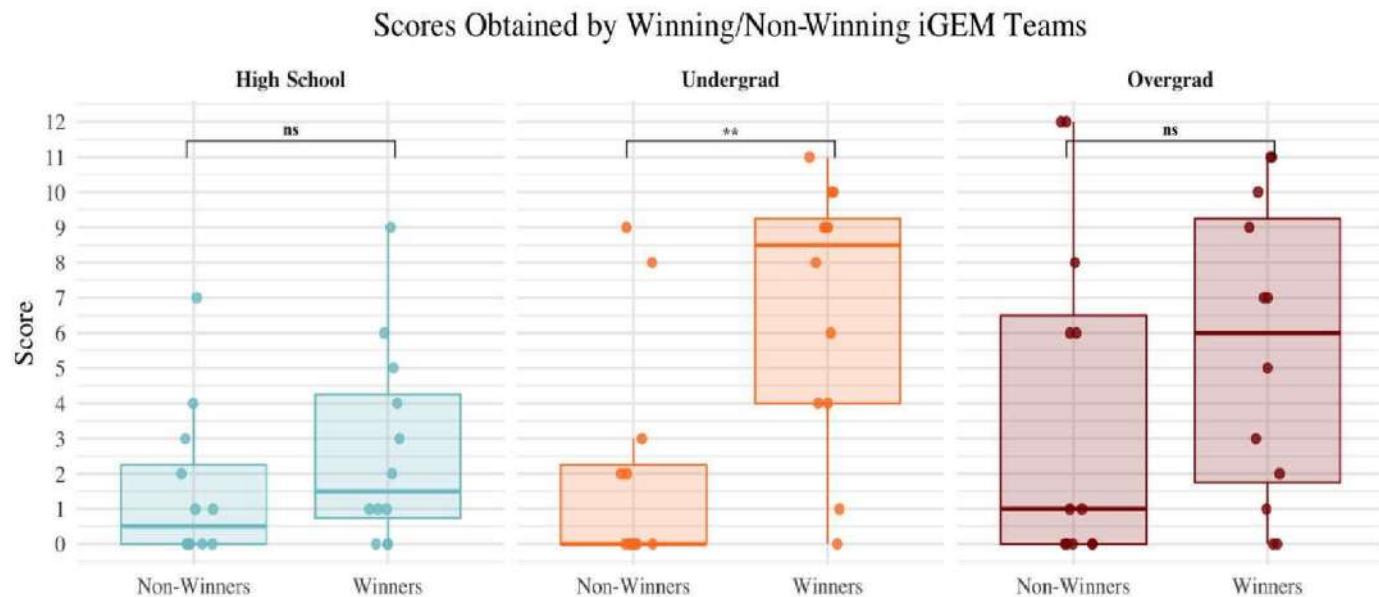


Figure 1. increased usage of concepts from Innovation Science are correlated with success in the iGEM competition for integrated Human Practices. Teams that won or were nominated for the best integrated Human Practices awards were compared to teams that were not nominated for that award. Three teams were selected per year, per competition bracket for both winners and non-winners, with the years 2020-2024 being included. Their wikis were scored on whether they included information in agreement with certain theories of Sustainable Transition Science by criteria from Table 1, and their score is plotted on the y- axis.

However, the interpretation of those results is not as straightforward, as it is clear that teams that provide more information about multiple facets relevant to HP should perform better in iGEM as well as score higher on our test. This could mean that the value of using a framework is as great as the sum of its parts. But even in that case we recommend iGEM teams to include such frameworks as it will help them identify different relevant aspects for their project.

Additionally, we think our work highlights that adapting such frameworks could help future teams structure information. This is because virtually no team provided the information necessary to meet scoring criteria succinctly in one part of their wiki. The unstructured presentation implies that it is hard for even high scoring teams to provide a coherent description and analysis of the concepts relevant to HP. A second implication is that there is a high degree of subjectivity involved in accrediting teams. This subjectivity is further intensified through the use of two people judging half of the teams each. To minimise any potential influence on the final data, we had each person judge both winners and non-winners, of alternating years.

Furthermore, not a single team met criteria 2 and 3 of the TIS, nor criterium 3 of the TDF. This makes sense, as those are criteria that are specific to the theory and would thus not likely be considered if not using those frameworks. There are two implications of this: first, it limits the maximum score of teams to 12, as can be seen in the data; secondly, iGEM teams are not likely to consider all dimensions of the current ability of a network of actors to drive innovation to a desired goal, design their project specifically to fill gaps in that ability, nor look at modifiable behavior and its impact on implementation routes. In our view, this means that TIS could be used as an alternative mindset to identify useful approaches to reach a goal through synthetic biology. In respect to TDF, we think it can be useful but does not fit nicely with the main objective of iGEM, as the focus of TDF is on modifying behavior whereas iGEM has a primary focus on innovation through biologically inspired products.

On a different note, teams often organise their HP based solely on chronological order of activities, which reduces coherency. We recommend that teams organise their HP page to logically convey their analysis of barriers, drivers and considerations of stakeholders' needs, and provide information on feedback, reflection and implementation separately for each aspect of their project in a chronological order on their integrated HP page.

Overall, we would recommend future iGEM teams to structure their HP around either multi-level perspective or strategic niche management frameworks. We recommend these two because they overlap most with the goals of iGEM teams in our view. Much of the work that is done in an iGEM project has the purpose to optimise the functionality of their

their solution, termed niche, and make sure that their solution can be easily incorporated into current standard practices, termed regime, without posing excessive risks at a socio-technological scale.

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Sticky Situation: A Review of Spider-Silk Proteins as Novel Materials and Parts in iGEM

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ABSTRACT

*In recent years, spider-silk proteins, or spidroins, have emerged as promising biomaterials in biotechnological research and iGEM due to their notable material and biomechanical properties. These properties vary amongst spidroins and are dependent on the sequence-structure relationship. Advances in synthetic biology have enabled the recombinant production of spidroins in organisms such as *E. coli*, providing an ethical and scalable alternative to spider farming. However, the widespread use of spidroins remains limited due to the fundamental challenges associated with their expression including their large size, repetitive sequence identity, codon usage biases, and challenging replication of native-self-assembly processes. This review explores and aims to summarise recent literature concerning the structure and properties of spidroins, as well as explore the use of spidroins within iGEM by previous teams, specifically highlighting the challenges and limitations associated with their recombinant expression. Highlighting and addressing these technical challenges associated with the recombinant production of spidroins will be essential in exploring the full potential of spidroins and will greatly facilitate future research efforts, and in turn, will broaden the usage of spidroins and allow for the introduction of novel functions and applications, within and outside of iGEM.*

Index Terms - Spider-silk Proteins, Spidroins, Dragline Silk, MaSp, Recombinant Proteins, iGEM

I. INTRODUCTION

In the face of a changing climate and increasing need for environmental protection, our modern systems of resource harvesting, materials production and waste management require reconsideration and inevitably, change. In particular, the production of textiles, construction materials, medical applications and food infrastructure, among many other sectors, greatly contribute to global greenhouse gas emissions and ecosystem pollution. On the stage of sustainable materials,

spider-silk proteins have risen in recent years as an ideal candidate for many applications, due to their impressive inherent biomechanical properties (Chen et al., 2024). Synthetic biology can provide a feasible and ethical avenue for their production.

As is often the case, investigating the interests and projects of young aspiring professionals, like the student researchers participating in iGEM, can help to predict and understand the future of technologies, industries and ideologies. Therefore, this review aims to catalogue and analyse the ways in which iGEM teams throughout the history of this competition have utilised and contributed to our understanding of spider-silk proteins as a novel tool.

II. METHODS

This literature review was conducted using data gathered from papers stored and available in two databases: 1) Google Scholar and 2) PubMed; excluding any papers published before 2000. The utilised search queries contained the following index terms: spider silk, spider-silk proteins, spidroins, major ampullate, MaSp, dragline silk, and recombinant spider-silk proteins. Additionally, the available database of iGEM wikis was used as the primary source for the second section of the paper addressing the use of spider-silk proteins in iGEM by previous teams.

III. SPIDER-SILK PROTEINS

Spiders are capable of producing up to seven different types of silks that make up the different functional sections of their webs, and are responsible for associated web properties, such as construction, capture of prey, and protection of eggs. (Chen et al., 2024) Among the different types of silks, dragline silk, produced in the major ampullate gland, is the most studied in current literature, due to its unparalleled combination of strength and elasticity (Chen et al., 2024). It is primarily composed of two different proteins, namely the major ampullate spidroin 1 (MaSp1) and 2 (MaSp2) (Xing et al., 2014). These proteins are characterised by non-repetitive

termini and an extensive repetitive ‘core’ region, which makes up more than 90 % of the sequence. The termini are thought to be less structured and are described as amorphous, while the repetitive domains assemble in a crystalline structure through the formation of β -sheets (Fig.1). Interestingly, the sequences responsible for the non-repetitive termini are evolutionarily conserved even across different species, whereas the repetitive region differs between different spidroin types (Liu & Zhang, 2014).

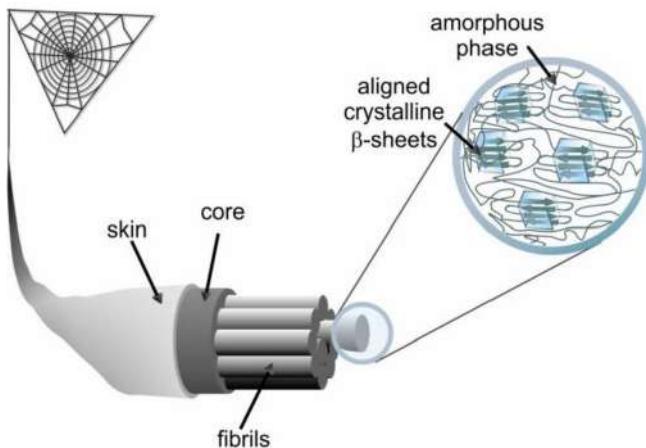


Figure 1. Structure of a Dragline Spider Silk Fibre. (Adapted from Humenik, M., Smith, A. M., & Scheibel, T. (2011)). Recombinant Spider Silks—Biopolymers with Potential for Future Applications. *Polymers*, 3(1), 640–661.

Phylogenetic analyses have identified five types of major ampullate spidroins but only the first two types, MaSp1 and MaSp2, have been studied extensively, making them the focus of this literature review (Sumalata et al., 2024).

In their core region, both types contain tandem repeats of poly-Ala blocks interspersed with Gly-rich repeats (Liu & Zhang, 2014). While Gly-Gly-X motifs are primarily found in MaSp1, MaSp2 additionally contains repeats rich in Pro and Gln, such as Gly-Pro-Gly and Gln-Gln motifs (Sumalata et al., 2024). The poly-Ala domains have been found to primarily assemble in a β -sheet conformation. In the spider’s gland, spidroins adopt a homodimeric structure linked through the C-terminal domain, in turn leading to the formation of micelles, which resemble droplets. As seen in Figure 2, they travel from the sac, through the duct, and a pH gradient from 7 to 5 induces a dimerisation of the C-terminal domains. The combination of shear force in the narrowing duct and the increasingly acidic environment leads to the destabilisation of the C-terminal domains and subsequent formation of the antiparallel β -sheets nanocrystals (Sumalata et al., 2024). These β -sheets are integrated in a semi-amorphous matrix composed of 3_{10} -helices and β -turns, which contribute to the mechanical and thermal properties of the silk (Zhang et al., 2015). The β -sheet structures are primarily responsible for the tensile strength and stiffness of dragline silk, whilst the Gly-rich amorphous regions are thought to confer high mobility and elasticity (Lu et al., 2023).

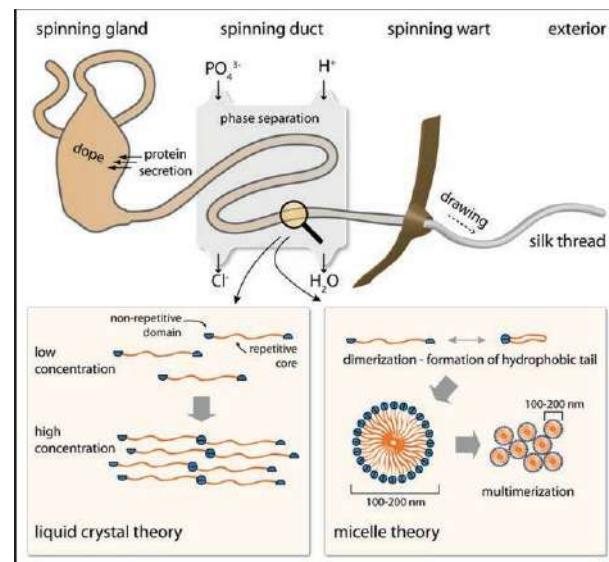


Figure 2. Self-assembly process of spider silk proteins. (Adapted from Römer, L., & Scheibel, T. (2008)). The elaborate structure of spider silk: structure and function of a natural high performance fiber. *Prion*, 2(4), 154–161.

Understanding the general structure of the constituent proteins allows insight into how their properties can be modulated.

I. STRUCTURAL PROPERTIES

Tensile Strength

Spider-silk proteins have impressive mechanical properties. According to Malay et al. (2024), the β -sheet structures within spider silk fibres confer high tensile strength. This is a result of the inter-strand hydrogen bonds present, which provide resistance to motion between neighbouring β -strands (Zhang et al., 2014). Moreover, Elices et al. (2006) shows that the sheer force used in the fiber induction step of silk production has an effect on the tensile properties of silk. Not only this, but the strength of spider silk fibers also depends on the orientation and the size of the nanocrystals formed by β -sheets, as shown by Du et al. (2006).

Elasticity

Spider silk sparks an interest in many fields as it combines an elastic character and a high toughness hinting towards an extraordinary durability (Römer & Scheibel, 2008). The elastic character results from structural properties, namely the amorphous regions (Keten & Buehler, 2010). Moreover, differences in physical properties, such as elasticity, between MaSp1 and MaSp2 have been suspected to be influenced by

the amino acid conformation (Savage & Gosline, 2008). For example, both the Pro and Gly content of sequence can determine the elastic and amyloid tendencies of material. With a suggested Pro/Gly threshold Rauscher et al. (2006) were able to explain the elasticity found in MaSp2 and toughness of MaSp1. Once this threshold is reached, the probability of elastic fibres increases substantially.

Young's modulus, which describes the resistance to stress before permanent deformation, has been observed to range up to 12 GPa, comparable to that of the human cortical bone (Beisekenov et al., 2025). This demonstrates that spider silk is strong, yet elastic.

Thermal Conductivity

Dragline silk stands out for its impressive mechanical properties, such as a high Young's modulus, reflecting spider silk's elastic character. According to a widely used kinetic model (Huang et al., 2012), which shows a proportional relationship between the thermal conductivity and the square root of Young's modulus, this structural rigidity may contribute to the remarkably high thermal conductivity of dragline silk. Huang et al. (2012) set out to explore the thermal properties of dragline silk produced by *Nephila clavipes* and measured thermal conductivity values up to 415.9 W/m K under strain, exceeding that of copper. Without strain, conductivity was measured at around 340 W/m K, which is still considered to be an exceptional value for a protein-based polymer (Xue et al., 2019). It is important to consider that this measurement is subject to debate due to a lack of replicability (Xue et al., 2019).

The antiparallel β -sheets nanocrystals, formed through hydrogen bonding between the poly-Ala residues, are thought to be responsible for the incredibly high thermal conductivity observed by Huang et al. (2012). These crystalline structures are hypothesised to provide continuous, ordered paths for phonon transport. Moreover, these hydrogen bonds allow for β -sheets to transport the phonons synchronously (Sun et al., 2012).

The aligned β -sheet architecture facilitates efficient propagation of vibrational energy, which accounts for the unusual thermal conductivity exhibited by dragline spider silk (Zhang et al., 2015).

II. APPLICATIONS

Recombinant spider-silk proteins are polymerised into silk fibres by emulating the *in vivo* process of silk production in spiders (Altman et al., 2002). These fibres can be woven to form textiles or solubilised in different solvents. After solubilisation, they can be processed into a variety of materials, including hydrogels, films, sponges and nanoscale fibres (Altman et al., 2002), all of which display varying properties, and are suitable for different applications in fields ranging from biomedical technology, material science, bioremediation to aerospace engineering (Altman et al., 2002).

III. CHALLENGES

While spider-silk proteins offer a high potential for diverse applications due to their polyvalent characteristics, several challenges and limitations are associated with their use.

Firstly, natural production through spider farming is unfeasible, as they are known to display cannibalistic behaviour, making them unsuitable for livestock breeding (Ramezaniaghdam et al., 2022). In consequence, recombinant production has emerged as the preferred strategy for spider silk protein production. However, major ampullate spidroins have an estimated molecular mass of around 250 kDa (Ramezaniaghdam et al., 2022), which is relatively high when compared to the typical molecular weight of proteins between 30-60 kDa (Milo & Phillips, 2015). In addition, their highly repetitive core sequence makes them difficult to express in full length, especially in prokaryotic hosts. These repeats render the genetic sequence unstable (Bhattacharyya et al., 2021). As previously mentioned, *E. coli* is the main expression system in current literature. Nonetheless, as spidroins are rich in Gly and Ala residues, this results in a high demand for glycyl-tRNA and alanyl-tRNA, which leads to codon usage bias. A lack of tRNA or codon choice may be the cause of premature ribosome stalling (Ramezaniaghdam et al., 2022; Maraldo et al., 2025). Collectively, this results in low expression levels, making *E. coli* a challenging, although common, host.

Furthermore, another critical challenge lies in mimicking the native self-assembly process (Ramezaniaghdam et al., 2022). Replicating the conformational switch and alignment of β -sheet structures, which *in vivo* occurs in a highly controlled environment, represents a complex task to reproduce *in vitro*. Due to the

hardship of imitating these physiological conditions, recombinant systems often lead to the production of non-functional or amorphous aggregates rather than highly ordered silk-like materials.

IV. SPIDER-SILK PROTEINS IN iGEM

While certainly not complete, this selection of iGEM projects which utilised spider-silk proteins still acts as a proxy and demonstrates the ways in which these proteins have been used, and associated challenges encountered.

Fashion and Cosmetics

The aim of the 2019 project *SPIDERMAN*, from GreatBay SZ, was the recombinant production of spider-silk proteins followed by fusing them with chromoproteins and natural dyes, in turn creating a sustainable coloured textile material (*SPIDERMAN*, 2019). The *Neoleathic Age* project from LINKS-China (2021) had similar sustainability goals in mind. The aim of the project was to create a leather-like biomaterial from bacterial cellulose and spider-silk proteins fused with cellulose-binding modules (*Neoleathic Age*, 2021). Both teams expressed the spider-silk proteins in *E. coli* but encountered similar issues: expression difficulties, low yields, and low solubility. They took similar approaches in mitigating the encountered issues, such as codon optimisation.

Medicine

BactoAid (SDU-Denmark 2016) (*Bactoaid*, 2016) was a project that worked on the development of antimicrobial wound dressings made of spider-silk proteins. The spider-silk proteins were used as scaffold material and infused with bacteriocins. A similar approach was taken by the 2022 RU-Bochum team. By coupling spider-silk threads with collagen, they aimed to create a scaffold material for biocompatible patches which would promote wound healing (*BioSpatch*, 2022). The team from RU Groningen (2013) (RU Groningen iGEM, 2013) addressed the issue of implant rejection and infection by creating a coating for medical implants which would promote biocompatibility and reduce immune rejection. They produced spider-silk proteins functionalized with cell-adhesion peptide sequences with the goal of mimicking the natural extracellular matrix environment. All teams encountered difficulties with protein solubility and low yields. These problems were addressed by changing the chassis strains, codon optimization, and reducing the size of the repetitive core region of the proteins to name a few.

BionExe, the 2022 project from Exeter, used spider-silk proteins to develop a silk-graphene composite scaffold material which could be used in numerous applications such as nerve regeneration and tendon repair (*BionExe*, 2022). In addition, they address issues associated with spider-silk production in all aspects of their design with the goal of optimising expression. From their choice of chassis, that being Rosetta *E. coli* as it overexpresses certain tRNAs necessary for spidroins synthesis, to maximalising the yield and modulating the solubility by encoding CycA and TrxA and a thioredoxin protein followed by a thrombin cleavage site respectively.

Bioremediation

The 2024 CNPEM-Brazil *B.A.R.B.I.E.* project tackled the issue of microplastic contamination of drinking water. The team designed a bio-based filtration system that captures microplastics by using specialized plastic-binding proteins (PBPs) embedded in a hydrogel-like scaffold made of spider-silk proteins. The spider-silk proteins were covalently linked to the PBPs via the *SpyTag-SpyCatcher* system (*B.A.R.B.I.E.*, 2024).

Biomanufacturing

Pichitecture, the 2022 project from BOKU-Vienna, created a sustainable building material with properties similar to those of cement. The material was a composite of gelatine, CaCO₃, polyhydroxybutyrate, construction waste and spider silk proteins. The proteins, expressed in *Pichia pastoris*, function as a binding scaffold, providing structural stability as well as flexibility (*Pichitecture*, 2022). As opposed to utilising waste in their spider silk product, the 2019 University of Edinburgh project *Remdyne*, engineered *E. coli* to degrade polluting textile dyes and used the resulting aromatic amines as a carbon source to produce spider silk proteins, specifically MaSp1 (*Remdyne*, 2019).

The Overgraduate Grand Prize Winner in the 2022 competition, *Netlantis* (UCopenhagen), created novel biodegradable alternatives to plastic fishing nets. These nets were constructed using the SnoopTag-SnoopCatcher system which allowed for the binding of mussel-foot proteins to spider silk resulting in a strong, yet flexible, composite fiber material (*Netlantis*, 2022).

VI. CONCLUSION

Spider-silk proteins have emerged as exciting and promising biomaterials due to their notable properties, such as their elasticity, tensile strength, and thermal conductivity. These result from their highly organised core structure of crystalline β-sheet structure, as well as their amino acid sequence, rich in Ala and Gly residues. These properties, in addition to advances in recombinant expression techniques of these proteins, have made them attractive to researchers and iGEM teams alike.

In the scope of iGEM, spider-silk proteins have been used in projects ranging from fashion and cosmetics to bioremediation, medicine and biomanufacturing. Despite the variety of areas and projects where they have been used, research surrounding spider-silk proteins is still in its infancy. Persistent challenges are associated with the replication of the native self-assembly process and recombinant expression of such proteins due to their large size, repetitive sequence, and codon usage biases, amongst others. Addressing and overcoming these challenges by improving existing processes and formulating novel approaches in gene design, host selection, and expression strategies will be critical in unlocking the full potential of spider-silk proteins, paving the way for their broader application in iGEM and synthetic biology.

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A Single-Stranded DNA Biosensor in the Form of a Capillary Tube

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ABSTRACT

The standard methods for detection of viruses in samples are expensive, time-consuming, and require access to specialized equipment. To address this problem, we aimed to design a point-of-care oligonucleotide sensor in the form of a capillary tube. This article discusses a proof-of-concept for this system as well as a modular phagemid system for us, and future iGEM teams, to safely validate viral-detection systems without the need to use pathogenic viruses.

Index Terms - Oligonucleotide biosensor, Viral diagnostics, DNA Modifications, Glass functionalization, Phagemids, Synthetic biology

I. INTRODUCTION

Avian influenza is a single-stranded RNA virus that has recently affected a range of animals (wild birds, cattle, poultry, humans, etc.) and gained the attention of the medical community because of its transmission and pathogenicity, especially Highly Pathogenic Avian Influenza (HPAI) (Charostad et al., 2023). This virus has caused the United States \$1.4 billion since 2022, and continues to follow the upward trend (Drake, 2025). Due to the extent to which this outbreak caused economic losses and its slow predicted transmission into other species, we designed this sensor for the poultry and cattle industry, and veterinary diagnostics markets. Current viral testing methods include PCR, antigen tests, RT-LAMP and more (Ginocchio & McAdam, 2011). These complex methods are expensive; time-consuming; require specialised equipment, reagents, and personnel (Ginocchio & McAdam, 2011). The Rumino biosensor serves as a faster and field-deployable alternative to such technologies.

The biosensor proposed here aims to achieve early detection of HPAI in the form of a capillary tube. The capillary

tube consists of an inner surface functionalized with DNA in which one of the strands (substrate) is covalently immobilized to the surface with one end, and has an overhang (toehold) in the other end. The other strand (incumbent) is hybridized to the substrate. The target sequence is detected through a TMSDR in which the target strand is fully complementary to the substrate strand, including the toehold overhang (Simmel, 2023). Given that the incumbent lacks the toehold region, it's more energetically favorable for the target strand to bind to the substrate, which causes the displacement and release of the incumbent strand (Simmel, 2023). To verify the TMSDRs, oligos modified with fluorophores were used to visualize the presence/absence of specific strands.

In order to prepare a TMSDR in a capillary tube, the substrate strand needs to be immobilized on glass. Modifying the surface with a chemical group that can be used to covalently bind the DNA to the surface is integral. The chemical 3-Aminopropyltriethoxysilane (APTES) is often used to functionalize these surfaces with primary amines for crosslinking other reagents to glass (Sypabekova et al., 2022). To optimize the reaction, the choice of solvent and temperature were tested as they are of special relevance to the reaction kinetics and monolayer quality (Sypabekova et al., 2022). Success of the reaction can be assessed by ninhydrin assays, water contact angle (WCA) measurements, and fluorescence assays. Ninhydrin reacts with the amine groups of APTES to form a compound known as Ruhemann's purple (RP), which absorbs light at 570 nm (Karade et al., 2021). WCAs serve as an indicator of the wettability of the glass surface, which is significantly altered due to APTES functionalization (Sypabekova et al., 2022). Fluorescein isothiocyanate (FITC) is a fluorophore that can be used to label primary amine, meaning that fluorescence would confirm the presence of these groups. The aminated surface can then be reacted with glutaraldehyde to create a surface that's reactive to primary amines (Sun et. al., 2025).

Another crucial step is to prepare single-stranded DNA (ssDNA) for immobilization onto the glass surface of a capillary tube. A water-soluble carbodiimide that activates the 5' end of DNA called 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) which can be used to modify the ends with primary amine groups (Wickramathilaka and Tao, 2019). A target sequence is needed to test the reaction and validate our developing platform, but the use of live viral samples is not permitted and poses a hazard to researchers. These challenges were addressed by designing a plasmid capable. The purpose of the phagemid is to produce laboratory-generated viruses carrying the target sequence, acting as a probe-virus to test and validate our platform safely, and at a low-cost production of the single-stranded DNA oligonucleotides.

II. METHODS AND MATERIALS

APTES Reaction Temperature Optimization

Glass slides were washed with aqua regia and then reacted with APTES (10 mM) in ethanol under three different conditions: Room temperature, 50°C and 70°C for 3-4 minutes. The reactions were then taken off heat and incubated for 24 hours while stirring. After rinsing with water, two 1cm x 2.5cm pieces were cut from each glass slide and washed with acetic acid (5%) as suggested by Sypabekova et al. (2022). Success was assessed using the ninhydrin test and WCA measurements.

APTES Reaction Solvent Choice Optimization

Glass slides were washed with aqua regia and air dried. The slides were then reacted with APTES (5 mM) and trace amounts of water in one of three solvents: Anhydrous ethanol, toluene, or a 9:1 (v:v) toluene:ethanol solution. Slides were reacted overnight submerged in the three solutions listed in separate containers at room temperature. Then rinsed with acetic acid (5%) and baked at 110°C for 30 minutes as suggested by Sypabekova et al (2022). Success was assessed using the ninhydrin test and WCA measurements.

Ninhydrin Validation

A volume of 5 mL of a ninhydrin solution (2% w/v) in ethanol was added to a beaker with a glass slide of interest, and then heated at 110°C for 10 minutes. Once cooled to room temperature, absorbance of the mixture at 570nm was measured using an acrylic cuvette and a UV-Vis spectrophotometer at 570nm. Values were assessed using a standard curve previously generated.

Water Contact Angle Testing

Volumes of 24 uL and 44 uL were pipetted onto glass slides. A picture of the droplets was taken using a phone camera. This image was processed by generating a circle whose circumference overlapped the outline of the water droplet, and drawing a radius to a point on the circle where the droplet meets the glass slide. A tangent was drawn to the radius, and the angle it made with the horizontal plane of the slide was analysed using an online protractor tool (Ginifab).

Capillary Tube Functionalization

Amine and glutaraldehyde functionalization procedures were adapted from Sun et al. (2025). Capillary tubes were rinsed with methanol, injected with APTES (10% (v/v)) in anhydrous ethanol, submerged in a water bath at 70°C, and left to react for 30 minutes. Amine functionalization was verified by reacting the tubes with 10⁻⁴ M fluorescein isothiocyanate (FITC) in dimethylformamide (DMF) for 30 minutes. Amine-functionalized tubes were rinsed with methanol, and then reacted with aqueous glutaraldehyde (50%) under the same conditions. Glutaraldehyde-reacted tubes were then rinsed with deionized water.

DNA Modifications

The one pot method developed by Wickramathilaka and Tao (2019) was performed for DNA modifications. DNA (13.5nM) in phosphate-buffered saline (PBS) buffer was mixed with imidazole (0.1 M), EDC (15 μM), and ethylenediamine (EDA, 100 nM) for 4 hours at room temperature to obtain aminated dsDNA.

For verification of the reaction, the modified DNA was then reacted with FITC and purified using the NEB Monarch® PCR & DNA Cleanup Kit according to the manufacturer's instructions. Fluorescence for the elution was then measured with a fluorometer.

Glutaraldehyde-functionalized capillary tubes were then reacted with the purified amine-functionalized dsDNA for 1 hour in a 70°C water bath. The reacted capillaries were washed with water and fluorescence was observed with a 365 nm UV-lamp in a dark-room.

Strand-Displacements

DNA oligos (S1) were purchased from IDT with the following modifications: 3'-NH₂ and a 5'-Cy3 for Substrate; 3'-Cy5 for Incubent; and 3'-6FAM for the Target strand. 1x10⁻⁸ M of the Substrate in nuclease-free water (NFW) was reacted with the glutaraldehyde-functionalized capillary tubes

for 30 minutes in a 70°C water bath. After rinsing with water, 1x10⁻⁸M of the Incubent in annealing buffer (100 mM Potassium Acetate, 30 mM HEPES) was added to the capillaries, heated at 95°C for 2 minutes, cooled down in room temperature, and rinsed with water. 1x10⁻⁷ M of the target strand in a Mg²⁺ and Tris (10 mM) buffer was prepared as according to Zhu et al. (2021). The target solution was then added to the capillary, heated in a water bath at 60°C for 5 minutes, and rinsed thoroughly with water. The capillaries were then visualized using an Alba Confocal Microscope.

Phagemid Design

The part design consists of two main components: The MS2 bacteriophage genome (NC_001417), and an insert site that is integrated into the phage genome. The part can be added to a vector with a *Lac Operon* so that the MS2 genome can be under the control of an inducible promoter. This way, when isopropyl β-D-thiogalactopyranoside (IPTG) is added to cells transformed with this sequence, the MS2 bacteriophage will be produced and released into the culture.

The insert site contains a constitutively-expressed *LacZα* gene flanked by *BsaI* sites. Through Type IIS assembly, the reporter can be swapped by another sequence, a process which can be easily confirmed with blue-white screening. This design allows for the insertion of any sequence desired into the progeny phages produced, serving as a controlled method of verification for viral sensors.

III. RESULTS

APTES Solvent Optimization

The ninhydrin test indicated that the 70°C group had the highest concentration of RP, while the room temperature group had a ten fold decrease in comparison (Table 1). Similarly, the 70 °C group had the lowest WCA, the room temperature group had the highest (Table 1). All three experimental groups had a smaller WCA than the negative control (Table 1). A one-way ANOVA test was performed for the WCA measurements, which shows a significant difference between the temperature groups ($p < 0.01$).

Table 1. Average values for the validation tests for ninhydrin tests in molar concentrations, and water contact angle measurements (in degrees) for the temperature groups.

| Temperature group | Ninhydrin test (M) | WCA (44uL) |
|-------------------|-------------------------|------------|
| Negative control | - | 25.4° |
| Room temp. (20°C) | 2.00*10 ⁻⁵ M | 55.4° |
| 50 °C | 5.14*10 ⁻⁴ M | 39.3° |
| 70 °C | 6.19*10 ⁻⁴ M | 36.9° |

APTES Solvent Optimization

The ninhydrin test shows that the ethanol group had the highest concentration of RP. Meanwhile, the toluene, and the mixed group had RP concentrations at a ten-fold decrease compared to the ethanol group, toluene having the lowest concentration. WCA measurements indicate that all three experimental groups had a noticeable increase in the wettability of the slide, compared to its negative control. The toluene and ethanol group had the greatest increase at 70.0°, followed by the toluene at 67.0°, and then finally the ethanol group with 63.2°. These results have been summarized in Table 2. A one-way ANOVA test was performed for the WCA measurements ($n = 3, p < 0.01$).

Table 2. Average values for the ninhydrin validation tests in molar concentrations and WCA measurements (in degrees) for the different solvent groups ($n = 3$ for each group).

| Solvent | Ninhydrin test (M) | WCA (44uL) |
|---------------------|-----------------------|------------|
| Negative control | - | 25.4° |
| Ethanol | 1.03x10 ⁻³ | 63.2° |
| Toluene | 2.38x10 ⁻⁴ | 67.0° |
| Toluene and ethanol | 7.52x10 ⁻⁴ | 70.0° |

DNA Modifications

When analyzed with a fluorometer, the reacted DNA samples showed an intensity at 517nm ($M = 47.75$ A.U) much higher than the control (Fig 1).

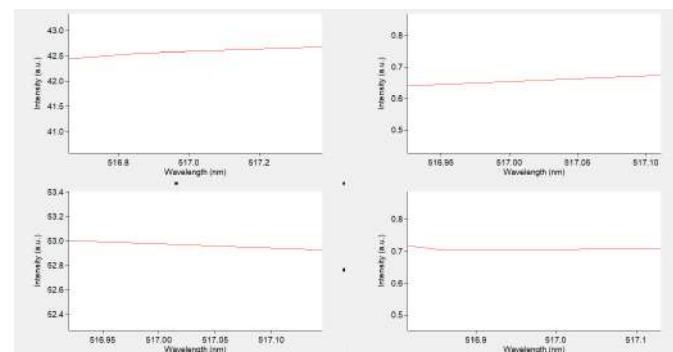


Figure 1. Spectra graphs of EDC reaction samples. Two graphs on the left represent reacted DNA samples. The two graphs on the right are control samples.

Confocal Microscopy of Capillaries

Capillary tubes with Substrate strands immobilized on the surface, and following the TMSDR showed a significantly higher average photon count for Cy3/6-FAM

emission light ($n = 3$, $\bar{x} = 1.31$, $SD = 0.22$, $p = 0.016$ versus control, Student's t test) when compared to unreacted capillaries ($n = 3$, $\bar{x} = 0.24$, $SD = 0.09$) (Fig 2A). No difference was observed for emission light corresponding to Cy5 (Fig 2B).

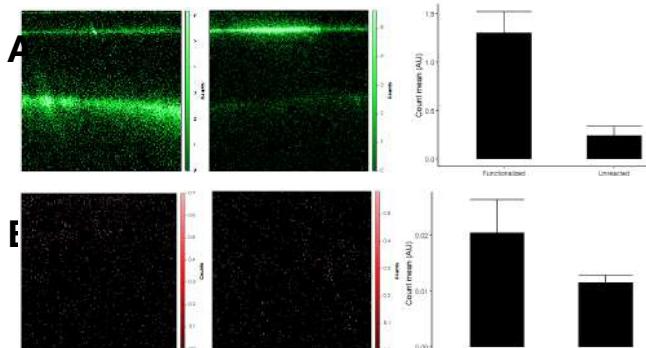


Figure 2. Confocal microscopy imaging and average count for TMSDRs in functionalized and unreacted capillary tubes (bottom light bands) corresponding to the emission wavelength of Cy3/6-FAM (A) and Cy5 (B).

IV. DISCUSSION

APTES Reaction Optimization

The ninhydrin tests suggest that in order to get the highest concentration of APTES functionalized onto the glass surface, the reaction should be carried out in Ethanol and at 70°C. This is consistent with the literature, which suggests that higher temperatures allow more water to hydrolyse the APTES in the initial step of the reaction, improving the overall yield (Pasternack et al., 2008).

DNA Modifications and Immobilization

The fluorescence intensity of the modified DNA at 517nm (Fig. 1) indicates that the reaction was successful, and the DNA was functionalized with primary amines. Likewise, the confocal microscope imaging of capillary tubes (Fig. 2) indicate that the immobilization of DNA on the surface was successful along with the strand-displacement reaction. Given that there was no significant difference in the Cy5 channel between the control and functionalized capillaries, it's likely that the TMSDR proceeded at nearly 100% completion.

For future directions, the system needs to be tested and better adapted for the conditions that it will face in the field. This includes testing the TMSDRs at lower temperatures and figuring out the limit of detection based on fluorescence assays. The MS2-production plasmid system also needs to be

experimentally validated to test for its efficiency and functionality.

V. CONCLUSION

The experiments and results described here lay out the bases for the development of this technology into a fully-fledged biosensor. The optimization of the APTES reaction allows us to maximize the yield of each reaction, which will eventually result in a higher sensitivity for the device. The DNA modifications sets up a scheme for in-house functionalization of oligonucleotides, which will allow us to cut costs in the future. The success of DNA immobilization on the capillary tubes and of the TMSDRs (and at a 100nM sensitivity) give good prospects for future development of this biosensor. Although there's still a lot more work to be done, Rumino has the potential to change the landscape for infectious disease control in Canada. Especially considering the current outbreaks of Highly Pathogenic Avian Influenza, where a quick affordable point-of-care device is in high demand. Rumino can fill a critical gap that will benefit farmers, veterinarians, and the general public overall.

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SUPPORTING INFORMATION

S1: [UCalgary iGEM Vector Journal 2025 Supplement](#)

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Nylon waste tackled: a synthetic biology approach to this environmental pollution problem

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ABSTRACT

Nylon, also known as polyamides, is the second most used synthetic fibre but suffers from low recycling rates (~2%). It is widely used in textiles, which often get discarded and shipped to non-EU countries. A new law passed by the European Commission [Regulation (EU) 2024/1157] has recently forbidden this practice, forcing EU countries to seek sustainable recycling alternatives. We propose a synthetic biology solution to this problem by combining microbial engineering of *Pseudomonas putida* with protein engineering, leveraging computational tools to discover enhanced nylon-degrading enzymes. In order to support a circular economy for nylon.

One of the biggest hurdles is the pretreatment of nylon waste, due to its crystallinity and heterogeneity. Our dry lab approach develops nylon-degrading enzymes with enhanced

transferred to a bioreactor, where the bacteria further metabolise them and upcycle into a high-value product, notably textile dyes like indigoidine.

Index Terms - nylon, textile waste, thermostability, *Pseudomonas putida*; enzyme

I. INTRODUCTION

Every year, more than 92 million tonnes of textile waste are produced, and this is estimated to increase to 134 million tonnes by 2030 (UNEP, 2020; Business Waste, 2023). Currently, less than 1% of textiles are recycled back into new textiles (fibre-to-fibre), meaning that almost all new synthetic fabrics are still made from virgin fossil fuels, according to Textile Exchange (Textile Exchange, n. d.).

There is a wide variety of synthetic fibres (acrylic, polyester,

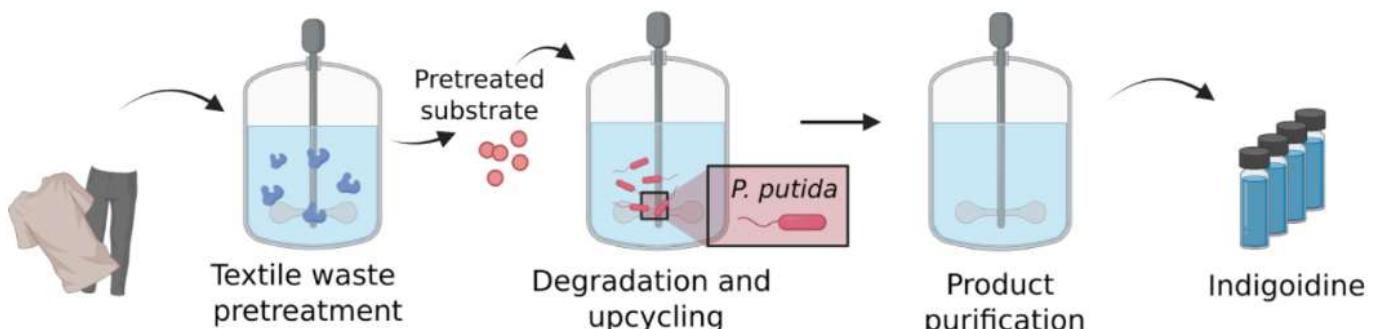


Figure 1. Schematic representation of our proposed biosolution for nylon degradation and upcycling.

activity and thermostability. The nylon oligomers are then

elastane, etc.), however, nylon remains particularly

challenging to recycle due to limitations in waste purity and chemical recycling technologies (Cornou, 2025). Considering all the difficulties nylon poses, we have engineered a strain of *P. putida* capable of growing on nylon-6 oligomers and upcycling it into indigoidine, a natural microbial dye (Figure 1). Our project has been built upon the previous work by J. de Witt et al. (2025).

DRY LAB

Sequence mining

Nylon-6 has a glass transition temperature (T_g) of 50–55°C, which causes enzymatic treatment to be sub-optimal, as NylC's thermostability (T_m) is 52°C (Negoro et al. 2012). Therefore, there is a need to find new thermostable nylon-degrading enzymes. Hoffman et al. (2025) identified TvgC as a suitable candidate after a sequence mining effort. This enzyme has significantly higher thermostability ($T_m = 93^\circ\text{C}$), paving the way to apply a similar strategy for our sequence mining. As part of our dry lab approach, we searched for thermostable NylC homologues in thermophiles using JackHMMER and Foldseek.

NylC's thermostability is challenged by its low thermostability, with a $T_m = 52^\circ\text{C}$ (Negoro et al., 2018) relative to the glass transition temperature of dry nylon 6, which is 48°C (De Naoum, K., 2022). This is relevant as enzymatic treatment is optimal above glass transition temperature. The discovery of TvgC in 2024 by Hoffman et al. (2025), with a significantly higher thermostability than wild-type NylC ($T_m = 93^\circ\text{C}$), pave the way to a sequence mining

thermostable NylC homologues in thermophiles using JackHMMER and Foldseek.

JackHMMER is a Hidden Markov Model (HMM) sequence-based tool that iteratively trains an HMM on protein sequences and uses it to query for homologs in a database (Potter et al., 2018). It has often been proven highly efficient in capturing subtle biological trends in protein sequences, being considered a very robust software for searching homologues. NylC was used as the query sequence, and JackHMMER was run for 5 iterations with maximum sensitivity and a bit-score threshold of 0.1.

Foldseek is a high-throughput software for structural homology search (Van Kempen et al., 2023). To the best of our knowledge, this is the first-ever application of structural homologue search in the context of NylC. A Boltz-1-generated (Wohlwend et al., 2024) structure of TvgC was used as input for Foldseek, and the matches were subsequently filtered based on their presence in thermophilic organisms acquired from ThermoBase (DiGiacomo et al., 2022). All matches were aligned in an MSA file together with a NylC reference, and all those without alignment to NylC's catalytic residues (Lys154, Asn185, Thr234, Asp273, Asp275) were discarded. Matches were also filtered to primarily include full proteins. These analyses yielded a reduced list of 276 enzymes.

Inspired by Hoffman et al.'s approach of filtering competent homolog matches using SSNs, we performed a parallel filtering of our subset of homologues. Using EFI-EST

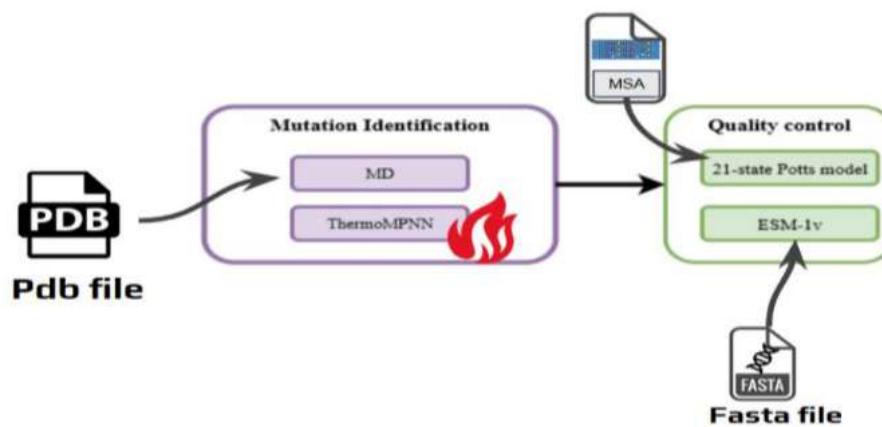


Figure 2: Pipeline for single-point mutations to increase activity and thermostability.

approach as a strategy to tackle the low efficiency of NylC. In this work, we tried a similar approach by searching

(Gerlt et al., 2016) and an alignment score threshold of 100, we created an SSN for the homologues matches and various

reference NylCs. The SSN was visualised in Cytoscape (Shannon et al., 2003), being able to isolate 27 sequences that clustered together with TvgC.

Protein engineering pipeline

Given the low activity and thermostability of NylC, we aimed to develop a pipeline to enhance the thermostability and activity of enzymes. As protein engineering for activity and thermostability is a common task for both iGEM teams and researchers, we designed an open-source pipeline, depicted in Figure 2.

ESM-1v is a protein language model (Meier et al. 2021) that is applied in our pipeline as a filter to score mutants at the quality control stage. The model is trained on evolutionary scale data and uses a deep neural network.

The Potts model is fitted on an MSA of NylC homologues identified by JackHMMER. For any input sequence, the Potts model can then output a Hamiltonian, which is an indication of how well this sequence fits into the MSA. To train the Potts model, we used GREMLIN (Balakrishnan et al., 2011).

First round of mutations

Prior to building the pipeline, mutations were manually produced for wet lab to subsequently test. In this round, single-point mutations were made for two separate objectives: thermostability and activity.

The ideal positions to mutate NylC were identified based on two factors: (i) the flexibility of them—in NylC, this is given by the B-factor and in TvgC, it was calculated using Cabsflex RMSF (Wróblewski et al., 2025), and (ii) ESM-1v cross entropy (Funk et al., 2024). ThermoMPNN predicts the $\Delta\Delta G$ of all possible mutations (Dieckhaus et al., 2024). Filtering for positions where the $\Delta\Delta G$ is less than -0.5, the theoretically thermostability-increasing mutations were obtained.

In the case of the activity-improving mutations, the strategy followed was different: ESM-1v and the Potts model were used, identifying mutations based on low Hamiltonian energy and a high zero-shot score.

WET LAB

The reasons behind the choice of *Pseudomonas putida* as chassis are its large genome and strong metabolic capabilities. It can already grow on polyamines, which makes it a good starting point for laboratory evolution. In the strains

used as starting point, only the nyl genes are heterologous, with the rest being native pathways that were switched on (De Witt et al., 2025).

After the dry lab's pipeline generated and narrowed down mutations in NylC and TvgC, they were expressed to test the activity and thermostability. Sitedirected mutagenesis was performed via PCR on the gene sequences optimized for *E. coli* (Federici et al., 2025). NylC variants were expressed in *E. coli* BL21 and purified via affinity chromatography, making use of a His-tag in their N-terminal.

To overcome the challenges of working with nylon and the current low enzymatic activity, a promising approach is to combine different hydrolysis methods, considering how other polymer degradation processes are carried out at an industrial level (Zhang et al., 2021). This allows a “rough chopping” of the polymer, followed by enzymatic “fine cutting”. In this context, research shows that NylC works best on oligomers of about 6 to 7 monomers in length (de Witt et al., 2025). Therefore, nylon was first broken down through cryomilling (Katiyar et al., 2021), followed by acid and heat treatments (de Witt et al., 2025), to later apply the enzymatic treatment.

To test enzymatic activity, an adaptation of the ninhydrin assay was followed, which is commonly used to quantify free amines (Stauß et al., 2024). This is the principle that was leveraged: when the enzyme cleaves the polymer, free amines are left at the ends (De Witt et al., 2025). By detecting the increase in free amines, enzymatic activity can be indirectly measured.

Once nylon is broken down into monomers, the team strives to upcycle it into high-value products that also contribute to a more sustainable and environmentally responsible fashionindustry. Firstly, the team was successful in the production of carotenoids as a proofof-concept, and later, in the production of indigoidine, a natural microbial dye that could replace the toxic and petrochemically derived indigo.

Carotenoids have previously been successfully produced in engineered *P. putida* strains (SánchezPascuala et al., 2019). For indigoidine expression, a plasmid has been constructed by inserting two codependent indigoidine genes into a backbone optimized for *P. putida*. The construction was performed in *E. coli* DH5alpha using PCR and Gibson Assembly (GA), followed by colony PCR and sequencing to select the indigoidine-producing colonies. The selected colonies were grown on a media containing inducer 3-methylbenzoate to biosynthesize the blue pigment.

II. CONCLUSION

While not all the work described in this paper has been brought to fruition yet, we strongly believe in the concepts laid out, and look forward to showcasing the final version of the pipeline, as well as the nylon-degrading capabilities of our *P. putida*, at the 2025 iGEM conference.

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TRAPS - A modular condensate-based RNA *in vivo* sensory platform

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ABSTRACT

*RNA is an essential biomolecule, with both functional and structural versatility. Understanding RNA dynamics, localization, and expression in living cells is therefore essential for decoding cellular function. Current RNA imaging methods often involve cell fixation or extensive genetic modifications and are limited in detecting low RNA concentrations. Here, we present RNA detection through Targeted RNA Activated Phase Separation (TRAPS). TRAPS is a novel, modular method for *in vivo* RNA detection using catalytically inactive Cas13 fused to GFP-tagged scaffold domains. With TRAPS, fluorescent condensates form upon multivalent binding of the target RNA by Cas13, allowing easy detection. This enables real-time detection of endogenous RNAs, advancing the toolbox to detect RNA in lower abundance and shorter lifetime. As a proof-of-concept experiment, we target mCherry RNA in *S. cerevisiae*. The Cas13 component of TRAPS offers easy adaptation to different target RNAs and varying *in vivo* conditions, making it a versatile method to investigate a range of biological processes, such as stress response, tissue morphogenesis, and progression of disease.*

Index Terms - RNA, Detection, Condensation, Phase Separation

I. INTRODUCTION

For the understanding of cellular function, it is crucial to comprehend the dynamics, localization, and expression patterns of RNA molecules within living cells. The roles RNA has in a cell reach far beyond its original discovery as just an intermediate molecule between DNA and protein. The earliest discoveries hinting at this were the existence of messenger RNA (mRNA), transcribed from only a small fraction of the full genome and responsible for translation (Brenner et al., 1961; Gros et al., 1961). Since then, non-coding RNA (ncRNA) has been found in a variety of forms

with catalytic activity, i.e. ribozymes, or capable of regulating gene expression, i.e. small interfering (siRNA) and microRNA (miRNA) (Fu, 2014; Strobel & Cochrane, 2007). Unsurprisingly, the versatility of RNA form and function leads RNA to play a role in most cellular processes. Consequently, RNA research has been and will continue to be crucial for a better understanding of biological processes such as development, stress response and more (Muñoz-Velasco et al., o. J.). In addition, RNA research has led a new avenue of therapeutics, diagnostics, and medicine with notably the mRNA-based vaccines during the global COVID pandemic (Barta & Jantsch, 2017; Chatterjee et al., 2023; Zhang et al., 2023). This makes RNA one of the most diverse and influential classes of biomolecules with growing global research interest, highlighted by four Nobel Prizes linked to RNA since 1965. Simultaneously, RNA detection methods have been developed and improved over the years to facilitate RNA research and elucidate its many functions in biology. In this paper, we will briefly describe the current RNA detection methods, their strengths and limitations, and propose a novel RNA detection method based on liquid-liquid phase separation (LLPS).

II. Common RNA Detection Methods

One of the most widely used RNA detection methods is realtime reverse transcription polymerase chain reaction (RT-PCR) (Afzal, 2020; Sullivan et al., 2023). Here, the cell is lysed and the RNA extracted, after which it is transcribed into cDNA with reverse transcriptase and amplified using PCR. Although RTPCR technique is very sensitive and provides fast results, it requires the cell to be lysed prior to analysis, making RT-PCR unsuitable for real-time expression studies (Afzal, 2020; Sullivan et al., 2023).

Another widely used technique is fluorescence *in situ* hybridisation (FISH). FISH utilizes fluorescently labelled oligonucleotides that hybridize with the targeted RNA (Singer & Ward, 1982). The presence and location of the target RNA in cells can then be observed using fluorescence microscopy. However, FISH generally requires heavy tissue preparation and fixing cells with formaldehyde

(Eltoum et al., 2001), limiting its applicability to study RNA dynamics *in vivo*. While both RT-PCR and FISH are limited in observing realtime transient RNA behaviour, several other systems were developed to address this limitation. These techniques often utilize oligonucleotides as molecular beacons (MB) (Cao et al., 2022). An MB is an oligonucleotide with an antisense stemloop that binds the target RNA. A fluorophore is added to one end of the loop and a quencher on the other end. The stem-loop ensures that the fluorophore and quencher remain in close proximity, suppressing fluorescence. The MB unfolds upon hybridization with the target RNA, separating the quencher and the fluorophore, resulting in a fluorescent signal (Marras, 2002). A limitation of MBs is the transport into the cells by protein carriers, making it prone to false positives due to nucleic acid degradation (Chen et al., 2007).

In recent years, novel *in vivo* RNA detection methods based on Cas13 have been developed to address some of these limitations. Cas13 can bind and cleave specific RNA sequences based on a highly modular guide RNA (Zhu et al., 2024). By mutating the catalytic center, RNA cleavage is deactivated (dCas13), resulting in a modular RNA binding platform. The dCas13-RNA binding can be visualized by fusing a fluorescent protein to the dCas13. Consequently, when target RNA is present, the localized fluorescence of dCas13 can be detected (Yang et al., 2019). This method is most effective at higher RNA concentrations, since a low amount of target RNA leads to only a fluorescence increase and a poor signal-to-noise ratio (SNR).

III. Improving signal strength by LLPS

In the previously discussed methods the signal was generated upon binding to the target RNA. The signal strength is therefore determined by the binding dynamics of the reporting probe to RNA, which following the law of mass action results in the fluorescent signal being linearly dependent on the concentration of the target. The lower the target's concentration, the lower the signal on average and more stochastic itself, making it harder to differentiate from background autofluorescence and measurement device noise. Liquid-liquid phase separation (LLPS) of biomolecules, like proteins and nuclear acid polymers leads to the formation of at least two coexisting phases with different compositions, usually appearing as droplets in a larger phase, and are used for a diverse set of cellular functions (Banani et al., 2017). The spontaneous mixing in multicomponent systems is driven by entropy, during phase separation an enthalpic contribution to the free energy, originating from molecular interactions can win over, causing the formation of separate phases.

Using the target molecule as a scaffold molecule, meaning it facilitates the interactions necessary for phase separation, its concentration determines when phase separation into droplets occurs and the size of the fluorescent droplets. This offers a less noise sensitive approach of

concentration measurements compared to fluorescence intensity based methods. Below the critical concentration required for LLPS there is only one mixed phase and the SNR behaves as described for the previous methods. Therefore the critical concentration of phase separation needs to be optimized to be as low as possible.

Biological condensate systems tend to use multivalent scaffold molecules, as having many binding sites lowers the critical concentration and increases the critical temperature, allowing bio-molecules to phase separate easier (Banani et al., 2017). Mechanistically having multiple binding domains causes cooperative binding dynamics and local clustering of the target RNA and the probes, facilitating stronger interactions among each other at lower concentrations. It should also be noted that very strong specific interactions are commonly related to the assembly of networks, while weaker less specific interactions in greater numbers (high valency macromolecules and intrinsically disordered domains) are suggested to play a major role in condensates maintaining their liquid like properties (Banani et al., 2017).

While previous methods work best at high RNA concentrations, lower amounts of target RNA lead to a decrease in fluorescence and worse signal-to-noise ratio (SNR). We aim to improve upon current techniques through liquid-liquid phase separation (LLPS) in the presence of the target RNA conditionally forming fluorescent condensates. The formation of these condensates significantly increases the local fluorophore concentration, thereby allowing us to detect RNA at a lower concentration.

IV. Design of phase separating RNA binding sensory system

The proposed system is based on the scaffold system engineered by Heidenreich et al. (2020). Their work presents a modular, synthetic system designed to investigate phase separation in living cells from first principles. Crucial for controlling condensation in this system is the toxin-antitoxin interaction with intermediate affinity. We adapted this scaffold system to the TRAPS system. The system is built on the same two protein interaction system, the toxin E9 and the immunity protein Im2 and also the tetramerization domain, 1AIE.

However, we also added a dCas13, functioning as an RNA Binding Protein (RBP). The E9 is fused to the tetramerization domain and green fluorescent protein GFP, forming a fluorescent tetramer. The RBP is fused to the Im2, connecting it to the tetrameric unit (Figure 1. A). The toxin and antitoxin will constantly bind and unbind, allowing LLPS. There are different gRNAs to bind multiple RNA target sites. If the RNA of interest is present, it will bind to the RBP and start connecting different tetrameric units of the system. This process forms a network and ultimately leads

to phase separation (Figure 1.C). By forming the condensates, the GFP fluorescence gets concentrated. As mentioned before, we want to use these effects to passively sense the signal of the target RNA.

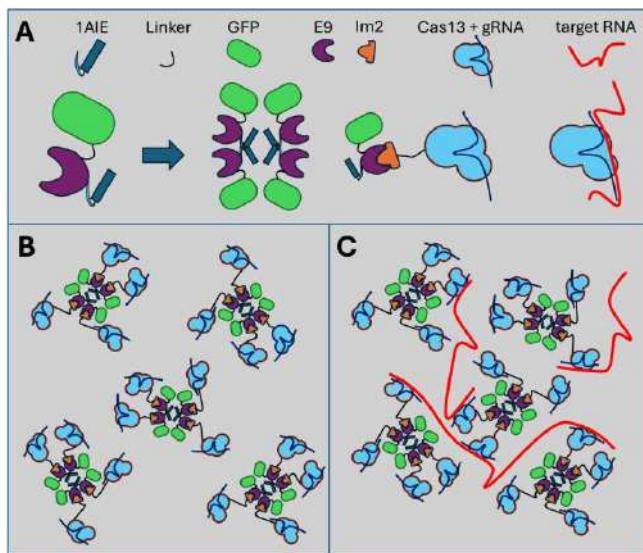


Figure 1. Visualisation of components and mechanisms of TRAPS system **A.** The constructs will form a tetramer, which will function as scaffold. The Cas13 and gRNA are connected through the E9-Im2 toxin-immunity binding to the scaffold structure. The gRNA/Cas13 complex will bind the RNA of interest. **B.** After expression the TRAPS system diffuses freely through the cytosol. **C.** In presence of the target RNA a network will form, leading to LLPS.

V. MATERIALS AND METHODS

ymCherry integration

To introduce *ymCherry* into W303 *S. cerevisiae* gateway cloning was performed. The *ymCherry* cassette was cloned into the pAG304GAL-ccdb plasmid from the pEntry-*ymCherry* plasmid using the LR-clonase reaction. The resulting pAG304GAL-*ymCherry* plasmid was linearized with MfeI and transformed into W303 *S. cerevisiae* for genomic integration into the tryptophan locus.

TRAPS-Cas13 integration

For the functional TRAPS-Cas13 system two fusion proteins and one gRNA cassette, coding for five gRNAs, were introduced into the *ymCherry* containing *S. cerevisiae*. Two individual plasmids were used for the transformation. Both the GFP-E9-Tetramer protein and Im2-Cas13 protein were ligated, introduced into one centromeric plasmid and transformed. pAG416 was used as the plasmid backbone. The cloning was performed by conventional cloning using the SacI

and MluI restriction sites in the plasmid. The gRNA coding cassette was similarly transformed using the pAG305 plasmids backbone. The plasmid was linearized using EcoRV and genomically integrated into the leucine locus. The yeast transformation was done with LiAc/Peg chemical transformation method.

Imaging

To specifically activate the RNA production the Gal-Promotor was induced by exchanging the carbon source from glucose to raffinose and finally galactose. The cells were imaged after activation. For imaging a Nikon ECLIPSE Ti2 was used with 1000x magnification for live yeast cell imaging.

Image analysis

Cell fluorescence intensities were obtained by segmenting the cells using Cellpose 2.0 (Stringer et al., 2021) with the cyto3 model and extracting the intensities from the corresponding fluorescence channels. For analysis the average fluorescence intensity of the non-cell areas was subtracted as background.

To test the functionality of the TRAPS system yeast optimized mCherry was integrated into a W303 *S. cerevisiae* under the control of a galactose-dependent promoter, resulting in significant mCherry fluorescence upon galactose induction (Figure 2.). Some fluorescence was observed in the vacuoles of the uninduced cultures, but not in the cytosol (Figure 2.B). In the induced *S. cerevisiae* cells, bright mCherry fluorescence is seen in the cytosol (Figure 2.D).

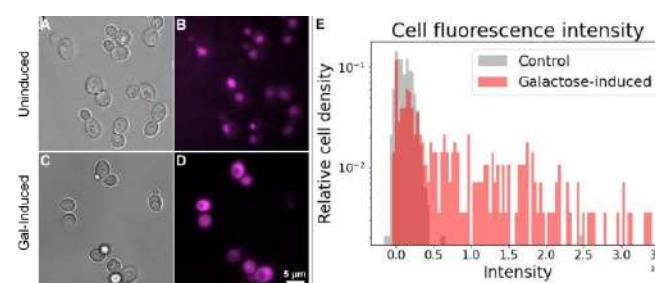


Figure 2. Fluorescence microscopy of the mCherry expression. **A-D** Brightfield and Fluorescence microscopy images and mCherry channel of the uninduced and induced cultures. **E** Histogram of summed mCherry fluorescence per cell. On the y-axis is the relative density of cells in a logarithmic scale. On the x-axis the sum intensity.

We introduced the TRAPS-Cas13 fusion proteins to investigate if the mCherry RNA can be captured and contribute to the formation of a condensate. In fluorescence microscopy droplets were observed (Figure 3.). These structures were independent of target RNA or gRNA presence (Figure 3. F,I,L,O).

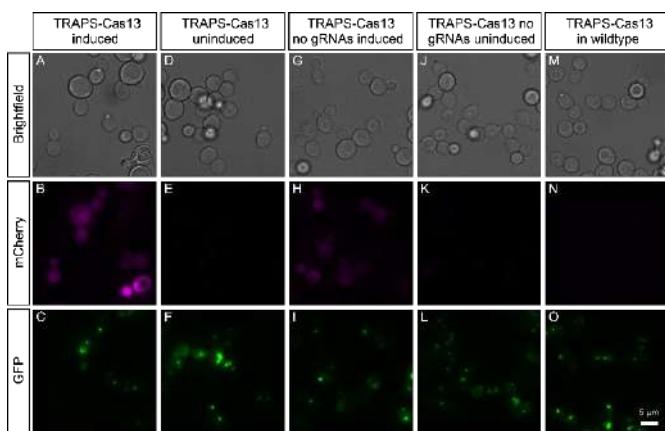


Figure 3. Fluorescence microscopy of the mCherry and GFP expression. **A-O** Brightfield and fluorescence microscopy images, mCherry and GFP channel of the mCherry RNA induced and uninduced cultures. The gRNAs were targeting the mCherry RNA.

VI. DISCUSSION

mCherry expression

The strong cytosolic fluorescence signal in the galactose induced samples indicates a successful *ymCherry* integration. If the yeast is continuously grown in glucose containing media no cytosolic mCherry is observable confirming the inactivity of the galactose dependent promoter. Interestingly, a fluorescent signal is observed in the vacuole of the respective cells.

This fluorescence can be attributed to two possible causes. First being, that adenine deficient *S. cerevisiae* strains, like the used W303, generally have a tendency for autofluorescence in the vacuole due to the accumulation of toxic metabolic intermediates in the vacuole (Park et al., 2014). A second possible cause is the accumulation of mCherry degradation products. The respective yeast cultures were grown overnight, diluted in the morning in new media and imaged in the evening. In the overnight culture a scarcity of glucose in the media during the stagnant growth phase can cause activation of the galactose promoter, which activates mCherry production. This overnight expressed mCherry will be degraded during the next hours in new glucose media, but degradation products might still be visible in the vacuole. This is usually unlikely but may happen when the glucose is fully depleted (Harrison et al., 2021).

In conclusion the conditional mCherry expression was achieved, but a promoter leak in the overnight growth is not ideal for the following tests, since this might already activate irreversible condensation of our proteins distorting results. To overcome this, it is necessary to add new media every 3-4 hours, even during overnight growth.

TRAPS-Cas13 functionality

The genes coding for the two fusion proteins composing the TRAPS-Cas13 system were transformed into mCherry containing and mCherry deficient yeast stains. Independent of the presence of our mCherry target RNA the system formed a droplet droplet in the cell (Figure 3.). The droplet might be a liquid-like condensate or a more solid-like aggregate. Since the formation of this droplet is independent of the RNA, protein-protein interactions are most likely the driving force. Here the current status of this project is reached and the following conclusions are assumptions.

Due to the RNA independent droplet formation, the observed droplets are most likely aggregates rather than the desired condensates. This assumption is also further supported by the low fluidity observed in the mobility assay.

In the heat-shock response of *S. cerevisiae*, a multitude of chaperone proteins are being expressed, that unfold and refold misfolded proteins. It has been shown that this response is also able to dissolve aggregates (Duennwald et al., 2012). This mechanism was also tested on the putative aggregates, but since no change was observed this approach was not further followed on.

Which of the two proteins is the cause of this aggregation is currently being investigated. It is unlikely that the tetramerization unit is the contributor of this aggregation since the unit has already been proven not to (Heidenreich et al., 2020). The working hypothesis is the aggregation of the Im2-Cas13 units.

Future outlook on the TRAPS platform

To determine and repair the error in the current iteration of the systems proteins are being transformed individually and the tendency to form aggregates is monitored. Additionally, the results of an upcoming western blot might give more insight into the cause of the aggregation. If the assumption that Cas13 is the main contributor is confirmed a new version of the fusion protein will be integrated switching from the current dCas13x variant to a more stable and yeast optimized rfxCas13d variant. Once the initial concept validation is successful, a multitude of further experiments are planned to investigate the efficiency and to adapt the platform to more than just sensing purposes. One important parameter of the system is the RNA copy number threshold at which no condensation occurs. To investigate this, an RNA titration experiment is planned. Additionally, the system will be tested on a native RNA Hfs1, which is highly upregulated during a heat shock response (Hahn et al., 2004). Further experiments like adding a translation initiation or inhibition factors to the scaffold, to regulate translation are also being evaluated.

VIII. CONCLUSION

We are establishing TRAPS as a novel RNA detection platform based on Cas13 driven phase separation. While we already successfully produce condensate-like structures, their formation seems to be independent of target

RNA presence. The current construct therefore needs improvement. Once refined, TRAPS is a promising platform with many possible adaptations to suit different purposes. As a detection platform it is a highly flexible and modular system, making it easy to adjust for different targets. It enables robust real-time *in vivo* RNA detection at low concentrations.

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CRIKIT: Developing a rapid CRISPR-based diagnostic kit for detecting antibiotic resistance

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ABSTRACT

Antibiotic resistance is one of the most critical emerging threats to global healthcare, with current projections indicating it will surpass cancer as a leading cause of mortality in the coming decades. Conventional methods for identifying antibiotic resistant infections are time-consuming, as they depend on culturing bacteria in the presence of antibiotics. This diagnosis delay often leads to trial-and-error treatment strategies, which not only compromise patient outcomes but also promote the spread of resistance. To address this challenge, we are developing CRIKIT, a rapid and versatile diagnostic tool based on CRISPR-Cas13 technology. CRIKIT uses programmable CRISPR guide RNAs (gRNAs) to target bacterial transcripts of specific antibiotic resistance genes. Upon recognition of its target, Cas13 is activated and initiates the collateral cleavage of nearby RNA. We make use of this property by adding a fluorophore quencher reporter molecule that will generate a measurable fluorescent signal when Cas13 is activated. This mechanism provides a functional read-out of gene activity in real time, offering insight into active resistance mechanisms in bacteria. This dynamic, expression-based approach will enable clinicians to make rapid, targeted decisions about antibiotic therapies, reducing the reliance on empirical treatments and improving clinical outcomes.

Index Terms - CRISPR-Cas system, Cas13, Diagnostics, Antibiotic resistance

I. INTRODUCTION

Antibiotic resistance (AR) is recognized as one of the top ten global health threats, with an estimated 1.27

million deaths each year and contributing to nearly five million more worldwide. The continual emergence of resistant bacteria poses a persistent challenge, undermining the effectiveness of treatments and creating significant biosafety risks for clinical procedures. Although hospitals and clinics implement measures such as controlled prescription and careful management of antibiotic dose, route, and duration, the overuse and misuse of antibiotics remain the principal drivers of resistance development (World Health Organization, 2023).

Resistance is an inevitable consequence of the selective pressure imposed by antibiotic exposure, allowing resistant bacteria to survive and outcompete non-resistant strains. The two primary genetic drivers of resistance acquisition are spontaneous gene mutations, often linked to the mode of action of a given compound, and the acquisition of foreign DNA carrying resistance determinants through horizontal gene transfer (Munita & Arias, 2016).

Addressing this problem requires coordinated action along three main fronts: the development of new antibiotics capable of overcoming existing resistance, the strengthening of AR surveillance to inform risk assessments and guide clinical decision-making, and the optimization of antibiotic use through the combination of drug usage with surveillance tools (World Health Organization, 2023).

At present, most clinical diagnostic laboratories rely on phenotypic methods for therapeutic decisions, because they are simple, reproducible, scalable, and relatively inexpensive. However, they are unsuitable for unculturable pathogens and demand considerable time and laboratory resources (Tong et al., 2025).

Molecular methods offer complementary advantages by directly identifying resistance genes and delivering results far more rapidly than phenotypic assays. However, these methods have inherent limitations beyond the need for more

advanced lab equipment. Some bacteria may carry resistance genes without expressing them, leading to false positives. Also, these methods can only detect known mechanisms of resistance, leaving novel or highly divergent genes undetected. Current technologies, such as PCR, although effective, still require refinement and simplification before they can be widely deployed in routine clinical settings (Woodford & Sundsfjord, 2005).

With the intent to overcome some limitations of the molecular methods while preserving their speed, we propose leveraging the powerful CRISPR-Cas13 system (Kellner et al., 2019) to develop a commercial diagnostic kit for rapid detection of AR. Furthermore, we adapted and optimized an innovative approach proposed by Team ARIA (UPF Barcelona, iGEM 2021), in which engineered *E. coli* are used as a self-growable factory of biosensors to detect AR genes.

II. METHODOLOGY AND EXPECTED RESULTS

We present a proof-of-concept, designed to test the mechanism underlying our proposed kit for detecting AR. By pairing the *Leptotrichia wadei* (Lw) Cas13a endonuclease with an array of guide RNAs we can target the mRNA transcript of different resistance genes, thereby identifying AR genes that are actively expressed. Specifically, we are evaluating a method using engineered *E. coli* that coexpress both the guide RNA (gRNA) and Cas13a protein, producing a self-contained testing system that is active upon lysis. Validating this is crucial, as it establishes the viability of the kit and lays the groundwork for more advanced and applied experiments. [Wet lab - Cas13a detection proof-of-concept](#)

In order to get targeted detection our kit requires three core components: 1) a system that detects whether the target RNA is present, 2) a reporter that generates a readable output, and 3) a coupling between detection and read-out. We aim to use the CRISPR-Cas13a system for detection, an RNA oligonucleotide fluorescent reporter to generate a detectable fluorescent signal, and the collateral activity of Cas13a as the link between them.

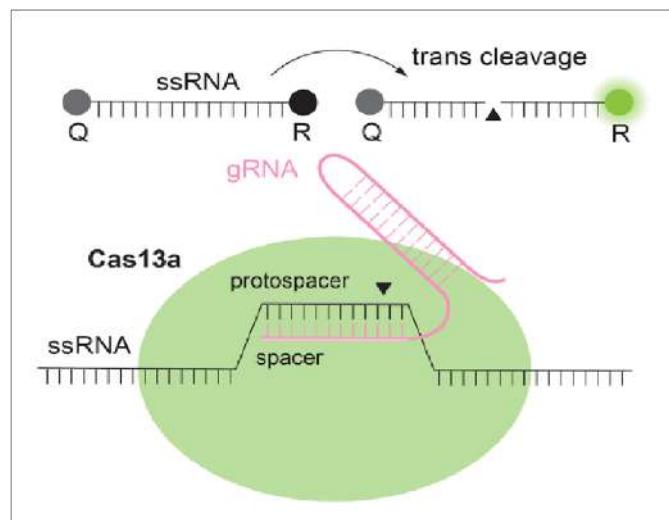


Figure 1. Cleavage of a ssRNA fluorescent reporter by active Cas13a

Note. Schematic of the active Cas13a-gRNA complex bound to the target RNA sequence (protospacer). The trans cleavage (or collateral cleavage) activity of Cas13a is represented by the cleavage of the fluorescent reporter that consists of a quencher (Q) and a fluorescein (R) linked by an RNA oligonucleotide. Cleavage of the reporter results in increased fluorescence.

When Cas13a finds an RNA complementary to its gRNA spacer, its endonuclease function is activated and it cleaves the target. After activation, Cas13a also non-selectively cleaves surrounding RNA, a property known as collateral (or trans) cleavage. This forms the basis of the detection mechanism, as the targeted activation of Cas13a by the presence of the target RNA can be coupled to the non-specific cleavage of RNA. By adding a commercial RNA oligonucleotide reporter, the presence of a specific AR transcript can be measured as a fluorescent output.

The fluorescent reporter contains a fluorescein (R) and a quencher (Q), linked by an RNA oligonucleotide. Before cleavage, any fluorescent signal is quenched by the close proximity of the quencher, but due to cleavage the fluorescence intensity of the sample will increase over time. However, since our Cas13a-gRNA system is produced and contained within *E. coli* cells, the fluorescence reporter will also be cleaved over time by endonucleases present in the cell. We expect that activated Cas13a will cleave the reporter faster than the endonucleases do. Therefore, by measuring the rate of fluorescence increase we can determine if the AR gene is present (rate is fast) or not (rate is slow).

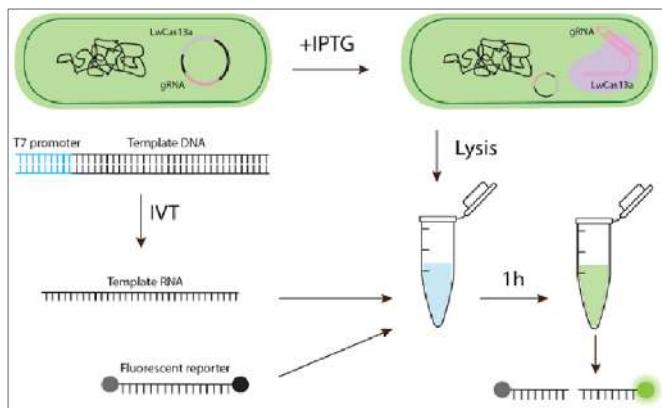


Figure 2. Detection protocol

Note. LwCas13a production is induced by addition of IPTG, then the cell lysate is combined with the fluorescent reporter and the template RNA (complementary to the spacer), produced by *in vitro* transcription (IVT) of the template DNA. After incubation the fluorescence of the sample increases due to the cleavage of the reporter.

In order to test this detection system we will follow a workflow as shown in Figure 2. Briefly, the Cas13a and gRNA are expressed in *E. coli* that are then lysed and added to the target RNA, that is complementary to the spacer, and the fluorescent reporter. Then the fluorescence output will be measured using a plate reader and the results interpreted by our analysis tool (see section “Dry lab - CRIKIT data analysis tool”).

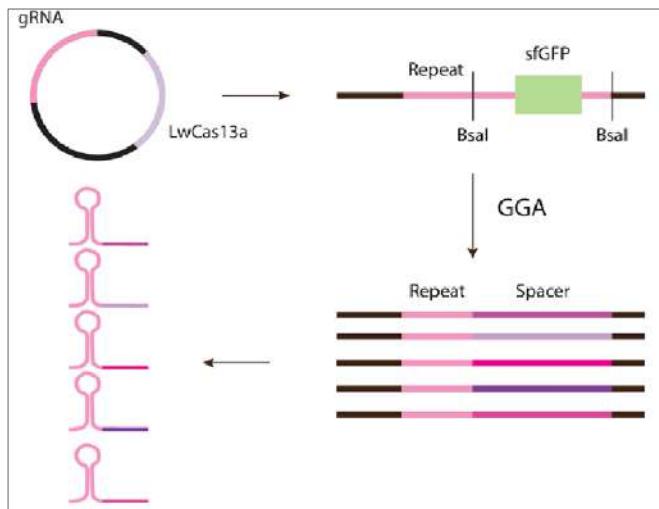


Figure 3. Golden Gate Assembly of gRNA spacers

Note. The designed plasmid encodes for the LwCas13a and gRNA, which is composed of a repeat region specific for LwCas13a and an sfGFP gene flanked by two BsaI sites. This

set up allows for Golden Gate Assembly (GGA) of multiple spacers into the plasmid, creating a library of gRNAs.

To use this technology as a kit we need the ability to screen for many antibiotic resistances, therefore we are designing a library of gRNAs. Our plasmid contains two BsaI restriction sites flanking a super folded GFP (sfGFP), allowing for easy green-white screening of colonies. Therefore, we can readily adapt to emerging resistance patterns and new resistance genes by adding new spacers to the plasmid and transforming them into *E. coli*.

Dry lab - CRIKIT data analysis tool

As discussed previously, Cas13a-mediated collateral cleavage cuts the RNA reporter, resulting in fluorescence signals corresponding to each AR gene detected across multiple time points. These signals need to be detected and analyzed to identify which AR gene are active or present in the sample. Detection can be performed by a plate reader, and to help interpret the results, we are developing an automated RShiny application capable of data preprocessing, statistical analysis, and visualization. The application will be hosted on GitLab as part of the kit, enabling researchers to efficiently analyze their results without requiring programming expertise.

Hardware - Portable fluorescence reader

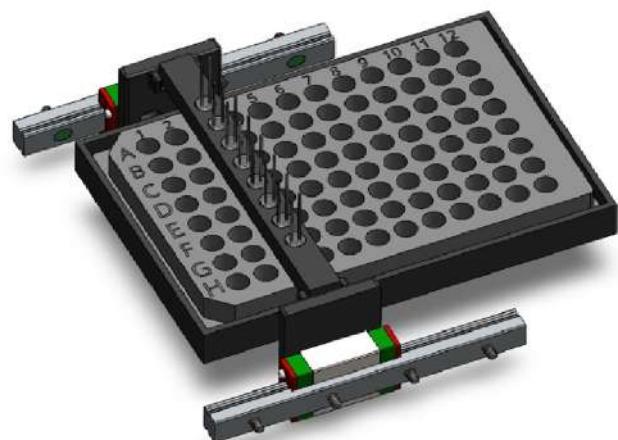


Figure 4. CAD model of the fluorescence reader

Note. The 96-well plate sits in a fixed grid for alignment of the wells. Around it, a rectangular scanning module moves row by row on linear rails. LEDs on the top side of the scanning module excite the samples, while photodiodes on the underside detect the emitted fluorescence, allowing the full plate to be read sequentially. The CAD model was created in SolidWorks (Dassault Systèmes). The MGN12 rail, 96-well plate, LED, and photodiode models were obtained from GrabCAD.

As part of the kit we are developing a lowcost fluorescence plate reader for 96-well assays. The device is designed to excite samples with blue light (~ 490 nm) from the top of the plate and record emission around 520 nm from below, through a band-pass filter. A detection frame spanning one row of eight wells is moved linearly across the plate to capture all twelve rows. Control, data acquisition, and wireless transfer are integrated in a single unit, with data output given in a standard fluorescence plate reader format and compatible with our CRIKIT webtool. The reader will be enclosed in a light-tight housing and is intended for use with standard black-walled microplates, allowing operation without laboratory infrastructure. We expect the system to clearly distinguish negative from positive wells, maintain uniform signal across each row, and deliver consistent results after normalization, enabling reliable use of CRIKIT assays in both laboratory and resource-limited settings.

III. DISCUSSION

CRIKIT relies on the recognition of bacterial RNA transcripts by Cas13a, which introduces several limitations. The assay is expression dependent; resistance genes must be actively transcribed at the time of testing to generate a detectable signal. Genes that are present but silent, or inducible genes expressed only under certain conditions, may therefore escape detection and create false negatives. RNA instability and natural expression variability further complicate interpretation. A possible future solution would be to complement Cas13a with a DNA-based detection strategy, allowing a distinction between genes that are present in the genome and those that are actively expressed. By combining this information, CRIKIT could provide a more complete diagnostic picture and improve applicability in both clinical and resource-limited settings.

CRIKIT demonstrates a low-cost and accessible approach to antibiotic resistance diagnostics by coupling CRISPR-based detection with a simple fluorescence reader. It establishes a framework that can be extended to increase robustness and be used in other settings where detection is applicable, such as agriculture and water sanitation. By lowering the technical and financial barriers to the detection of resistance, CRIKIT highlights the potential of portable synthetic biology tools to support diagnostics in both established laboratories, and resource-limited settings.

The combination of specificity from molecular diagnostics with real-time clinical applicability, means CRIKIT could improve patient outcomes, reduce the risk of resistance perpetuation, and help tackle antibiotic resistance.

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From probiotics to precision tools: Engineering *Lactobacillus crispatus* as a live biotherapeutic

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ABSTRACT

The vaginal microbiome plays a critical role in reproductive health by acting as a microbial barrier against pathogens. In healthy women, it is predominantly composed of *Lactobacillus* species, which maintains an acidic environment and produce antimicrobial compounds. A decline in these protective lactobacilli can lead to vaginal dysbiosis, allowing opportunistic pathogens to establish a reservoir. These pathogens may then ascend to cause further infection in the urinary tract. With rising antimicrobial resistance and the detrimental impact of antibiotics on commensal communities, alternative treatments are needed. Probiotic *Lactobacillus* strains represent a promising alternative, though their effectiveness is often limited by competition for host adhesion sites. This review outlines a synthetic biology strategy to enhance the competitiveness of probiotics, using *Lactobacillus crispatus* - a key species in the healthy vaginal microbiota - as a chassis. We detail the design of high-affinity peptide ligands that neutralize pathogen-specific adhesins. This approach aims to precisely target early-stage infection, reinforcing the protective function of the vaginal microbiome without disrupting beneficial resident communities.

Index Terms – antibiotic resistance, *Lactobacillus crispatus*, live biotherapeutic (synthetic biology), peptide inhibitors, vaginal dysbiosis, vaginal microbiome

I. INTRODUCTION

Vaginal dysbiosis is characterised by an imbalance in the microbial ecosystem. *Lactobacillus* spp., the most prevalent

members, play a key role in modulating the pH of the vaginal microenvironment (Boskey et al., 1999; Petrova et al., 2017). A reduction in these protective lactobacilli allows opportunistic pathogens like *Gardnerella*, *Bacteroides*, and *Prevotella* to invade. Following initial adhesion, these opportunistic pathogens colonise and form robust biofilms that shield them from antibiotics. In doing so, the biofilms promote the horizontal gene transfer of resistance traits such as enzymatic drug inactivation, target modification, elevated efflux pump activity, and metabolic alterations that inhibit prodrug activation (Bjarnsholt et al., 2009; Swidsinski et al., 2005).

Since the discovery of antibiotics in 1928, they have become a powerful tool to treat bacterial infections (Fleming, 1929). As a result, they are typically prescribed as the first-line of defence against bacterial infections in response to vaginal dysbiosis and urinary tract infections. Despite, the crucial role of antibiotics in clinical settings, its long-term efficacy is in jeopardy due to the emergence and spread of antibiotic-resistant bacteria (Swidsinski et al., 2005). Within dysbiotic vaginal communities, a high prevalence and recurrence of conditions like bacterial vaginosis - which affects up to 30% of women in the United States and over 50% in sub-Saharan Africa - have been linked to antibiotic-resistant bacteria (Allsworth & Peipert, 2007; Kenyon et al., 2013).

Conventional antibiotics often fail to eliminate resistant strains, enabling their spread towards the urinary tract while inadvertently targeting the native microbiome. The subsequent loss of beneficial microbes opens adhesion sites for opportunistic pathogens to re-establish, leading to high recurrence rates of 50% to 80% (Bradshaw & Sobel, 2016).

There are currently few remaining antibiotics that are used as a last resort treatment (e.g. carbapenems and colistin) and no new antimicrobial drugs have been discovered since 1987 (Silver, 2011). As a result, alternative treatment options are being studied in wake of the antibiotic resistance crisis.

Consequently, probiotics are often studied as an alternative to antibiotics. *Lactobacillus crispatus* (*L. crispatus*) has emerged as a promising probiotic to potentially improve the immune defense of the vagina (Lepargneur, 2016). *L. crispatus* is the most dominant species in the vaginal microbiome and a key member (Petrova et al., 2013). It is known that modulating the vaginal microbiome environment prevents infection by establishing protective mucosal barriers, producing antimicrobial agents, and maintaining a low vaginal pH that inhibits pathogen growth (Boskey et al., 1999). However, clinical studies show that probiotics are overall are ineffective and inconsistent due to poor colonization and specificity in the vagina. (Buggio et al., 2019 and Colodner et al., 2003).

Adhesion is the first key step to pathogen colonization, therefore targeting their adhesive mechanisms can improve probiotic efficacy. A key mechanism of adhesion in *L. crispatus* is the use of special surface proteins (S-layer proteins) to adhere to the vaginal epithelial cells, preventing pathogens from establishing a foothold (Decout et al., 2024 & Hu et al., 2011). S-layer proteins can also modulate the host immune system to reduce levels of pro-inflammatory cytokines by interacting with the anti-inflammatory receptor DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) (Decout et al., 2024). To improve the efficacy of probiotics, genetic modifications could be made to the S-layer proteins (of *L. crispatus* or other *Lactobacillus* spp.) to fuse enzymes or inhibitors that specifically binds to pathogen of interest (Ilk et al., 2011). This design approach directly neutralizes pathogens to prevent adhesion in the vagina and provides a promising avenue for a targeted and specific therapeutic approach. To that end, this review will discuss the design principles of modifying the S-layer proteins of bacterial cells towards improving immune defenses and how engineering live biotherapeutics could improve the vaginal immunity.

II. LITERATURE SUMMARY

Use of naturally occurring Lactobacillus in live biotherapeutics

Live biotherapeutics products (LBPs) contain live microorganisms and is an emerging strategy to prevent, treat, or cure a disease. *Lactobacillus* comprises many vaginal probiotics and non-antimicrobial treatments because it constitutes the majority of the normal vaginal flora population (Xiang Ng et al., 2018). A meta-analysis was conducted on nine clinical trials where *Lactobacillus* spp. were used as prophylaxis against recurrent urinary tract infections (rUTIs) and found that it significantly reduces rUTIs in females. They noted *Lactobacillus crispatus* CTV-05 as one of the treatments that were particularly effective against uropathogens and greatest efficacy for UTI prophylaxis (Czaja et al., 2007). As the data in this meta-analysis were published between 1960 and 2017, there remains significant opportunity for further advancement in live biotherapeutic development.

LACTIN-V is a vaginally administered live biotherapeutic from Osel, Inc. (Santa Clara, CA) that delivers the naturally occurring human vaginal strain *L. crispatus* CTV-05. It is designed to replenish protective *Lactobacillus* population in women with bacterial vaginosis following conventional antibiotic treatment with 0.75% metronidazole (Hemmerling et al., 2009; Cohen et al., 2020). Phases 1 and 2a clinical trials successfully established the safety, tolerability, and colonization efficiency of *L. crispatus* CTV-05 (Hemmerling et al., 2009; Hemmerling et al., 2010). Building on this, a phase 2b trial demonstrated that treatment with LACTIN-V significantly reduced the recurrence of bacterial vaginosis (Cohen et al. 2020). One hundred and fifty-two (152) women underwent Among the 152 women treated, *L. crispatus* CTV-05 colonized 79% of participants at 12 weeks. While colonization declined to 48%, compared to 2% rate observed in the placebo group. This result further supports the selection of *L. crispatus* as an ideal chassis for a live biotherapeutic, given its natural presence in the vaginal flora and proven efficacy in improving vaginal health.

A clinical pilot study by Pino et al. (2021) further supports the use of *Lactobacillus* as a treatment. The study investigated the efficacy of the *Lacticasebacterium rhamnosus* TOM 22.8 strain in women with vaginal dysbiosis. Results indicated that both oral and vaginal administration effectively attenuated clinical symptoms and restored eubiosis (Pino et al., 2021). The treatment also significantly reduced viable pathogens, including *Candida* spp. and anaerobic bacteria.

Synthetic live biotherapeutics and biocontainment measures

The studies highlighted above reported the use of naturally occurring strains; both LACTIN-V (*L. crispatus* CTV-05) and *L. rhamnosus* TOM 22.8 seem to be naturally occurring microbes that were derived from healthy individuals. However, it is possible to engineer *Lactobacillus* strains for biotherapeutic applications (Klotz & Barrangou, 2018). A pioneering example is a synthetic live biotherapeutic developed by Isabella et al. (2018) to treat phenylketonuria, a genetic disorder characterized by the inability to metabolize phenylalanine. The researchers genetically engineered *Escherichia coli* Nissle 1917 to express genes for phenylalanine-metabolizing enzymes in the gut using a lambda red recombineering approach. This method utilizes lambda bacteriophage proteins to facilitate precise homologous recombination into the bacterial genome. Crucially, as a biocontainment measure, they also deleted an essential gene for cell wall biosynthesis, rendering the engineered bacteria auxotrophic and dependent on an exogenous compound not found in the environment. This safety feature prevented the uncontrolled proliferation of the modified strain outside the controlled gut environment.

III. THE RATIONAL DESIGN OF INHIBITOR PEPTIDES FOR TARGETED NEUTRALIZATION OF VAGINAL PATHOGENS

In silico peptide design to improve specificity

A proof of principle that can be used to improve *L. crispatus* as a probiotic would be to engineer this bacterium's S-layers to express peptides that bind and inhibit pathogens. Alternatively, these peptides can be expressed separately fused to S-layers and added exogenously to the bacteria (Hu et al., 2011). The UManitoba 2025 team is currently attempting to engineer pathogen-binding peptides into *L. crispatus* and validate this design principle (not published) as a potential biotherapeutic treatment. To effectively enhance the specificity of *L. crispatus* against vaginal pathogens, adhesive proteins on the pathogen surface must first be identified, as they are readily accessible for binding. It is important that both protein and its inhibitor ligands are soluble once purified for subsequent quantitative binding assays, limiting the selection process. Alternatively, truncations can be to the amino acid sequence of protein targets to exclude non-essential hydrophobic regions and potentially improve their solubility. Once target proteins have been selected, their potential binding pockets can be identified using computational tools such as P2Rank (Krivák & Hoksza, 2018). Key amino acid residues,

often located on α -helices, typically provide stable and reliable interaction sites for inhibitor design. The three-dimensional structures of these sites can be analyzed using visualization software like PyMOL (e.g, version 3.1.4) or ChimeraX (Goddard et al., 2018).

For *de novo* design of peptide inhibitors, RFdiffusion (Watson et al., 2023) can generate protein backbones guided by spatial constraints derived from key residues. Subsequently, Protein Message Passing Neural Network (ProteinMPNN) (Dauparas et al., 2022) can be employed applied to design amino acid sequences that promote proper folding and enhance binding affinity. The stability and structural consistency of the designed peptides can be evaluated using AlphaFold (Abramson et al., 2024) with reliability assessed through predicted Local Distance Difference Test (pLDDT) and predicted aligned error (PAE) scores.

Protein-peptide docking can be performed using the High Ambiguity Driven protein-protein DOCKing (HADDOCK) computational tool to assess binding compatibility (Dominguez et al., 2003). Further refinement of the peptide–protein interactions can be optimized via energy minimization and dynamics-based scoring. Finally, molecular dynamics simulations using tools such as GROningen MAchine for Chemical Simulations (GROMACS) tool can evaluate the stability of the peptide–protein complexes under physiological conditions, providing insights into the longevity and robustness of binding (Berendsen et al., 1995).

Quantitative binding assays to assess ligand binding

Designed inhibitors and their corresponding targets should be expressed and purified. Quantitative binding assays are then required to measure the interaction (binding) between the peptide inhibitor ligand and its target. Isothermal titration calorimetry (ITC) is considered the gold standard for characterizing molecular interactions, as it directly measures the heat change associated with binding (Bastos et al., 2023). Under constant temperature and pressure, ITC records the heat released or absorbed after each incremental addition of one reactant. From this data, the dissociation constant (K_D) can be derived to quantify binding affinity.

Alternatively, microscale thermophoresis (MST) offers another method to quantify the interaction between the inhibitors and targets (Huang & Zhang, 2020). This biophysical technique detects binding by monitoring changes

in the thermophoretic movement of a fluorescently labeled molecule through a temperature gradient. An infrared laser creates a localized temperature gradient within capillary-loaded samples. When a non-fluorescent ligand binds to the fluorescent target, it alters properties such as size, charge, or hydration shell, resulting in a detectable shift in thermophoretic behaviour. This movement is used to calculate the K_D constant. In MST, a kinetic model, described by mass-action equation can be used to quantitatively and statistically determine the K_D constant (Jerabek-Willemsen et al., 2011). A low K_D value – typically in the nanomolar range – indicates strong binding between the ligand and its target protein.

IV. THE CHALLENGES OF DEVELOPING ENGINEERED BACTERIA TOWARDS IMPROVING VAGINAL IMMUNE DEFENCES

I. Reduced efficacy

Traditional *Lactobacillus*-based probiotics (LBPs) face significant challenges related to efficacy, delivery, and safety. As live therapeutics, they must overcome host physiological barriers - including low pH, digestive enzymes, and immune responses to reach their target site (Cook et al., 2012). Even upon arrival, their therapeutic potential, which includes immune modulation, metabolite production, and competitive exclusion of pathogens, is often limited by poor colonization and persistence (Reid et al., 2003). This is particularly challenging in the vaginal environment, where LBPs must compete with both resident microbiota and pathogens for limited adhesion sites (Mei & Li, 2022).

I. Delivery of live biotherapeutic agent

Engineering native *Lactobacillus* strains to bind pathogen adhesion proteins offer a promising strategy to prevent initial colonization (Table 1). This targeted, temporary inhibition can disrupt the cycle of infection and create a window for the native microbiome to recover. LBPs are commonly administered through two primary routes: orally via encapsulated formulations or locally using topical applications such as hydrogels. In both cases, maintaining bacterial viability is critically dependent on precise formulation and strict environmental control throughout the manufacturing process (Anal & Singh, 2007).

| Aspect | Strengths | Weaknesses |
|---------------------------------|--|---|
| Antibiotic resistance footprint | Minimal resistance risk when using non-antibiotic selection (e.g., auxotrophy, toxin - antitoxin systems). | Requires strict waste management to avoid resistance dissemination. Engineered bacterial strains can interact with the host's microbiota via horizontal gene transfer. |
| Efficacy | High specificity and tunability for target organisms. Neutralizes pathogens in controlled model systems. | Performance can drop in complex real-world settings. Vulnerable to host immune defense or environmental stress. The efficiency also depends on the patient's medical condition. |
| Safety | Low systemic toxicity (ex. designed to act locally, reducing risk of systemic side effects). Containment strategies can prevent survival and spread of live therapeutics in the environment (ex. engineering auxotrophic strain and kill switches). | Potential unforeseen interactions with microbiota or ecosystems. Genetic mutations may bypass safety features. Strict waste handling is needed to ensure genetic material is not exchanged within bacterial populations. |
| Regulatory compliance | Design can follow GMO containment guidelines (e.g., BSL-1/2 compliance). | Therapeutic dose may not be universally safe, as individual variability can lead to potential adverse effects in certain patients. Lengthy, costly approval process for live biological agents. |
| Stability | Transparent genetic construct design to indicate introduced modifications enables better assessment of safety and compliance with biosafety standards. Genetic stability achievable with chromosomal integration. | Lack of standardized regulations complicates deployment. Plasmid-based stability can be lost or effectiveness reduced by mutation over time. |
| Delivery | Encapsulation or freeze-drying can protect bacterial viability, extending shelf-life and improving practicality for clinical use. Multiple delivery routes possible (ex. oral, topical, environmental release). Mechanism of delivery can be encapsulated or immobilized for targeted release. | Environmental stressors can compromise viability. Delivery efficiency may be reduced by physical barriers (e.g., biofilms, host tissues). Requires protection from degradation before reaching the target site. |

Table 1. Analysis of live biotherapeutics towards improving vaginal immune defence.

Note. This analysis examines the perceived strengths and weaknesses of a live biotherapeutic agent as a treatment option for vaginal health. The information was obtained from Team Athens, 2022, *Biocomputing Applications and Interleukin -10 in Autoimmune Diseases*, TU- Eindhoven.

II. Lack of standardized regulation to ensure safety (Safety, stability, and regulatory challenges)

Safety and stability remain key challenges for live biotherapeutic products (LBPs). Although many LBPs are derived from commensal microbes, introducing engineered strains at high densities may lead to unforeseen ecological interactions or competitive dynamics (Table 1). The potential for replication and horizontal gene transfer (HGT) raises concerns regarding genetic stability and the possibility of disseminating engineered traits into indigenous microbial communities (van Reenen & Dicks, 2011). Risk mitigation strategies—such as the incorporation of biocontainment

circuits (e.g., kill switches) and stable genomic integration of genetic constructs—can help alleviate these concerns (Table 1). Additionally, proactive engagement, transparent communication about benefits and risks, and a steadfast commitment to ethical principles are essential to navigate societal concerns regarding the use of live biotherapeutics.

IV. CONCLUSION

Vaginal dysbiosis, characterized by a decline in protective *Lactobacillus* species and a rise in opportunistic pathogens, presents a mounting therapeutic challenge in the era of antimicrobial resistance. Conventional antibiotic treatments often fail due to biofilm formation, recurrence, and their disruptive impact on the native microbiota. While traditional probiotics offer a promising alternative, their efficacy is frequently limited by poor colonization and a lack of specificity. This review presents a novel synthetic biology strategy to address those challenges: engineering the native commensal *Lactobacillus crispatus* to function as a precision live biotherapeutic. By displaying high-affinity peptide inhibitors on its surface, this engineered chassis is designed to selectively neutralize pathogen adhesins, preventing the initial colonization that leads to dysbiosis without resorting to broad-spectrum antimicrobial activity.

Looking forward, the clinical translation of this and similar advanced LBPs hinges on overcoming interconnected scientific and societal hurdles. Scientifically, the path involves refining design principles - using tools like RFdiffusion and ProteinMPNN for inhibitor design and rigorous *in vitro* and *in vivo* validation of binding affinity and safety. Implementing robust biocontainment strategies will be crucial for environmental safety and regulatory approval. From a regulatory perspective, the current fragmented and ambiguous landscape, exemplified by the divergent approaches of Health Canada and the FDA, must evolve toward harmonized, predictable pathways that encourage innovation while ensuring safety. The success of engineered microbes heavily depends on earning public trust to ensure these technologies are safe and can fulfil their potential to redefine women's health.

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InkSight - Turning tattoos into diagnostic tools

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ABSTRACT

Tattoos, historically used as markers of identity, social belonging, and therapeutic practice, provide a meaningful backdrop for their use in medical diagnostics. Here, we present InkSight, a modular, mammalian cell-based tattoo biosensor designed for real-time, equipment-free detection of circulating biomarkers directly in the interstitial fluid. The system employs synthetic melanosomes made of encapsulin-tyrosinase nanocages to achieve a safe, localized melanin production that, coupled with modular synthetic receptors, enables a contrast shift between light and dark states in response to specific biomarkers. We describe strategies to ensure high contrast and specific tyrosinase activity, as well as ongoing molecular approaches to mediate encapsulin nanocage behaviour. Preliminary findings on public perceptions of safety and relevant regulatory frameworks complement these technical efforts, while perceived areas of use guide our approach to different targets. To our knowledge, InkSight is the first example of a continuous, eye-visible biosensor tattoo based on mammalian cells, marking a significant advance in wearable synthetic biology. This platform opens new possibilities for continuous and decentralized health diagnostics, with envisioned applications in personalized medicine and remote health monitoring.

Index Terms - Tattoo, Synthetic Melanosomes, Tyrosinase, Melanin, Diagnostic Tools

I. INTRODUCTION

Tattoos are one of the oldest forms of body modification, historically used to mark social status, group membership, and rites of passage, as well as to express deep personal and cultural meanings, in ways that widely vary across different cultures (Deter-Wolf et al., 2016). Archaeological evidence reveals that in some ancient cultures, tattooing was also employed for therapeutic or medical purposes, with tattoo markings located along

acupuncture points associated with the treatment of pain and arthritis (Hawkin, 2022). As societies transformed, so did the meanings attached to tattoos. They have been associated with religious devotion, but also as markers of criminal punishment or punitive stigmatization (Alker & Shoemaker, 2022; Bloch, 2024). Nowadays, they have become widely accepted forms of self-expression and creativity, increasingly normalized across generations and social groups. Drawing from the rich history of tattoos as both symbolic and medical tools, recent advances in the field of synthetic biology have explored the diagnostic potential of cell-based tattoos, also called “living tattoos”. These developments emphasize tattoos’ position at the intersection of cultural practice and biomedical innovation.

Current Synthetic Biology-based Tattoos

Regarding current approaches on cell-based tattoos, Tastanova et al. (2018) developed an implantable biomedical tattoo using engineered mammalian cells to monitor long-term hypercalcemia, a marker often associated with the asymptomatic development of different types of cancer. Their system is based on a melanin-induced color change, a product of a signaling cascade that results in the over-expression of transgenic tyrosinase, leading to melanin production and visible skin darkening. While this approach leads to a stable and permanent pigmentation, as demonstrated *in vivo* in mice, this very stability significantly limits the reversibility and versatility of the diagnostic tool.

More recently, Allen et al. (2024) presented a modular dermal tattoo biosensor based on the use of engineered *E. coli* encapsulated in microfluidic hydrogels. These bacterial systems are capable of responding to various biochemical (such as IPTG) and biophysical (like temperature) stimuli, generating a fluorescent signal when applied to a skin mimic. However, while encapsulated, the use of engineered bacteria as a diagnostic tool remains controversial, opening up questions about immunogenicity, safety, and ethics that need to be explored in future work. At the same time, both systems rely on the encapsulation of cells in alginate beads, which might further restrain their

reversibility and adaptability, as the material is known for its complex biodegradability and limited mechanical strength (Dell et al., 2022). Together, these studies clearly demonstrate the potential of cell-based tattoos as future biosensors, while also highlighting the need for novel approaches that combine clear visual readouts with real-time, reversible detection of clinically relevant biomarkers.

In that context, we present InkSight, a mammalian cell-based tattoo capable of reversible biomarker detection in the interstitial fluid (ISF). Using mammalian cells rather than bacteria allows for improved biocompatibility, reduced immunogenic risk, and the ability to support more complex synthetic gene circuits *in vivo*. Furthermore, by focusing on the interstitial fluid (ISF) as the diagnostic space, InkSight enables a dynamic monitoring of circulating biomarkers that is less invasive compared to current methods that rely on constantly taking blood samples (Wu et al., 2024). In the next sections, we describe the mechanism of InkSight based on existing research, followed by a discussion on future directions regarding the safety, legislation, and possible targets around the use of tattoos as diagnostic tools.

II. OUR SYSTEM - InkSight

We designed InkSight to act as an optical biosensor made of engineered mammalian cells that are introduced into the dermis via tattooing. Upon detection of biomarkers in the ISF, it triggers a signaling cascade, which leads to a contrast change in the form of a visual output detectable with the naked eye (Figure 1).

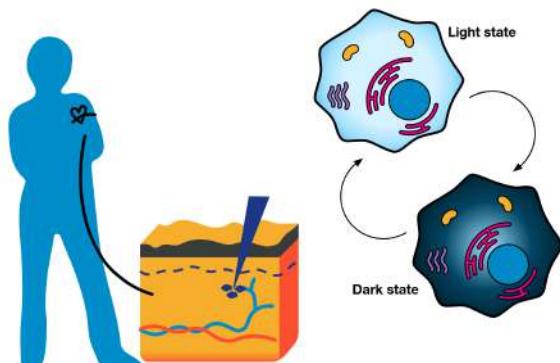


Figure 1. Schematic overview of InkSight biosensor system. Engineered mammalian cells embedded in dermis function as biosensors detecting biomarkers in the ISF. InkSight uses synthetic melanosomes to display a melanin-dependent contrast change between dark (pigmented) and light (faded) states.

Synthetic Melanosomes

Melanin is a naturally occurring pigment in the skin, but its accumulation at high concentrations can become cytotoxic (Cabaço et al., 2022). To address this challenge, at the core of our system, we are using synthetic melanosomes: encapsulin nanocages with tyrosinases localized to their

interior, enabling robust yet spatially confined melanin production (Figure 2). This strategy allows for high concentrations of melanin to be specifically sequestered within these compartments, therefore, preserving cell viability by keeping overall cellular melanin concentration within physiologically safe levels (Plonka & Grabacka, 2006).

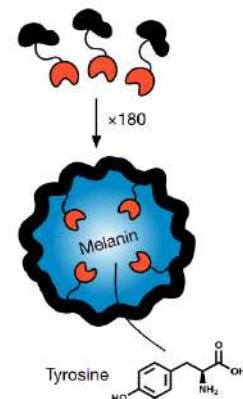


Figure 2. Assembly of a synthetic melanosome. Schematic of 180 subunits of the Encapsulin-Tyrosinase (black and red, respectively) protein fusion self-assembling into a nanocage. The structure catalyzes melanin production in the presence of tyrosine. (Adapted from [Sigmund et al., 2018](#)).

Figure 2 presents the assembly of a synthetic melanosome, consisting of self-assembling bacterial encapsulin proteins (black) fused to tyrosinases (red). This approach catalyses localized melanin synthesis from tyrosine, without inducing strong cellular toxicity ([Sigmund et al., 2018](#)). We developed multiple strategies to ensure high contrast change and specificity of tyrosinase activity: (i) a split tyrosinase that reconstitutes the active form upon nanocage formation, (ii) activity modulation through caddie proteins, and (iii) LID domains that inhibit tyrosinase activity until cleaved by TEV protease (TEVp). We are currently prototyping these strategies and evaluating the most effective approach for robust and specific melanin-based visual output.

Strategies for Contrast Change

Fundamental to our design is the programmable synthetic receptor, MESA (Daringer et al., 2014). Upon ligand binding, the receptor dimerizes, triggering either the release of a transcription factor or the downstream cascade activation through proteolytic TEV reconstitution (Figure 3).

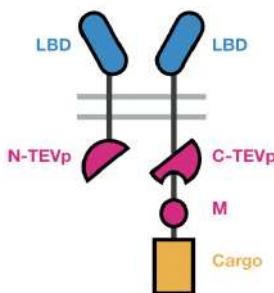


Figure 3. Modular Extracellular Sensor Architecture (MESA) Receptor. Parts of the receptor include: LBD –ligand binding domain; N-TEVp and C-TEVp – N-terminus and C-terminus of TEV protease; M – TEVp cleavage site; Cargo – output domain (e.g. transcription factor).

Hence, ligand-induced visible contrast change can be achieved through several engineered molecular strategies governing encapsulin nanocage behaviour: (i) expression, (ii) assembly, and (iii) agglomeration (Figure 4).

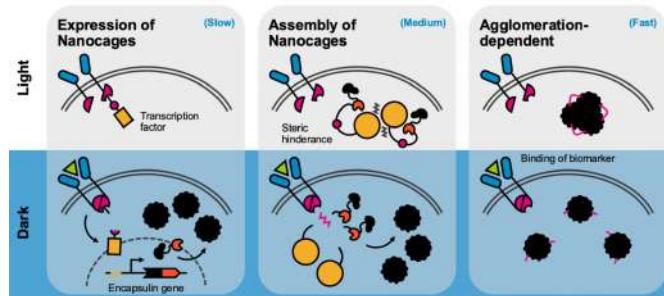


Figure 4. Contrast-change strategies upon ligand binding to MESA receptor. Schematic illustrating three approaches, based on (i) expression, (ii) assembly, and (iii) agglomeration of nanocages. The annotations on the top of frames indicate the speed of contrast change following ligand binding.

In the first strategy, ligand binding to MESA activates a transcriptional cascade via the release of a TF that induces expression of an encapsulin-tyrosinase fusion protein. In the assembly-dependent strategy, constitutively expressed encapsulin-tyrosinase monomers are prevented from

assembling into a functional nanocage by a steric block. Ligand binding to MESA reconstitutes TEVp, which cleaves the hindrance domain and permits nanocage assembly. The third approach facilitates a rapid contrast shift by switching pre-assembled nanocages between dispersed and agglomerated states. It is inspired by the split-protease-cleavable orthogonal-CC-based (SPOC) logic circuits system (Fink et al., 2019), in which externally displayed P3/P4 domains are initially masked by autoinhibitory segments. Upon ligand binding, TEVp cleavage unmasks these domains, enabling rapid contrast change.

I. ON SAFETY AND REGULATIONS

To further explore the potential implications of a mammalian cell-based tattoo, we conducted an online survey to collect empirical data on practical, personal, and ethical considerations relevant to the design and implementation of health monitoring technologies. Although results will be discussed in greater detail in future work, the demographic profile of respondents reflects the perspectives of a predominantly young adult population, with nearly half reporting experience with wearable health devices and most having at least some familiarity with genetic engineering¹. We present findings in relation to participants' perceptions of safety after being informed that the system uses mammalian cells (Figure 5):

How does knowing that the biosensor tattoo uses mammalian cells affect your perception on its safety?

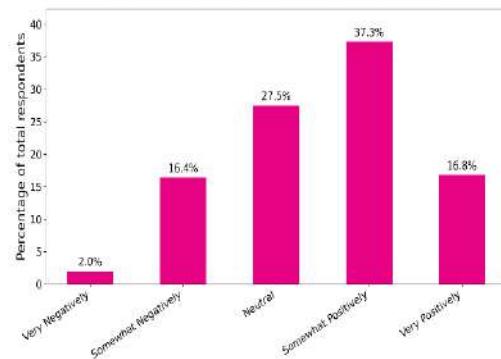


Figure 5. Percentage distribution of participant perceptions regarding the safety of a biosensor tattoo using mammalian cells ($N = 246$).

Regarding genetic engineering, 33% had moderate knowledge, 30% little knowledge, 17% basic knowledge, 9% professional experience, and 10% no knowledge with it. Further demographic details are available at the iGEM Munich 2025 Wiki (<https://2025.igem.wiki/munich/human-practices>).

¹ A total of 246 individuals completed the online survey: 57% were aged 18–24 and 25% aged 25–34. Gender was 58% female, 40% male, and 2% other or undisclosed. Nearly half (47%) reported current or past use of wearable health devices.

Note for Figure 5. Participants were asked: “How does knowing that the biosensor tattoo uses mammalian cells affect your perception of its safety?”, preceded by a brief explanatory text describing the genetic modification and type of cell utilized.

As shown in Figure 5, most participants (81.6%) report either neutral or positive perception of safety upon learning that the biosensor tattoo uses mammalian cells. This suggests that the use of mammalian cells is not broadly perceived as a significant safety concern among respondents, however, it is important to emphasize that this finding is limited to the surveyed population and should not be overgeneralized. Subsequent questions related to the safety and open-text responses indicated concerns regarding the longevity and long-term specificity of the engineered cells. These insights have directly informed the design and implementation of a functional kill switch as an added safety measure.

We also examined which application areas or use-cases respondents identified as sufficiently compelling to consider adopting a biosensor tattoo (Figure 6):

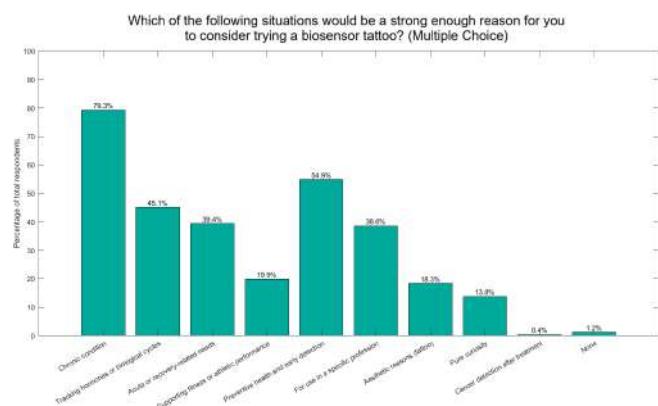


Figure 6. Percentage distribution of participant responses for potential application areas of a biosensor tattoo ($N = 246$). Participants were asked: “Which of the following situations would be a strong enough reason for you to consider trying a biosensor tattoo?” Respondents could select multiple options.

Figure 6 shows that the most relevant application areas for respondents are management of chronic conditions, early detection or preventive health monitoring, and tracking hormones or biological cycles. In direct response to this, we

have prioritized research on biomarkers such as: cardiac troponin I (cTnI, TNNI3) for its critical role in detecting and possibly preventing myocardial infarction (Thygesen et al., 2018), and progesterone for its importance in tracking hormonal cycles and reproductive health (Sun et al., 2023) as possible targets for the InkSight system.

Further considerations being explored in ongoing work include regulatory frameworks for the governance of mammalian-cell-based tattoos. Given its combination of genetically engineered mammalian cells and a hydrogel scaffold, our system would likely be classified as a combined Advanced Therapy Medicinal Product (ATMP) under Regulation (EC) No 1394/2007. The hydrogel itself would fall under EU Regulation (EU) 2017/745, which classifies hydrogels as Class III Medical Devices that require strict conformity to risk management assessments (Catoira et al., 2020).

IV. CONCLUSION

InkSight offers a promising and revolutionary approach for the continuous tracking of biomarkers directly in the ISF through an intuitive visual readout. This technology has the potential to reshape current medical capabilities in healthcare, empowering individuals to monitor aspects of their physiology in real time. Possible applications include supporting the management of anemia following cancer treatment by monitoring erythropoietin levels, preventing cardiac events by early detection of cardiac troponin, and providing visibility into hormonal fluctuations via progesterone tracking. These developments will contribute to quicker, more accessible, and patient-centered diagnostic approaches, bringing some monitoring capabilities from the clinic into the palm of one's hand.

Our ongoing research is focused on validating InkSight's performance, optimizing its sensing precision and visual output by testing our different agglomeration strategies and exploring different MESA receptors. At the same time, we are constantly reshaping our targets and experimental designs based on practical, ethical, and user-raised concerns observed in our work. Ultimately, with InkSight, we are laying the groundwork for merging the timeless artistic symbolism of tattoos with cutting-edge synthetic biology, reimaging them as a relevant and personal future diagnostic tool.

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Exploring the development of a continuous lactate monitoring system

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ABSTRACT

We aim to develop a wearable, patch-based lactate sensor that continuously monitors interstitial lactate levels without painful blood sampling and disposable strips, or interrupting training flow. Building on the success of continuous glucose monitors, our device will embed lactate-oxidising enzymes (e.g., lactate oxidase) within a microneedle array to catalyse lactate in dermal interstitial fluid into pyruvate and hydrogen peroxide. An integrated electrode will then oxidise the generated hydrogen peroxide, producing a current which correlates directly with lactate concentration. By tracking these electrical signals in near real time, athletes can optimise training and recovery on the fly while reducing recurring costs and biological waste. The market potential is also significant, not only to athletes in Norway but has a broader appeal to European athletes and sport enthusiasts as well. The Continuous Lactate Monitor (CLM) has already gained strong endorsements from elite coaches and institutions like Olympiatoppen, NIH (Norwegian School of Sport Sciences). Over the summer, we will express and immobilise the enzyme, prototype the microneedle-electrode interface, and validate sensor performance in laboratory conditions, laying the groundwork for a low-cost, sustainable sports biosensor. Wearable fitness technology continues growing rapidly, and our goal is to develop a device to empower athletes to push just right and reach new heights, through data-driven training.

Index Terms – electrochemistry, fitness research, lactate, technology, biosensors, genetic engineering

I. INTRODUCTION

During strenuous exercise, insufficient oxygen delivery will eventually lead to the shift from aerobic to

anaerobic respiration in muscle cells (Brooks, G. A et al., 2009). As a byproduct of glucose metabolism, lactate accumulates in the blood during exercise¹. Thus, lactate levels are a good indicator of exercise intensity and can be used to predict acute fatigue as well as efficacy of interval training (Huang T et al., 2025). As of today, athletes often rely on lactate analysers to monitor blood lactate levels between exercise intervals (Swart J, et al., 2004). This instrument requires a blood sample and will only inform of current lactate levels. Over the years, interest has grown for the potential development of continuous lactate monitoring (CLM) methods. The potential of such an instrument has been tested to moderate success. A common minimally invasive approach involves a biosensor embedded in a patch containing microneedles that penetrate the skin outer layer. By sampling interstitial fluid (ISF), the device enables lactate monitoring, setting it apart from conventional blood lactate meters. Such a method can be found in the paper by Freeman et al. (2023). Of course, the main issue with this is the known delay of lactate levels accumulating in the ISF due to diffusion from the blood, but this is something than will have to be accommodated for by using i.e. machine learning to optimise pattern recognition over time.

We aim to develop a proof-of-concept minimally delayed CLM system that uses enzyme-coated microneedles to convert lactate in the ISF into a measurable signal. To achieve this, two types of experiments need to work in parallel:

- (1) cloning, expression, large-scale production, and purification of functional enzymes suitable for this reaction.
- (2) developing a standardised electrochemical protocol to detect the enzyme-catalysed reaction and quantify the signal for different lactate concentrations. Two enzymes were selected for this purpose: lactate dehydrogenase and lactate

oxidase, as both can efficiently catalyse the conversion of lactate in the body.

II. MATERIALS AND METHODS

Cloning, expression and production

To acquire the enzymes to be used for creating a detectable signal proportional to lactate levels, two sequences were ordered – lactate dehydrogenase (LDH) and lactate oxidase (LOD). Following miniprep to isolate a pET28a vector from *Escherichia coli* (*E. coli*), the sequences were cloned using a Gibson assembly into TOP10 *E. coli*. Miniprep was done to isolate the plasmids containing the sequences. Validation was done using a gel, and in parallel, the plasmids were sent to a commercial sequencing facility to verify the identity correctness of the inserted gene sequences. The validated plasmids were transformed into BL21 (DE3) *E. coli*. After initial incubation, the bacteria were allowed to grow for 1.5h and OD₆₀₀ was tested to induce expression at mid-log phase (OD₆₀₀ ≈ 0.5–0.6).

OD₆₀₀ measurements were repeated at 4h and 16h under different conditions (37°C and 20°C, 200 rpm shaker) after induction with IPTG. An SDS-PAGE gel was run with standardised samples of the lysed bacteria under different conditions to identify optimal growth and expression conditions. Large-scale production (1L of bacteria) was then performed under optimal conditions, and the resulting solutions were spun down, and pellets were washed and frozen down. To purify the proteins following lysis of the bacteria, high-performance liquid chromatography (HPLC) and immobilised metal affinity chromatography (IMAC) will be performed to purify the proteins.

Electrochemistry

Prior to this step, enzymes need to be acquired and tested for functionality. LDH will be tested by adding a mixture of NADH and pyruvate with the enzyme to a microplate reader and testing absorbance at 340 nm, which is the absorbance of NADH (Igwe et al., 2023). If the enzyme is functional, the OD of the mixture should decrease as NADH is oxidised to NAD⁺. Several articles also explore the substantial difference in pH optimum for protein stability and activity, so this will have to be considered when performing a functionality assay (Esa et al., 2023). For LOD, one can bypass the need for a different assay through the combined use with LDH. LOD will convert lactate to pyruvate, and this mixture can then be added a functional supply of LDH and NADH,

either commercially available or a self-produced one. As the LDH reaction is reversible, pyruvate should be converted back to lactate while oxidising NADH, leading to a decrease in 340 nm absorbance.

For the length of the project, a PalmSens potentiostat was acquired to measure the desired reaction. Using PalmSens 2mm gold-plated screen-printed electrodes, a 60 µL droplet of 0.5 M H₂SO₄ in H₂O was applied to both working, reference and counter electrode, and cyclic voltammetry was used to clean the electrode of oxidative species. Then, 2 mg/mL of the enzyme was added onto the working electrode (3 µL droplet), and put in a 37°C incubator for 30 min. Following this, the electrode was rinsed 3X with PBS. A different mixture was applied onto the coated the electrode dependent on type of enzyme. For the LDH, different physiologically relevant concentrations of lactate and NAD⁺ was applied to allow for conversion to pyruvate. Cyclic voltammetry was performed, and small peaks of oxidation was found at 0.25V. This might correspond to NADH or lactate oxidation, thus chronoamperometry was performed at this voltage, testing different concentrations of lactate within the range of 0.5–10 mM to create a concentration curve.

III. RESULTS

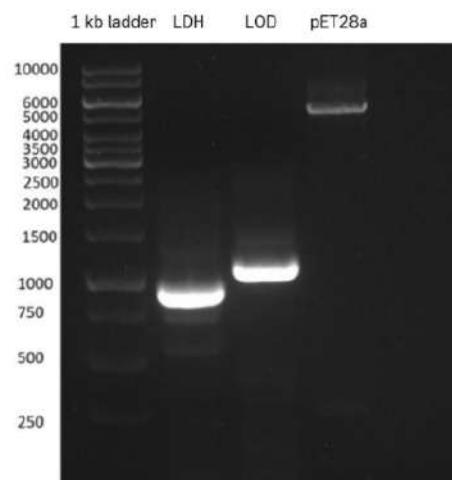


Figure 1. Initial agarose gel run of the gene sizes.

Sizes of the amplified gene sequences were verified with an agarose gel as shown in Figure 1. Following the transformation of TOP10 *E. coli*, the plasmid constructs LOD1 and LDH2 showed both correct sizes and sequences (Figure 2).

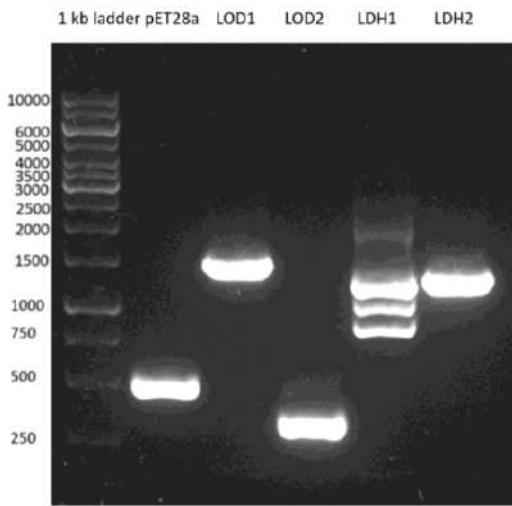


Figure 2. Agarose gel run of the plasmid constructs of genes with pET28a. Two replicates were prepared for each gene.

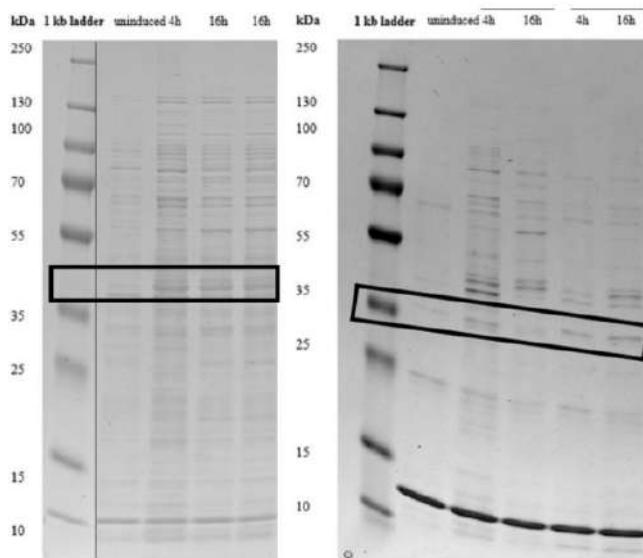


Figure 3. (left) and 4 (right). SDS-PAGEs of the proteins of lysed bacteria following transformation and expression induction. Figure 3 depicts the bacteria transformed with LOD and marked are the potential bands of the protein. Figure 4 shows the bacteria transformed with LDH, including the potential protein bands in each condition. Note that LOD was also tested initially for optimal conditions (see Table 1), though results were not consistent. In the case of LOD different loading volumes were used – depicted are the 5 μ L volumes, which were also used in the case of LDH. The ladder used was the PageRulerTM Plus from Thermo Fisher (#26619).

These plasmids were isolated with miniprep and used to transform BL21 (DE3) E. coli. As presented in Figures 3 and 4, conditions in Table 1 were the most optimal for growth, and large-scale production of both proteins was performed. Though successful in both cases, the bacteria expressing LOD took quite a while to enter exponential growth phase.

Table 1. Growing conditions of growth for bacteria transformed with the LDH and LOD constructs.

| | LDH-expressing | LOD-expressing |
|--|----------------|----------------|
| Incubation period before OD600 reaches 0.6 | 1.5h | 3.5h |
| Optimal temperature for expression | 37°C | 37°C |
| Optimal time of expression | 16h | 4h |

Figure 5. shows a close-up of cyclic voltammetry using LDH-coated electrodes. The oxidation peak might imply a reaction occurring, as this peak was absent from non-coated electrodes.

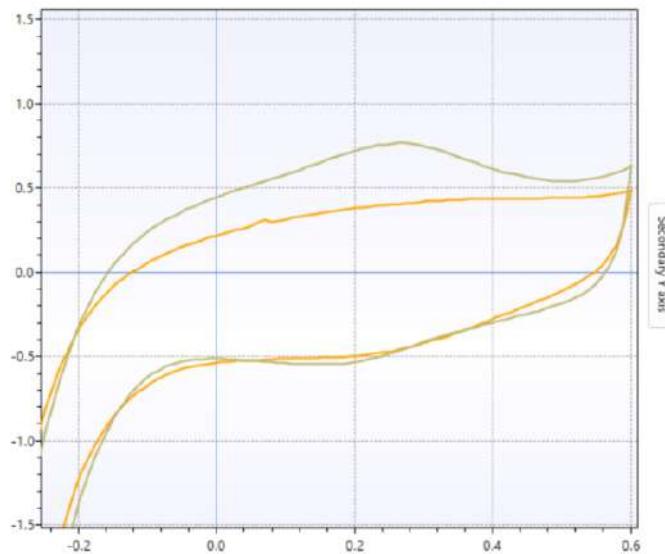


Figure 5. Cyclic voltammetry of 0.33 mg/mL LDH coating with 5 mM lactate and NAD⁺ in PBS (green) versus control (yellow).

Shown in Figures 6 and 7 are the concentration curves generated by performing chronoamperometry using LDH coated electrodes versus non-coated ones, showing that some

differences are observed even without LDH function. This will need to be taken into consideration.

Figure 6. The measurement of different lactate concentrations using chronoamperometry. The electrode was not coated.

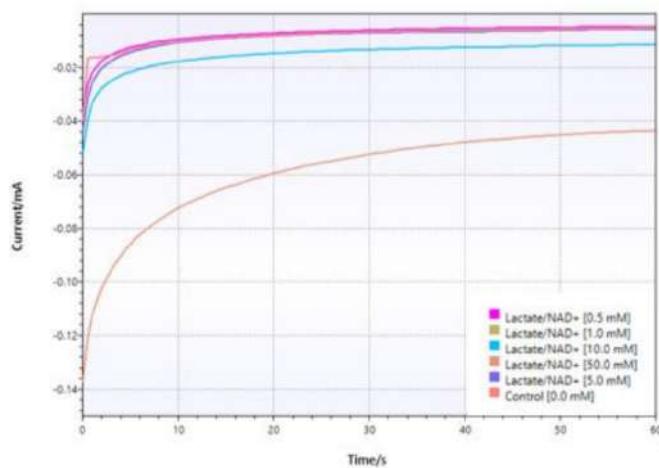
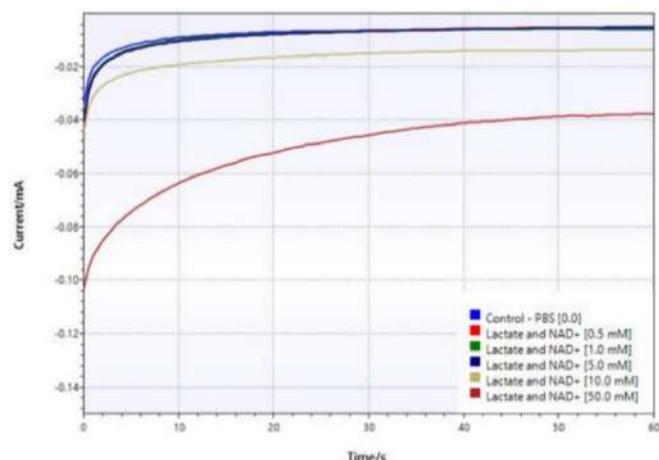


Figure 7. The measurement of different lactate concentrations using chronoamperometry. The electrode was coated with 2 mg/mL LDH.

IV. DISCUSSION

Enzyme production and purification

Regarding the production and purification of enzymes, LDH and LOD were successfully produced on large-scale and are soon to be purified. The optimal protocol for this workflow is presented in Table 1. LDH required less bacteria sample to show more intense SDS-PAGE bands than LOD and was found to express optimally at 16h incubation at 37°C after induction. As for LOD, 4h incubation at 37°C after induction

was chosen for mass production as the bands imply there is a possibility that the protein is unstable over longer periods. This will need to be tested using Western Blot after purification, and functional assays will show whether the enzymes produced are functional.

Electrochemistry

The electrochemical detection of the enzymatic reactions in vitro turned out to be challenging. It was observed that the coating step of the electrode was the limiting factor of these experiments. Weak oxidation peaks were observed for the electrochemical tests, implying some enzyme function, yet it was too faint to conclude anything substantial. The current hypothesis is that drying out the enzyme on the electrode destroys the enzyme structure. Yet, leaving the enzyme coating on the electrode without drying will prove problematic as the reaction might find place near the counter and reference electrodes, as opposed to solely being present at the working electrode.

Literature researched for this project made use of more complex coating components such as a PANI surface and Nafion to keep the enzyme encapsulated and hydrated on microneedle surfaces, as well as to both protect the electrode and mediate electron transfer (Rathee et al., 2016; Freeman et al., 2023). Thus, Nafion was acquired for future experiments to ensure the protein structure is attained. If no results are made from this, PANI might need to be produced to easier manipulate the enzyme's positioning. After all, an enzyme's specific conformation is important for function, as access to the active site and proper alignment for the reaction to take place are required.

Additionally, the chronoamperometry method was not reliable. Initial results showing differences between different lactate concentrations at -0.7V turned out to be present regardless of enzyme presence, implying that despite cleaning of the electrodes, some oxidative species are still present. Experiments in the future need to focus on cyclic voltammetry to find correct parameters to use for chronoamperometry. To reduce sources of error, NAD⁺ will be removed from the solution tested electrochemically, as the electrodes will work as both electron donor and acceptor – at least if the coating method used allows for direct electron transfer.

V. CONCLUSION

Regarding the transformation of *E. coli* and mass production of the proteins, the LDH and LOD genes were relatively easy to work with. Future lab work will be focused on the purification of these proteins and their use in the electrochemical aspect of the project. However, the bacteria transformed with LOD exhibited unusual behaviour as observed both during initial testing of expression as well as during large-scale production, requiring several attempts. The difference in the bands of induced bacteria growing for 4h versus 16h observed in the SDS-PAGE of Figure 3 seem to imply that the protein might degrade over time, and the slow growth of the large-scale bacteria solution might indicate that the protein is somewhat toxic to the bacteria. However, a Western Blot is needed to confirm the exact protein bands belonging to both LDH and LOD. To further study the characteristics of both proteins, future experiments could focus on making growth curves for both transformed types of bacteria in addition to conducting functional assays.

As for electrochemistry, further experimentation is needed on in vitro testing of redox reactions catalysed by enzymes before further conclusions can be drawn. As of now, the experience is that a higher complexity of protein coating is needed even for the low volumes used on 2 mm screen-printed electrodes.

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HydroGuard: A controlled release hydrogel-phage delivery system to combat catheter-associated urinary tract infections

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ABSTRACT

*Catheter-associated urinary tract infections (CAUTIs) rank as the fourth most significant nosocomial infection globally. CAUTIs cause 80% of infections in 40% of hospitalized patients, representing nearly one-third of all device-associated infections and significantly increasing morbidity and mortality. Urinary catheters serve as a hub for bacteria to stick on and form tough biofilms. Biofilm-forming pathogens such as *Enterococcus (E.) faecalis* reduce the effectiveness of treatments like antibiotics, while also impairing the immune system from effectively fighting infection. Therefore, tackling biofilm formation at the catheter surface represents a crucial step in mitigating the occurrence of CAUTIs. This project, entitled HydroGuard, addresses biofilm formation by engineering a Gelatin Methacryloyl (GelMA)-based hydrogel injected with bacteriophages, designed to provide targeted, enzyme-triggered antimicrobial activity. The hydrogel exploits the presence of gelatinase (*gelE*), an enzyme produced by *E. faecalis* during biofilm formation. The *gelE*-mediated degradation of the hydrogel matrix triggers localized release of bacteriophages that specifically lyse *E. faecalis*, creating a controlled release system. *GelE* was expressed in *Escherichia (E.) coli*, as a Biosafety Level 1 proof-of-concept with a well-studied host and phage system. Plasmids were cloned with recombinant *gelE* and transformed into *E. coli* for expression. Additionally, they were tested on dynamically grown biofilms, to simulate the urinary tract. Mathematical models were developed to predict phage release profiles and environmental sensitivities of the overall system. Currently, the *E. coli* proof of concept has shown slight inhibition when tested against the hydrogel*

product, however testing is still being continued with different phages and phage combinations. By integrating synthetic biology, tissue engineering, and modeling, HydroGuard offers a novel, nonantibiotic pathway for CAUTI prevention.

Index Terms – antimicrobial resistance, bacteriophage therapy, catheter-associated urinary tract infections, hydrogels

I. INTRODUCTION

Urinary tract infections (UTIs) are among the most common bacterial infections worldwide, affecting millions of individuals annually (Mlugu, 2023). In healthcare settings, the risk of infection is significantly heightened for patients who require longterm urinary catheterization (Potugari et al., 2020). Catheterassociated urinary tract infections (CAUTIs) account for approximately 75% of all hospital-acquired UTIs, representing a major public health and economic burden (Centers for Disease Control and Prevention [CDC], 2025). Between 15–25% of hospitalized patients undergo catheterization, and prolonged use inevitably increases the likelihood of bacterial colonization, biofilm development, and infection (Stickler, 2008).

The pathogenesis of CAUTIs arises from the ability of microorganisms to bypass the body's natural defense mechanisms through the catheter, adhering to surfaces and forming biofilms within the urinary tract. Biofilms are complex microbial communities encased in an extracellular matrix that enhances bacterial survival and antimicrobial resistance (AMR). Once established, catheter-associated biofilms are extremely difficult to eradicate, resulting in recurrent or persistent infections that contribute to significant patient morbidity and mortality (CodeliaAnjum et al., 2023). In the United States alone, CAUTIs account for over 13,000 deaths annually (Podkovik, 2019), contribute to

longer hospital stays, and generate an estimated \$1.6 billion in additional healthcare costs each year (Simmering, 2017).

While *E. coli* causes 80% of all UTI cases, it typically enters through gastrointestinal or oral routes rather than catheter-associated pathways, rendering it less relevant for catheter-driven biofilm infections. In contrast, *E. faecalis* is a Gram-positive, facultative anaerobic bacterium that thrives in the catheterized urinary tract environment, where it adheres to catheter surfaces, forms biofilms, and produces a zinc metalloproteinase called gelE (Del Papa et al., 2007). GelE degrades host proteins such as fibrinogen into peptides, which bacteria utilize for nutrition, while simultaneously promoting biofilm growth and persistence (Codelia-Anjum et al., 2023; Ch'ng et al., 2019). Furthermore, *E. faecalis* has strong AMR capabilities, often necessitating repeated or prolonged courses of antibiotics, exacerbating the global crisis of AMR (Del Papa et al., 2007). These properties make *E. faecalis* a critical pathogen in CAUTIs, despite its lower prevalence relative to *E. coli*.

Current clinical strategies to mitigate CAUTIs focus primarily on improved hygiene, catheter management protocols, and antibiotics. These approaches do not eliminate the underlying issue of biofilm formation, and infections remain common, particularly among elderly, critically ill, and chronically catheterized patients (CDC, 2025). As a result, CAUTIs continue to strain the healthcare system by causing significant patient discomfort, hospital readmissions, and clinical complications (Maharjan et al., 2018; Zhang et al., 2019). Addressing biofilm-associated infections at the site of infection can substantially improve patient outcomes while minimizing AMR. Currently, there are studies on both hydrogel formation for treatments and phage therapy, however our approach combines the two, using gelE as a strain specific-approach.

This study presents a novel GelMA hydrogel system embedded with a bacteriophage (phage) cocktail, designed to release phages in response to gelE-mediated degradation and directly target *E. faecalis* biofilms on catheter surfaces. GelMA is a biocompatible and biodegradable polymer derived from gelatin, easily functionalized via ultraviolet (UV) photocrosslinking (Sun et al., 2018; Dörterler et al., 2024; Zhao et al., 2016). Current conflicts in knowledge include if this can be used for other biofilm producing bacteria, as it is unknown if gelE is produced in other UTI-causing strains. A bioinformatic analysis is being conducted to investigate gelE production in other bacteria.

II. MATERIALS AND METHODS

This project employed a combined wet-lab and computational modelling approach to design and evaluate a bioresponsive hydrogel system to prevent and treat CAUTIs.

The methodology is centred around three interconnected components: gelE production and characterization, GelMA hydrogel fabrication, and phage encapsulation and release. The system was further optimized with bioinformatics analyses and computational modelling.

Gelatinase Production and Biofilm Simulation

GelE was selected as the enzymatic trigger for hydrogel degradation. To enable proof-of-concept testing under BSL-1 conditions, *E. coli* DH5α was genetically engineered to express gelE using three plasmid backbones: pUC19, pATEXP2, and pATEXP4.

Biofilms were grown on glass slides, with continuous shaking and media replenishment. This mimicked a dynamic environment with shear stress and was intended to replicate the catheter environment. They were grown for three days in LB, as infectious biofilms often develop in patients within 1-3 days of catheterization (Gunardi et al., 2021).

Hydrogel Fabrication and Characterization

The hydrogel was fabricated using GelMA, which was prepared by reacting gelatin dissolved at 10% w/v in PBS (pH = 7.8) with methacrylic anhydride at an optimum temperature of 40°C to introduce photo-crosslinkable methacryloyl groups. The resulting GelMA was dialyzed against deionized water for 48 hours, and the dialysis water was refreshed every 12-16 hours. Post-dialysis, the product was lyophilized for 6 days. The GelMA was then redissolved in PBS at 10%, 15%, and 20% w/v and supplemented with 0.5% Irgacure 2959 prior to photocrosslinking. The solution was cured under UV light at 365 nm for 30 minutes. Resulting hydrogels were mechanically tested for strength, malleability, and resistance to chemical degradation. The hydrogels were injected with a cocktail of lytic phages, targeting *E. faecalis*. Hydrogel degradation in the presence of gelE will be assessed by measuring weight loss, crosslink density changes, and release kinetics of embedded phages under physiological conditions.

Bacteriophage Cocktail Design

Whitelisted *E. coli* phages were donated to the team. A lytic phage cocktail comprising T7, T4, T2, and P1 was selected for incorporation into the hydrogel matrix. These phages were chosen for their broad host range and demonstrated activity against biofilm-forming bacteria. Propagation was done to increase concentration, as well as genomic extractions and digestions to ensure the correct phages were obtained.

Computational Modelling

Several computational modelling approaches were developed to complement the wet-lab experiments. Biofilm growth was simulated using an agent-based model that incorporated nutrient availability, pH, temperature, and flow dynamics, and was validated against experimental biofilm

assays. To describe phage transport within the hydrogel, a poroelastic partial differential equation framework was constructed, capturing diffusion behaviour in a degrading GelMA matrix with time-dependent geometry and swelling effects. Finally, phage–bacteria interactions were modelled using Michaelis-Menten kinetics, enabling predictions of bacterial suppression by phage cocktails. Together, these modelling approaches provided a multiscale understanding of biofilm colonization, hydrogel response, and phage-driven bacterial clearance.

Bioinformatics and Protein Design Optimization

A genomic analysis of *E. faecalis* strains was conducted to investigate gelE co-expression with other virulence and biofilm-associated genes. Collagen-like peptide motifs (GPH repeats) were computationally redesigned using MPNN sequence optimization, with thousands of variants screened in AlphaFold2 for folding confidence, stability, and protease accessibility. Structural validation was performed with PyRosetta and Poisson-Boltzmann electrostatics simulations, enabling the design of synthetic peptides with tunable crosslinking and degradation properties.

III. RESULTS

Phage Propagation and Validation

Seven *E. coli* phages (T2, T4, T7, P1, λvir, and ΦX174) were successfully propagated and tested for lytic activity. Clear lysis zones on *E. coli* lawns confirmed infection by all phages (Figure 1).

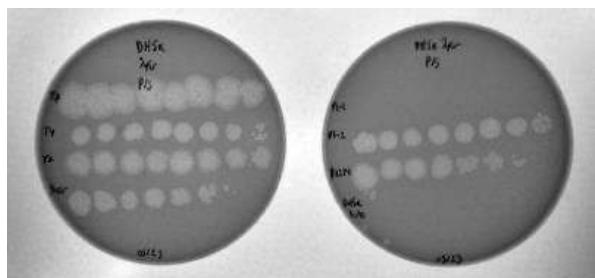


Figure 1. Genome Extractions of Titred *E. coli* Phages T2, T4, T7, P1, λvir, and ΦX174.

Agarose gel electrophoresis of extracted genomic DNA revealed strong bands for T2, T4, T7, and P1, while λvir and ΦX174 yielded faint or degraded bands. Restriction digestion produced distinct banding patterns for T2, T4, T7, and P1 that matched in silico predictions (Figure 2). λvir digestion produced weak but interpretable fragments, whereas ΦX174 remained inconclusive due to persistently low DNA yield.

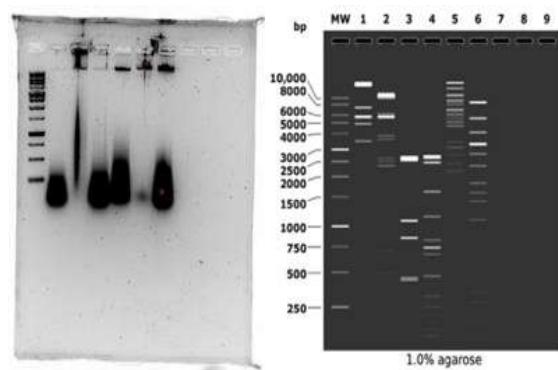


Figure 2. Gel Electrophoresis of Extracted Genome Digestions and Simulated Gel.

Plasmid Validation for GelE Expression

Plasmids pATEXP2, pATEXP4, and pUC19 were amplified using PCR and validated and purified through gel extraction (Figure 3). Codon-optimized gelE fragments were also amplified for each plasmid. pUC19 with the gelE fragment was constructed using Gibson Assembly. Recombinant plasmid pUC19 was then transformed into DH5 α competent *E. coli* cells for cloning, and then into BL21 competent cells for optimal protein expression, via heat shock. The transformation was screened and cultured, to obtain a freezer stock of BL21 containing the construct. Ultimately, *E. coli* cells that express the gelE fragment were obtained; these cells were then used to mimic the behaviour of biofilm-forming *E. faecalis* cells for testing against the constructed hydrogel.

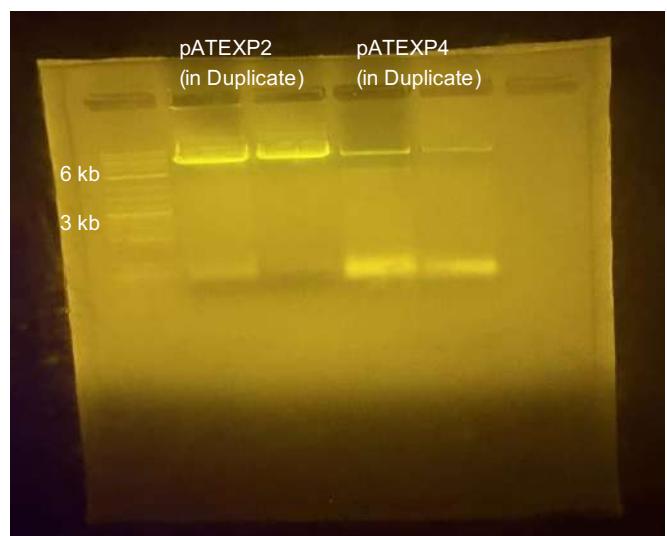


Figure 3. Gel Visualization of pATEXP2 and pATEXP4, respectively.

Hydrogel Diffusion Modelling

To characterize phage release dynamics, a poroelastic PDE model was developed to simulate protein diffusion from a degrading GelMA slab. The model incorporated time-dependent geometry and variable porosity to reflect enzymatic degradation by gelE. Simulations predicted a nonlinear release profile, with rapid diffusion during early degradation stages followed by slower release as hydrogel thickness decreased (Figure 4). Sensitivity analysis revealed that increasing crosslinking density reduced effective diffusivity, delaying phage release, while larger pore sizes accelerated release kinetics. These results highlight tunable hydrogel parameters that directly influence therapeutic phage availability during infection.

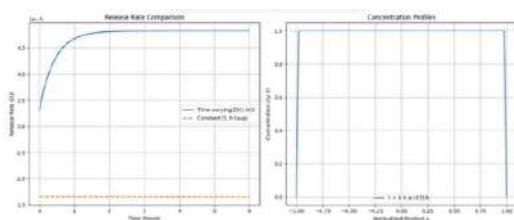


Figure 4. Simulation of Protein Diffusion from Degrading Hydrogel Over Time.

Protein Design Modelling

In parallel, protein modelling was conducted to design synthetic collagen-like peptides to reinforce GelMA hydrogels by modulating crosslinking and degradation rates. Starting from GPH repeating motifs, thousands of peptide variants were generated using constrained MPNN redesign, followed by structural predictions in AlphaFold and electrostatic stability analysis via Poisson–Boltzmann simulations. Candidate sequences were filtered based on structural similarity to parent peptides, fold confidence, and stability under physiological pH. Final candidates were validated in silico using PyRosetta. The resulting designs suggest that select engineered peptides can increase hydrogel stability while maintaining gelE-degradable motifs, optimizing hydrogel responsiveness (Figure 5).



Figure 5. Design Process of a Synthetic Collagen-like Peptide to Improve Hydrogel Stability, Degradation Rates and CrossLinking Efficiency.

IV. DISCUSSION

This study demonstrates the feasibility of a hydrogel-based, enzyme-responsive phage delivery platform to address CAUTIs. By combining wet-lab phage propagation and genetic engineering with computational modeling of hydrogel degradation, phage–bacteria dynamics, and protein design, several critical components of the system were validated.

Phage propagation confirmed that lytic *E. coli* phages T2, T4, T7, and P1 can be reliably amplified and characterized, providing the foundation for a GelMA-embedded phage cocktail.

All plasmids and fragments were successfully amplified and gel extracted. Currently, only the pUC19 construct has been transformed into *E. coli*, further steps for this study include Gibson Assembly for the pATEXP2 and pATEXP4 fragments. Nevertheless, construction of gelE-expressing plasmids remains essential for simulating *E. faecalis* virulence in *E. coli*, enabling enzymatic degradation studies in a BSL-1 environment prior to pathogenic testing.

Biofilms were successfully developed after 72 hours of cultivation. Currently, testing of these biofilms is underway. The T7 phage alone has been currently tested on a biofilm and has showed slight inhibition of *E. coli* growth. Ongoing tests include a cocktail of phages on the biofilm.

Hydrogel diffusion modeling demonstrated strong dependence of phage release on degradation dynamics, highlighting the importance of cross-linking density and composition to balance early antimicrobial action with controlled delivery. Protein modeling was used to propose synthetic collagen-like peptides with optimized folding, stability, and accessible gelE cleavage sites for enhanced hydrogel durability and performance.

Together, these results highlight the potential of enzymeresponsive hydrogels as a dual-function therapeutic, capable This strategy addresses several key shortcomings of current CAUTI management, including antibiotic overuse, inability to penetrate biofilms, and high recurrence rates. The approach is also extensible, as the modular design of the hydrogel and phage cocktail allows for adaptation against other uropathogens that secrete degradative enzymes.

Beyond its technical novelty, this work holds considerable clinical and societal relevance. A system that releases phages only when infection is detected has the potential to minimize unnecessary antimicrobial exposure while eradicating target biofilms. Reducing the incidence of CAUTIs could lower patient morbidity, prevent recurrent infections, and decrease hospitalizations, particularly among populations that are elderly, chronically ill, or dependent on long-term catheterization. The economic implications are equally important, as CAUTI-related hospitalizations can

reach thousands of dollars per case. Effective prevention and treatment would reduce reliance on expensive antibiotics and shorten inpatient stays, alleviating pressures on healthcare systems.

Environmental and biosafety considerations were incorporated into the design. GelMA is biodegradable and safe for disposal and the phages used are lytic and non-toxin-producing. These features provide assurance of clinical and environmental safety, supporting the translational potential of this system.

Overall, the work presented here establishes a foundation for further optimization, including validation of gelE-triggered release in vitro, and expanded testing in biofilm models. The modularity of this approach also suggests its adaptability to other pathogens that secrete degradative enzymes, providing a broader platform for development of antimicrobial biomaterials and device-associated infection treatments.

V. CONCLUSION

This presented study outlines a novel gelE-responsive hydrogel for phage delivery, offering a targeted strategy to prevent and treat CAUTIs. By coupling enzyme-triggered hydrogel degradation with lytic phage release, the system addresses limitations of antibiotics in eradicating biofilms. The demonstrated feasibility, combined with favourable biosafety and translational potential, positions the project as a promising intervention for device-associated infections.

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Engineering a Systematic Pipeline for Site-Specific Lipid Functionalization of Enzymes

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ABSTRACT

Protein therapeutics have revolutionized modern medicine; however, their efficacy is often limited by rapid clearance from the body. L-asparaginase, a chemotherapeutic enzyme used to treat the most commonly diagnosed childhood cancer, exemplifies this challenge. Advances in synthetic biology now allow incorporation of nonstandard amino acids (nsAAs) via an orthogonal translation system (OTS), facilitating copper(I)-catalyzed azide-alkyne Huisgen cycloaddition (click chemistry), which introduces an alternative to bioactivity-limiting terminal end modifications like PEGylation. However, selecting sites for nsAA incorporation in catalytic enzymes remains challenging, especially for multisite modifications, due to the sensitivity of protein folding and function. To address this, we used a conserved sequence database and variant feature analysis to prioritize candidate sites, then employed Golden Gate assembly to generate modified L-asparaginases with single-codon nsAA reassignments, experimentally assessing their catalytic kinetics to identify activity-preserving sites. Multiple sites of non-standard incorporation were found to preserve bioactivity comparable to the wild-type. Using our experimental results as ground truth, we executed a comprehensive computational ensemble pipeline involving molecular dynamics, molecular docking, and protein stability analyses to develop a neural network model that predicts bioactivity upon nsAA incorporation. This integrated pipeline establishes a novel methodology that bridges experimental validation with computational prediction, laying the foundation for accelerated protein engineering efforts by dramatically reducing experimental screening requirements.

Index Terms – nsAAs, ncAAs, L-Asparaginase, chemotherapy, acute lymphoblastic leukemia, copper-mediated click chemistry, palmitic acid, lipid-functionalization, molecular dynamics, orthogonal translation systems, bioactivity, azide-alkyne click, PEGylation alternatives

I. INTRODUCTION

L-asparaginase, a protein chemotherapy, is a primary treatment for acute lymphoblastic leukemia (ALL), the most commonly diagnosed childhood cancer [1]. This enzyme facilitates the breakdown of the vital amino acid L-asparagine into its components: L-aspartic acid and ammonia. By depleting L-asparagine required for protein synthesis in cancer cells, the treatment leads to cell death, thereby controlling the infamously unrestrained proliferation characterizing cancer [2].

Recent synthetic biology advances have shown that clicking fatty acids onto nonstandard amino acids (nsAAs) has the potential to improve the notoriously weak pharmacokinetic profile of L-asparaginase. These derivatives of the natural 20 amino acids can precisely incorporate modifying groups through an azide–alkyne Huisgen cycloaddition (click chemistry). However, previous attempts using the phenylalanine derivative para-azidophenylalanine (pAzF) presented major issues such as azide degradation [3]. Another significant limitation was the sole incorporation of functional groups at a terminal end, which reduced bioactivity [4].

In this study, we sought to conserve L-asparaginase activity when replacing native amino acids with nsAAs at select target sites. We hijacked the native translation system of a recoded organism, C321, to read our orthogonal translation system (OTS) to site-specifically incorporate the non-standard amino acid p-propargyloxy-l-phenylalanine (pPaF), which will later serve as specific target sites to conjugate an azide-based palmitic acid [5, 6]. Limited effect of site switches on function would demonstrate that intra-protein conjugation is more favorable than the termini integration strategies like PEG-asparaginase and previous lipid functionalized treatments. We developed an MDDSEM (Molecular Dynamics, Docking, and Stability Ensemble Model) on our bioactivity results to predict potential sites of incorporation within L-asparaginase. Ultimately, these comparative studies can be used to make protein therapeutic treatment less frequent, more effective, and more compatible with the human body.

II. MATERIALS AND METHODS

Bacterial Strains & Variant Creation

Wild-type optimization tests were performed with the genetically recoded organism (GRO) (*E. coli* C321.A, CP006698.1, GI:54981157). Experimental tests involved a modified C321 strain with the orthogonal translation system (OTS) containing plasmid pAcFRS.1.t1(DG.OTS). The L-asparaginase gene was modified through Site-Directed Mutagenesis, then chemically transformed into the GRO as per NEB instructions.

Protein Expression, Purification, and Bioactivity Testing

Firstly, 5mL each of LB media with appropriate antibiotics was inoculated with the appropriate variant and incubated overnight shaking. Then, the control cultures were back-diluted with LB media with antibiotics to OD 0.1. The variant cultures were back-diluted with LB media with antibiotic to OD 0.1, 0.2% arabinose, 1mM pPaF, and 2mM HCl. Once OD 0.45-1.2 was met, the culture was induced with 100ng/µL aTc, then incubated.

The culture was then transferred into a Falcon tube and spun down. The pellets were then resuspended in Bugbuster with protease inhibitor, lysed then spun. The soluble portion was then purified with the Zymo Research HIS-spin Miniprep kit as per the manufacturer's instructions. The Negative Control strain was not purified. The final elution was then collected and buffer exchanged through a 30kDa

MWCO Amicon filter as per instructions until a 1:1000 dilution.

Lastly, the resulting elutions were run through an SDS Page as per BioRad's Guide to Polyacrylamide Gel Electrophoresis and Detection and the Activity Assay. The bioactivity of the produced proteins was tested by measuring the OD570 colorimetric output of the BioVision Asparaginase Activity Assay Kit (ab107922) as per the manufacturer's instructions within a 96-well plate. The Red Alexa Fluor F647-Azide and a CuAAC Biomolecule Reaction Buffer Kit (THPTA-based) from Jena Biosciences was used to click a fluorescence tag onto one variant with pPaF incorporation.

Computational Prediction Pipeline for nsAA Incorporation Sites

We developed MDDSEM, an *in silico* ensemble model that utilizes three orthogonal approaches to molecular modeling to ascertain a common mechanism of the loss of catalytic activity in L-Asparaginase. We extracted learned patterns from a multilayer perceptron model and made predictions for novel incorporation sites.

Molecular dynamics simulations utilized Schrodinger's Maestro to introduce nsAA mutations into our monomeric structure (PDB: 6EOK:A) and Schrodinger's Protein Preparation Wizard for protein preparation. We used Schrodinger's Desmond suite for system solvation, system relaxation, energy minimization, and simulation. Molecular dynamics simulations utilized the TIP3P solvation model and ran for 250 nanoseconds. We extracted protein-wise Root Mean Squared Deviation (RMSD), residue-wise Root Mean Squared Fluctuation (RMSF), Radius of Gyration (Rg), and Solvent Accessible Surface Area (SASA) quantities to define a dynamic profile for each mutant.

Molecular docking simulations utilized the ComBind methodology, which refines the poses generated by Schrodinger's Glide suite [7]. By providing four crystal structures of a homodimeric EcII L-Asparaginase co-crystallized with known ligands, we fine-tuned docking predictions through feature extraction of binding behavior. Thus, docking can be calibrated for new ligands, especially if newly docked ligands are similar in structure to ligands in our reference library. We created mutant homodimers by using phenylalanine as a proxy for the nsAA within AlphaFold3 before mutating the residue to the nsAA using Maestro. We then assembled the dimer by aligning the post-MD monomer

with experimental crystal structures to form the active site and relaxed the system. We docked according to the ComBind workflow, extracting hydrogen bond contacts, van der Waals contacts, binding distances, and the ComBind score to define a docking favorability profile. Protein stability simulations involved using $\Delta\Delta G$ values for incorporation of the nsAA relative to the wild-type monomeric protein as a proxy for incorporation stability. We also performed ΔpI analysis to observe the change in isoelectric point for the protein at every possible residue, as the protein's overall charge can affect interactions with nearby solvent.

All extracted features were combined to train a multilayer perceptron model predicting mutant bioactivity, using Adam optimization and a sigmoid activation function, validated with our observed bioactivity. We also annotated active site residues and buried residues critical to the core structure of the protein and extended the model using transfer learning, retraining the network with a random forest model on residue-level annotations from PDB entries. This allows the model to predict dynamic, docking, and stability profiles from residue-level features beyond our limited wet lab dataset.

RESULTS AND DISCUSSION

Bioactivity at 3 Sites is Conserved with pPaF Incorporation

After purification, we used the BioVision Asparaginase Activity Assay Kit to measure aspartate output over time.

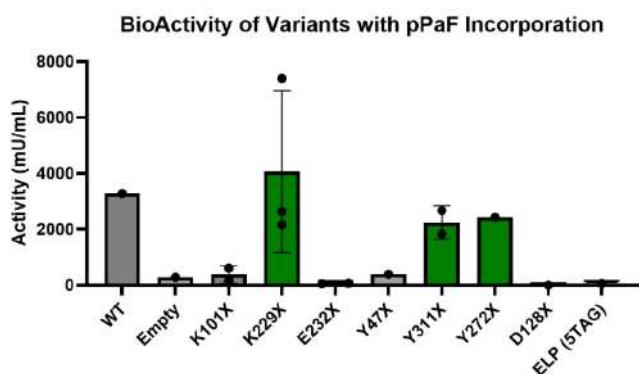


Figure 1. Activity Assay Demonstrates Bioactivity is Conserved at K229, Y311, and Y272. Analysis of plate reader data demonstrates the activities of the modified protein produced by each variant where mU/mL is defined as nmol of Aspartate converted per minute per original volume in mL.

Preliminary results testing purified samples suggest that the sites K229, Y311, and Y272 are strong candidates for fatty acid conjugation, as bioactivity is largely conserved. Furthermore, the conservation of bioactivity confirms the ability of DG.OTS to incorporate the linear alkyne pPaF, suggesting further promiscuity than previously thought. Additionally, these results also support our site selection methodology using protein database analysis [8]. A comparison of the selection methodology can be further confirmed once all target variant strains are cloned. For one experimental site, an azidal fluorophore was copper-clicked, suggesting successful integration of the alkynal pPaF. This will be repeated for the rest of the variants in future experimentation.

MDDSEM Feature Extraction Reveals Novel Association Between Dynamics, Stability, and Bioactivity

To evaluate MDDSEM, we applied the ensemble model to novel incorporation sites using the same workflow described in Methods, integrating MD, docking, and stability simulations. Using MDDSEM, we identified four potential incorporation sites with at least a 0.7 probability for maintaining catalytic activity. In Figs. 2B and 2C, MD simulations of these predicted-active mutants featured active site residues that exhibited intermediate magnitudes of flexibility that closely aligned with those of the wild-type enzyme and other experimentally validated bioactive mutants, implying that our MD information includes informative features to our model of whether a mutant features a similar dynamic profile to wild-type. Fig. 2A demonstrates the critical nature of our stability analysis, positing that more structured dynamics are integral to maintain catalytic activity. Internally, we employed leave-one-out cross-validation (LOOCV) as an estimator of model effectiveness. Our model correctly predicted the bioactivity of 89% of our input mutants with this approach.

Importantly, the multi-modal approach—combining MD, docking, and stability features—provided distinct value: while docking and stability scores were not the highest-ranked features individually, excluding both reduced cross-fold validation accuracy, demonstrating that orthogonal analyses improve predictive performance. Additionally, residues contributing to the homodimer interface in these mutants displayed lower fluctuations relative to inactive mutants, highlighting the importance of interface stability in preserving bioactivity.

Future direction involves clicking a palmitic acid on sites of interest, then comparing the bioactivity of fatty-acid conjugated L-asparaginase to the C-terminally conjugated variants and non-clicked variants. Furthermore, additional sites from the MDDSEM will be cloned to further test the machine learning model. Ultimately, this research demonstrates the successful selection of intraprotein sites susceptible to modification with minimal decrease in bioactivity. By optimizing the stability of L-asparaginase, this research seeks to reduce dosing frequency and mitigate adverse reactions associated with current modifications like PEGylation, offering a promising avenue for safer and more effective ALL therapy.

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Reviewer Feedback

All opinions expressed below are solely given in a personal capacity and do not reflect the views of AAAS/Science. These comments should in no way be construed as relating to evaluation of Science papers.

Dr. Michael Funk's Feedback for the Top Three Papers, as per the Peer Review:

Leiden University: A Novel Method for Protein Engineering: Post-Translational Modifications Using Self Integrating Scaffolds

van Neer et al. conducted experiments on a split intein system with results including production of the plasmid constructs and preliminary expression of a model protein for use in FRET-based trans-splicing assays. The introduction of this article gives a clear account of the challenge and motivation, which is production of homogeneous glycan-modified proteins using established split intein technology. The results so far are a promising start and demonstrate the authors' ability to follow standard molecular biology protocols for generating functional expression constructs. There are two main issues for improvement: 1) The schematics and methods do not directly correspond to the results presented, which makes it hard to understand what was done. The authors appear to have built several plasmid constructs and show DNA gels, but these results need to be explained in more detail so that it is clear what is being presented and why. Although the schematics in fig 1 and 2 are helpful for understanding the planned assays, it was unclear how the "MBP-HA-GFP" construct related to either of these schematics. Additionally, the figure numbering needs to be checked as the results refer to fig 4 and 5 instead of fig 3 and 4. 2) Although advance planning is admirable, it does not seem appropriate to include methods if there are no corresponding results presented. An alternative would be to go through your plan for future experiments in the discussion. This would help avoid confusion as to what was done so far. I appreciated the discussion of limitations and generally thought the authors had done a good job highlighting recent work in this field that might be useful to consider when planning how to achieve their ultimate goal.

McMaster University: A Review on Acute Graft Rejection Detection: Current Methods and Emerging Solutions

Bui et al. present a review on kidney transplant acute rejection, primarily covering diagnostic approaches that serve as an alternative to invasive biopsy. The review is focused on very recent developments and includes a number of personal communications from clinicians. This draft article could be well suited to development into a news feature instead of a scholarly review. However, I think this work is well presented and do not have any major comments for improvement.

Queens University: Foundations for an AHL-Responsive Genetic Circuit in Eukaryotic Cells: A Biofilm-Disrupting Therapeutic for Cystic Fibrosis

Leuchter et al. used docking simulations to characterize acyl homoserine lactone-binding transcriptional regulatory proteins involved in bacterial quorum sensing. The article explains the motivation for this work is the use of such proteins as eukaryotic cell biosensors for pathogens such as *Pseudomonas aeruginosa*. The core work presented in the paper is in silico analysis of the protein constructs to assess their ability to bind ligands and DNA. One important issue the authors should discuss further is what data to provide in the main text vs. the supplemental figures. The paper has only one figure and one table despite referencing multiple supplemental figures that cover key parts of the construct design. Additional discussion of the docking procedure and figures showing the docking pose with ligands would be helpful to evaluate this part of the results. The project is currently at an early stage, so clear explanation of the approach, goals, and design principles are necessary. Although the authors include some references in the introduction and methods as motivation, the discussion would be stronger if the authors could reflect on some of the limitations of the approach and compare to other recent work that addresses similar problems in different ways.

Reviewer Feedback

All opinions expressed below are solely given in a personal capacity and do not reflect the views of AAAS/Science. These comments should in no way be construed as relating to evaluation of *Science* papers.

Dr. Yevgeniya Nusinovich's Feedback for the Top Three Papers, as per the Peer Review:

Leiden University: A Novel Method for Protein Engineering: Post-Translational Modifications Using Self Integrating Scaffolds

In this project, van Neer et al. set out to design a system for targeted protein modification, which would allow researchers to introduce glycosylation sites into proteins on demand. This type of system could be very useful for the production of therapeutic antibodies, where the glycosylation patterns can play important roles in their overall function, and potentially also for other protein-based therapeutics. To achieve this, the authors designed a self-integrating scaffold system based on a split intein mechanism, then began to construct the individual components of the system. They do a nice job of explaining what they want to do and why, as well as highlighting some potential challenges and limitations of this approach, but the experimental part of the study is still very preliminary at this stage. One thing the authors could consider doing is extending the discussion of their future research plans further out to help bridge the gap between the early data being presented and the comments about this method's potential relevance for therapeutic development, but that part would necessarily be just speculative until the project reaches more advanced stages.

McMaster University: A Review on Acute Graft Rejection Detection: Current Methods and Emerging Solutions

The review article by Bui et al. focuses on the clinical needs, challenges, and ongoing advances in the timely detection of kidney transplant rejection. This is an important topic in clinical practice, and the authors provided an information-rich writeup that pulls together a large amount of content on a variety of research advances, as well as some key societal considerations, underlining the relevance of this material. To help convey this trove of information to the audience, the article could benefit from some improvements in organization and presentation. For one thing, it would be important to state what kind of graft you are going to talk about from the very beginning, and incorporate some mention of the kidney into the title and abstract to avoid confusion. It would also be helpful to delineate more clearly what is currently standard practice and what is still work in progress. To that end, it would help to move the overview of current practices into the introduction section, and reserve the main part of the manuscript for the emerging and future technologies. Finally, it may be best to minimize the reliance on personal communications or even avoid them altogether and instead gather equivalent information from published sources such as textbooks and clinical guidelines, so as not to detract from the strong scientific grounding in other parts of the review article.

Queens University: Foundations for an AHL-Responsive Genetic Circuit in Eukaryotic Cells: A Biofilm-Disrupting Therapeutic for Cystic Fibrosis

Leuchter et al. report on their efforts to engineer biological systems to disrupt *Pseudomonas aeruginosa* biofilms that can be deadly for patients with cystic fibrosis. The goal of the work is to engineer eukaryotic cells that would detect bacterial proteins involved in quorum sensing that drives biofilm formation, and then trigger a therapeutic response to the detected biofilm. The work is at a relatively early stage so far, but the authors are meticulous and systematic in their efforts to isolate the relevant components for their desired biological circuits, modify them for use in eukaryotic systems, and then test each piece in silico at first, then eventually in vitro and in vivo. The therapeutic component of the circuit is not yet connected to the detection component, but each of them is being individually designed and optimized, and the authors have clear plans for how to move forward and eventually connect the pieces once the individual components are in place. What isn't clear from the writeup at this stage is why it would be beneficial to do this in a eukaryotic system, however, and what practical advantages that might offer, as compared to introducing a similar biological circuit in prokaryotes (which has been done before) and then delivering those engineered prokaryotes into patient lungs as a living therapeutic.

Acknowledgements

The revival and continuation of *Vector* for its 4th edition would have been impossible without the participation and hard work of all of this year's participating teams. As such, we extend our deepest gratitude to all of the teams whose papers make-up this edition of *Vector*. We understand that writing a scientific paper is not an easy task, especially doing it in addition to the extensive required workload that comes with working on an iGEM project. Thank you for your cooperation, for sticking with us through all of the steps of the process and the associated deadlines. We hope that you sharpened your academic skills, explored some of the core tenets of iGEM - collaboration and teamwork, and most importantly, enjoyed being a part of this initiative. Congratulations on your papers, you should be proud!

To our reviewers, Dr. Yevgeniya Nusinovich and Dr. Michael Funk, by lending your time, expertise, insight and feedback, you have enriched the quality of this journal as a whole, but also, provided a unique teaching opportunity for all of the young aspiring researchers and scientists who submitted their work. It is certain that the guidance you have shared will stick with the authors and continue to inform and aid their scientific and academic journeys for a long time. We are thankful for your support and openness to work with us on this project and look forward to the possibility of collaborating again in the future.

The whole of our iGEM journey, including this very journal, would not have been possible without the continued support of our sponsors and partners, as well as many others who contributed via individual personal donations. Thank you for believing in our idea, our project, and our goal. Thank you for believing in *us*.

To Hassan A. Tahini and the rest of the Science Brush team, thank you for your help in designing and creating this year's *Vector* cover page. To Ridderprint, thank you for covering the cost of printing this journal. Your expertise and work transformed the idea of the 4th edition of *Vector* into a reality.

We would also like to issue a thank you to Anirudh Rajesh, CEO of *Aestuarium* - the start-up founded by MSP iGEM's 2023 team. He encouraged us to continue with *Vector* and provided us with helpful materials and advice.

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We hope this is just the beginning!



Afterword

While a healthy dose of scepticism is more than necessary when engaging with potentially impactful technology like genetic engineering, we encourage our readers to leave this year's issue of *Vector* with an optimistic outlook on the contributions of young researchers to the direction of scientific development.

The collaborative collection of research presented in this journal is part of a larger story of how synthetic biology is changing the face of scientific innovation, sustainable development and the world at large. From acute graft rejection detection to vanillin synthesis, and from reviews on human practices within iGEM to tattoos as diagnostic tools, this issue showcases a diverse and impressive range of applications of synthetic biology, and this anthology is proof that these aspiring scientists and successful authors have bright, interesting and impactful careers ahead of them. We can only commend them for their hard work and scientific integrity, and we hope that you, the reader, recognise the importance of global scientific collaboration, towards a better future.

While it is important to consider the wider impacts of the scientific work which we do, and the potential for it to be misused, it is equally as important to bear in mind that *real* fundamental change could be just one DNA set away from reality.



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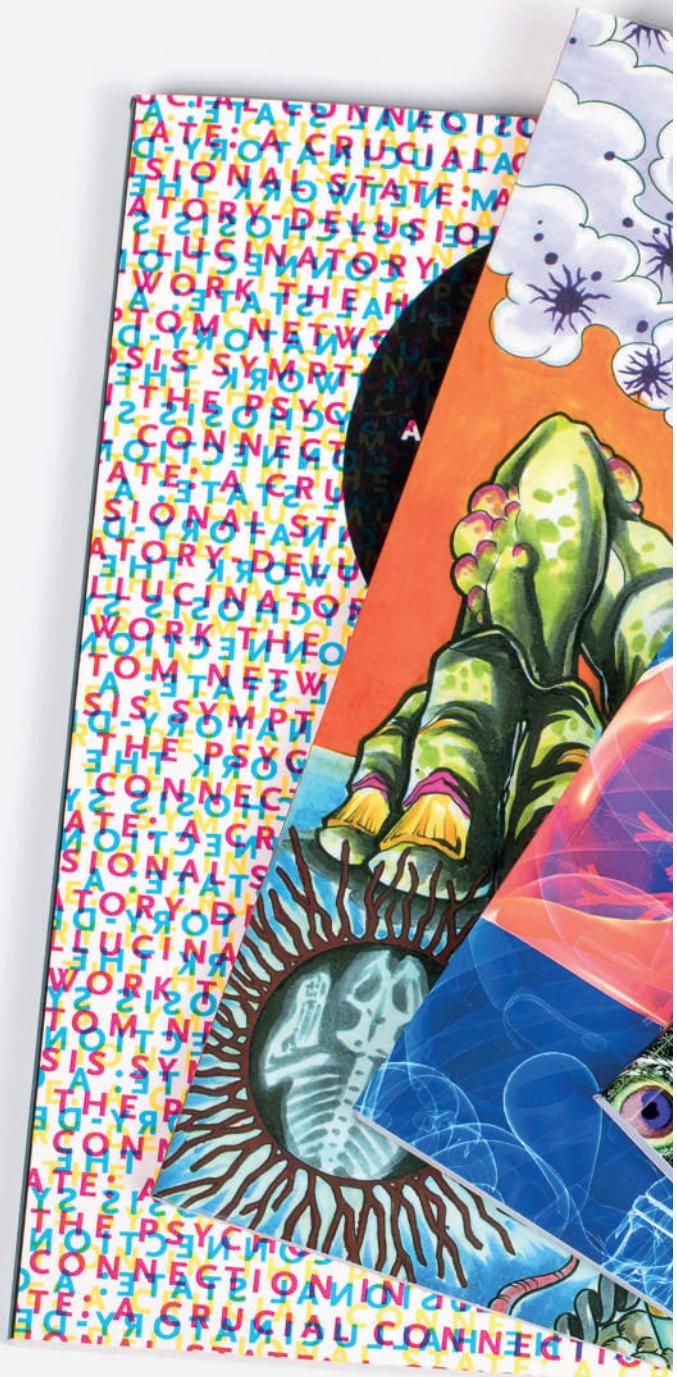
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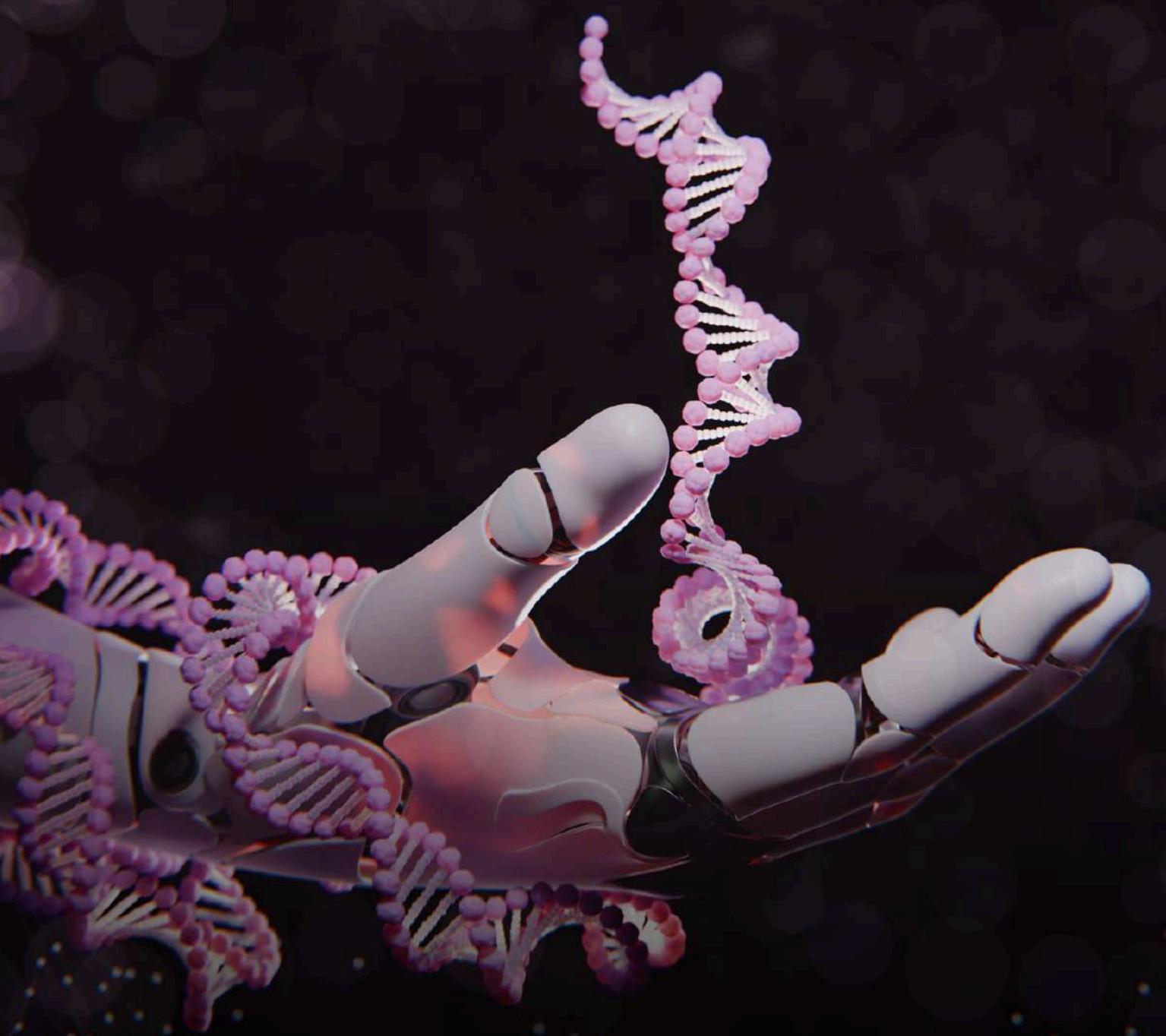
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