MSP-Vector

The unofficial iGEM Proceedings Journal 2022

Research is what I'm doing when I don't know what I'm doing.

WERNHER VON BRAUN

October 2022, Vol 3., created by MSP-Maastricht, mspvector.wordpress.com

Dear Fellow-iGEMers and scientifically interested people,

Before you delve into the exciting articles that you will find in this journal, we would like to introduce our team.
We are a group of international students from Maastricht, a quaint city in the Netherlands caught between Belgium and Germany. We are all studying at the Maastricht Science Programme, a bachelor implementing a Liberal Arts and Sciences degree. This allowed for the formation of an iGEM team with different scientific backgrounds, giving rise to a polyvalent and scientifically creative team.

The MSP-Maastricht 2022 iGEM team is called *Aestuarium*, which is defined as the place where a river meets the sea. This name was chosen to reflect the focus of the project, namely the desalination of water by cyanobacteria. Saline water comprises 97% of water on Earth, leaving only 3% of freshwater. Out of this small percentage, only 1% is destined for human use, while the major part is used for industrial and agricultural purposes. Due to the climate crisis, this percentage is being depleted. Our project aims to increase the amount of freshwater available by converting saltwater into freshwater by using transformed cyanobacteria. Salt will be extracted by inserting halorhodopsin, a green light activated Cl^- pump, and an Na^+/H^+ antiporter in the cyanobacteria.

As iGEM projects often do, *Aestuarium* is looking to become a startup once iGEM is over. With this in mind, it is necessary to think about how our project could be applied in the real world. Desalination of water using biological organisms is not novel. However, our project also focuses on chemical immobilization of the bacteria to a surface in order to facilitate extraction from the desalinated water. The method chosen for this is encapsulation in Alginate Beads crosslinked by calcium ions. Overall, this method is critical to the applicability of our project. In fact, all first world countries have strict laws about the containment of GMOs, particularly in regard to the food industry. Therefore, immobilization is a promising pathway for the safe implementation of our product.

Having concluded this introduction, we wish you a pleasant read and hope you will appreciate the content we chose to put forward!

The MSP-Vector

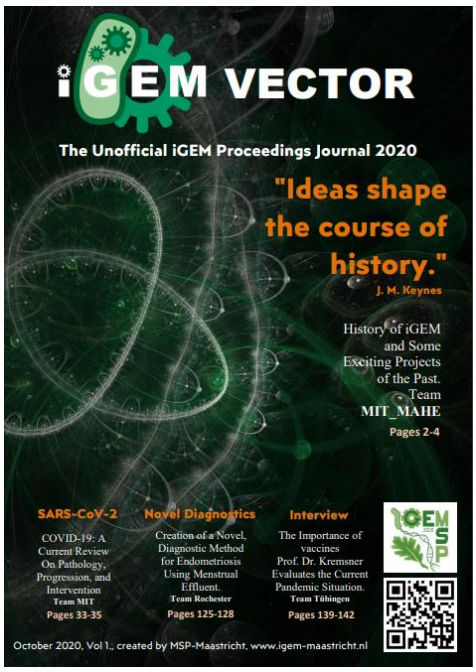
The MSP-Vector owes its existence to the first MSP-Maastricht iGEM Team, Oakshield, founded in 2020.
It was based on a simple idea: to give the opportunity to iGEM teams to develop their scientific writing and critical thinking. In order to do this, they decided to found an iGEM-specific journal dedicated to put forward the best research of the year. This was not an easy process, as peer reviews had to be designed and the teams had to be contacted. As you can see, the team succeeded in establishing the tradition that is the MSP-Vector.

Since 2020, all MSP-Maastricht iGEM teams have had a team dedicated to the improvement and growth of the journal and its reputation. In 2021, the major improvement was the establishment of a peer review form that ensured thorough and standardized evaluation of the articles submitted by the teams.

This year's journal team has been dedicated to improve the MSP-Vector to the best of its abilities. To this end, major features were added.

The most notable one was the addition of expert reviewers. This change in particular led to a series of adjustment and tweaks that had to be made to accommodate for it. These experts, namely Dr. George Church, Dr. Michael Funk and Dr. Yevgeniya Nusinovich reviewed what the participants deemed to be the three most deserving articles. In order to do this, a standard grading system had to be developed, based on the previous year's peer review form. With the help of members from the MSP teaching staff specialized in academic writing, the form was repurposed to fit our needs. The new grading form ensured transparency in the selection process. A given team had to review three other articles by filling the review form. Based on these results, the final grades were calculated by the editors. The outcome of the selection was then announced, and the three articles were sent to the panel of reviewers for individual feedback.

As a team, our hope is that this tradition will continue in the next years, and that the future teams will be able to benefit and build upon the work that has been done by Aestuarium.



The MSP-Vector Team

Lucrezia Gheno (she/her)
Lucrezia is a third year student from Italy at the Maastricht Science Programme.
Thanks to Lucrezia's comradeship and self-reliance, the journal team managed to publish a successful journal filled with information on synthetic biology. A lot of dedication was invested by her to collaborate with other iGEM teams and experts in the field.

Contact: l.gheno@student.maastrichtuniversity.nl

Anirudh Rajesh (he/him)
Anirudh is a second year student from India at the Maastricht University Programme.
Anirudh was actively engaged in spreading the knowledge of synthetic biology. With his interest, the team was able to reach out and educate young minds. Troubleshooting challenges we met especially in the lab was Anirudhs' strong suit.

Contact: a.rajesh@student.maastrichtuniversity.nl

Mathis Ben Harira (he/him)
Mathis is a third year student from the France at the Maastricht Science Programme.
Mathis' ambitious attitude allowed the team to build a trustworthy network. This has also provided potential for the long run of this project. Having such valuable contacts thanks to Mathis has allowed the team to understand a better implementation of our project.

Contact: m.benharira@student.maastrichtuniversity.nl

Bastien Vassort (he/him)
Bastien is a third year student from France at the Maastricht Science Programme.
With the knowledge Bastien has in the labs, he has been a backbone for troubleshooting. His organization also allowed the team to do their work efficiently. He was also involved in making sure our teams' journal collaboration was a success.

Contact: b.vassort@student.maastrichtuniversity.nl

The Reviewers

Dr. George Church

Dr. George Church is widely recognized for his contributions to genomic science, chemistry and biomedicine. His most notable innovations include the development of the first direct genomic sequencing method, helping in the initiation of the Human Genome Project and Personal Genome Project.

Dr. George Church is currently leading Synthetic Biology at Harvard's Wyss Institute, and he is the Director of the U.S. Department of Energy Technology Center and Director of the National Institutes of Health Center of Excellence in Genomic Science. Additionally, he is Professor of Genetics at Harvard Medical School and Professor of Health Sciences and Technology at Harvard and the Massachusetts Institute of Technology (MIT).

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.

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.

Content

Literature Reviews

Research Articles

A Review On Extraordinary Biocomputing Applications

Aristotelis Anastopoulos*, Nikoletta Drazinaki, Anthi Kapsali**, Anny Mais**, Marianna Mitsioni***, Sofia Oikonomou****, Emmanouil Anastasios Serlis*****, Panteleimon Marios Stragalis******, Aspasia Vozi*******

*Team Athens, National and Kapodistrian University of Athens, Medical School
**Team Athens, National and Kapodistrian University of Athens, Biology Department
***Team Athens, National Technical University of Athens, School of Chemical Engineering
****Team Athens, National Hellenic Research Foundation, Laboratory of Enzyme and Synthetic Biotechnology
*****Team Athens, National Technical University of Athens, School of Electrical & Computer Engineering
******Team Athens, National and Kapodistrian University of Athens, Department of Pharmacy
*******Team Athens, National and Kapodistrian University of Athens, Department of Informatics and Telecommunications

***Abstract*- Unconventional computing has yielded a lot of attention in recent years. The notion of biocomputing was first introduced in late '50s and today constitutes a promising field of technology that combines biology, engineering and computer science. The increasing limitations of currently used computers, with respect to performance, manufacturing cost and environmental footprint, lead to the need for alternative solutions that can perform complex computational tasks in a faster, more robust, reproducible and affordable way. Until today, various software and hardware applications of biocomputing have been developed. The scope of this review is to provide an overview of the field by presenting some of the most exceptional biocomputing applications.**

Index Terms - Biocomputing, Biomolecular systems, DNA Computing, Genetic engineering

I. INTRODUCTION

Computation is defined as any type of arithmetic or non-arithmetic calculation that is based on a predetermined model, i.e., an algorithm. Most computational algorithms are being developed, tested and launched via silicon-based devices, such as personal computers, phones, electronic sensors etc. Until recent years, such technologies have offered a combination of speed, reliability and affordable cost. However, recent obstacles in silicon technology, from the increasing environmental footprint to the slow-down of Moore's Law along with the need for faster, in vivo computational systems, have given rise to alternative methods of implementing computational algorithms, leading to the emergence of biocomputing.

Biocomputing is defined as the process of building computational systems that use biological materials, mimic biological organisms or are used to gather data in them. The systems developed are called biocomputers and use biologically derived molecules, such as DNA, RNA and proteins, to perform computations.The biocomputing approach implements the gathering and the processing of data simultaneously inside the living organism, in contrast to the methods used in traditional biomedical science that separate these two stages. The first steps in biocomputing were proposed by Jacob and Monod (Jacob & Monod, 1961), who presented a biomolecular computation scheme, where the presence of lactose and the lack of glucose in the bacterial growth medium would induce the Lac operon and elevate the expression of genes *lacA, lacY and lacZ*, leading to the logical function: *Lactose AND NOT Glucose → lacA, lacY, lacZ*. Another fundamental study on biocomputing was that of Knight and Sussman, who proposed a biochemically plausible mechanism for constructing digital logic signals and gates of significant complexity within living cells (Knight & Sussman, 1998). Based on that research, Knight introduced the concept of BioBrick parts, which are DNA parts that conform to a restriction-enzyme assembly standard, in an effort to standardize systematic genetic engineering of *Eschericichia coli* (Knight, 2002). The BioBrick assembly would form the basis of the iGEM Competition and lead to the foundation of The Registry of Standard Biological Parts, one of the largest synthetic biology databases, with over 20,000 Parts (Marchisio & Stelling, 2009).

The present work provides an overview of the most eye-catching software and hardware applications of biocomputing, from tunable oscillators to biological encryption systems. This short and comprehensive analysis aims to help researchers and welcome newcomers to the field of biocomputing.

II. RESEARCH ELABORATIONS

For this review, we performed a search in the following databases; IEEE Xplore and PubMed using the keywords "biocomputing", "biocomputing applications", "biocomputing algorithms", "biological computation", "DNA computing" from 2000 to 2022. 1893 to 1972 papers were retrieved from

the IEEE Xplore and PubMed databases similarly. After reviewing the abstracts of the papers that were gathered, we excluded biocomputing review papers and ended up with the papers presented here.

III. RESULTS AND FINDINGS

A. *Software related biocomputing applications*

"Smart" bacterial consortia. Since McCulloch & Pitts set the foundations for the development of artificial neural networks (ANNs) (McCulloch & Pitts, 1943), artificial intelligence has reached an impressive level of complexity and a broad range of applications. ANNs are computational networks inspired by the way brain neurons work. Recently, researchers, inspired by the architecture of ANNs, attempted to create novel living systems able to perform complex computational tasks. Sarkar et al. created a single layer ANN structure in a microbial consortium to perform multiple computational tasks (Sarkar et al., 2021). The rules for the function of the biological neural network are set by characterizing and tuning genetic devices via manipulating gene regulation modules (promoter and RBS engineering). Li et al. created a network in bacterial consortia for pattern recognition, imitating a perceptron algorithm (Li et al., 2021). Inspired again by the perceptron algorithm, Pandi et. al built a whole-cell and a cell-free single layer metabolic perceptron-like network that perform rapid and multiplex analog computation to classify metabolic states in a weighted manner (Pandi et al., 2019). All of the above work provides a new perspective on engineering cells to process information beyond the dominant logic-gate inspired systems.

Membrane computing for solving Sudoku and DNAzymes that calculate square roots. A family of different membrane P-systems was used in order to solve a large amount of Sudoku problems (Diaz-Pernil et al., 2010). A human-like approach was developed, where the P-system computes the Sudoku solution via a reset-checking cycle and the computational procedure is initiated only if at least one unique candidate exists in all partial solutions. Despite its success in a variety of Sudoku setups, the method proposed is still unable to tackle the hardest Sudoku problems, implying the need for more sophisticated P-systems. A research team designed a method that could calculate the square root using 10bit square root logic operations via DNA strand displacement (TDSD) (Zhou et al., 2019). Their approach used nanoindicators (DNAzymes) because of their capability to create large-scale logic circuits. Nanoindicators use Fluorescence Resonance Energy Transfer in order to measure the square root of different numbers, based on three basic modules: the cognitive module, the biocomputing module and the thresholding module. The innovation this system offers is the projection of TDSD in the field of creating complicated computing circuits.

An encryption system implemented using DNA-computing. Cui et al. developed a DNA-computing implementation of an encryption system, using the technologies of DNA synthesis, PCR amplification and DNA digital coding (Cui et al., 2008). The encryption model is the RSA Algorithm, a public key cryptography method, where the sender translates the original or plaintext with a public key to the encrypted or ciphertext before sending it to the receiver. Next, the receiver decrypts the ciphertext to the plaintext via a private key. In the implemented biocomputing approach, the two keys correspond to two pairs of PCR primers that the two parties send to each other. Next, the sender converts the plaintext to a binary sequence and then to a DNA one, using the given encryption PCR primers. Similarly, with the correct decryption primers, the receiver can reversely extract the binary sequence from the DNA one and, finally, the initial plaintext. Some advantages of the process include the different ciphertexts that can be derived from the same plaintext, the high confidential strength, as well as the ever-decreasing cost due to the progress of the various biological technologies used.

Solving two NP-complete, one NP-hard problems. In 1994, Adleman encoded and solved the directed Hamiltonian Path Problem via DNA computing (Adleman, 1994). A DNA computing 5-step algorithm was implemented in order to identify the correct Hamiltonian path. The proposed method compared greatly with the supercomputers of the early '90s, since it allowed not only for a thousandfold more operations per second, but also for a more energy efficient way of executing those operations. Furthermore, the use of DNA molecules as storage media led to a dramatic increase in information density. Following Adleman, Lipton in 1995, proposed a generalized method that solves NP-complete problems by solving the Boolean Satisfiability Problem using DNA computing once more and highlighting the advantages of the parallelism inherent by it (Lipton, 1995). Based on the Adleman-Lipton model, Wang et al., solved the Capacitated Vehicle Routing problem, a well-known NP-hard problem (Wang et al., 2019).

B. *Hardware related biocomputing applications*

Foundational Research. In contrast to modern electronic devices which are based on microchip technology, biocomputing uses molecular and synthetic biology parts to implement its various algorithms. These parts follow specific predefined rules, which are being deployed by designers and engineers, in order to assemble novel genetic circuits. One fundamental paper on the hardware aspect of biocomputing is that of Gardner et al. who engineered a genetic toggle switch composed of two repressible promoters arranged in a mutually inhibitory network (Gardner et al., 2000). Each promoter was inhibited by the repressor that was transcribed by the opposing promoter. Switching was accomplished by transiently

introducing an inducer of the currently active repressor, since the inducer permitted the opposing repressor to be maximally transcribed until it stably repressed the originally active promoter. Another ground-breaking approach was that of Weiss et al., who implemented an in-vivo NAND gate via a genetic regulatory network (Weiss et al., 2002). Logic signals were represented by the synthesis rates of cytoplasmic DNA binding proteins and the NAND gate consisted of structural genes for output proteins, fused to promoter/operator regions that were regulated by input proteins. The implementations above would become the inspiration for a series of electronic-inspired bioengineering applications in the following years.

Further Advancements in Circuitry Biotechnology. In the domain of cellular digital circuits, a worth-mentioning work is that of Strack et al., who constructed a biomolecular keypad lock (Strack et al., 2008). The system consisted of three consecutive AND logic gates and each gate required the presence of two inputs, a biocatalyst and a chemical compound, to activate the subsequent reaction in a chain-like manner, where the product of each biochemical conversion served as the substrate of the following one. Were all the reactants present and in the right order, the biocomputing network was "unlocked" and a coloured agent was synthesized (output "1"), otherwise the "dialed password" was recognized as incorrect, and the "alarm" was set off (output "0"), hence the resemblance with an electronic keypad lock. A similar biomolecular configuration based on fluorescent devices has also been reported (Margulies et al., 2007) Another electronic component implemented in-vivo was an oscillator analogue created in *E.coli* (Stricker et al., 2008). The genetic oscillator was composed of *araC*, *lacI* and *yemGFP* genes, under the control of three identical copies of the hybrid promoter plac/ara-1, to form three co-regulated transcription modules using positive and negative feedback loops. Through the biochemical interaction of these components and under the influence of IPTG and arabinose, the yemGFP concentration was recorded to fluctuate periodically. Several conditions such as temperature and inducer concentration were used to tune the period of oscillation, which could be as low as 13 minutes.

Modern Approach. State-of-the-art biocomputing hardware aims to take advantage of the fundamental genetic circuits and research of the past, in order to synthesize more complex computing systems. One such example is the development of a CRISPR-Cas 9 system that controlled gene expression with sophisticated logic gates (Kim et al., 2019). In particular, the researchers used single guide RNAs (gRNAs) as a genetic software to develop an ON and an OFF system. Both systems consist of an inactive form of Cas 9 (dCas 9) that binds to the gRNA-relying DNA and regulates the transcription of the target, with dCas 9 inhibiting the expression of the reporter in the OFF system and activating it in the ON system. Those two systems, with the proper gRNA inputs, implemented a range of computations such as Boolean logic gates and arithmetic operations, thus leading to the design of dual-core CPUs. Other approaches aim to create biological systems of modern electronic components, such as bio-memristors utilizing the slime mold *Physarum polycephalum* (Miranda & Braud, 2017). *P.polycephalum* was cultured in designated receptacle chambers to manufacture such a device that is based on biological architecture, since the aforementioned unicellular organism displays memristive properties. When AC voltage is applied to its plasmodium, *P.polycephalum* produces the distinctive current vs. voltage non-linear plot of a memristor, called a pinched hysteresis loop. That kind of breakthrough in memristive technology could be beneficial for the future development of multilayer biological Neural Networks, since memristors are already a complexity-and-power friendly hardware implementation of such systems (Adhikari et al., 2015).

IV. DISCUSSION

As presented above, biocomputing is an emerging field that has already offered a wide range of applications, both in the software and hardware domains. The main advantages of biocomputing include the synthesis of low energy, parallel computational systems, that are easily integrated in the biological environment of interest. This ever increasing research interest in biocomputing applications (Figure 1) could revolutionize the field of biosensors, since it leads to devices that implement the 'sense-process-act' procedure. Under this scheme, a variety of different applications could take advantage of biocomputing tools, from theranostic and personalized drug-delivery systems (Tregubov et al., 2018) to water quality assessment biosensors (Jung et al., 2022).

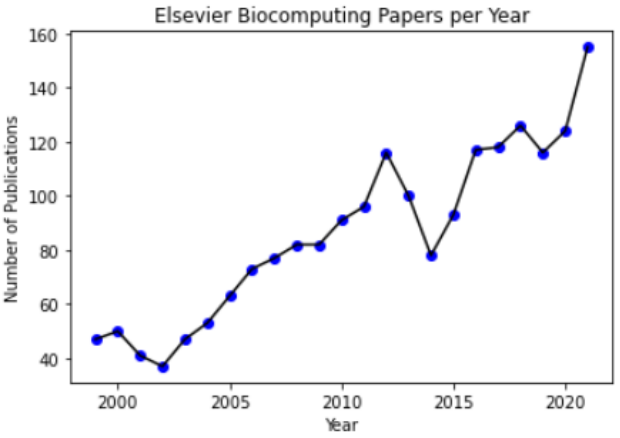


FIGURE 1.
Number of Elsevier publications related to biocomputing applications (1999-2021)

However, there are still hurdles that should be acknowledged, the most significant one being the low-scale information processing ability that even modern biocomputers offer. This threshold in computing power turns biocomputers insufficient for building a general-purpose computer that will replicate silicon-based microelectronic devices of the past decades. Furthermore, questions about the scale-up demand, production and commercialization of such technologies should be asked. Apart from these limiting factors, biocomputing is still an area of growth and well-suited in situations that require constant operation and adaptation to external changes.

V. CONCLUSION

Biocomputing has been a recent field of research interest, that aims to execute efficient computations at a molecular level. Due to the immediate relation of such systems to synthetic biology principles, plenty of iGEM Teams have already offered quality projects related to biocomputing, such as information storage and transferring systems (iGEM Team Groningen, 2016) and innovative synthetic circuit technologies (iGEM Team EPFL, 2016). Under such context, this review may serve future iGEM Teams and researchers with the information necessary to comprehend the main aspects, rules and applications of biocomputing in their effort to further brainstorm and contribute in this domain with their own projects and research work.

REFERENCES

Adhikari, S. P., Kim, H., Budhathoki, R. K., Yang, C. & Chua, L. O. (2015). A Circuit-Based Learning Architecture for Multilayer Neural Networks With Memristor Bridge Synapses. IEEE Transactions on Circuits and Systems I: Regular Papers, 62(1), 215–223. https://doi.org/10.1109/TCSI.2014.2359717

Adleman, L. M. (1994). Molecular Computation of Solutions to Combinatorial Problems. *Science*, *266*(5187), 1021–1024. https://doi.org/10.1126/science.7973651

Cui, G., Qin, L., Wang, Y. & Zhang, X. (2008). An encryption scheme using DNA technology. *2008 3rd International Conference on Bio-Inspired Computing: Theories and Applications*, 37–42. https://doi.org/10.1109/BICTA.2008.4656701

Diaz-Pernil, D., Fernandez-Marquez, C. M., Garcia-Quismondo, M., Gutierrez-Naranjo, M. A., & Martinez-del-Amor, M. A. (2010). Solving sudoku with Membrane Computing. *2010 IEEE Fifth International Conference on Bio-Inspired Computing: Theories and Applications (BIC-TA)*, 610–615. https://doi.org/10.1109/BICTA.2010.5645195

Gardner, T. S., Cantor, C. R., & Collins, J. J. (2000). Construction of a genetic toggle switch in Escherichia coli. *Nature*, *403*(6767), 339–342. https://doi.org/10.1038/35002131

iGEM team EPFL. (2016). Wiki iGEM Team EPFL 2016. https://2016.igem.org/Team:EPFL

iGEM team Groningen. (2016). Wiki iGEM Team Groningen 2016. https://2016.igem.org/Team:Groningen

Jacob, F., & Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *Journal of molecular biology*, (3), 318-356. https://doi.org/10.1016/S0022-2836(61)80072-7

Jung, J. K., Archuleta, C. M., Alam, K. K., & Lucks, J. B. (2022). Programming cell-free biosensors with DNA strand displacement circuits. *Nature Chemical Biology*, *18*(4), 385–393. https://doi.org/10.1038/s41589-021-00962-9

Kim, H., Bojar, D., & Fussenegger, M. (2019). A CRISPR/Cas9-based central processing unit to program complex logic computation in human cells. *Proceedings of the National Academy of Sciences*, *116*(15), 7214–7219. https://doi.org/10.1073/pnas.1821740116

Knight, T. (2002). *DARPA BioComp Plasmid Distribution 1.00 of Standard Biobrick Components*. MIT Artificial Intelligence Laboratory. http://hdl.handle.net/1721.1/21167

Knight, T. F., & Sussman, G. J. (1998). Cellular Gate Technology. *First International Conference on UNCONVENTIONAL MODELS OF COMPUTATION, Auckland, New Zealand*. http://hdl.handle.net/1721.1/29793

Li, X., Rizik, L., Kravchik, V., Khoury, M., Korin, N., & Daniel, R. (2021). Synthetic neural-like computing in microbial consortia for pattern recognition. *Nature Communications*, *12*(1), 3139. https://doi.org/10.1038/s41467-021-23336-0

Marchisio, M. A., & Stelling, J. (2009). Computational design tools for synthetic biology. *Current Opinion in Biotechnology*, *20*(4), 479–485. https://doi.org/10.1016/j.copbio.2009.08.007

Margulies, D., Felder, C. E., Melman, G., & Shanzer, A. (2007). A Molecular Keypad Lock: A Photochemical Device Capable of Authorizing Password Entries. *Journal of the American Chemical Society*, *129*(2), 347–354. https://doi.org/10.1021/ja065317z

McCulloch, W. S., & Pitts, W. (1943). A logical calculus of the ideas immanent in nervous activity. *The Bulletin of Mathematical Biophysics*, *5*(4), 115–133. https://doi.org/10.1007/BF02478259

Miranda, E. R., & Braund, E. (2017). A Method for Growing Bio-memristors from Slime Mold. *Journal of Visualized Experiments*, *129*, 56076. https://doi.org/10.3791/56076

Pandi, A., Koch, M., Voyvodic, P. L., Soudier, P., Bonnet, J., Kushwaha, M., & Faulon, J.-L. (2019). Metabolic perceptrons for neural computing in biological systems. *Nature Communications*, *10*(1), 3880. https://doi.org/10.1038/s41467-019-11889-0

Sarkar, K., Bonnerjee, D., Srivastava, R., & Bagh, S. (2021). A single layer artificial neural network type architecture with molecular engineered bacteria for reversible and irreversible computing. *Chemical Science*, *12*(48), 15821–15832. https://doi.org/10.1039/D1SC01505B

Strack, G., Ornatska, M., Pita, M., & Katz, E. (2008). Biocomputing Security System: Concatenated Enzyme-Based Logic Gates Operating as a Biomolecular Keypad Lock. *Journal of the American Chemical Society*, *130*(13), 4234–4235. https://doi.org/10.1021/ja7114713

Stricker, J., Cookson, S., Bennett, M. R., Mather, W. H., Tsimring, L. S., & Hasty, J. (2008). A fast, robust and tunable synthetic gene oscillator. *Nature*, *456*(7221), 516–519. https://doi.org/10.1038/nature07389

Tregubov, A. A., Nikitin, P. I., & Nikitin, M. P. (2018). Advanced Smart Nanomaterials with Integrated Logic-Gating and Biocomputing: Dawn of Theranostic Nanorobots. *Chemical Reviews*, *118*(20), 10294–10348. https://doi.org/10.1021/acs.chemrev.8b00198

Wang, Z., Ren, X., Ji, Z., Huang, W., & Wu, T. (2019). A novel bio-heuristic computing algorithm to solve the capacitated vehicle routing problem based on Adleman–Lipton model. *Biosystems*, *184*, 103997. https://doi.org/10.1016/j.biosystems.2019.103997

Weiss, R., Homsy, G. E., & Knight, T. F. (2002). Toward in vivo Digital Circuits. In L. F. Landweber & E. Winfree (Eds.), *Evolution as Computation* (pp. 275–295). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-55606-7_14

Zhou, C., Geng, H., Wang, P., & Guo, C. (2019). Programmable DNA Nanoindicator‐Based Platform for Large‐Scale Square Root Logic Biocomputing. *Small*, *15*(49), 1903489. https://doi.org/10.1002/smll.201903489

AUTHORS

First Authors

– Aristotelis Anastopoulos, National and Kapodistrian University of Athens, Medical School aristotelis.anasto@gmail.com.

– Nikoletta Drazinaki, undergraduate student, National and Kapodistrian University of Athens, Department of Biology, nicoledrazi01@gmail.com.

– Anthi Kapsali, undergraduate student, National and Kapodistrian University of Athens, Department of Biology, anthi.kapsalis@gmail.com.

– Anny Mais, undergraduate student, National and Kapodistrian University of Athens, Department of Biology, maisannie@hotmail.com .

– Marianna Mitsioni, undergraduate student, National and Technical University of Athens, School of Chemical Engineering, mitsionimarianna@gmail.com.

– Sofia Oikonomou, BSc, graduate student, University of Patras, Department of Biology, intern, National Hellenic Research Foundation, Laboratory of Enzyme and Synthetic Biotechnology, sofialoikonomou@yahoo.com.

– Emmanouil Anastasios Serlis, undergraduate student, National and Technical University of Athens, School of Electrical Engineering, manosserlis@gmail.com.

– Panteleimon Marios Stragalis, undergraduate student, National and Kapodistrian University of Athens, Department of Pharmacy, pantelistra.pharm@gmail.com.

– Aspasia Vozi, MD, master's student, National and Kapodistrian University of Athens, Department of Informatics and Telecommunications, aspasia.vozi@gmail.com.

Correspondence Author – Aspasia Vozi, aspasia.vozi@gmail.com, igemathens2022@gmail.com, +306970380088.

Interleukin-10 as a potential therapeutic in autoimmune diseases

Famke Klop,[1] Elisa Passet,[2] Femi Hesen,[3] Jolien Marcelis,[4] Wouter Langers,[5] Jakob Scheele,[6] Floor van Boxtel[6] Kim Wintraecken,[7] Rian Driedijk[1]

[1]TU-Eindhoven, Dept. Biomedical Engineering, Eindhoven University of Technology, The Netherlands.
[2]TU-Eindhoven, Dept. Systems biology and metabolic disease, Eindhoven University of Technology, The Netherlands.
[3]TU-Eindhoven, Dept. Protein Engineering, Eindhoven University of Technology, The Netherlands.
[4]TU-Eindhoven, Dept. Synthetic Biology , Eindhoven University of Technology, The Netherlands.
[5]TU-Eindhoven, Dept. Precision medicine, Eindhoven University of Technology, The Netherlands
[6]TU-Eindhoven, Dept. Chemical biology, Eindhoven University of Technology, The Netherlands
[7]TU-Eindhoven, Dept. Industrial Design, Eindhoven University of Technology, The Netherlands

ABSTRACT

Interleukin-10 is a cytokine that plays a central role in dampening the immune system. Autoimmune diseases can be caused by or can result in dysregulation of interleukin-10, which causes sudden or chronic inflammation. Administration of this cytokine to normalize the interleukin-10 concentration could therefore be used as a potential therapeutic. However, there is a lack of knowledge on the exact mechanism of interleukin-10 within autoimmune diseases. Furthermore interleukin-10 cannot be easily administered due to its short half-life and side effects. Despite these facts interleukin-10 presents a viable option in creating novel treatments for autoimmune diseases.

Index Terms - Interleukine-10, therapeutic agents, autoimmune diseases, immune response, iGEM

I. INTRODUCTION

Interleukin-10 (IL-10) is a cytokine active in regulating the immune response. Its primary role is to deactivate or dampen the immune response without damaging surrounding tissue (Howes et al., 2014). This is done by suppressing antigen-specific immunity and thus allowing for T cell tolerance (Akdis & Blaser, 2001). IL-10 was first described in mice as inhibiting cytokine production by Th1 cells. Soon thereafter came the discovery of BCRF, an Epstein-Barr virus gene, which closely resembled the IL-10 cytokine. Research suggested that the virus uses the IL-10 homolog, BCRF, as a means to evade the immune system (Moore et al., 2001), characterizing the central role of IL-10 in the human immune system.

IL-10 is the founding member of its cytokine family which consists of nine members: IL-10, 5 members of the IL-20 subfamily and 3 cytokines that are commonly classified as type III interferons. The members of the IL-10 family are all produced by both adaptive and native immune cells and regulate the immune response (Ouyang & O'Garra, 2019). The involvement of specifically IL-10 is demonstrated in many different disease states, both in animal models and humans with mutations in IL-10 (Iyer & Cheng, 2012). However, how this cytokine can be used as a therapeutic agent is still being reviewed.

Since IL-10 lowers the inflammatory host response, it is co-responsible for preventing inflammation in autoimmune pathologies. Hence why dysregulation of IL-10 is known to be the cause of some autoimmune diseases. However, because of the broad diversity in autoimmune diseases, IL-10 plays a varying role. Systemic Lupus Erythematosus patients have shown high levels of IL-10, while during allergic Asthma reactions, the relevant cells have shown to produce reduced IL-10 levels (Iyer & Cheng, 2012). The unbalance, however, suggests the potential of (artificial) IL-10 administration in autoimmune disease patients.

In this review, the questions that currently still exist regarding IL-10 biology will be discussed. We will discuss the latest developments related to the metabolic regulation of IL-10. Furthermore, we summarize the applications in which IL-10 has the most potential and is already in use. Finally, we highlight the challenges we are facing while using IL-10 as a possible therapeutic agent in autoimmune diseases.

II. METABOLIC REGULATION OF IL-10

IL-10 is produced by a wide range of cells including, but not limited to, monocytes, macrophages, CD4+, CD8+ and dendritic cells (Couper et al., 2022). Although always present in low concertation (Sarris et al., 1999), Il-10 production can be initiated by various stimuli (Saraiva et al., 2019). Production in myeloid cells for example can be caused by the presence of a microbial product recognized on pattern recognition receptors or can be caused by the presence of cytokines, like type I interferon, produced by other immune cells. T cells, on the other hand cannot directly react to the presence of pathogens and instead solely depend on the presence of cytokines (Board et al., 2016). However, the exact mechanisms triggering these systems remain largely unclear.

Since IL-10 plays a central role within the regulation of the immune response, an IL-10 deficiency could lead to sudden, chronic or excessive infections, as pathogens are let through the human immune mechanisms. Furthermore, a heightened concentration of IL-10 has been shown to increase the risk of autoimmune diseases.(Iyer & Cheng, 2012).

The way Il-10 minimizes the host's infection is dependent on the affected cell. For example , IL-10 inhibits the cytokine production of Th 2 cells, which in turn regulates the production of inflammatory cytokines such as, IL-4, IL-5 and IL-13. Another example is IL-10 inhibiting the expression of most inducible chemokines that cause inflammation (Moore et al., 2001). These two examples are simplifications, and the full effect of IL-10 as an anti-inflammatory cytokine is much more complex and involves many more positive, negative and self-regulatory mechanisms (Saraiva et al., 2019).

III. APPLICATIONS OF IL-10 AS A THERAPEUTIC AGENT

IL-10 has inhibitory effects on multiple proinflammatory cytokines by indirectly inhibiting their production such as tumor necrosis factor (TNF) (Clarke et al., n.d.). Besides, IL-10 promotes wound healing and down-regulation of MHC class II cells (Ouyang & O'Garra, 2019; Peranteau et al., 2008) Therefore IL-10 is able to adjust the function of proinflammatory cytokines in vivo and in vitro. As a result, IL-10 has been viewed as a potential agent to treat multiple inflammatory diseases (Minshawi et al., 2020).

Enhancing IL-10 levels to treat diseases is being researched for a number of different diseases but is best understood in inflammatory bowel disease (IBD) and cancer, see TABLE 1. The relation between IL-10 and IBD was shown when IL-10 deficient mice developed colitis (Kühn et al., 1993). Due to the ability of IL-10 to induce repair of the epithelial cell layer (Quiros et al., 2017) caused by the inflammation and inhibition of the inflammatory responses during IBD, the treatment with IL-10 is favorable. Besides, pegylated IL-10 stimulates CD8+T cell expansion and activation, which in turn triggers IFN-ɣ secretion and therefore solid tumor suppression.

Furthermore, researchers show that for different autoimmune diseases, the corresponding IL-10 response is strikingly diverse (Iyer & Cheng, 2012). Meaning that the efficiency of IL-10 administration differs among the disorders. One explanation for this is the difference in IL-10 surplus and deficiency. Both are common in auto-immune diseases yet, IL-10 deficiency as seen in IBD suggests, and has shown (Fedorak et al., 2000), to yield better and more efficient results after manual IL-10 administration. In other cases IL-10 effects might not be strong enough to cancel the pro-inflammatory workings of other cytokines present during the disease. Lastly, it has been suggested that individual phenotype and severity of the disease influences effectiveness of IL-10 (Marlow et al., 2013).

Role of IL-10	**Application area**
Repairing the epithelial cell layer	Inflammatory bowel disease (IBD)
Triggering IFN-ɣ secretion	Cancer tumour suppression

TABLE 1: Applications of IL-10 as therapeutic agent and its specific roles in treating the disease.

IV. CHALLENGES RELATED TO IL-10 AS THERAPEUTIC AGENTS

Although the potential of IL-10 in a clinical application has been demonstrated, many challenges still remain. No clinically approved therapy manipulates IL-10 family cytokines (Ouyang & O'Garra, 2019).

A significant drawback, which withholds clinicians from using IL-10, is that the biologically active form of such a molecule is an unstable homodimer. As a result, IL-10 has a short half-life and is easily degraded in vivo (Minshawi et al., 2020). To use IL-10 as a potential therapeutic agent, it is therefore necessary to continually administer small doses to maintain the wanted concentration.

Furthermore, research has shown that, although small concentrations are safe and overall well tolerated, high doses of IL-10 are associated with systemic side effects like fever, headache and malaise (Tilg et al., n.d.). Most of these side-effects however are temporary and mild to moderate and disappear after the cancelation of the treatment (Colombel et al., 2001).

The last difficulty that presents itself is the complexity of the IL-10 mechanism. New research suggests that IL-10 is not solely immune inhibitory but can also stimulate the immune response in some immune diseases (Colombel et al., 2001), and cancers (Naing et al., 2016). A proposed explanation is that IL-10 also induces pro-inflammatory cytokine production, which may be heightened in presence of other proteins, like disease-specific antibodies (Lu et al., 2021). This is an unwanted effect when using IL-10 as a therapeutic agent. However, much remains uncertain on this effect of IL-10.

V. CONCLUSION

There are still obstacles that need to be addressed before IL-10 can be used clinically. The main challenges are the complexity and thus uncertainty regarding some of IL-10's mechanisms and the lack of representative immune system models in mice. Therefore, there needs to be a focus on further understanding of the exact mechanisms of each autoimmune disease in humans before it can be used as a therapeutic. Despite the current challenges, IL-10 has large potential as a therapeutic agent when filtering out the patients with a disease phenotype that causes dysregulation in the IL-10 mechanisms and consequently an IL-10 deficiency.

VI. RESEARCH ELABORATIONS

The information and data obtained for this review was obtained using google scholar and PubMed.

VII. CONFLICTS OF INTERESTS

There are still obstacles that need to be addressed before IL-10 can be used clinically. The main challenges are the complexity and thus uncertainty regarding some of IL-10's mechanisms and the lack of representative immune system models in mice. Therefore, there needs to be a focus on further understanding of the exact mechanisms of each autoimmune disease in humans before it can be used as a therapeutic. Despite the current challenges, IL-10 has large potential as a therapeutic agent when filtering out the patients with a disease phenotype that causes dysregulation in the IL-10 mechanisms and consequently an IL-10 deficiency.

VIII. ACKNOWLEDGEMENTS

Many thanks to prof.dr. Maarten Merckx, prof.dr.ir. Luc Brunsveld and prof.dr.ir. Tom de Greef for the guidance and discussions. Thanks to postdoc. Alexander Gräwe, doctoral candidate Bryan Nathalia and Anna-Maria Makri Pistikou for the many insightful discussions and guidance during our iGEM project. Finally, the iGEM TU Eindhoven advisors Luke Rossen, Fleur Kalberg, Josephina Smits and Daniek van Hoorn for the advice during the iGEM competition related to this work.

REFERENCES

Akdis, C. A., & Blaser, K. (2001). Mechanisms of interleukin-10-mediated immune suppression. Immunology, 103(2), 131. https://doi.org/10.1046/J.1365-2567.2001.01235.X

Board, E., Cohen, I. R., & Lambris, J. D. (2016). Regulation of Interleukin-10 Expression. Advances in Experimental Medicine and Biology, 941, 89–116. http://www.springer.com/series/5584

Clarke, C. J. P., Hales, A., Hunt, A., & Foxwell, B. M. J. (n.d.). IL-10-mediated suppression of TNF-> production is independent of its ability to inhibit NF B B activity. https://doi.org/10.1002/(SICI)1521-4141(199805)28:05

Colombel, J. F., Rutgeerts, P., Malchow, H., Jacyna, M., Nielsen, O. H., Rask-Madsen, J., van Deventer, S., Ferguson, A., Desreumaux, P., Forbes, A., Geboes, K., Melani, L., & Cohard, M. (2001). Interleukin 10 (Tenovil) in the prevention of postoperative recurrence of Crohn's disease. Gut, 49(1), 42–46. https://doi.org/10.1136/GUT.49.1.42

Couper, K. N., Blount, D. G., & Riley, E. M. (2022). Infection IL-10: The Master Regulator of Immunity to. J Immunol References, 180, 5771–5777. https://doi.org/10.4049/jimmunol.180.9.5771

Fedorak, R. N., Gangl, A., Elson, C. O., Rutgeerts, P., Schreiber, S., Wild, G., Hanauer, S. B., Kilian, A., Cohard, M., LeBeaut, A., Feagan, B., Fedorak, R.

N., Gangl, A., Gasche, C., Elson, C. O., Rutgeerts, P., Schreiber, S., Wild, G., Hanauer, S. B., … Gibson, P. (2000). Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. The Interleukin 10 Inflammatory Bowel Disease Cooperative Study Group. Gastroenterology, 119(6), 1473–1482. https://doi.org/10.1053/GAST.2000.20229

Howes, A., Gabryšová, L., & O'Garra, A. (2014). Role of IL-10 and the IL-10 Receptor in Immune Responses. Reference Module in Biomedical Sciences. https://doi.org/10.1016/B978-0-12-801238-3.00014-3

Iyer, S. S., & Cheng, G. (2012). Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease. Critical Reviews in Immunology, 32(1), 23. https://doi.org/10.1615/CRITREVIMMUNOL.V32.I1.30 Kühn, R., Löhler, J., Rennick, D., Rajewsky, K., & Müller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell, 75(2), 263–274. https://doi.org/10.1016/0092-8674(93)80068-P

Lu, L., Zhang, H., Dauphars, D. J., & He, Y. W. (2021). A Potential Role of Interleukin 10 in COVID-19 Pathogenesis. Trends in Immunology, 42(1), 3–5. https://doi.org/10.1016/J.IT.2020.10.012

Marlow, G. J., van Gent, D., & Ferguson, L. R. (2013). Why interleukin-10 supplementation does not work in Crohn's disease patients. World Journal of Gastroenterology : WJG, 19(25), 3931. https://doi.org/10.3748/WJG.V19.I25.3931

Minshawi, F., Lanvermann, S., McKenzie, E., Jeffery, R., Couper, K., Papoutsopoulou, S., Roers, A., & Muller, W. (2020). The Generation of an Engineered Interleukin-10 Protein With Improved Stability and Biological Function. Frontiers in Immunology, 11, 1794. https://doi.org/10.3389/FIMMU.2020.01794/BIBTEX

Moore, K. W., de Waal Malefyt, R., Coffman, R. L., & O'Garra, A. (2001). INTERLEUKIN-10AND THEINTERLEUKIN-10 RECEPTOR. Annual Review of Immunology, 19(1), 683–765. https://doi.org/10.1146/annurev.immunol.19.1.683

Naing, A., Papadopoulos, K. P., Autio, K. A., Ott, P. A., Patel, M. R., Wong, D. J., Falchook, G. S., Pant, S., Whiteside, M., Rasco, D. R., Mumm, J. B., Chan, I. H., Bendell, J. C., Bauer, T. M., Colen, R. R., Hong, D. S., Van Vlasselaer, P., Tannir, N. M., Oft, M., & Infante, J. R. (2016). Safety, Antitumor Activity, and Immune Activation of Pegylated Recombinant Human Interleukin-10 (AM0010) in Patients With Advanced Solid Tumors. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology, 34(29), 3562–3569. https://doi.org/10.1200/JCO.2016.68.1106

Ouyang, W., & O'Garra, A. (2019). IL-10 Family Cytokines IL-10 and IL-22: from Basic Science to Clinical Translation. Immunity, 50(4), 871–891. https://doi.org/10.1016/j.immuni.2019.03.020

Peranteau, W. H., Zhang, L., Muvarak, N., Badillo, A. T., Radu, A., Zoltick, P. W., & Liechty, K. W. (2008). IL-10 Overexpression Decreases Inflammatory Mediators and Promotes Regenerative Healing in an Adult Model of Scar Formation. Journal of Investigative Dermatology, 128(7), 1852–1860. https://doi.org/10.1038/sj.jid.5701232

Saraiva, M., Vieira, P., & O'Garra, A. (2019). Biology and therapeutic potential of interleukin-10. The Journal of Experimental Medicine, 217(1). https://doi.org/10.1084/jem.20190418

Sarris, A. H., Kliche, K. O., Pethambaram, P., Preti, A., Tucker, S., Jackow, C., Messina, O., Pugh, W., Hagemeister, F. B., McLaughlin, P., Rodriguez, M. A., Romaguera, J., Fritsche, H., Witzig, T., Duvic, M., Andreeff, M., & Cabanillas, F. (1999). Interleukin-10 levels are often elevated in serum of adults with Hodgkin's disease and are associated with inferior failure-free survival. Annals of Oncology: Official Journal of the European Society for Medical Oncology, 10(4), 433–440. https://doi.org/10.1023/a:1008301602785

Zhu, J., Ying, S.-H., & Feng, M.-G. (2016). The Na+ /H+ antiporter Nhx1 controls vacuolar fusion indispensible for life cycles in vitro and in vivo in a fungal insect pathogen. Environmental Microbiology, 18(11), 3884–3895. https://doi.org/10.1111/1462-2920.13359

AUTHORS

First author - Famke Klop, Eindhoven University of Technology, Biomedical Engineering and iGEM@tue.nl

Second author - Elisa Passet, BSc., Eindhoven University of Technology Dept. Systems biology and metabolic disease and iGEM@tue.nl

Third author – Femi Hesen, BSc., Eindhoven University of Technology, Dept. Protein Engineering and iGEM@tue.nl

Fourth author – Jolien Marcelis, BSc., Eindhoven University of Technology, Dept. Synthetic Biology and iGEM@tue.nl

Fifth author – Wouter Langers, BSc., Eindhoven University of Technology, Dept. Precision medicine and iGEM@tue.nl

How single-cell bacterial bioluminescence opens up a new research area

ROBLIN Tom*, MATHIEU Théo*, CECCHI Mathilde*

* INSA_Lyon1, INSA Lyon, Université Lyon 1

***Abstract*- Observing bacterial development in real time has always been a major challenge in research. In this context, bacterial bioluminescence is a useful tool for studies. It is commonly used as a biosensor for toxic chemical detection. Different genetic constructions, involving mainly the *Lux* operon, were created and tested over time to improve the bioluminescence system, modifying bacteria so that they could produce their own light. However, those genetic constructions were always too weak to allow single-cell imaging. A new genetic construction called *iLux*, an improvement of the *Lux* operon, was designed by Carola Gregor and her team in 2017 to address this problem. Thus, bacterial bioluminescence can now be used to study bacterial development and spread inside host organisms. This is a promising advance for a better understanding of bacterial pathogenesis pathways, with a focus on *Dickeay* in this article, and for testing new cures against bacterial diseases. An improvement of *iLux* operon to make it more adaptable is in progress.**

***Index Terms*-** bioluminescence, bacteria, *iLux*, pathogenesis, luciferase

I. INTRODUCTION

Observing bacteria in vivo has always been a big challenge within the global scientific community, and its understanding would help fight infections or diseases to protect any type of living organisms. A real challenge would be to be able to follow bacterial infections in real time, with non-intrusive methods in order to collect the most representative data to understand them accurately. Until now, to observe and understand bacteria development, scientists have been performing many types of studies using fluorescence, with the broadly used GFP reporter gene. However, this method does not always provide quantitative and accurate results concerning *in vivo* studies of bacteria, since living organisms produce autofluorescence, which can interfere with the GFP fluorescence detected and hence distort the reality (Teuscher et al., 2018). Thus, researchers tried to develop single cell bioluminescence, which could overcome the autofluorescence problem and could allow for the development of highly luminescent tools to be used for *in vivo* studies in the future.

In 2017, single-cell bioluminescence was made possible thanks to the design of the *iLux* operon by Carola Gregor and her team (Gregor et al., 2018). The goal of our iGEM team, *Fiat Lux*, is to use synthetic biology to create an improved, more adaptable, version of the *iLux* operon. Hence, it would be easier for any scientific team to use the *iLux* operon for single-cell imaging on any bacterial strain.

The advantage of single-cell imaging is the possibility of observing bacterial spread directly through living organisms' tissues thanks to a highly sensitive Charge Coupled Device camera (highly sensitive CCD camera). Thus, an all new research area can now be investigated, principally in crop sciences, animal and even human health. Understanding bacterial development across time and its response to treatments will be more precise. Some crop diseases still don't have any treatment because of the difficulties in understanding phytobacteria evolution inside the host, as for example the soft rot diseases caused by *Dickeya solani* on potato plants (Czajkowski et al., 2011). The *iLux* operon is a hope for finding new cures.

II. RESEARCH ELABORATIONS

A. *Research design and databases*

Research of articles was made through Pubmed's platform. The main key words used to find articles were "*iLux* operon", "bioluminescence" and "*Dickeya*". Reviews of articles for "bioluminescence", "*Dickeya*" and "soft rot diseases" were spotted to have a summary of known research so far. In order to have the newest articles, no articles before the year 2000 were selected.

B. *IVA PCR*

Details of the protocol are not available yet because of a confidential agreement.

C. *OE-PCR*

Details of the protocol are not available yet because of a confidential agreement.

D. *Synthesized oligonucleotides*

Details of the protocol are not available yet because of a confidential agreement.

III. RESULTS AND FINDINGS

A. *iLux's functioning*

The *iLux* operon was designed by Carola Gregor and her team in 2017, as a better version of the *Lux* operon (Gregor et al., 2018). The *Lux* operon is made of a core of five genes. Because it has evolved in different organisms, other genes were added in the *Lux* operon in some species of bacteria (Brodl et al. 2018). However, the *iLux* operon is only using the five genes from the *Lux* operon, which are *lux A, lux B, lux C, lux D* and *lux E*. *LuxA* and *luxB* code for an enzyme, a

heterodimeric luciferase, while *luxC*, *luxD* and *luxE* code for a fatty acid reductase complex (Fig.1). The luciferase catalyzes the mono-oxygenation of an aliphatic aldehyde using FMNH2 as a redox cofactor. This reaction needs O2 and the excited FMN releases its energy under the form of light with a wavelength of 490 nm. The fatty acid reductase complex is a complex of three different proteins which are an acyl-reductase (*luxC*), an acyl-transferase (*luxD*) and an acyl-synthetase (*luxE*). In order to produce light in a continuous way, the bacterium needs to recycle FMN and the mono-oxygenated aliphatic aldehyde, into FMNH2 and an aliphatic aldehyde respectively. The fatty acid reductase complex regenerates the fatty acid, while FMN is reduced by either another *Lux* operon gene (*lux G*) or by a genomic bacterium gene. In order to improve the reduction of FMN, Gregor and her team added the *frp* gene after *luxCDABE*. The *frp* gene is a NADPH-flavin oxidoreductase from *V. campbellii*. The operon assembling *luxABCDE* from *P. luminescence* and *frp* was mutated using error-prone PCR.
The *iLux* operon created is therefore a combination of *luxA* with K22E, T119A and S178A mutations ; *luxB* with S13P, V121A and N259D mutations ; *luxC* with N10T, N59D, E74D, S256P, M355T and N230D mutations ; *luxD* and *luxE* without any mutations ; *frp* with M213L, R242L and K256R mutations.

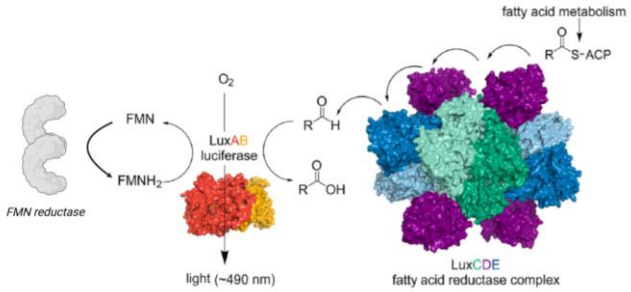


FIGURE 1.
Bioluminescence pathway of iLux operon. (Adapted from Brodl and al., 2018)

B. *Improvement of iLux's adaptability*

Our iGEM team, *Fiat Lux*, decided to adjust the *iLux* operon according to iGEM's competition in order to create a universal bioluminescence system. It will satisfy the BioBrick convention, making the change of plasmid and the use in different bacterial strains easier (Shetty et al., 2008). Directed mutagenesis was successfully realized using IVA PCR to erase EcoRI and XbaI restriction sites within the operon (Fig.2) and adding a BioBrick suffix and prefix around it. Because of the operon's size, six parts were created through that IVA PCR. Thanks to an overlap extension PCR (OE-PCR), their numbers were reduced to three : one with *luxCD*, one with *luxABE* and one with *frp*. They were supposed to be joined again using OE-PCR. However, this time OE-PCR wasn't successful. Hence, a different approach was taken. *LuxABE* was kept and it was joined through OE-PCR to *frp* and *luxCD*, which we synthesized artificially without problematic restriction sites. Thanks to the BioBrick prefix and suffix, the change of vector for the *iLux* operon is now easier.

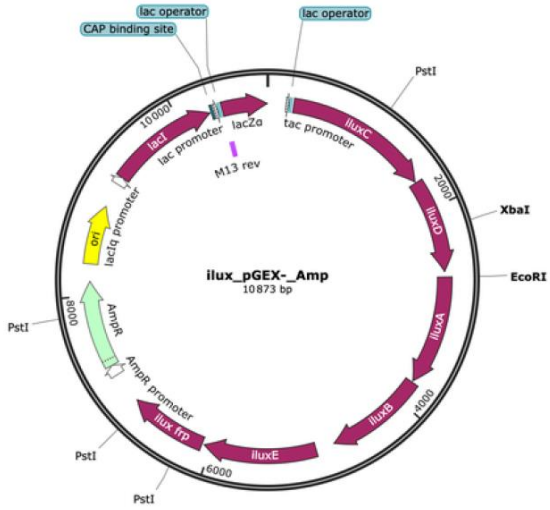


FIGURE 2.
iLux operon in pGex(-)xAmp vector containing problematic restriction sites.

C. *Dickeya's pathogenesis pathway*

Dickeya's bacterial strains are known for being phytopathogens. In Europe, one of the most common *Dickeya* strain found in fields is *Dickeya solani* which can infect potato plants (Blin et al., 2021). *Dickeya solani*'s and other *Dickeya*'s phytopathogenic pathways aren't fully understood yet. The phytopathogenic mechanisms are known, but the spread mechanism of bacteria inside the host isn't.
For now, the indicator of *Dickeya*'s infection and spread are side effects of the infection. "The *Dickeya* virulence strategy is based on its capacity to dissociate the plant cell wall and, for this, the bacteria secrete an extensive set of polysaccharide degrading enzymes, composed mostly of pectinases." (Hugouvieux-Cotte-Pattat, 2016). Hence, the examination of plant tissues, which is the only way to locate the infection for now, is based on the recognition of soft rot tissue caused by cell wall dissociation.

IV. DISCUSSION

iLux operon has the advantage of being autonomous, producing its own enzymes and regenerating the products into new reactives allowing "infinite" bioluminescence as long as the bacterium has enough ATP. The presence of FMNH2 is not problematic because this luciferin is present in every bacterium. It is produced through a biosynthetic pathway in which riboflavin is a precursor (García-Angulo et al., 2017). Gregor and her team inserted their *iLux* operon into the vector pQE(-) and expressed it in *E.coli* Top10. They used this vector because it was the one that increased the bioluminescence activity for the Top10 cells the most. However, this construction is not suitable for every bacterial strain. Indeed, each bacterial strain has a relative affinity for each plasmid, modulating the induced bioluminescence activity. The conformation of the *iLux* operon was not convenient to allow its change to another vector. Firstly, synthesizing the *iLux* operon is not an easy solution because of its size (6605 bp). Usually, oligonucleotide synthesized fragments are 1kb long. To synthesize bigger DNA fragments, ligation and/or recombination steps are necessary (Notka et al., 2011). It represents a considerable cost in terms of money and

time for teams to adapt *iLux* into a specific vector for the bacterial strain they will use. Secondly, extracting the *iLux* operon from pQE(-) and inserting it in another vector is complex. In fact, lots of restriction sites are contained within the operon. A need for the standardization of the operon became apparent to help further studies, which was fulfilled by the BioBrick convention.
The improvement of *iLux*'s adaptability was successful. But even if inserting the new *iLux* operon is quick and easy, additional research should be done to understand, for each bacterial strain, which vector should be used. In fact, the bioluminescence pathway is ATP-consuming which can negatively affect either growth or cell division. Hence, using the *iLux* operon to study and try out treatments on a phytopathogenic bacterial strain is possible if the vector carrying the operon is in a quantity that doesn't use too much ATP. Otherwise, growth or spread pathways could be negatively regulated, falsifying results. However, if the vector doesn't negatively affect those two pathways, then using a high sensitive CCD camera allows the tracking of the infection without interfering with the host integrity. Knowing over time where bacteria are spreading, how long they live in an infected tissue, and how long it takes for the soft rot phenotype to appear, are key pieces of information to find cures. Moreover, when a treatment (biological or chemical) needs to be tested, it will be easier to understand the effects on the phytopathogenic bacterial strain over time. Knowing with precision the consequence of treatments on bacteria is also promising for environmental protection. Indeed, it will be easier to find the best concentrations of a product to apply for its efficacy to be at the minimum needed level, preventing the use of unnecessary products in fields, which could possibly cause environmental problems.

V. CONCLUSION

The creation of the *iLux* operon is a breakthrough for the pathogenic research field. Thanks to the BioBrick convention, the *iLux* operon modified by *Fiat Lux* allows teams to work with it, in an easier and more efficient way. An open-source data collection inventorying each typical bacterial strain and the corresponding vector, in which inserting the iLux operon would not impact the bacterial life pathway, would be a great help for the scientific community.
Furthermore works on the *iLux* operon could be done, focussing particuliary on the luciferase coded by *luxA* and *luxB*, which kinectics datas are still undocumented so far. Diseases, such as the soft rot disease in potato plants caused by *Dickeya solani*, still do not have any treatments nowadays. The use of the *iLux* operon would allow the collection of data on infection evolution through time and treatments to better understand the general pathogenic pathway of bacteria. With a better understanding of pathogenic bacteria, the *iLux* operon gives hope to find new treatments.

REFERENCES

Blin, P., Robic, K., Khayi, S., Cigna, J., Munier, E., Dewaegeneire, P., Laurent, A., Jaszczyszyn, Y., Hong, K.-W., Chan, K.-G., Beury, A., Reverchon, S., Giraud, T., Hélias, V., & Faure, D. (2021). Pattern and causes of the establishment of the invasive bacterial potato pathogen Dickeya solani and of the maintenance of the resident pathogen D. dianthicola. *Molecular Ecology*, *30*(2), 608-624. https://doi.org/https://doi.org/10.1111/mec.15751

Brodl, E., Winkler, A., & Macheroux, P. (2018, 2018/01/01/). Molecular Mechanisms of Bacterial Bioluminescence. *Computational and Structural Biotechnology Journal*, *16*, 551-564. https://doi.org/https://doi.org/10.1016/j.csbj.2018.11.003

Cormac, G. M. G. (2012). The Bacterial lux Reporter System: Applications in Bacterial Localisation Studies. *Current Gene Therapy*, *12*(1), 12-19. https://doi.org/http://dx.doi.org/10.2174/156652312799789244

Czajkowski, R., Pérombelon, M. C. M., van Veen, J. A., & van der Wolf, J. M. (2011). Control of blackleg and tuber soft rot of potato caused by Pectobacterium and Dickeya species: a review. *Plant Pathology*, *60*(6), 999-1013. https://doi.org/https://doi.org/10.1111/j.1365-3059.2011.02470.x

García-Angulo, V. A. (2017, 2017/03/04). Overlapping riboflavin supply pathways in bacteria. *Critical Reviews in Microbiology*, *43*(2), 196-209. https://doi.org/10.1080/1040841X.2016.1192578

Gregor, C., Gwosch, K. C., Sahl, S. J., & Hell, S. W. (2018). Strongly enhanced bacterial bioluminescence with the ilux operon for single-cell imaging. *Proceedings of the National Academy of Sciences*, *115*(5), 962-967. https://doi.org/doi:10.1073/pnas.1715946115

Hugouvieux-Cotte-Pattat, N. (2016). Chapter Four - Metabolism and Virulence Strategies in Dickeya–Host Interactions. In M. San Francisco & B. San Francisco (Eds.), *Progress in Molecular Biology and Translational Science* (Vol. 142, pp. 93-129). Academic Press. https://doi.org/https://doi.org/10.1016/bs.pmbts.2016.05.006

Notka, F., Liss, M., & Wagner, R. (2011). Chapter eleven - Industrial Scale Gene Synthesis. In C. Voigt (Ed.), *Methods in Enzymology* (Vol. 498, pp. 247-275). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-385120-8.00011-5

Shetty, R. P., Endy, D., & Knight, T. F. (2008, 2008/04/14). Engineering BioBrick vectors from BioBrick parts. *Journal of Biological Engineering*, *2*(1), 5. https://doi.org/10.1186/1754-1611-2-5

Teuscher, A. C., & Ewald, C. Y. (2018, 2018/07/20). Overcoming Autofluorescence to Assess GFP Expression During Normal Physiology and Aging in Caenorhabditis elegans. *Bio-protocol*, *8*(14), e2940. https://doi.org/10.21769/BioProtoc.2940

AUTHORS

First Author – ROBLIN Tom, Biosciences engineering student, INSA Lyon, tom.roblin@insa-lyon.fr .

Second Author – MATHIEU Théo, Biosciences engineering student, INSA Lyon, theo.mathieu@insa-lyon.fr .

Third Author – CECCHI Mathilde, Biosciences engineering student, INSA Lyon, mathilde.cecchi@insa-lyon.fr .

Metallic Nanoparticles for Photothermal Therapy

Moor de Waal*, Anna Golova*, Mirthe Zandbelt*, Eva Pakvis*

* Team iGEM Leiden

***Abstract*- Photothermal therapy (PTT) is a promising novel treatment for head and neck cancers. In PTT, metallic nanoparticles (NPs) are used to convert light energy of a laser to heat, which causes cell death in the surrounding tumour tissue. The composition, morphology, and size of these NPs influences the safety and efficacy of PTT. This review concludes that the optimal NPs for PTT have a bimetallic composition with a silver core and golden shell, a star-shaped morphology, and a size between 5 and 150 nm. Depending on size, these nanoparticles have properties required for PTT, such as an optimal photothermal conversion efficiency in the near infrared region (NIR), efficient tumour uptake, low accumulation in other organs, and prevention of immediate clearance. Before metallic NPs can be applied in PTT, a suitable method of synthesis must be developed, next to tumour targeting strategies and coatings. The efficacy and safety should be determined using animal models, organoids, and clinical trials.**

***Index Terms*-** Head and neck cancer, metallic nanoparticles, near infrared light, photothermal therapy

I. INTRODUCTION

Yearly, over 3000 patients in the Netherlands get the diagnosis of head and neck cancer (HNC) (Nederlandse Kankerregistratie, 2022). The treatment of HNC is very dependent upon the type and stage of the cancer, but it often includes a combination of radiotherapy, surgery and systemic therapy (Mody et al., 2021). This can come with severe consequences, such as toxic side-effects or (partial) loss of speech.

A promising new treatment for HNC is photothermal therapy (PTT). PTT is based on the conversion of light energy of a near infrared light (NIR) laser to heat, which causes cell death in the surrounding tissue (Chen et al., 2019). Multiple photothermal agents can be used to achieve this effect, and important traits for these agents are a specificity for tumour cells and high photothermal conversion efficiency (PCE). (Han & Choi, 2021).

Metallic nanoparticles (NPs) can be used as a photothermal agent for PTT due to surface plasmon resonance (SPR). This describes the oscillation of free electrons at the surface of a metal in resonance with an electromagnetic field applied to its particles. The oscillation of electrons is converted to localized heat (Lv et al., 2021). The use of NPs makes it possible to utilise the SPR effect by targeting tumour cells specifically.

Several clinical trials have been conducted with nanomaterial-mediated PTT by the company AuroLase®, using silica-gold nanoshells (Han & Choi, 2021). A trial for prostate cancer showed promising results in tumour reduction (NCT02680535), which indicates the effectiveness of PTT as a treatment for cancer.

A trial on refractory and/or recurrent HNC tumours was not completed as patients showed side-effects (NCT00848042). These clinical trials show that PTT is a promising new therapy, where optimization of the treatment is needed to increase the efficacy and decrease the side effects.

By optimising the NPs in PTT treatment, a more successful cancer therapy could be found for this group. The composition, morphology and size of the NPs influence the light to heat conversion due to a change in the SPR effect. The composition of the NPs also influences their toxicity and stability. The aim of this literature review is therefore to discuss the optimal morphology, size and composition of metallic NPs for the use in PTT.

II. METHODS

The Google Scholar search engine is used, with the following key words in varying combinations: bimetallic, nanoparticles, photothermal therapy, surface plasmon resonance, synthesis, gold, silver, head and neck cancer, morphology, shape, composition, size. Included studies must have been published in the past 15 years and be peer-reviewed. They can be experimental studies or reviews. Studies published before 2007 and non-English publications are excluded from this review.

III. RESULTS AND FINDINGS

A. Composition

Metallic NPs can be used in PTT due to the SPR effect which describes the conversion of light into heat by oscillation of electrons. Noble metals have a strong SPR effect, which makes them effective in producing heat and therefore a good candidate for use in PTT (Lv et al., 2021).

Gold is the most explored noble metal for PTT because the metal requires low radiation energies due to its optical-thermal conversion efficiency. The low radiation energies make PTT less invasive (Jabeen et al., 2014). In addition, gold nanoparticles have an excellent photostability, low cytotoxicity and are biocompatible, which is beneficial for their use as a medical treatment (Hwang et al., 2014). Silver materials have also gained attention recently for the use in PTT due to low toxicity and a better heat conductivity than other metals (Boca et al., 2011). Combining silver and gold into bimetallic NPs offers unique optical properties, which cannot be found in monometallic NPs. The SPR effect of these bimetallic NPs is stronger and the absorption spectra are broader (Boote et al.,

2014). To obtain NPs which have the advantages of silver and gold, without the drawbacks of both metals, the core of the particle should be silver and the so-called shell should be gold (Calderon et al., 2021). These properties make bimetallic NPs preferred for the application of PTT.

B. Morphology

The morphology of metallic NPs significantly influences their physical properties and therefore affects the possible application in photothermal therapy. The optimal absorption of the NPs is in the near infrared region (NIR) between 750 and 900 nm, due to the maximal penetration depth in the tissue and the minimal absorption by biomolecules in the skin and the blood (Hwang et al., 2014). Next to this, NPs with an optimal absorption in the NIR region can be activated with an infrared laser, which makes it possible to apply the treatment at a specific place in the body (Lv et al, 2021). A great diversity of NP shapes is possible, varying from spheres to cubes, rods, wires, and cages (Xie et al., 2010).

Spherical NPs made of only gold have a characteristic absorption at 500-600 nm, which is in the visible spectrum. However, changing the shape to a non-spherical one can shift the absorption to the NIR region between 750 and 900 nm (Xie et al., 2010). The most common morphology of pure gold NPs with absorption in the NIR region are nanorods. These NPs exhibit two wavelength bands, caused by longitudinal and transverse oscillation of electrons, of which the stronger longitudinal wavelength band is in the NIR region (Huang et al., 2007). Gold nanorods have been shown to be effective *in vivo* in ablating ovarian tumour cells (Jang et al., 2012).

Gold nanocages are cubes with hollow interiors and porous walls. NPs of this shape have a variable SPR effect depending on the amount of added metal precursor and can therefore be tuned to exhibit absorbance in the NIR region (Skrabalak et al., 2018). The use of golden nanocages in PTT has been shown to be promising with an *in vivo* study. In this study, golden nanocages of 45 nm were used with an LSPR of 810 nm. They were coupled to an antibody targeting the epidermal growth factor (EGFR) that is overexpressed on breast cancer cells. This caused cellular death of breast cancer cells after irradiation with a laser (Chen et al., 2007).

Another morphology of gold NPs showing absorption in the NIR region are so-called nanostars, or branched NPs. They consist of a core with sharp tips, where the size of the tips influences the optical properties (Hao et al., 2007). Applied electrical fields can be enhanced near the tips of the stars, leading to heat generation. Therefore, a nanostar with long tips, called a nano-urchin, can cause effective photothermal ablation (Liu et al., 2013). Next to this, nanohexapods (nanostars with 6 spikes) have shown greater tumour uptake and photothermal conversion efficiency than gold nanorods and nanocages (Vines et al., 2019)

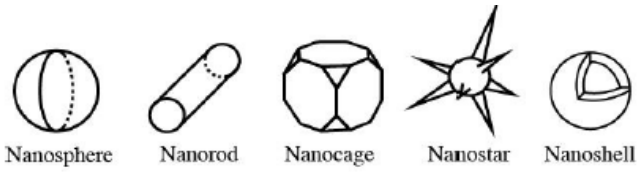


FIGURE 1.
Schematic representation of possible shapes of nanoparticles. The shapes are based on the review of De Berardis et al. (2020).

Besides gold NPs, the shift of absorbance from the visual spectrum to the NIR region also occurs in bimetallic NPs, such as those with an iron oxide core and a gold nanoshell. These particles in solution have been irradiated with NIR light with a centre wavelength of 808±10 nm leading to an increase of the temperature of the solution (Ji et al, 2007). Nanostars made of silver and gold also exhibit absorption in the NIR region as these show an absorbance peak at 949 nm (Joseph et al., 2019).

To conclude, the absorbance of gold and bimetallic NPs can be shifted from the visual spectrum to the NIR region by changing the shape from spherical to a nanorod, nanoshell, nanocage, or nanostar, where nanohexapods are the most optimal morphology for PTT due to their great tumour uptake and photothermal conversion efficiency (Vines et al., 2019).

C. Size

suitable the particles are for photothermal therapy. Firstly, the nanoparticle distribution in the body after intravenous injection is influenced by the size of the NPs. NPs larger than 200 nm get removed by the reticuloendothelial system, which causes direct clearance and thereby an ineffective therapy (de Barros, 2012). Research by Moghimi et al. (2012) suggested that filtration by the reticuloendothelial system can be avoided by using NPs smaller than 150 nm. However, NPs of a size under 10 nm, are also unsuitable for photothermal therapy, since they quickly get filtered out of the body by the renal system due to their small size (Zuckerman, 2012).

Additionally, accumulation of NPs at sites outside of tumour tissue is undesirable, since this can have cytotoxic effects. The largest accumulation of NPs occurs in the heart, the liver, and the spleen (Dreaden, 2012). Several studies have been conducted to discover at what size NPs accumulate least. In the research of Jong et al. (2008) mice were intravenously injected with gold NPs of size 10, 50, 100 and 250 nm. After 24 hours, the amount of gold NPs in the blood, heart, lung, thymus, liver, spleen, kidney and brain was measured and it was observed that most NPs accumulated in the liver and spleen. The particles with a size of 10 nm accumulated in almost every organ, while the larger particles were only found in the blood, liver, and spleen. Interestingly, the particles with size of 50 nm showed the lowest accumulation in the liver and spleen compared to all other sizes. Research of Sonavane et al. (2008) found similar results after injecting mice with 15, 50, 100, and 200 nm NPs. 15 nm NPs accumulated most throughout the tissues, while the 50 nm NPs had the lowest accumulation in the liver and spleen.

Another factor that influences the efficiency of PTT is how well the particles are able to reach the tumour cells. Due to the leaky vasculature and the poor lymphatic drainage around tumours,

the interstitial fluid pressure in tumours is high (Hoffman, 2006). This pressure is higher at the core of the tumour, making it harder for NPs to reach the centre. A model of tumour tissue has shown that NPs of 12 nm are better at penetrating the tumour than larger particles (Chauhan, 2012). An *in vivo* study by Natarajan et al. (2008) confirmed these results, in which tumour targeting was compared for NPs of size 20, 30, and 100 nm. They found that the 20 nm particles were more efficient at targeting the tumour than their bigger-sized counterparts. However, the NPs of 20 nm did have a lower heating capacity than their bigger-counterparts, which is another aspect that needs to be considered.

Hence, the range for NPs suitable for PTT lies between 10 and 150 nm and within this range certain sizes are more favourable for certain aspects. While NPs of 50 nm show lower accumulation in the liver and spleen, NPs around 20 nm are best at reaching tumour tissue. Therefore, it is not agreed upon what the 'ideal' size for NPs is.

IV. DISCUSSION

In order to realise NPs with optimal composition, shape and size, a suitable method of synthesis must be developed. With current techniques this is hard to accomplish chemically, for the reason that golden spikes will be reduced quicker than silver, which leads to the formation of NPs with a golden core and silver spikes. These NPs are less preferred for PTT as explained earlier (Calderon et al., 2021). Moreover, the preparations of NPs are usually carried out by various physical and chemical methods like laser ablation, pyrolysis, lithography, chemical vapour deposition, sol-gel technique, and electrodeposition which are very expensive and hazardous. Using these methods also leads to the presence of some toxic chemicals absorbed on the surface that may have adverse effects in applications (Roopan et al., 2014).

After completing synthesis of the NPs, several aspects need to be further researched and taken into account. Firstly, different methods for the administration of the NPs need to be explored. Some known strategies are intra-tumour injection, active targeting, biometric targeting and programmed targeting (Zhao et al., 2021). In addition, different coatings should be tested. Polyethylene glycol is a coating with high potential to use successfully and safely on golden NPs for drug purposes (Zamora-Justo et al., 2019). Over and above that, the distribution through the body and the natural clearance of the NPs should be checked in animal models. Additionally, the cytotoxicity could be tested on organoids before entering the clinical trials (Angela L Caipa Garcia, 2021). After finalising the preclinical experiments, the newly developed therapy for PTT should be tested in the clinical trials, including additionally needed devices, e.g. NIR lasers. In the course of these trials efficacy and adverse effects will be tested on test patients.

V. CONCLUSION

To conclude, the optimal NPs for PTT have a bimetallic composition with a silver core and golden shell, combining the best of both worlds.The optimal morphology is likely to be a nanohexapod due to their good tumour uptake and photothermal conversion efficiency. NPs of a size between 10 and 150 nm are optimal, where NPs of 50 nm show lower accumulation in organs, and NPs under 30 nm are best at reaching the tumour tissue. More research is needed on the synthesis of the optimal NPs. Before the NPs can be utilized in PTT, the options for administration and coatings should be explored, and research is needed on the distribution through the body and the cytotoxicity. might elaborate on the importance of the work or suggest applications and extensions.

REFERENCES

Angela L Caipa Garcia, Volker M Arlt, David H Phillips, Organoids for toxicology and genetic toxicology: applications with drugs and prospects for environmental carcinogenesis, Mutagenesis, Volume 37, Issue 2, April 2022, Pages 143–154, https://doi.org/10.1093/mutage/geab023

Boca, S. C., Potara, M., Gabudean, A. M., Juhem, A., Baldeck, P. L., & Astilean, S. (2011). Chitosan-coated triangular silver nanoparticles as a novel class of biocompatible, highly effective photothermal transducers for in vitro cancer cell therapy. Cancer Letters, 311(2), 131–140. https://doi.org/10.1016/j.canlet.2011.06.022

Boote, B. W., Byun, H., & Kim, J. H. (2014). Silver–Gold Bimetallic Nanoparticles and Their Applications as Optical Materials. Journal of Nanoscience and Nanotechnology, 14(2), 1563–1577. https://doi.org/10.1166/jnn.2014.9077

Calderon, I., Alvarez-Puebla, R. A., & Pazos-Perez, N. (2021). Gold-spiked coating of silver particles through cold nanowelding. Nanoscale, 13(8), 4530-4536.

Chauhan, V., Stylianopoulos, T., Martin, J. et al. Normalization of tumour blood vessels improves the delivery of nanomedicines in a size-dependent manner. Nature Nanotech 7, 383–388 (2012). https://doi.org/10.1038/nnano.2012.45

Chen, J., Ning, C., Zhou, Z., Yu, P., Zhu, Y., Tan, G., & Mao, C. (2019). Nanomaterials as photothermal therapeutic agents. Progress in Materials Science, 99, 1–26. https://doi.org/10.1016/j.pmatsci.2018.07.005

Chen, J., Wang, D., Xi, J., Au, L., Siekkinen, A., Warsen, A., Li, Z. Y., Zhang, H., Xia, Y., & Li, X. (2007). Immuno Gold Nanocages with Tailored Optical Properties for Targeted Photothermal Destruction of Cancer Cells. Nano Letters, 7(5), 1318–1322. https://doi.org/10.1021/nl070345g

De Barros, A. B., Tsourkas, A., Saboury, B., Cardoso, V. N., & Alavi, A. (2012). Emerging role of radiolabeled nanoparticles as an effective diagnostic technique. EJNMMI research, 2(1), 39. https://doi.org/10.1186/2191-219X-2-39

De Berardis, B., Marchetti, M., Risuglia, A., Ietto, F., Fanizza, C., & Superti, F. (2020). Exposure to airborne gold nanoparticles: a review of current toxicological data on the respiratory tract. Journal of Nanoparticle Research, 22(8), 1-41.

De Jong, W. H., Hagens, W. I., Krystek, P., Burger, M. C., Sips, A. J., & Geertsma, R. E. (2008). Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials, 29(12), 1912–1919. https://doi.org/10.1016/j.biomaterials.2007.12.037

Dreaden, E. C., Austin, L. A., Mackey, M. A., & El-Sayed, M. A. (2012). Size matters: gold nanoparticles in targeted cancer drug delivery. Therapeutic delivery, 3(4), 457–478. https://doi.org/10.4155/tde.12.21

Han, H. S., & Choi, K. Y. (2021). Advances in Nanomaterial-Mediated Photothermal Cancer Therapies: Toward Clinical Applications. Biomedicines, 9(3), 305. https://doi.org/10.3390/biomedicines9030305

Hao, F., Nehl, C. L., Hafner, J. H., & Nordlander, P. (2007). Plasmon Resonances of a Gold Nanostar. Nano Letters, 7(3), 729–732. https://doi.org/10.1021/nl062969c

Hofmann, M., Guschel, M., Bernd, A., Bereiter-Hahn, J., Kaufmann, R., Tandi, C., Wiig, H., & Kippenberger, S. (2006). Lowering of tumor interstitial fluid pressure reduces tumor cell proliferation in a xenograft tumor model. Neoplasia (New York, N.Y.), 8(2), 89–95. https://doi.org/10.1593/neo.05469

Hong, H., Zhang, Y., Sun, J., & Cai, W. (2009). Molecular imaging and therapy of cancer with radiolabeled nanoparticles. Nano today, 4(5), 399–413. https://doi.org/10.1016/j.nantod.2009.07.001

Huang, X., El-Sayed, I. H., Qian, W., & El-Sayed, M. A. (2007). Cancer Cells Assemble and Align Gold Nanorods Conjugated to Antibodies to Produce Highly Enhanced, Sharp, and Polarized Surface Raman Spectra: A Potential Cancer Diagnostic Marker. Nano Letters, 7(6), 1591–1597. https://doi.org/10.1021/nl070472c

Hwang, S., Nam, J., Jung, S., Song, J., Doh, H., & Kim, S. (2014). Gold nanoparticle-mediated photothermal therapy: current status and future perspective. Nanomedicine, 9(13), 2003-2022.

Jabeen, F., Najam-ul-Haq, M., Javeed, R., Huck, C., & Bonn, G. (2014). Au-Nanomaterials as a Superior Choice for Near-Infrared Photothermal Therapy. Molecules, 19(12), 20580–20593. https://doi.org/10.3390/molecules191220580

Jang, B., Park, S., Kang, S. H., Kim, J. K., Kim, S. K., Kim, I. H., & Choi, Y. (2012). Gold nanorods for target selective SPECT/CT imaging and photothermal therapy in vivo. Quantitative imaging in medicine and surgery, 2(1), 1.

Ji, X., Shao, R., Elliott, A. M., Stafford, R. J., Esparza-Coss, E., Bankson, J. A., Liang, G., Luo, Z. P., Park, K., Markert, J. T., & Li, C. (2007). Bifunctional Gold Nanoshells with a Superparamagnetic Iron Oxide−Silica Core Suitable for Both MR Imaging and Photothermal Therapy. The Journal of Physical Chemistry C, 111(17), 6245–6251. https://doi.org/10.1021/jp0702245

Liu, R. S., Cheng, L. C., Huang, J. H., Chen, H. M., Lai, T. C., Hsiao, M., Chen, C. H., Yang, K. Y., Tsai, D. P., & Her, L. J. (2013). Highly efficient urchin-like bimetallic nanoparticles for photothermal cancer therapy. SPIE Newsroom. https://doi.org/10.1117/2.1201301.004676

Joseph, D., Baskaran, R., Yang, S. G., Huh, Y. S., & Han, Y. K. (2019). Multifunctional spiky branched gold-silver nanostars with near-infrared and short-wavelength infrared localized surface plasmon resonances. Journal of Colloid and Interface Science, 542, 308–316. https://doi.org/10.1016/j.jcis.2019.01.132

Lv, Z., He, S., Wang, Y., & Zhu, X. (2021). Noble Metal Nanomaterials for NIR-Triggered Photothermal Therapy in Cancer. Advanced Healthcare Materials, 10(6), 2001806. ISO 690

Mody, M. D., Rocco, J. W., Yom, S. S., Haddad, R. I., & Saba, N. F. (2021). Head and neck cancer. The Lancet, 398(10318), 2289–2299. https://doi.org/10.1016/s0140-6736(21)01550-6

Moghimi, S. M., Hunter, A. C., & Andresen, T. L. (2012). Factors controlling nanoparticle pharmacokinetics: an integrated analysis and perspective. Annual review of pharmacology and toxicology, 52, 481–503. https://doi.org/10.1146/annurev-pharmtox-010611-134623

Natarajan, A., Gruettner, C., Ivkov, R., DeNardo, G. L., Mirick, G., Yuan, A., Foreman, A., & DeNardo, S. J. (2008). NanoFerrite particle based radioimmunonanoparticles: binding affinity and in vivo pharmacokinetics. Bioconjugate chemistry, 19(6), 1211–1218. https://doi.org/10.1021/bc800015n

Nederlandse Kankerregistratie (NKR), IKNL. Obtained through iknl.nl/nkr-cijfers, on 20-07-2022.

Roopan, S.M., Surendra, T.V., Elango, G. et al. Biosynthetic trends and future aspects of bimetallic nanoparticles and its medicinal applications. Appl Microbiol Biotechnol 98, 5289–5300 (2014). https://doi.org/10.1007/s00253-014-5736-1

Saion, E., Gharibshahi, E., & Naghavi, K. (2013). Size-controlled and optical properties of monodispersed silver nanoparticles synthesized by the radiolytic reduction method. International journal of molecular sciences, 14(4), 7880–7896. https://doi.org/10.3390/ijms14047880

Shafiqa, A.R. & Abdul Aziz, Azlan & Mehrdel, Baharak. (2018). Nanoparticle Optical Properties: Size Dependence of a Single Gold Spherical Nanoparticle. Journal of Physics: Conference Series. 1083. 012040. 10.1088/1742-6596/1083/1/012040.

Skrabalak, S. E., Chen, J., Sun, Y., Lu, X., Au, L., Cobley, C. M., & Xia, Y. (2008). Gold Nanocages: Synthesis, Properties, and Applications. Accounts of Chemical Research, 41(12), 1587–1595. https://doi.org/10.1021/ar800018v

Sonavane, G., Tomoda, K., & Makino, K. (2008). Biodistribution of colloidal gold nanoparticles after intravenous administration: Effect of particle size. Colloids and Surfaces B: Biointerfaces, 66(2), 274–280. https://doi.org/10.1016/j.colsurfb.2008.07.004

Vines, J. B., Yoon, J. H., Ryu, N. E., Lim, D. J., & Park, H. (2019). Gold Nanoparticles for Photothermal Cancer Therapy. Frontiers in Chemistry, 7. https://doi.org/10.3389/fchem.2019.00167

Xie, J., Lee, S., & Chen, X. (2010). Nanoparticle-based theranostic agents. Advanced drug delivery reviews, 62(11), 1064-1079.

Zamora-Justo, J. A., Abrica-González, P., Vázquez-Martínez, G. R., Muñoz-Diosdado, A., Balderas-López, J. A., & Ibáñez-Hernández, M. (2019). Polyethylene Glycol-Coated Gold Nanoparticles as DNA and Atorvastatin Delivery Systems and Cytotoxicity Evaluation. Journal of Nanomaterials, 2019, 1–11. https://doi.org/10.1155/2019/5982047

Zhao, L., Zhang, X., Wang, X., Guan, X., Zhang, W., & Ma, J. (2021). Recent advances in selective photothermal therapy of tumor. Journal of Nanobiotechnology, 19(1), 1-15.

Zuckerman, J. E., Choi, C. H., Han, H., & Davis, M. E. (2012). Polycation-siRNA nanoparticles can disassemble at the kidney glomerular basement membrane. Proceedings of the National Academy of Sciences of the United States of America, 109(8), 3137–3142. https://doi.org/10.1073/pnas.1200718109

AUTHORS

First Author – Moor de Waal, Undergraduate Life Science & Technology Student, Leiden University, moordewaal@gmail.com.

Second Author – Anna Golova, Undergraduate Mathematics Student, Leiden University, anna.golova@gmail.com.

Third Author – Mirthe Zandbelt, Undergraduate Life Science & Technology Student, Leiden University, mirthezandbelt@gmail.com.

Fourth Author - Eva Pakvis, Undergraduate Biology Student, Leiden University, eva-pakvis@hotmail.nl.

Literature review and evaluation of previous iGEM biodesalination projects

Lara Anderegg*, Mathis Ben Harira*, Lucrezia Gheno*, Anirudh Rajesh*, Helène Rulkens*, Neha Silva*, Natalie Varahram*,

* MSP-Maastricht iGEM 2022 team, Maastricht University

ABSTRACT

This year, as a part of the iGEM competition, MSP-Maastricht has decided to tackle the ongoing issue of water scarcity, both locally and globally, by developing a biosynthetic approach to water desalination. The aim is to accomplish this by genetically modifying marine bacteria, such that the organism will be able to take up larger quantities of salt through two cellular pumps. The NpHR pump will be utilised for the removal of Cl^-, whilst a pH-dependent antiporter will be responsible for the removal of Na^+. The pump activity will be controlled by exposing light of a specific wavelength to the bacteria, which will be immobilised and embedded in a sol-gel medium, facilitating potential industry applications. This paper addresses previous iGEM teams' attempts at similar projects, their shortcomings, and how MSP-Maastricht aims to address these in this year's project. Furthermore, this paper functions as a literature review, providing an overview of the design of MSP-Maastricht's project.

Index Terms - Biodesalination, iGEM, cyanobacteria, genetic engineering, literature review

I. INTRODUCTION

Water scarcity is today more than ever a modern issue. Climate change has led not only to a decrease in the amount of freshwater available, but it has impacted its quality as well (Ma et al., 2022). Moreover, the impact has gotten larger and more urgent, not only in developing countries but in countries such as Italy, Spain, and Portugal as well, which are currently affected by extreme droughts (Cresswell-Clay et al., 2022; Jones, 2022). To combat this, restrictions in usage of freshwater are being implemented (Schauenberg, 2022). As this issue grows in urgency, so does the global interest towards innovative, sustainable solutions that could solve the water scarcity issue. Various iGEM teams have attempted to address this challenge, by developing projects in which saline water could be converted into freshwater through the process of biodesalination (Minas et al., 2015).

This article is a literature review and evaluation of the past iGEM teams' biodesalination projects, followed by future prospects and the current biodesalination project being conducted by the MSP-Maastricht team. For the final evaluation of the current state of research, various factors will be kept into account, such as the chassis organism chosen and the gene(s) that have been integrated in said organism. Through secondary research and expert interviews, it is clear that for a synthetic approach to be made into a scalable prototype for biodesalination some criteria must be met. Firstly, an ideal organism possessing features such as being axenic, easy manipulation, a naturally high salt tolerance and a sequenced genome must be chosen. Secondly, the energy costs needed for the biodesalination methods should also be as low as possible, in order to make it competitive with the costs and sustainability of current desalination methods (Kevin Watkins, 2006).

II. RESEARCH ELABORATIONS

A. Research design

Research was done through the use of the projects database provided by igem. Additionally, databases such as "WebofScience" or "GoogleScholar" were employed. When using these search engines, specific keywords were used, such as "biodesalination", "cyanobacteria" or "Na^+ influx pumps".

III. RESULTS AND FINDINGS

Colorado State University 2013

Colorado State University was the first iGEM team to attempt biodesalination. The project involved the genetic modification of *S. cerevisiae* to remove all pumps responsible for efflux of ions out of the cell, with particular attention to those removing Na^+. Additionally, light activated channelrhodopsins (ChR2 and ChEF) were used to increase sodium uptake ability of the cell (Lin, 2011; Nagel et al., 2003). The imported salt would have then been stored in the vacuole. In addition to channelrhodopsins, Na^+/H^+ antiporters were introduced in the cell. The NHX1 antiporter was chosen, since it is already present in the vacuole membrane of the organism (Zhu et al., 2016). However, NHX1 is less specific than other antiporters, meaning that the sodium movement rate across the vacuole membrane was lower. Thus, NHX1 was overexpressed in the chosen organism in order to compensate for its low specificity. The final product was not tested, as the Gibson assembly was not yielding positive results.

Shanghai 2015

The Shanghai 2015 iGEM team took inspiration from Amezaga 2014 et al. Their idea was to modify *Synechocystis sp.* PCC 6803 to express a Cl^- halorhodopsin pump (NpHR) and more Na^+ ion channels. Two different NpHR promoters were chosen, namely PcpcG2 and Pdark. The former induces transcription upon green light exposure, the latter upon absence of light; these two promoters were chosen to ensure efficiency in both presence or absence of light. In their results the team mainly focuses on the *Synechocystis* with the Pdark promoter then the ones with PcpcG2 promoter. In the first sets of data, Na^+ and Cl^- were measured at different time intervals during the desalination stage. It was observed that there was an obvious decrease at the beginning, followed by a rise, which was consistent with the expected process controlled by Pdark. After adjusting the measurement, it was shown that Na^+ concentration decreased by

1.75 g/L and Cl^- concentration by 3 g/L after 10 hours with the Pdark promoter.

Aachen 2017

In 2017, the Aachen team worked on modifying yeast in order to increase salt accumulation into yeast vacuoles. The chosen organism was *S. cerevisiae*, previously used by CSU in 2013. Na^+ efflux mechanisms (ENA1 and NHA1) were knocked out, while native Na^+ and Cl^- ion transporters (NHX1 and GEF1 respectively) were overexpressed. This would lead to an increase in NaCl uptake into the prevacuolar compartment, and reduced leakage of said ions back into the external environment (Gaxiola et al., 1999). Additionally, a vacuolar ion transporter from a salt tolerant plant was incorporated into their gene construct (AtNHXS1) (Sottosanto et al., 2007). This gene codes for an antiporter that exchanges H^+ ions with Na^+ ions to move Na^+ ions against their concentration gradient into the vacuole (Rodríguez-Rosales et al., 2009; Sottosanto et al., 2007). In order to maintain this exchange, elevated levels of H^+ had to be maintained in the vacuole. To achieve this, the AVP1 gene was inserted in the gene construct. This protein has many capacities, however here it was chosen for its property of vacuole acidification, which would allow high H^+ levels in the vacuoles (Schilling et al., 2017). This project had a rather successful outcome, as salt content of seawater was decreased by ~39%.

Kyoto 2018

In this project, yeast, namely *S. cerevisiae*, was once again used to desalinate water by transporting sodium ions into the vacuoles of yeast. However, a yeast used to brew soya sauce, *Zygosaccharomyces rouxii*, was also used. This yeast possesses naturally high salt tolerance; therefore, it was chosen with the intention of incorporating some of the genes yielding this high salinity stress resistance into *S. cerevisiae* (Hou et al., 2013). As glycerol was found to be an important molecule in regulating the osmotic stress of *S. cerevisiae*, the genes chosen were ZrGPD1, a glycerol-3-phosphate dehydrogenase, and ZrFPS1, a glycerol transporter. (Costenoble et al., 2000). Additionally, Mangrin, a mangrove protein, was included in the gene construct as well, for its ability of repairing other cellular proteins damaged by salt, (Yamada et al., 2002).

As in previous projects, Na^+ efflux pumps were knocked out, namely NHA1, ENA1, ENA2 and ENA4. Furthermore, AtNHXS1 and SseNHX1, Na^+/H^+ antiporter genes, were added. The AVP1 gene (previously mentioned) increases H^+ concentration within the vacuole, allowing the antiporters to work as efficiently as possible. However, during the project it was found that overexpressing plasmids led to ERAD (Endoplasmic Reticulum-Associated Degradation) which significantly inhibited growth of colonies, or rendered it impossible in some cases (Vembar & Brodsky, 2008). Despite this issue, it was still observed that when lacking all Na^+ efflux pumps, the cells were able to reduce the salt concentration of the medium by 80 mM Na^+.

Linkoping 2021

Linkoping aimed to use phototrophic organisms to desalinate saltwater for agricultural use. *Synechocystis sp.* PCC 6803, a cyanobacterium, was chosen as a chassis organism (Yu et al., 2013). This cyanobacterium was engineered to express halorhodopsin, an inward directed Cl^- pump, channelrhodopsin, an ion channel incorporated to allow influx of Na^+, and CBD, a cellulose binding domain, meaning a surface protein that binds to cellulose (Deisseroth & Hegemann, 2017; Dutta et al., 2015; Linder & Teeri, 1996). The combination of these parts would have led to inward pumping of Cl^-, which would have in turn created an electrochemical gradient within the cell that would have caused Na^+ to flow into the cell. Additionally, expression of a native ion channel, MscL, was induced, to regulate osmotic pressure (Booth & Blount, 2012).

MSP-Maastricht 2022

In 2014 Amezaga et. al, published a paper on a potential method of biodesalination, in which the idea of genetically modifying cyanobacteria to express halorhodopsin NpHR was introduced. NpHR is a Cl^- pump activated upon exposure to 580 nm wavelength of light, which corresponds to the yellow/green visible light spectrum. Amezaga found this to be beneficial for biodesalination, as it reduces an organism's ability to pump Na^+ back out of the cell. This is due to the naturally occurring active Na^+-ATPase pumps, which depend on ATP produced by the organism during photosynthesis to remove Na^+ ions. When NpHR-expressing cells are exposed to green light (exclusively), their ability to photosynthesise is reduced, hence lowering ATP production. As a consequence, the cell is starved of energy, and Na^+ efflux is reduced. Furthermore, the green light activating NpHR provides the cells with the ability to take up more sodium than the osmotic potential allows it. With the influx of negative Cl^- ions, the cell's potential becomes negative, driving the accumulation of more positive ions, such as Na^+. This paper served as inspiration and basis for the MSP-Maastricht 2022 project.

The chassis organism chosen for this project was a marine cyanobacterium, *Synechococcus sp.* Given the marine origins of the organism, compensating for salt tolerance would not be a concern. The chosen organism strains were PCC 7002 and PCC 11901. Both strains are axenic, meaning that they can survive independently of other organisms in the ecosystem. (Zhang et al., 2021). Additionally, they are photosynthetic, meaning that a significantly lower quantity of nutrients is required; lastly, their genomes have been sequenced (PC7002 GenBank assembly accession: GCA_000019485.1; PC11901 GenBank assembly accession: GCA_005577135.1) allowing us to be able to work with these particular strains. Another reason for this choice was the high growth rate of both strains, with a doubling time of 2.6 hours for PCC7002 and 2 hours for PCC11901 (Włodarczyk et al., 2020; Ludwig et al., 2012).

The aim of the project is to express Cl^- and Na^+ pumps in *Synechococcus sp.* to desalinate saline water into fresh water. This project will use a membrane pump halorhodopsin that is activated by light. This halorhodopsin pump will pump in Cl^- ions into the cell (Lanyi, 2012). Thanks to its light-activation properties, NpHR was chosen, as this would allow for regulation of the pump activity. For the removal of Na^+, Na^+/H^+ antiporters will be used, namely NhaS3, NhaS4 and NhaA. All antiporters will be tested independently, to determine the most efficient combination.

The regulating factor of these three antiporters is the pH gradient; additionally, the magnitude of the electrochemical potential difference will decide the direction of ion exchange (Hunte et al.,2005). As seawater shows a pH of around 8.1 and the intracellular pH of *Synechococcus* PCC 7942 being 7.16 ± 0.03 to 7.55 ± 0.02., the gradient is maintained (Marion et al., 2011 Ritchie, 2012). Kaplan et al. (1989) also observed that

when the pH of the medium containing the bacteria was changed from 7.0 to 8.5, there was a higher intake of Na^+ via the NhaS3 antiporter.

It should be considered that cyanobacteria possess mechanisms that pump out excess Na^+, since when present in high quantity it could lead to cell death through various pathways (Huflejt et al., 1990; Allakhverdiev and Murata, 2008; Bhargava et al., 2003). To achieve this, cyanobacteria use ATP to actively pump out excess Na^+ (Marin et al., 2004; Wiangnon et al., 2007). However, it is possible to reduce this activity by reducing the rate of photosynthesis, and with it ATP production. Liu & van Iersel (2021) observed that green light corresponds to the lowest rate of photosynthesis. As previously mentioned, the Cl^- pump halorhodopsin is also activated at green light providing us with an advantageous working set up

Moreover, MSP-Maastricht has considered how this could potentially be scaled up and applied in an industrial context. Given the biosafety concerns and strict regulations surrounding GMOs, there would have to be a simple and safe way to introduce and remove the cyanobacteria to and from the water. The solution would have to meet several criteria, it would have to; allow the bacteria to adhere to the surface non-covalently; not interfere with ions; and be easily producible. This is attainable when using surface imprinting techniques. Such techniques are mostly applied in biosensors, in which they create synthetic receptors for bioparticle detection (Eersels et al., 2016). In surface imprinting, a template solution, containing bacteria, and a polymerization mixture, containing monomers, cross-linkers and initiators, are mixed (Cai et al., 2018; Eersels et al., 2016). The polymerization mixture engulfs the template and is cross-linked. After curing, the template particles can be removed, resulting in a polymer layer containing indentations that can rebind target bacteria by shape recognition.

Amongst several, sol-gel-derived films can be used as a surface imprinting technique (Cai et al., 2018; Eersels et al., 2016). The sol-gel process converts monomers into a colloidal solution (sol). This colloidal solution acts as the precursor for forming an integrated soft network of polymers (gel) (Cai et al., 2018). Compared to other imprinting techniques, sol-gel-derived films have the advantage that they have a low processing temperature, fairly good adhesion, and are easy to manufacture (Betancor et al., 2005). Therefore, the team decided to use a silica-based sol-gel to imprint the genetically modified (GM) cyanobacteria. This sol-gel, including the imprinted GM *Synechococcus*, will be transferred to a container filled with seawater. After the maximum salt absorption is reached in GM *Synechococcus*, the sol-gel with the imprinted GM *Synechococcus* will be transferred to a container filled with a solution that removes the bacteria. After removal, a new layer of GM *Synechococcus* is bound onto the sol-gel template surface (this is analogous to two jigsaw pieces fitting together). This results in a reusable sol-gel surface that can easily bind and unbind bacteria.

Figure 1
Light microscope image of E.coli stained with Safranin-O adherent to a silica-based sol-gel containing TMOS, taken during testing of the immobilisation technique. (*Source:* Author, 2022).

IV. DISCUSSION

Some of the previous (similar) attempts saw varying degrees of success. For starters, Colorado State University 2013 failed to get past the gibson assembly phase. The hypothesised reason for this failure is that the insert protein sequences contained a number of various restriction site sequences. To avoid such an error from occurring in MSP-Maastricht's project, the insert sequences were all screened.

Shanghai 2015 was one of the more successful teams. As aforementioned, the team managed to use their system to reduce the Na^+ concentration from 8.5g/L to ~6.75g/L (approx. -1.75g/L) and Cl^- concentration from ~15g/L to ~12g/L (approx. -3g/L). This is equal to a roughly 20% reduction in both Na^+ and Cl^- levels. However, Shanghai only inserted one pump - halorhodopsin - and a sodium channel. Shanghai (successfully) aimed to import Na^+ through the artificial creation of an electrochemical gradient. That being said, in order to maximise NaCl sequestration, MSP-Maastricht intends to pair the NpHR pump with one of three sodium/hydrogen antiporters, to actively pump both ions into cells. The most successful of the three combinations will then be determined and optimised. This should prove more efficient than pumping one ion and depending on a concentration gradient for the other one to be absorbed.

Although Aachen 2017 also obtained very promising results, there were still some areas for improvement with the device. Firstly, using yeast as a chassis organism means that one must supply the cultures with nutrients. This adds an - avoidable - extra level of complexity to the maintenance process. Secondly, to remove the free floating yeast from the water an ultrafiltration system was proposed. This means once the yeast have absorbed the maximum amount of salt, the tank would need to be drained of water, the filter would need to be cleaned or changed, and the surfaces would need to be cleaned of yeast. The cleaning process would have to be one that does not cause cell lysis, as this would release the salt content and render the entire biodesalination process redundant. The MSP-Maastricht immobilisation solution offers a much simpler and convenient solution, where a continuous flow of water could be applied as GM containing slides are removed/introduced into the water tank. Finally, with Aachen 2017's system, salt accumulation cannot be stopped ensuring that the organisms die and cannot be reused. This also means the water would have to be drained before the cells die to

limit lysis and subsequent release of salts back into the water. Hence, the tank would have to be cleaned out after each volume of water desalinated.

Kyoto 2018 was another team to report somewhat successful results. After developing multiple different mutant yeast strains, the team obtained a maximum sodium uptake of 80mM from the medium. Assuming the concentration of NaCl to be 0.6M in seawater (Huh et al., 2014), then this would be a ~13.3% reduction in sodium levels. Unfortunately, since chloride levels were not measured, no definite conclusions can be drawn regarding the net movements of this ion. Kyoto determined that translation of exogenous proteins could have been limited by Endoplasmic Reticulum-Associated Degradation. Furthermore, over-expression of exogenous proteins likely had cytotoxic effects on the host organism, limiting colony growth. Kyoto hypothesised that these effects occurred due to the use of high expression plasmids. This resulted in very limited growth of the colonies containing them, or no growth at all. In order to not run into the same issue, MSP-Maastricht's plasmid uses a mid-strength constitutive promoter to avoid over-expression of the inserted protein sequences.

Conversely to Shanghai, Linkoping 2021 found halorhodopsin to be toxic to the host organism (despite using identical strains and proteins). This was determined to be caused by poor conservation of the sequence after transformation into *E. Coli* and *V. Natriegens*. The proposed reasoning for this was that the gene was attacked by the host organism since it was compromising its survival, which led to the shorter and potentially toxic halorhodopsin proteins being translated. This, in turn, was a strongly limiting factor in the survival of colonies being grown with these plasmids as Linkoping hypothesised the misconserved proteins to be toxic to the cells. That being said, the team only applied the developed plasmid on non-cyanobacteria (*E. Coli* and *V. Natriegens*). The MSP-Maastricht project intends to transform cyanobacteria which are closely related to the species where the NpHR pump originates from. Therefore, a much more accepting response by the host cell is expected.

V. CONCLUSION

In conclusion, having identified and attempted to address weaknesses of previous iGEM teams with similar projects, the MSP-Maastricht 2022 team is hopeful that with careful execution, the project can yield strong initial results. These can then be continuously expanded upon and further investigated beyond the context of iGEM, to perhaps one day contribute as a viable, easily accessible desalination alternative.

REFERENCES

Eersels, K., Lieberzeit, P., & Wagner, P. (2016, 2016/10/28). A Review on Synthetic Receptors for Bioparticle Detection Created by Surface-Imprinting Techniques—From Principles to Applications. ACS Sensors, 1(10), 1171-1187. https://doi.org/10.1021/acssensors.6b00572

Austin, S., & Mayer, A. (2020). Phosphate Homeostasis - A Vital Metabolic Equilibrium Maintained Through the INPHORS Signaling Pathway. Front Microbiol, 11, 1367. https://doi.org/10.3389/fmicb.2020.01367

Betancor, L., López-Gallego, F., Hidalgo, A., Fuentes, M., Podrasky, O., Kuncova, G., Guisán, J. M., & Fernández-Lafuente, R. (2005, 2005/03/01). Advantages of the Pre-Immobilization of Enzymes on Porous Supports for Their Entrapment in Sol−Gels. Biomacromolecules, 6(2), 1027-1030. https://doi.org/10.1021/bm0493077

Booth, I. R., & Blount, P. (2012, Sep). The MscS and MscL families of mechanosensitive channels act as microbial emergency release valves. J Bacteriol, 194(18), 4802-4809. https://doi.org/10.1128/jb.00576-12

Cai, W., Li, H.-H., Lu, Z.-X., & Collinson, M. M. (2018). Bacteria assisted protein imprinting in sol–gel derived films [10.1039/C7AN01509G]. Analyst, 143(2), 555-563. https://doi.org/10.1039/C7AN01509G

Chintapalli, V. R., Kato, A., Henderson, L., Hirata, T., Woods, D. J., Overend, G., Davies, S. A., Romero, M. F., & Dow, J. A. (2015, Sep 15). Transport proteins NHA1 and NHA2 are essential for survival, but have distinct transport modalities. Proc Natl Acad Sci U S A, 112(37), 11720-11725. https://doi.org/10.1073/pnas.1508031112

Costenoble, R., Valadi, H., Gustafsson, L., Niklasson, C., & Franzen, C. J. (2000, Dec). Microaerobic glycerol formation in Saccharomyces cerevisiae [Article]. Yeast, 16(16), 1483-1495. https://doi.org/10.1002/1097-0061(200012)16:16<1483::Aid-yea642>3.0.Co;2-k

Cresswell-Clay, N., Ummenhofer, C. C., Thatcher, D. L., Wanamaker, A. D., Denniston, R. F., Asmerom, Y., & Polyak, V. J. (2022, 2022/07/01). Twentieth-century Azores High expansion unprecedented in the past 1,200 years. Nature Geoscience, 15(7), 548-553. https://doi.org/10.1038/s41561-022-00971-w

Deisseroth, K., & Hegemann, P. (2017, Sep 15). The form and function of channelrhodopsin. Science, 357(6356). https://doi.org/10.1126/science.aan5544

Desnos-Ollivier, M., Ragon, M., Robert, V., Raoux, D., Gantier, J. C., & Dromer, F. (2008, Oct). Debaryomyces hansenii (Candida famata), a rare human fungal pathogen often misidentified as Pichia guilliermondii (Candida guilliermondii). J Clin Microbiol, 46(10), 3237-3242. https://doi.org/10.1128/jcm.01451-08

Dutta, S., Weiner, L., & Sheves, M. (2015, 2015/05/26). Cation Binding to Halorhodopsin. Biochemistry, 54(20), 3164-3172. https://doi.org/10.1021/acs.biochem.5b00039

Gaxiola, R. A., Rao, R., Sherman, A., Grisafi, P., Alper, S. L., & Fink, G. R. (1999, Feb 16). The Arabidopsis thaliana proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. Proc Natl Acad Sci U S A, 96(4), 1480-1485. https://doi.org/10.1073/pnas.96.4.1480

Hou, L. H., Wang, M., Wang, C., Wang, C. L., & Wang, H. Y. (2013, Jul). Analysis of Salt-Tolerance Genes in Zygosaccharomyces rouxii [Article]. Applied Biochemistry and Biotechnology, 170(6), 1417-1425. https://doi.org/10.1007/s12010-013-0283-2

Huh, J.-H., Kim, S. H., Chu, J. H., Kim, S. Y., Kim, J. H., & Kwon, S.-Y. (2014). Enhancement of seawater corrosion resistance in copper using acetone-derived graphene coating. Nanoscale, 6(8), 4379–4386. https://doi.org/10.1039/c3nr05997a

Jones, T. (2022). Quiet flows the Po: the life and slow death of Italy's longest river. The Guardian. https://www.theguardian.com/environment/2022/jul/10/po-river-italy-drought-climate

Kevin Watkins, L. C., Daniel Coppard, Ricardo Fuentes, Arunabha Ghosh, Chiara Giamberar-dini, Claes Johansson, Papa Seck, Cecilia Ugaz, Shahin Yaqub. (2006). Beyond scarcity: Power, poverty and the global water crisis (Human Deveopment Reports, Issue. P. Macmillan. https://hdr.undp.org/system/files/documents//human-development-report-2006-english.human-development-report-2006-english

Lin, J. Y. (2011, Jan). A user's guide to channelrhodopsin variants: features, limitations and future developments. Exp Physiol, 96(1), 19-25. https://doi.org/10.1113/expphysiol.2009.051961

Linder, M., & Teeri, T. T. (1996, Oct 29). The cellulose-binding domain of the major cellobiohydrolase of Trichoderma reesei exhibits true reversibility and a high exchange rate on crystalline cellulose. Proc Natl Acad Sci U S A, 93(22), 12251-12255. https://doi.org/10.1073/pnas.93.22.12251

Ma, A., Cui, W., Wang, X., Zhang, W., Liu, Z., Zhang, J., & Zhao, T. (2020, 2020/04/01/). Osmoregulation by the myo-inositol biosynthesis pathway in turbot Scophthalmus maximus and its regulation by anabolite and c-Myc. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 242, 110636. https://doi.org/https://doi.org/10.1016/j.cbpa.2019.110636

Ma, B., Hu, C., Zhang, J., Ulbricht, M., & Panglisch, S. (2022, 2022/02/11). Impact of Climate Change on Drinking Water Safety. ACS ES&T Water, 2(2), 259-261. https://doi.org/10.1021/acsestwater.2c00004

Minas, K., Karunakaran, E., Bond, T., Gandy, C., Honsbein, A., Madsen, M., Amezaga, J., Amtmann, A., Templeton, M. R., Biggs, C. A., & Lawton, L. (2015, 2015/09/04). Biodesalination: an emerging technology for targeted removal of Na+ and Cl− from seawater by cyanobacteria. Desalination and Water Treatment, 55(10), 2647-2668. https://doi.org/10.1080/19443994.2014.940647

Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P., & Bamberg, E. (2003). Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proceedings of the National Academy of Sciences, 100(24), 13940-13945. https://doi.org/doi:10.1073/pnas.1936192100

Nakamura, A., Fukuda, A., Sakai, S., & Tanaka, Y. (2006, Jan). Molecular cloning, functional expression and subcellular localization of two putative vacuolar voltage-gated chloride channels in rice (Oryza sativa L.). Plant Cell Physiol, 47(1), 32-42. https://doi.org/10.1093/pcp/pci220

Rodríguez-Rosales, M. P., Gálvez, F. J., Huertas, R., Aranda, M. N., Baghour, M., Cagnac, O., & Venema, K. (2009, Apr). Plant NHX cation/proton antiporters. Plant Signal Behav, 4(4), 265-276. https://doi.org/10.4161/psb.4.4.7919

Schilling, R. K., Tester, M., Marschner, P., Plett, D. C., & Roy, S. J. (2017, 2017/02/01/). AVP1: One Protein, Many Roles. Trends in Plant Science, 22(2), 154-162. https://doi.org/https://doi.org/10.1016/j.tplants.2016.11.012

Sottosanto, J. B., Saranga, Y., & Blumwald, E. (2007, Apr 5). Impact of AtNHX1, a vacuolar Na+/H+ antiporter, upon gene expression during short- and long-term salt stress in Arabidopsis thaliana. BMC Plant Biol, 7, 18. https://doi.org/10.1186/1471-2229-7-18

Vembar, S. S., & Brodsky, J. L. (2008, 2008/12/01). One step at a time: endoplasmic reticulum-associated degradation. Nature Reviews Molecular Cell Biology, 9(12), 944-957. https://doi.org/10.1038/nrm2546

Yamada, A., Saitoh, T., Mimura, T., & Ozeki, Y. (2002, Aug). Expression of mangrove allene oxide cyclase enhances salt tolerance in Escherichia coli, yeast, and tobacco cells. Plant Cell Physiol, 43(8), 903-910. https://doi.org/10.1093/pcp/pcf108

Yu, Y., You, L., Liu, D., Hollinshead, W., Tang, Y. J., & Zhang, F. (2013, Aug 13). Development of Synechocystis sp. PCC 6803 as a phototrophic cell factory. Mar Drugs, 11(8), 2894-2916. https://doi.org/10.3390/md11082894

Zhu, J., Ying, S.-H., & Feng, M.-G. (2016, 2016/11/01). The Na+/H+ antiporter Nhx1 controls vacuolar fusion indispensible for life cycles in vitro and in vivo in a fungal insect pathogen [https://doi.org/10.1111/1462-2920.13359]. Environmental Microbiology, 18(11), 3884-3895. https://doi.org/https://doi.org/10.1111/1462-2920.13359

AUTHORS

Lara Anderegg – 2nd year (undergraduate) MSP student, Maastricht University
l.anderegg@student.maastrichtuniversity.nl

Mathis Ben Harira – 2nd year (undergraduate) MSP student, Maastricht University
m.benharira@student.maastrichtuniversity.nl

Lucrezia Gheno – 2nd year (undergraduate) MSP student, Maastricht University
l.gheno@student.maastrichtuniversity.nl

Anirudh Rajesh – 1st year (undergraduate) MSP student, Maastricht University
a.rajesh@student.maastrichtuniversity.nl

Helène Rulkens – 2nd year (undergraduate) MSP student, Maastricht University
h.rulkens@student.maastrichtuniversity.nl

Neha Silva – 2nd year (undergraduate) MSP student, Maastricht University
neha.silva@student.maastrichtuniversity.nl

Natalie Varahram – 2nd year (undergraduate) MSP student, Maastricht University
n.varahram@student.maastrichtuniversity.nl

A review on Endocrine Disrupting Chemicals: Properties, Effects, Detection and Degradation.

Hevia-Audrey[*], Cameras-Frida Lizeth[*], Higadera-Saúl[*], Volkov-Yaroslav[*], Culebro-Mirelle[*], Patiño-Rebeca[*], Marín-Karen Itzel[*], Martínez-Ana Paula[*], Muñoz-José Antonio[*], Ortiz-Jorge Arturo[*], Quintero-Ximena[*].

[*] iGEM Tec CEM, Tecnológico de Monterrey Campus Estado de México, School of Engineering and Sciences

***Abstract*- Endocrine disruptors are toxic chemical compounds that, when found in irrigation water, are capable of causing adverse damage to agricultural crops, at a cellular level and visible phytotoxic effects. The effects of endocrine disruptors that are present in wastewater used for irrigation of crops are evaluated thought out various authors, such as plants where it can be observed changes of color, decrease in root, leaf and stem growth; in animals with relevant reproductive and development consequences and in humans interfering with the synthesis, action and metabolism of steroid hormones. Detection protocols for Endocrine disruptors are evaluated as well as proven research for degrading such compounds.**

***Index Terms*-** Endocrine disruptors, effects, wastewater, irrigation crops.

I. INTRODUCTION

Endocrine Disrupting Chemicals (EDCs) are defined by the U.S. Environmental Protection Agency (EPA) as exogenous agents that interfere with synthesis, secretion, transport, metabolism, binding action, or elimination of natural hormones produced and present in the body; therefore disrupting homeostasis, reproduction, and developmental processes in the human body. Furthermore, these agents present a wide array of mechanisms through which they interact and exert their effect in the metabolism, thus making a single system of detection and degradation ineffective due to the great diversity between these agents.(Evanthia, DK. 2019)

EDCs, unlike naturally produced hormones, are not natural ligands and do not interact with affinity and/or specificity with hormone receptors in the body, making their effects quite unpredictable and making it hard for detection systems to measure the levels of damage that exposure to these agents produce. Nevertheless, the Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals (EDC-2) has come to the conclusion that chronic exposure to such agents is detrimental to human health and that further research regarding hormonal signaling pathways and mechanisms of action, with emphasis in the basic biology of hormones and their function during fetal development.(Gore, A. 2015).

In addition to describing some of the most discussed effects that EDCs have on biological systems, this review seeks to also discuss known methods of degradation and elimination of these agents both from the environment and living organisms. Such methods are mainly based on a detection-trigger-elimination system, and EDCs are usually eliminated by degradation through chemical means; however, special effort is being put on the research of the use of enzymes for the degradation of these agents.

II. RESEARCH ELABORATIONS

The information and data was obtained principally from google scholar, we selected the information with the following criterias: the research was filtered with the published date, such dates range from 2015 up to 2022, in addition, the minimal number of references has to be 15 and finally, the principal key words are: endocrine disruptors compounds, food, animals, vegetables, agriculture, detection, elimination and degradation.

III. RESULTS AND FINDINGS

A. EDCs general properties

Endocrine-Disrupting Chemicals (EDCs) are substances that interfere with hormone biosynthesis, metabolism, or action resulting in a deviation from normal homeostatic control or reproduction. (Diamanti, E. 2022) EDCs are highly heterogeneous and include compounds that are often distantly related to endogenous ligands in terms of size or chemical structure. (Melanie, S. 2022)

Endocrine disruptors act via nuclear receptors, non-nuclear steroid hormone receptors, nonsteroid receptors, orphan receptors, enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that converge upon endocrine and reproductive systems. An endocrine-disrupting substance is either natural or synthetic that becomes harmful.(Diamanti, E. 2022)

B. Effects in plants

Studies have shown that at high concentration of BPA (an EDC), as would be values like 1000 mg/kg of soil in *Vigna radiata,* rooth length and surface area decreased. (Dokyung, k. 2018) It's been demonstrated that different crops can be affected also in their levels of chlorophyll and that is directly related to how BPA affects the machinery in photosynthesis. (Dokyung, k. 2018; Zhang, J. 2015). The number and size of leaves decreased in the presence of BPA and it also showed necrosis at the end of the experiment. Generally, the impact of EDCs in crops are copious and includes phototoxic effects, morphological changes and biochemical damages in plants that are either irrigated with contaminated water or grown in soil with accumulation of EDCs. (Laura, J. 2014). In **Figure 1** are shown the illustrations of some of these effects, comparing the guard cells, vessels and leaves without the presence of EDCs (a, c, and e) and contaminated with the chemicals (b, d, and f).

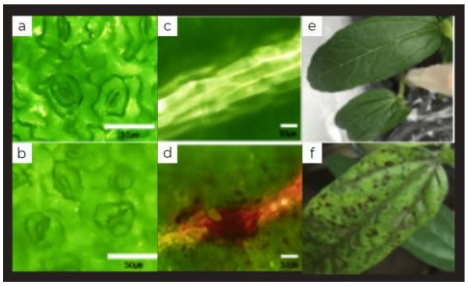


FIGURE 1. *Effects on guard cells, vessels and leaves.(Dokyung, k. 2018)*

C. Effects in some animals

It has been proven that EDCs have estrogenic, androgenic, anti-androgenic and antithyroid effects in animals; all of which disrupt their reproduction and development. Animals in contact with EDCs undergo subtle and permanent alterations such as abnormalities in organs, physiology and behavior (changes in sexual behavior, alterded immune function and disturbed sex differentiation with feminized or masculinized sex organs). Alligators are another example since exposure to EDCs causes the following: significantly reduced plasma testosterone concentration, aberrant testicular morphology, small penis size and ovarian abnormality associated with reduced fertility and embryonic mortality. *(Street, M. 2018).*

D. Effects in humans

EDCs may interfere with the synthesis, action and metabolism of steroid hormones, triggering development and fertility problems, from reduced semen quality to polycystic ovarian syndrome, as well as hormone-based cancers in both men and women. They also interfere with the hypothalamic pituitary-thyroid and adrenal systems. (Yilmaz, B. 2020). These compounds have also been associated with child and adult obesity, diabetes, childbirth prematurity, endometriosis, reduced birth weight, amongst many others.(Kahn, L. 2020).

E. Detections protocols

As previously mentioned, the effects of EDC's highlight the urgency of an effective and reliable method to detect these chemicals, even at low concentrations. According to La Spina, they developed a SPR biosensor capable of detecting EDC's-ligands, through their binding to a rationally designed ER□, enabling the detection of these chemicals even at low levels of ppb. (La Spina, R. 2017) The method was used in spiked real water, demonstrating a promising assessment for EDC's presenting estrogenic activity, monitored by a SPR sensor. In this study, conformation-sensitive peptides were used as amplification agents. Additionally mutants of ER□ were produced to enhance and favor a strong binding affinity towards the EDC's. After testing with SPR the peptide-specific recognition of the complex wt-ER□ (and mutants) with its agonists, the change of SPR signal was determined with a ratio (R) (1):

$$R = \frac{Intensity_{ER\alpha\ -ligand}^{LBD} - Intensity_{free-ER\alpha}^{LBD}}{Intensity_{free-ER\alpha}^{LBD}} \quad (1)$$

This ratio represents the differential amount of ER□ as an active complex, normalized to free ER□. This assay is able to detect different compounds at the same time by using ER□ mutants and various channels. Furthermore, this method promises the screening of EDC's in field by regenerating the platform and adapting to a portable SPR equipment. (La Spina, R. 2017).

F. Degrading or eliminations protocols

In the literature, it is possible to find several protocols that report the use of the enzyme laccase for endocrine disruptors degradation. According to Singh et. al. research has started exploring and exploiting the potential for laccase enzymes because it can catalyze the oxidation of a wide range of substrates, structurally comparable to EDCs, by a radical-catalyzed reaction mechanism, with corresponding reduction of oxygen to water in an electron transfer process. Further, in the presence of certain mediators, the substrate range of laccases can be further enhanced to non-aromatic substrates. So the removal of EDCs using this enzyme has been proven worthy in water purification. (Singh, J. 2018).

Likewise, it is worth mentioning that there are certain limitations in the elimination of EDCs from water when conventional treatment methods are used, so alternatives based on the use of laccase have been developed and studied, which have proven to be effective and low polluting. (Ramírez, L. 2014).

IV. DISCUSSION

The different effects that EDCs cause to living beings have been reported, as well as the different ways to detect, quantify and even eliminate them. However, it is a problem that lacks attention worldwide, which increases its harmful effects on society. With all these tools, it is time to start acting and apply all this knowledge to not only continue to innovate in the study of these EDCs but also put them into practice, reducing risks for everyone.

That is why a project is currently being developed based on the implementation of a biosensor for the detection and quantification of EDCs in liquid samples and, in turn, on a filter for their elimination. Which would help countless people. Mainly to farmers in the prevention of these contaminants in their crops, which would eliminate the risk of being consumed through food. It would also help chemical industries in quality control and even households for their health care.

V. CONCLUSION

Among this work we have seen the different effects of the EDC´s on the living beings, and the ways in which these prevent the full development of endless environmental factors. Likewise, some methods of detection and degradation of the compounds have been put forward in order to highlight this area of opportunity. However, we also shed light on the fact that it is a problem with little attention worldwide. This is why it is up to us as a scientific community to start acting in this situation and contribute from our knowledge to caring for the environment. Through projects like the one mentioned during the discussion.

ACKNOWLEDGEMENT

We would like to extend our greatest greetings to our iGEM team supervisors and colleagues. Also, we would like to show gratitude to Tecnológico de Monterrey Campus Estado de Mexico for the facilities.

REFERENCES

Diamanti, E. et. al. (2022). Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocrine Review. 30(4):293–342.doi: 10.1210/er.2009-0002.

Dokyung K., Jin Il K., Youn-Joo A. (2018) Effects of bisphenol A in soil on growth, photosynthesis activity, and genistein levels in crop plants (Vigna radiata), Chemosphere, 209(875-882), ISSN 0045-6535, https://doi.org/10.1016/j.chemosphere.2018.06.146.

Evanthia DK, Jean-Pierre B, Giudice LC, Russ H, Prins GS, Soto AM, et al.. (2009) Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocr Rev 30:293–342. doi: 10.1210/er.2009-0002

Fate and Uptake of Pharmaceuticals in Soil–Plant Systems Laura J. Carter, Eleanor Harris, Mike Williams, Jim J. Ryan, Rai S. Kookana, and Alistair B. A. Boxall Journal of Agricultural and Food Chemistry 2014 62 (4), 816-825 DOI: 10.1021/jf404282

Gore A.C., Chappell V.A., Fenton S.E., Flaws J.A., Nadal A., Prins G.S., Toppari J., Zoeller R.T. (2015). Executive Summary to EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocr. Rev.36:593–602. doi: 10.1210/er.2015-1093.

Kahn, L. G., Philippat, C., Nakayama, S. F., Slama, R., & Trasande, L. (2020). Endocrine-disrupting chemicals: implications for human health. The lancet Diabetes & endocrinology, 8(8), 703-718.

La Spina, R., et. al. (2017). Label-Free Biosensor Detection of Endocrine Disrupting Compounds Using Engineered Estrogen Receptors. 23 de julio de 2022, de NCBI.https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5872049/

Melanie,S. Jean-Luc, mP. Gilles, L. William, B. (2022). In Silico Predictions of Endocrine Disruptors Properties. Endocrinology 160(11):2709–2716. doi: 10.1210/en.2019-00382.

Ramírez-Cavazos, L. I., Junghanns, C., Ornelas-Soto, N., Cárdenas-Chávez, D. L., Hernández-Luna, C., Demarche, P., ... & Parra, R. (2014). Purification and characterization of two thermostable laccases from Pycnoporus sanguineus and potential role in degradation of endocrine disrupting chemicals. Journal of Molecular Catalysis B: Enzymatic, 108, 32-42.

Singh, J., Saharan, V., Kumar, S., Gulati, P., & Kapoor, R. K. (2018). Laccase grafted membranes for advanced water filtration systems: a green approach to water purification technology. Critical reviews in biotechnology, 38(6), 883-901.

Street, M., Angelini, S., Bernasconi, S., Burgio, E., Cassio, A., Catellani, C., Cirillo, F., et al. (2018). Current Knowledge on Endocrine Disrupting Chemicals (EDCs) from Animal Biology to Humans, from Pregnancy to Adulthood: Highlights from a National Italian Meeting. International Journal of Molecular Sciences, 19(6), 1647. http://doi.org/10.3390/ijms19061647

Yilmaz, B., Terekeci, H., Sandal, S., & Kelestimur, F. (2020). Endocrine disrupting chemicals: exposure, effects on human health, mechanism of action, models for testing and strategies for prevention. Reviews in endocrine and metabolic disorders, 21(1), 127-147.

Zhang, J., Wang, L., Li, M., Jiao, L., Zhou, Q., & Huang, X. (2015). Effects of bisphenol A on chlorophyll fluorescence in five plants. Environmental Science and Pollution Research, 22(22), 17724-17732.

AUTHORS

First Author – Audrey Hevia, B.Sc. in Nanotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México, A01745154@tec.mx

Second Author – Frida Lizeth Cameras, B.Sc. in Nanotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México, A01752070@tec.mx

Complementary Author – Saúl Higadera, B.Sc. in Biotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México.

Complementary Author – Yaroslav Volkov, B.Sc. in Biotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México.

Complementary Author – Mirelle Culebro, B.Sc. in Biotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México.

Complementary Author – Rebeca Patiño, B.Sc. in Biotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México.

Complementary Author – Karen Itzel Marín, B.Sc. in Biotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México.

Complementary Author – Ana Paula Martínez, B.Sc. in Biotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México.

Complementary Author – José Antonio Muñoz, B.Sc. in Biotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México.

Complementary Author – Jorge Arturo Ortiz, B.Sc. in Biotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México.

Complementary Author – Ximena Quintero, B.Sc. in Biotechnology Engineering junior year, Tecnológico de Monterrey Campus Estado de México.

Review on Phage Therapy

Ruchir Sahni*

* IISER-Pune-India, IISER Pune

Abstract **- Phage Therapy, which uses bacteriophages to combat a bacterial infection, has been around since the 1920s but was overshadowed by the success of antibiotics. The rise in antibiotic resistance has regenerated curiosity into the potential of this practice. Phage Therapy involves the introduction of lytic phages which will attach to the pathogenic bacterial cell, inject their genome and produce their progeny. The progeny are released by lysis of the bacterium, resulting in the death of the pathogen. This review highlights the current progress of the therapy along with listing the challenges lying ahead.**

Index Terms- Antibiotic Resistance, Bacteriophages, Phage Therapy, Phage Safety

I. INTRODUCTION

Soon after discovering the first antibiotic, Penicillin, Alexander Fleming had warned about the rise of antibiotic resistance. Today, it is seen as one of the greatest threats to public health, with 700,000 people dying every year due to antimicrobial resistance(World Bank, 2021). The world has entered the post-antibiotic era(CDC, 2019) and this has increased the interest in alternatives. Phage Therapy has been the main focus of this piqued interest.

Phage Therapy has existed before the emergence of antibiotics(D'Hérelle F, 1931) but was pushed aside by the golden age of antibiotics. Though it was still practiced in some Eastern European countries like Georgia, the West has only recently rediscovered it, with most of the research coming in the last decade.

This review takes an overview of the topic with the main focus being on the current situation and the obstacles needed to be crossed. It also dwells into the science behind this theory and goes onto listing the advantages of bacteriophages as a theory.

The search engines used for this article are Google Scholar and Pubmed(NCBI). The keywords used for the search were Phage Therapy, Bacteriophages, Clinical Trials and Antibiotic Resistance. The articles were then filtered by the time of publication : anything older than the 1980s were discarded. This is due to the fact that Phage Therapy is a rapidly evolving and recent research hotspot.

II. OVERVIEW OF THE THERAPY

A. Biology of Bacteriophages and the Therapy

There are two types of Bacteriophages: lytic and lysogenic. Only lytic phages are used in phage therapy. Lytic phages are used to attack bacteria. These phages will attach their receptors to the cell surface of the bacterium. On attaching, it will insert its genomic material into the bacterium. The viral particles undergo replication using the bacterium's resources and enzymes. On the formation of phage particles, they are released by the lysis of the bacterium. This process is then repeated by the new phage particles until no more bacteria are left(Kortright et al., 2019)

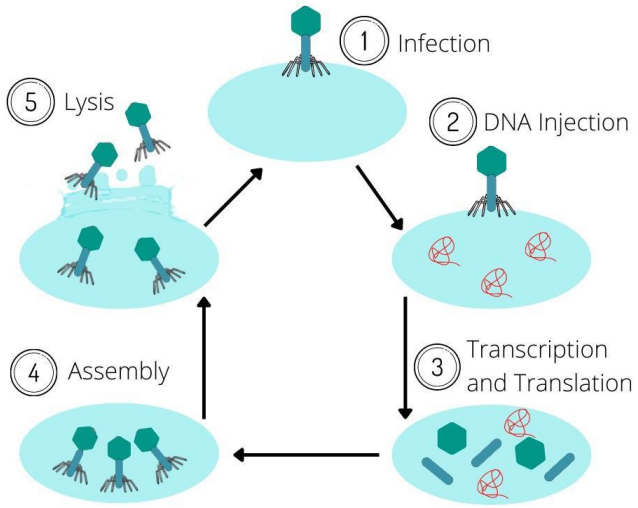


FIGURE 1.
Lytic Phage Cycle

.There are two approaches to phage therapy(Pirnay et al., 2010) :

1) Prêt-à-porter : fixed cocktails of several phages so that at least one that will be effective on the patient
2) Sur-mesure : phages that are actually active on the strain are selected in the hospital and are administered. The patient is monitored and when the patient becomes resistant to the phages : either training of a stored phage occurs or a new phage is isolated.

B. Advantages of Phage Therapy

1. Auto-dosing

Phages can increase their number significantly at the site of infection.This reduces the number of doses needed to be given. The timing of these doses, however, is critical to the treatment.

2. Low toxicity

Phages, mostly consisting of proteins and nucleic acid, are inherently non-toxic. Phages can interact with the immune system, which can potentially be harmful, but there is little evidence it is a concern during the treatment(T. Abedon et al., 2011;Liu et al., 2021)

3. High Specificity

Phages are very specific with the range of infecting only a few strains of a species to relatively close bacterial genus(Hyman et al., 2010). Due to the high specificity, phages only minimally impact the microbiome in contrast to broad-spectrum antibiotics. This high specificity also avoids generating resistance to a particular phage in a large number of species(Loc-Carrillo et al.,2011).

4. Less time and money in development

Phages can be discovered easily, especially from regions with high bacterial concentrations. Programs have been developed like the SEA-PHAGES program, which has led to an ease in the discovery of phages with very low cost. This is in contrast to antibiotics, whose cost estimate for development at US$1.5 billion over 10-15 years(Towse, A. et al., 2017).

5. Biofilm Clearance

Phages can penetrate into biofilms, which shows high resistance to antibiotics. It is possible by lysing one layer at one time or due to biofilm exopolymer-degrading depolymerases(Abedon et al., 2011).

III. PRESENT SITUATION

This section provides a glimpse into the current usage of the therapy. It lists the animal studies performed along with their results. This is followed by a brief description of human case studies and clinical trials.

A. Animal Studies

Animal studies can bridge the gap between in-vitro studies and actual clinical phage therapy. These studies have been done in different types of infection :

1. Systemic Infections

Several studies have investigated the usage of phages against Systemic infections. Capparelli et al., 2007 observed that successful protection of mice with a systemic Staphylococcus aureus infection depended on phage dose. Biswas et al., 2002 observed similar results of dose-dependent success in a mouse model of vancomycin-resistant *Enterococcus faecium* bacteremia. In a systemic disease model of *Vibrio vulnificus*, successful control of disease was only achieved when bacterial infection and phage treatment were administered simultaneously (Cerveny et al., 2002). Thus the success of the therapy in systemic infections depends on many factors, and these factors must be thoroughly examined.

2. Local Infections

Phage therapy for localized infections (e.g., otitis, urinary tract infections, infected burns) is recognised for its potential to replace chemical antibiotics. It is also important to note that the use of chemical antibiotics for surgical and hospital-acquired infections is limited, as these often constitute the strains with the greatest antibiotic resistance, making phage therapy a valuable tool(Kortright et al., 2019).

Watanabe et al., 2007 observed 92% survival of mice with an intraperitoneal *P. aeruginosa* infection treated simultaneously with phage. A similar study of *S. aureus* abscesses in mice by Capparelli et al., 2007 observed the reduction in bacterial load resulting from phage therapy. In a mouse model of *P. aeruginosa* infection of burn wounds, phage treatment improved survival rate from 6% in the untreated controls to 88% when phages were administered via an intraperitoneal injection after 72 h post-infection (McVay et al., 2007). It has also been observed that the intraperitoneal mode of delivery of phages has shown better results than subcutaneously or intramuscularly(McVay et al., 2007).

3. Gastrointestinal Infections

Using phages for gastrointestinal infections can reduce the colonies of the virulent bacteria without affecting the gut microbiome.

Galtier et al. (2017) observed that a preventative treatment of phage, four days after an *E. coli* challenge, was able to reduce bacterial colonization in the gut of dextran sodium sulfate-treated mice and prevented the progression of colitis symptoms. Nale et al., 2016, Yen et al., 2017 have used prophylactic treatment of phages which has shown favorable results. While prophylactic studies have shown favorable results, more studies that provide treatment after bacterial challenge, such as Galtier et al., 2017, are needed.

4. Lung Infections

Phage therapy for the treatment of lung infections, particularly chronic lung infections, which are common in those with cystic fibrosis (CF), has seen renewed interest recently with the increase in MDR bacteria associated with the lung. Waters et al., 2017 , Morello et al., 2011 observed that phage treatment significantly improved the survival rate of mice when administered intranasally.

A 100-fold decrease in bacterial load was observed by Semler et al., 2014 when phage was administered via nebulisation, while no decrease was observed when administered via intraperitoneal injection.

Phage-loaded polymeric microparticles deposited throughout the lung via dry powder inhalation effectively reduced P. aeruginosa infections in wild-type and cystic fibrosis trans-membrane-conductance-regulator knockout mice(Agarwal, R et al., 2018).

B. *Human Case Studies*

Thirty-five case reports/series of phage therapy were published between 2008–2021. Most involved the combined use of phages with antibiotics, targeting a variety of pathogens. The conditions treated included cystic fibrosis exacerbation, bone/joint infection, pneumonia, bacteremia, urinary tract infection (UTI), endocarditis, cardiothoracic surgery-related infections, aorto-cutaneous fistula, necrotising pancreatitis, skin infection, brain infection, diabetic foot ulcers, corneal abscess, lung transplant-related infection, an intestinal infection.. Very few cases reported any adverse effects(Liu et al., 2021).

C. *Clinical Trials*

The first investigation into the bioavailability of oral *E. coli* phage T4 in 2005 involving fifteen healthy humans did not identify any adverse events(Britain at al., 2005). Since 2008 there have been 14 clinical trials of phage therapy investigating a multitude of bacterial infections, including endocarditis, sepsis, rhinosinusitis, UTI, venous leg ulcers, chronic otitis media, acute bacterial diarrhea, and burn wounds. Very few adverse events were observed, none of which were linked to the Phage treatment(Liu et al., 2021).

IV. CHALLENGES AND POSSIBLE OPPORTUNITIES

A. *Regulatory Framework*

There is no specific regulation around phages. Since 2011, phages have been considered as a drug in the United States (Fauconnier, 2019) or a medicinal product in the European Union (Directive 2001/83/EC European Parliament). This qualification of phages as a medicinal product for human use was endorsed on 8 June 2015 at an EMA workshop, even though those present at the event who were all working on phage development warned that such categorisation was inadequate. Following this meeting, they expressed their opposition in a short two-page letter, mentioning two aspects in particular: (i) the need to create a new regulatory framework, which would (ii) counterbalance the lack of public and private investment in phages (Debarbieux et al., 2015).
However, in Georgia, phage therapy is embedded in the healthcare system as a standard medical application (Kutateladze, 2015). Specifically, the Eliava Institute of Bacteriophages, Microbiology and Virology has several phage preparations readily available (over-the-counter) and a broader range of products, specifically supplied to medical practitioners (Kutter et al., 2010; Kutateladze, 2015). Similarly, Poland has the Hirszfeld Institute of Immunology and Experimental Therapy, although this center supplies personalized phage products directly to physicians using a more tailored approach (Kutter et al., 2010).
In the United States, patients can be treated by phages following the emergency investigational new drug (eIND) pathway of the FDA (expanded access program).
These regulations make clinical applications tough and time-consuming.IPATH got 785 requests from 1 June 2018, to 30 April 2020, out of which phage therapy was administered to 17 of 119 patients in whom it was recommended(Saima et al., 2020)

We need a new framework regarding phage therapy. Calls for two regulatory pathways were proposed in Verbeken et al., 2014, including product-market placement of natural phage-based products and hospital exemption pathways for tailored phage therapy. The consensus among surveyed stakeholders in Furfaro et al., 2018 was the need for a dedicated new regulatory framework for phage therapy and one which acknowledges the specific properties of phages and their interactions, in addition to the role of hospitals as providers of phage therapy.

B. *Clinical Trials*

Clinical trials done to date have multiple safety factors missing. Only 14 of the 66 studies in Liu et al.,2021 reported the level of endotoxin contamination. The bacterial host DNA was reported in only four of the evaluated studies. Other toxins and contaminants such as lipoteichoic acid, superantigens, or cesium chloride were rarely considered in most studies. Additional quality controls regarding shelf life, pH, visual appearance were sporadically mentioned. More research is needed on these factors.

Clinical Trials of Phage Therapy in itself has many obstacles. Several articles published by proponents of phage therapy in Europe during the last few years focus on two main elements: (i) the inadequacy of clinical trials to demonstrate the efficacy of phages because of their specificity and, therefore, the personalized nature of the treatment (Pirnay et al., 2019); and (ii) the major difference between what is evaluated in trials and what occurs in real life (Patay et al., 2019).

C. *Endotoxin Release*

The release of endotoxin during treatment places limits on the phage treatment of gram-negative systemic infections(Kutter, 2008).Slopek et al. , though otherwise observing few side effects, did report that some patients experienced several hours of pain in the liver area 3 to 5 days into treatments. This might conceivably reflect phage-mediated liberation of endotoxins and other bacterial pathogenicity factors in the course of bacterial lysis. Lysates from Gram-positive organisms, when injected, also can lead to “side effects ranging from mild to severe”, as discussed by Sulakvelidze and Kutter, 2005,.It is also possible to modify phages to prevent lysis, so that toxin release is less of a concern, but that removes one of the basic advantages of phage therapy—the ability to move throughout

the organism and multiply where needed(Goodridge et al., 2010).

D. Harmful Genes

Phages may carry virulence factors or toxin genes. The in vivo phage-associated conversion of *Tox Streptococcus* pyogenes into *Tox+* bacteria (Broudy and Fischetti, 2003) is an example of the phage-related dangers encountered in nature, and it can serve as a warning of the possible side effects of bacteriophage therapy. A full knowledge of phage genome sequences would be helpful to evaluate possible complications during phage therapy(Skurnik et al., 2006).

E. Neutralization by Immune Response

Dedrick et al., 2021 observed that on treating an 81-year old immunocompetent patient with *Mycobacterium abscessus*, the *M. abscessus* decreased initially but after two months increased as the patient mounted a robust IgM- and IgG-mediated neutralizing antibody response to the phages. This raises questions on clinical applications on chronic infections in immunocompetent patients.

F. Transduction of Antibiotic Genes

Leclerc et al.,2021 estimated that in a co-culture of two strains of Methicillin-resistant *Staphylococcus aureus*, each harboring a different antibiotic resistance gene : one in every 108 new phages released was a transfusing phage carrying an Antimicrobial gene. Though this number is low and often ignored, it presents an obstacle which needs to be studied more.

V. CONCLUSION

Antibiotic Resistance is arguably the biggest threat to public health. Phage therapy is well suited to be part of the multidimensional strategies to fight against it. Phage therapy itself has cycled between being the subject of optimism, skepticism, disinterest, and now renewed curiosity(Barr et al., 2019).There is a plethora of research activity in this field with many animal models and case studies showing positive results. This has resulted in the therapy being seen as a future alternative to chemotherapy.

However significant challenges remain, which will require coordinated, multidisciplinary efforts to overcome(Hesse et al., 2019). The regulatory framework remains the largest hurdle to cross and would require efforts from both scientists and policy-makers. The other challenges listed above are active research topics and we are making great strides towards resolving these challenges. Hopefully, this century-old idea will finally be part of the tools against microbial invaders.

REFERENCES

Abedon, S. T. (2011). *Bacteriophages and Biofilms: Ecology, Phage Therapy, Plaques*. Nova Science Publishers.

Abedon, S. T., Kuhl, S. J., Blasdel, B. G., & Kutter, E. M. (2011, Mar). Phage treatment of human infections. *Bacteriophage*, *1*(2), 66-85. https://doi.org/10.4161/bact.1.2.15845

Agarwal, R., Johnson, C. T., Imhoff, B. R., Donlan, R. M., McCarty, N. A., & García, A. J. (2018, Nov). Inhaled bacteriophage-loaded polymeric microparticles ameliorate acute lung infections. *Nat Biomed Eng*, *2*(11), 841-849. https://doi.org/10.1038/s41551-018-0263-5

Altamirano, F. L. G., & Barr, J. J. (2019). Phage Therapy in the Postantibiotic Era. *Clinical Microbiology Reviews*, *32*(2), e00066-00018. https://doi.org/doi:10.1128/CMR.00066-18

Antibiotic resistance threats in the United States, 2019. (2019). [Report]. https://stacks.cdc.gov/view/cdc/82532

Aslam, S., Lampley, E., Wooten, D., Karris, M., Benson, C., Strathdee, S., & Schooley, R. T. (2020, Sep). Lessons Learned From the First 10 Consecutive Cases of Intravenous Bacteriophage Therapy to Treat Multidrug-Resistant Bacterial Infections at a Single Center in the United States. *Open Forum Infect Dis*, *7*(9), ofaa389. https://doi.org/10.1093/ofid/ofaa389

Biswas, B., Adhya, S., Washart, P., Paul, B., Trostel, A. N., Powell, B., Carlton, R., & Merril, C. R. (2002). Bacteriophage Therapy Rescues Mice Bacteremic from a Clinical Isolate of Vancomycin-Resistant Enterococcus faecium. *Infection and Immunity*, *70*(1), 204-210. https://doi.org/doi:10.1128/IAI.70.1.204-210.2002

Broudy, T. B., & Fischetti, V. A. (2003, Jul). In vivo lysogenic conversion of Tox(-) Streptococcus pyogenes to Tox(+) with Lysogenic Streptococci or free phage. *Infect Immun*, *71*(7), 3782-3786. https://doi.org/10.1128/iai.71.7.3782-3786.2003

Bruttin, A., & Brüssow, H. (2005, Jul). Human volunteers receiving Escherichia coli phage T4 orally: a safety test of phage therapy. *Antimicrob Agents Chemother*, *49*(7), 2874-2878. https://doi.org/10.1128/aac.49.7.2874-2878.2005

Capparelli, R., Parlato, M., Borriello, G., Salvatore, P., & Iannelli, D. (2007). Experimental Phage Therapy against Staphylococcus aureus in Mice. *Antimicrobial Agents and Chemotherapy*, *51*(8), 2765-2773. https://doi.org/doi:10.1128/AAC.01513-06

Cerveny, K. E., DePaola, A., Duckworth, D. H., & Gulig, P. A. (2002). Phage Therapy of Local and Systemic Disease Caused by Vibrio vulnificus in Iron-Dextran-Treated Mice. *Infection and Immunity*, *70*(11), 6251-6262. https://doi.org/doi:10.1128/IAI.70.11.6251-6262.2002

d'Herelle, F. (1931, May). Bacteriophage as a Treatment in Acute Medical and Surgical Infections. *Bull N Y Acad Med*, *7*(5), 329-348.

Debarbieux, L., Pirnay, J.-P., Verbeken, G., De Vos, D., Merabishvili, M., Huys, I., Patey, O., Schoonjans, D., Vaneechoutte, M., Zizi, M., & Rohde, C. (2015). A bacteriophage journey at the European Medicines Agency. *FEMS Microbiology Letters*, *363*(2). https://doi.org/10.1093/femsle/fnv225

Dedrick, R. M., Freeman, K. G., Nguyen, J. A., Bahadirli-Talbott, A., Smith, B. E., Wu, A. E., Ong, A. S., Lin, C. T., Ruppel, L. C., Parrish, N. M., Hatfull, G. F., & Cohen, K. A. (2021, 2021/08/01). Potent antibody-mediated neutralization limits bacteriophage treatment of a pulmonary Mycobacterium abscessus infection. *Nature Medicine*, *27*(8), 1357-1361. https://doi.org/10.1038/s41591-021-01403-9

Directive 2001/83/EC of the European Parliament and the Council on the Community code relating to medicinal products for human use.

Fauconnier, A. (2019). Phage Therapy Regulation: From Night to Dawn. *Viruses*, *11*(4), 352. https://doi.org/https://doi.org/10.3390/v11040352

Fleming, A. (1945). Penicillin's finder assays its future. The New York Times, 26 June 1945. A21.

Furfaro, L. L., Payne, M. S., & Chang, B. J. (2018). Bacteriophage Therapy: Clinical Trials and Regulatory Hurdles. *Front Cell Infect Microbiol*, *8*, 376. https://doi.org/10.3389/fcimb.2018.00376

Galtier, M., De Sordi, L., Sivignon, A., de Vallée, A., Maura, D., Neut, C., Rahmouni, O., Wannerberger, K., Darfeuille-Michaud, A., Desreumaux, P., Barnich, N., & Debarbieux, L. (2017, Jul 1). Bacteriophages Targeting Adherent Invasive Escherichia coli Strains

as a Promising New Treatment for Crohn's Disease. *J Crohns Colitis*, *11*(7), 840-847. https://doi.org/10.1093/ecco-jcc/jjw224

Goodridge, L. D. (2010, Jan). Designing phage therapeutics. *Curr Pharm Biotechnol*, *11*(1), 15-27. https://doi.org/10.2174/138920110790725348

Hesse, S., & Adhya, S. (2019). Phage Therapy in the Twenty-First Century: Facing the Decline of the Antibiotic Era; Is It Finally Time for the Age of the Phage? *Annual Review of Microbiology*, *73*(1), 155-174. https://doi.org/10.1146/annurev-micro-090817-062535

Hyman, P., & Abedon, S. T. (2010). Bacteriophage host range and bacterial resistance. *Adv Appl Microbiol*, *70*, 217-248. https://doi.org/10.1016/s0065-2164(10)70007-1

https://www.worldbank.org/en/topic/health/brief/antimicrobial-resistance-amr

Kasman, L. M., Kasman, A., Westwater, C., Dolan, J., Schmidt, M. G., & Norris, J. S. (2002, Jun). Overcoming the phage replication threshold: a mathematical model with implications for phage therapy. *J Virol*, *76*(11), 5557-5564. https://doi.org/10.1128/jvi.76.11.5557-5564.2002

Kortright, K. E., Chan, B. K., Koff, J. L., & Turner, P. E. (2019). Phage Therapy: A Renewed Approach to Combat Antibiotic-Resistant Bacteria. *Cell Host & Microbe*, *25*(2), 219-232. https://doi.org/10.1016/j.chom.2019.01.014

Kutateladze, M. (2015, Feb). Experience of the Eliava Institute in bacteriophage therapy. *Virol Sin*, *30*(1), 80-81. https://doi.org/10.1007/s12250-014-3557-0

Kutter, E. (2008). Phage therapy: bacteriophages as natural, self-replicating antimicrobials. In *Practical Handbook of Microbiology* (pp. 733-750). CRC Press.

Kutter, E., De Vos, D., Gvasalia, G., Alavidze, Z., Gogokhia, L., Kuhl, S., & Abedon, S. T. (2010, Jan). Phage therapy in clinical practice: treatment of human infections. *Curr Pharm Biotechnol*, *11*(1), 69-86. https://doi.org/10.2174/138920110790725401

Leclerc, Q. J., Wildfire, J., Gupta, A., Lindsay, J. A., & Knight, G. M. (2021). The dual nature of bacteriophage: growth-dependent predation and generalised transduction of antimicrobial resistance. *bioRxiv*, 2021.2007.2024.453184. https://doi.org/10.1101/2021.07.24.453184

Liu, D., Van Belleghem, J. D., de Vries, C. R., Burgener, E., Chen, Q., Manasherob, R., Aronson, J. R., Amanatullah, D. F., Tamma, P. D., & Suh, G. A. (2021). The Safety and Toxicity of Phage Therapy: A Review of Animal and Clinical Studies. *Viruses*, *13*(7), 1268. https://www.mdpi.com/1999-4915/13/7/1268

Loc-Carrillo, C., & Abedon, S. T. (2011, 2011/03/01). Pros and cons of phage therapy. *Bacteriophage*, *1*(2), 111-114. https://doi.org/10.4161/bact.1.2.14590

McVay, C. S., Velásquez, M., & Fralick, J. A. (2007, Jun). Phage therapy of Pseudomonas aeruginosa infection in a mouse burn wound model. *Antimicrob Agents Chemother*, *51*(6), 1934-1938. https://doi.org/10.1128/aac.01028-06

Morello, E., Saussereau, E., Maura, D., Huerre, M., Touqui, L., & Debarbieux, L. (2011). Pulmonary Bacteriophage Therapy on Pseudomonas aeruginosa Cystic Fibrosis Strains: First Steps Towards Treatment and Prevention. *PLOS ONE*, *6*(2), e16963. https://doi.org/10.1371/journal.pone.0016963

Nale, J. Y., Chutia, M., Carr, P., Hickenbotham, P. T., & Clokie, M. R. J. (2016, 2016-August-31). 'Get in Early'; Biofilm and Wax Moth (Galleria mellonella) Models Reveal New Insights into the Therapeutic Potential of Clostridium difficile Bacteriophages [Original Research]. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01383

Patey, O., McCallin, S., Mazure, H., Liddle, M., Smithyman, A., & Dublanchet, A. (2018, Dec 28). Clinical Indications and Compassionate Use of Phage Therapy: Personal Experience and Literature Review with a Focus on Osteoarticular Infections. *Viruses*, *11*(1). https://doi.org/10.3390/v11010018

Pirnay, J.-P., De Vos, D., & Verbeken, G. (2019). Clinical application of bacteriophages in Europe. *Microbiology Australia*, *40*(1), 8-15. https://doi.org/https://doi.org/10.1071/MA19010

Pirnay, J. P., De Vos, D., Verbeken, G., Merabishvili, M., Chanishvili, N., Vaneechoutte, M., Zizi, M., Laire, G., Lavigne, R., Huys, I., Van den Mooter, G., Buckling, A., Debarbieux, L., Pouillot, F., Azeredo, J., Kutter, E., Dublanchet, A., Górski, A., & Adamia, R. (2011, Apr). The phage therapy paradigm: prêt-à-porter or sur-mesure? *Pharm Res*, *28*(4), 934-937. https://doi.org/10.1007/s11095-010-0313-5

Semler, D. D., Goudie, A. D., Finlay, W. H., & Dennis, J. J. (2014, Jul). Aerosol phage therapy efficacy in Burkholderia cepacia complex respiratory infections. *Antimicrob Agents Chemother*, *58*(7), 4005-4013. https://doi.org/10.1128/aac.02388-13

Skurnik, M., & Strauch, E. (2006). Phage therapy: facts and fiction. *International Journal of Medical Microbiology*, *296*(1), 5-14.

Slopek, S., Durlakowa, I., Weber-Dabrowska, B., Kucharewicz-Krukowska, A., Dabrowski, M., & Bisikiewicz, R. (1983). Results of bacteriophage treatment of suppurative bacterial infections. I. General evaluation of the results. *Arch Immunol Ther Exp (Warsz)*, *31*(3), 267-291.

Sulakvelidze Aet al. (2005) Bacteriophage therapy in humans. In: Kutter E, Sulakvelidze A, editors. Bacteriophages: Biology and Application. Boca Raton, FL:

CRC Press; 2005. pp. 381–436

Towse, A., Hoyle, C. K., Goodall, J., Hirsch, M., Mestre-Ferrandiz, J., & Rex, J. H. (2017, 2017/10/01/). Time for a change in how new antibiotics are reimbursed: Development of an insurance framework for funding new antibiotics based on a policy of risk mitigation. *Health Policy*, *121*(10), 1025-1030. https://doi.org/https://doi.org/10.1016/j.healthpol.2017.07.011

Watanabe, R., Matsumoto, T., Sano, G., Ishii, Y., Tateda, K., Sumiyama, Y., Uchiyama, J., Sakurai, S., Matsuzaki, S., Imai, S., & Yamaguchi, K. (2007, Feb). Efficacy of bacteriophage therapy against gut-derived sepsis caused by Pseudomonas aeruginosa in mice. *Antimicrob Agents Chemother*, *51*(2), 446-452. https://doi.org/10.1128/aac.00635-06

Waters, E. M., Neill, D. R., Kaman, B., Sahota, J. S., Clokie, M. R. J., Winstanley, C., & Kadioglu, A. (2017, Jul). Phage therapy is highly effective against chronic lung infections with Pseudomonas aeruginosa. *Thorax*, *72*(7), 666-667. https://doi.org/10.1136/thoraxjnl-2016-209265

Yen, M., Cairns, L. S., & Camilli, A. (2017, 2017/02/01). A cocktail of three virulent bacteriophages prevents Vibrio cholerae infection in animal models. *Nature Communications*, *8*(1), 14187. https://doi.org/10.1038/ncomms14187

AUTHOR

First Author – Ruchir Sahni, IISER Pune

Email : ruchirsahni@yahoo.com

MEtaPhos: Modified Enzymes targeting Phosphate-recycling

Nathalie Zehetner*, Laurin Röder*, Leonie Hümmeler*, Laurin Poro *

* iGEM Team Aachen 2022, RWTH Aachen University

***Abstract*- In our society, there is a huge demand for phosphorus, an essential nutrient that – in phosphate bonds – is necessary for the physiological function and growth of all living organisms. However, it is also a finite resource. For this reason, the recycling of phosphate is becoming more and more important. The phosphate must be recovered from the wastewater, otherwise it can lead to eutrophication of lakes. Most of the phosphate is currently removed from wastewater through chemical precipitation and cannot be recycled. For this purpose, we want to modify a phosphate binding protein in such a way that after it has bound the phosphate, it releases it again through certain stimuli (light and pH) and can be used reversibly. To determine the effectiveness of the modified proteins we add a phosphate solution (simulating wastewater) to the immobilized and modified proteins. To recover the bound phosphate, the proteins are then exposed to the different stimuli. Then, a colour assay is used to determine the amount of phosphate recovered. Because polyphosphate is worth ten times more than simple phosphate, we have decided to convert the extracted phosphate into polyphosphate. To this end, we use Saccharomyces cerevisiae which fermentatively converts phosphate into polyphosphate. Hereafter we will obtain polyphosphate in powder form, which could be used directly in agriculture.**

***Index Terms*-** Phosphate recovery, Protein engineering, QuickChange, Recycling economy

I. INTRODUCTION

Phosphor is an essential element of life, as it is a part of both DNA and the biological "energy currency" ATP. Thus, phosphate is an important and irreplaceable component of fertilizers in agriculture. With a growing population and hence growing demands for food and fertilizers the demand for phosphate is also increasing. However, phosphate is a finite resource, and its extraction mainly occurs through surface mining in a limited number of countries. Significant quantities of phosphate can be found in wastewater. In the event of excessive concentration of phosphate in water bodies, eutrophication might occur, and this must be prevented. Thus, phosphate must be removed from wastewater. According to the Water Framework Directive in Europe, the maximum allowable concentration of phosphate in effluent should decrease to 0.1 mg*P/L in municipal wastewater treatment.

Current techniques used in wastewater treatment aim to remove phosphate rather than to recover it. One widely used technology removing phosphate from the liquid phase of municipal wastewater is chemical precipitation with Mg/Ca materials. This technical method is highly stable and efficient.

An effective method to recover phosphate from wastewater rather than removing it would both avert eutrophication and produce fertilizers. Therefore, iGEM Team Aachen 2022 aims to develop a sustainable method for the recovery of phosphate from wastewater. To this end, a cyclic process is set up. In the first phase of this process, phosphate is removed from wastewater through binding to immobilized phosphate binding proteins (PBPs). In the second phase of the process, the phosphate is released from the PBPs through external stimuli and into an extraction solution. The immobilized PBPs are to be reused in further iterations of the cyclic process. The extraction solution enriched with phosphate is to be used to produce fertilizers. Specifically, polyphosphate shall be produced using *Saccharomyces cerevisiae*, creating a much more valuable resource for industrial usage.

PBPs are modified in different approaches all aiming to a single goal: making the binding of phosphate through these proteins reversible and controllable. In total, three approaches are pursued, all using protein-engineering to modify natural PBPs to make their binding of phosphate to reach both mentioned goals. The first two are light-driven approaches using the VVD-domain and the SOPP3-protein respectively. The third aims to release phosphate from the binding protein through mild alternations in pH-conditions, based on the principle used by Venkiteshwaran et al., 2018. Hoping to achieve phosphate release in milder pH values than the ones used by Venkiteshwaran et al. (pH 11.4 and 12.5), the QuikChange method is used to exchange amino acids in the binding pocket of the protein by histidines.

II. MATERIALS AND METHODS

a. CHOICE OF GENES

For our experiments, we wanted to use different PBPs, so we could compare the results the different approaches provide. We chose the PstS protein originated from *Escherichia coli* and the PfluDING originated from *Pseudomonas fluorescens* for our pH-driven approach. For the light-driven approach, we chose the SOPP3-protein as the photosensitizer and fused it to the PstS. To perform a reversible light driven approach, we created a fusion construct of VVD and the colicilin M immunity protein (CMI), because the CMI is a dimeric protein. This should allow the VVD domain to induce the dimerization upon stimuli. We purchased the PstS gene from Invitrogen, the PfluDING and the fusion constructs of SOPP3-PstS and VVD-CMI were purchased from BioCat.

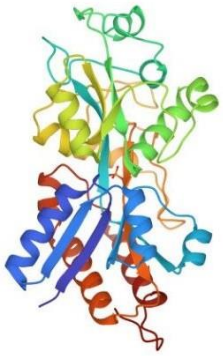


FIGURE 1: *2ABH – Phosphate-Binding Protein (Re-Refined)*

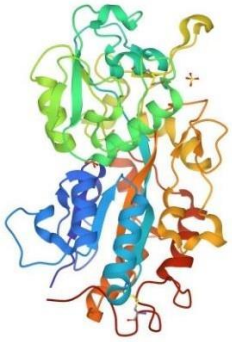


FIGURE 2: *4F1V – Subatomic resolution structure of a high affinity periplasmic phosphate-binding protein (PfluDING) bound with phosphate at pH 8.5*

b. PROTEIN EXPRESSION AND IMMOBILIZATION

The pet-28a (+)-vector was used for cloning and for protein expression *E. coli* BL 21 DE3 gold were used. eziG beads®, sponsored by EnginZyme, and Sepharose beads, purchased from SigmaAldrich were used for protein immobilization. Steps were carried out as given in the respective manuals.

c. BINDING AND ELUTION OF PHOSPHATE

After immobilization, the beads are washed with phosphate containing solutions with different phosphate concentrations. At first, a phosphate solution with a concentration of around 1500 mM is used. After binding of phosphate to the beads out of that solution had been observed, the phosphate concentration in the stock solution is reduced to around 900 mM and a second time to around 120 mM. After the wash cycles with the phosphate-containing solutions the beads are transferred into an extraction solution (ddH_20) and the stimulus is activated.

pH-driven approach

Following the methods used by Venkiteshwaran et al., the phosphate is bound from the phosphate-containing solution at pH 7. Subsequently, the beads are transferred to the extraction solution. To release the phosphate from the proteins, the pH is then increased. This process of binding phosphate from wastewater and releasing it into the extraction solution is repeated several times.

SOPP3-driven approach

For the light driven approach using the SOPP3-PstS construct, blue light emitting LEDs are used, providing blue light with a wavelength of λ=400 nm. This leads to the production of reactive oxygen species (ROS). The ROS destroy the structure of the PBP, and the Phosphate is no longer bound.

VVD-driven approach

For the light driven approach using the VVD-domain, the LED stripes are used to induce the dimerization of the PBP. After the phosphate is bound to the PBP while the proteins are illuminated, the LED are turned off. As soon as the illumination is stopped, the PBP loses its dimeric form, and the phosphate binding ability is no longer maintained. The phosphate is set free. The light driven approaches are both been performed under light exclusion.
To measure the phosphate concentration in the solution before and after triggering the integrated switch, samples of the solutions are taken and analyzed using the Phosfinity® assay (Aminoverse), provided by the Schwaneberg group at ABBt.

d. PRODUCTION OF POLYPHOSPHATE

To create a valuable industrial resource out of the recycled phosphate, we used *Saccharomyces cerevisiae*. This process is called polyphosphate hyperaccumulation (Christ and Blank 2019). Saccharomyces cerevisiae can be used to produce naturally polyphosphate-rich yeast extract. To increase the polyphosphate yield in S. cerevisiae, the cells are first incubated in phosphate-free medium and then in medium containing phosphate. As a result, the uptake of phosphate is increased, as well as the synthesis of polyphosphate.
For measuring the amount of polyphosphate created, the Phosfinity® assay (Aminoverse) was used.

III. DISCUSSION

The MetaPhos project aims to achieve a proof of concept regarding the extraction of phosphate from wastewater using immobilized PBPs. Since no specific experiments regarding the properties of the PBPs and their ability to recover phosphate have been completed at this point in the project, there are no results to discuss the viability of the methods yet.
In total, there are three different approaches being pursued. The SOPP3-approach only allows for every PBP to be used once, due to the destruction of the protein structure caused by the release of ROS. This is problematic for the profitability of the method, considering the costs of synthesizing one protein and the value of a single phosphate molecule. Nevertheless, it could be an important step to prove that PBPs can in fact be made switchable via light and present other research groups to pursue such a path.

To overcome the limitations of the SOPP3 approach, two reversible approaches (pH- and VVD-approach) are being tested additionally. The reusability of the proteins in these two approaches is a key factor to increase the number of phosphate molecules recovered for every protein used and thus increasing both the profitability and the sustainability of the recovery method.

IV. CONCLUSION

As it is discussed at length above, phosphate is essential for life, which makes it greatly desirable to recycle. The goal is to push the boundaries of phosphate recovery a little further and show the direction in which the phosphate economy eventually needs to go.
The possibilities of these methods are great and provide a possible solution to a problem that will otherwise only grow in the next decades. Ultimately, there needs to be a recycling economy for phosphate. This would allow a much more sustainable, ecological use of phosphate in the various ways it can be used. The core concept of engineering binding proteins to be switchable through external stimuli could be a key method for the sustainable management of natural resources in the future.
To keep up with the development of MetaPhos, feel free to look at igem.rwth-aachen.de!

REFERENCES

Alberts et al. (2001). Lehrbuch der Molekularen Zellbiologie, 2. Auflage, Pages 102-104, ISBN 3-527-30493-2.

Christ, Jonas Johannes and Blank, Lars (2019) Saccharomyces cerevisiae containing 28% polyphosphate and production of a polyphosphate-rich yeast extract thereof. FEMS Yeast Research 2019 Vol. 19, Issue 3. https://doi.org/10.1093/femsyr/foz011

Correl, D.L. (1998). The role of phosphorous in the Eutrophication of Receiving Waters: A Review. Retrieved from: https://acsess.onlinelibrary.wiley.com/doi/pdfdirect/10.2134/jeq1998.00472425002700020004x

Salm, C., Kros, J., Vries, W. (2016). Evaluation of different approaches to describe the sorption and desorption of phosphorus in soils on experimental data, Science of The Total Environment, Volume 571, Pages 292-306, ISSN 0048-9697. https://doi.org/10.1016/j.scitotenv.2016.07.004.

Shepherd, J., Sohi, S.P., Heal, K.V. (2016). Optimising the recovery and re-use of phosphorus from wastewater effluent for sustainable fertiliser development, Water Research, Volume 94, Pages 155-165, ISSN 0043-1354. https://doi.org/10.1016/j.watres.2016.02.038.

Venkiteshwaran, K., Pokhrel, N., Hussein, F., Antony, E., Mayer, B.K. (2018). Phosphate removal and recovery using immobilized phosphate binding proteins, Water Research X, Volume 1, 100003, ISSN 2589-9147, https://doi.org/10.1016/j.wroa.2018.09.003.

Williams, G. (2020). World´s Top 10 Phosphate Mining Production Countries, Phosphate Investing News. Retrieved from: https://phosphateprice.com/worlds-top-10-phosphate-mining-production-countries/

Ye, Y., Ngo, H.H., Guo, W., Liu, Y., Li, J., Liu, Y., Zhang, X., Jia, H. (2017). Insight into chemical phosphate recovery from municipal wastewater, Science of the total Environment, Volume 576, Pages 159-171, ISSN 0048-9697. https://doi.org/10.1016/j.scitotenv.2016.10.078.

AUTHORS

First Author – Nathalie Zehetner, B.Sc. Student in Biology, RWTH Aachen University, nathalie.zehetner@rwth-aachen.de

Second Author – Laurin Röder, B.Sc. Student in Molecular and applied Biotechnology, RWTH Aachen University, laurin.roeder@rwth-aachen.de

Third Author – Leonie Hümmeler, B.Sc. Student in Molecular and applied Biotechnology, RWTH Aachen University, leonie.huemmeler@rwth-aachen.de

Fourth Author – Laurin Poro, B.Sc. Student in Molecular and applied Biotechnology, RWTH Aachen University, laurin.poro@rwth-aachen.de

Redefining lung cancer diagnosis with the use of DNA nanostructures.

Bochalis Eleftherios[*], Konidi Garifalia[**], Dereki Irene[***]

[*] Patras Medicine, University of Patras
[**] Patras Medicine, University of Patras
[***] Patras Medicine, University of Patras

Abstract- Lung cancer is one of the most common types of cancer, with more than 2.2 million cases recorded in 2020 (Cancer Today, 2020a). Furthermore, it is the deadliest cancer type, with approximately 1.8 million deaths worldwide and a mortality rate of 81.4% (Cancer Today, 2020b). This high percentage is due to the late diagnosis of the disease since the current diagnostic techniques lack the potential to be used as effective screening methods. Lately, liquid biopsies have been successfully applied to cancer screening and diagnosis, targeting specific biomarkers such as circRNAs. In the context of liquid biopsies, we propose an innovative diagnostic tool based on Synthetic Biology, combining Rolling Circle Amplification and molecular beacons. We aim to create a Linear DNA nanostructure to detect a panel of over-expressed circular RNAs secreted in lung cancer patients' circulation. Our tool offers a simple and cost-effective way to diagnose lung cancer in just 70 minutes using standard lab equipment. Therefore, the early diagnosis provided by this technique can increase a patient's survivability by 80-90%.

Index Terms- Circular RNAs, Linear DNA Nanostructure, Liquid Biopsies, Lung Cancer Diagnosis, Hairpin Probes.

I. INTRODUCTION

Lung cancer is a type of cancer that originates in the pulmonary parenchyma and is divided into two main categories and some less frequent subtypes. Non-small cell lung cancer (NSCLC) is the most common subtype, applying for more than 80% of all cases, whereas small cell lung cancer (SCLC) accounts for approximately 10% of all cases but is the deadliest. According to the World Health Organization, lung cancer causes approximately 1.8 million deaths annually, amounting to a mortality rate of 81.4% (Nasim et al., 2019), (Siegel et al., 2022).

Late diagnosis is considered the number one factor for exceptionally high mortality. Tissue biopsy has been the established lung cancer diagnostic method, in which lung cells are surgically removed and examined for the identification of tumors. Additionally, imaging tests have also been developed and approved for detecting the disease at the early stages, when it is still curable. Among these, the most commonly used procedures are Chest X-ray, CT scan, and Positron emission tomography (PET) scan (Nooreldeen & Bach, 2021). However, none of the previously mentioned processes can be effectively used as a screening test due to a lack of accuracy and specificity (Pinsky, 2014).

On the other hand, liquid biopsy is an upcoming technique based on the intake of body fluids that has excellent prospects of functioning as a screening test. Multiple biomarkers, including circulating tumor cells and nucleic acids, can be targeted, and examined via liquid biopsies. In addition, this technique is less invasive, rapid, and easily performed, providing early diagnosis, and consequently increasing survival rates (Li et al., 2022).

Circular RNAs (circRNAs) are a large class of non-coding RNAs (ncRNAs) produced by an event named back splicing. Backsplicing is a non-canonical splicing event in which a downstream splice-donor site is covalently linked to an upstream splice-acceptor site. They are single-stranded RNA molecules that lack a 3'-poly-A tail and a 5'-prime cap. Due to the biogenesis of circRNAs, they possess a characteristic domain called back splice junction. The Backsplice junction (BSJ) site is where the canonical 5' splice site sequence is joined to the upstream 3' splice site sequence. BSJ is the only region distinct for circRNAs and does not appear in their linear RNA counterparts (Kristensen et al., 2019).

Circular RNAs may have the following functions:

They can act as miRNA sponges protecting target mRNAs from miRNA-dependent degradation. Some circRNAs contain RNA binding protein (RBP) motifs and can act as sponges for these proteins and indirectly regulate their functions. They can interact and enhance the function of specific proteins, e.g., the RNA polymerase II (Pol II) complex containing the small nuclear ribonucleoprotein U1 (snRNP) and other proteins. Some circRNAs have been shown to function as protein scaffolds, facilitating the localization of enzymes (phosphatases, acetylases, and ubiquitin ligases) and their substrates to influence reaction kinetics. circRNAs can also recruit specific proteins or even be translated under certain conditions.

When it comes to cancer, circRNAs, play an oncogenic or suppressive role and affect cellular functions. An abnormal increase or decrease in their expression in cancer cells makes them regulators of cell proliferation, immigration, apoptosis, the induction of multidrug resistance, regulating the tumor microenvironment, and immunoevasion.

Because of these actions, they can be used as potential diagnostic, prognostic, or therapeutic biomarkers. Although the detection of circRNAs has been mainly conducted in tissues and cell lines, many studies have focused on detecting circRNAs using less invasive and more accessible methods, such as liquid biopsy. circRNAs are ideal candidates as liquid biopsy

biomarkers as their expression is often tissue and developmental-stage related. They are found in large amounts not only in tissues and cells but also in body fluids. Due to the high stability of their covalently closed ring structure, circRNAs cannot be cleaved by most ribonucleases compared to linear RNAs. Combining circRNAs with traditional cancer biomarkers can confer higher diagnostic accuracy than single traditional biomarkers (Wang et al., 2020).

II. RESEARCH ELABORATIONS

Materials and Methods

All DNA sequences were synthesized by Eurofins Scientific and are listed in Appendix Supplementary Information S1. T4 DNA ligase, Exonuclease I, Exonuclease III, and Phi29 DNA polymerase were purchased from New England Biolabs. DEPC water was obtained from Sigma Aldrich. The PCR purification kit was obtained from Macherey-Nagel. dNTPs were purchased from Promega. The sequences for the probes were tested for their thermodynamic properties using the NUPACK (http://www.nupack.org/) and ViennaRNA (https://www.tbi.univie.ac.at/RNA/) (Zadeh et al., 2010) (Lorenz et al., 2011) softwares. Their secondary structures were obtained and visualized using NUPACK and mfold web services (http://www.unafold.org/) (Zuker, 2003). The thermodynamic properties and secondary structures can be found in Appendix Supplementary Information S2. The sequence of the target circRNA, upon which the hairpin probes H1 and H2 probes were created, was obtained from circBase (http://www.circbase.org/) (Glažar et al., 2014), an online database with gene coordinates and the mature sequences for circRNAs.

Circular DNA template formation:

First, 10 μL of the phosphorylated template (10 pmol/μL), 10 μL of DNA ligation buffer (10×), and 50 μL of primer (10 pmol/μL) were mixed in a PCR tube. RNase-free water was added until the volume of 100 μL. The mixture was heated to 95 °C for 5 minutes and left at 25 °C for 30 minutes. Then, 5 μL of T4 DNA ligase (400.000 U/mL) was added, and the mixture was incubated at 16 °C overnight. To purify the circular product, Exonuclease I and Exonuclease III were used for the removal of single-stranded and double-stranded linear by-products, respectively, according to the corresponding NEB protocols. PCR product purification kit was used to obtain the circular product.

Rolling Circle Amplification:

First, 50 μL of circular DNA template, 10 μL of primer (10 pmol/μL), and 2.5 μL of Phi29 DNA polymerase buffer (10×) were mixed in a PCR tube. The tube was heated to 95 °C for 5 minutes and immediately placed on ice. After 5 min, 7.5 μL of Phi29 DNA polymerase buffer (10×), 20 μL dNTPs (10 pmol/μL), 10 μL Recombinant Albumin (10%) and 2.5 μL of Phi29 DNA polymerase (10.000U/mL) were added to the mixture. The mixture was incubated at 37 °C for 45 minutes, followed by enzyme deactivation at 65 °C for 10 minutes. To obtain the RCA product, we used the PCR product purification kit.

Long DNA Nanostructure Assembly:

The RCA product, H1, and H2, were first heated to 95 °C for 5 minutes, left at 25 °C for 30 minutes, and placed in the refrigerator (4 °C) for 10 minutes. 25 μL of H1 (10 μM), 25 μL of H2 (10 μM), and 125 μL of Tris-MgCl2 buffer (500 mM, pH 8.0) were mixed to create the H1-H2 pool mixture. Next, 25 μL of the RCA product was added to the mixture. Following that, the mixture was incubated for 2 hours at 37 °C.

Detection testing:

Samples were prepared as follows: 3 μL LDN, 12 μL of synthetic BSJ (100 nM), and 15 μL of Tris-MgCl2 buffer (500 mM, pH 8.0) as the positive sample, and 3 μL LDN, 12 μL RNase-free water and 15 μL of Tris-MgCl2 buffer (500 mM, pH 8.0) as the negative control. Mixtures were incubated at 37 °C for 100 min and then transferred to a black 384-well microtiter plate to collect fluorescence emission at 576 nm by excitation at 554 nm. In addition, fluorescence emission spectra between 568 and 700 nm were also collected by excitation at 554 nm.

III. RESULTS AND FINDINGS

The main principle for the designed assay is divided into four steps, which are illustrated in Scheme 1. Firstly, the cyclization of the phosphorylated DNA template takes place. The corresponding circular DNA template is synthesized using T4 DNA ligase and a primer specific for the start and end domains of the DNA template (Step 1).

Setting that as a starting point and by using phi29 DNA polymerase that possesses great strand displacement activity and a corresponding primer, a long linear DNA molecule is created through Rolling Circle Amplification (RCA). That molecule possesses multiple repeats of the phosphorylated DNA template sequence (Step 2). The circular DNA template carries two hairpin probe installation sites for the H1 and H2 hairpin probes. The long linear DNA scaffold carries multiple hairpin probe installation sites. The hairpin probes H1 and H2 are installed to the DNA scaffold because one of their two "hanging tails" is complementary to the installation site. This way, multiple pairs of H1 and H2 probes are installed in the DNA scaffold, thus creating the Linear DNA Nanostructure (LDN) (Step 3).

The detection of the circRNA targets is carried through by a group of hairpin probes, previously mentioned H1 and H2. H1 probe "recognizes" the back-splice junction (BSJ) site of the circular RNA biomarker to distinguish the linear isoforms RNA in the sample and initiates a cascade reaction. On the other hand, the H2 hairpin probe is responsible for signal generation, as it contains a fluorophore and a quencher molecule. In the absence of the circRNA target, the quencher absorbs the signal emitted by the fluorophore due to its close distance, and the cross-reactivity between H1 and H2 is not optimal due to the protection of complementary regions within the hairpin stems. When the target molecule is present, it hybridizes to an H1 probe (installed in the LDN) through a toehold-mediated strand displacement reaction. The transformation of the two probes is initiated, with H2 hybridizing to H1. This way, the fluorophore is distanced from the quencher leading to fluorescent signal restoration that can be detected and quantified (Step 4).

The released circRNA target can hybridize with adjacent H1/H2 groups leading to an isothermal. Nonenzymatic signal amplification reaction along the LDN. Also, one circRNA molecule can catalyze the luminescence of multiple LDNs, causing a second signal amplification, showing a strong signal, and simultaneously lowering the detection limit.

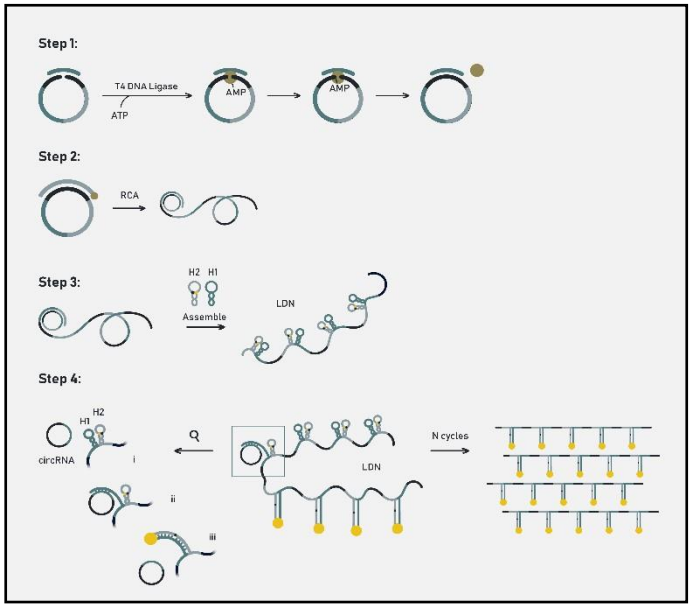


FIGURE 1.
Schematic representation of the four steps surrounding the assembly and the Linear DNA Nanostructure (LDN) function.

Testing the feasibility of the proposed assay:

To prove the feasibility of our design, we must test each step separately. As shown in Figure 1A, the preparation of the circular DNA template and the long DNA scaffold can be verified by agarose gel electrophoresis experiments. The long DNA scaffold remains at the top of the electrophoresis gel due to the low electrophoretic ability caused by its high molecular weight. The successful synthesis of LDN can also be verified by agarose gel electrophoresis. As shown in Figure 2B, after adding the long DNA scaffold, two bands appear, one accounting for the LDN synthesis and one accounting for the H1 and H2 uninstalled probes.

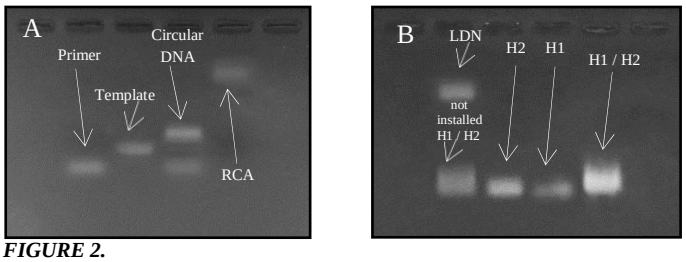


FIGURE 2.
(A) Agarose gel electrophoresis analysis of successfully synthesized RCA products. (B) Agarose gel electrophoresis analysis of successfully assembled LDNs and their corresponding probes.

At the time of writing this paper, not all our experiments are carried through, and we have not been able to test the LDN's ability to identify target circRNA molecules. However, we have carried out experiments targeting a synthetic BSJ site. We used the BSJ site for hsa_circ_0102533, a circRNA with the potential to be used as an early-stage Non-Small Cell Lung Carcinoma biomarker (Huang et al., 2022). We tested the LDN's ability to successfully identify the target molecule by fluorescent spectroscopy. Thermo Scientific VarioSkan LUX was used for the detection of the signal. As can be observed in Figure 3, there was a significant increase in fluorescent intensity when the BSJ site was present compared to the absence of the target, indicating a low background signal. This result confirms that the target successfully triggered a cascade strand displacement reaction in LDN (Jiao et al., 2020).

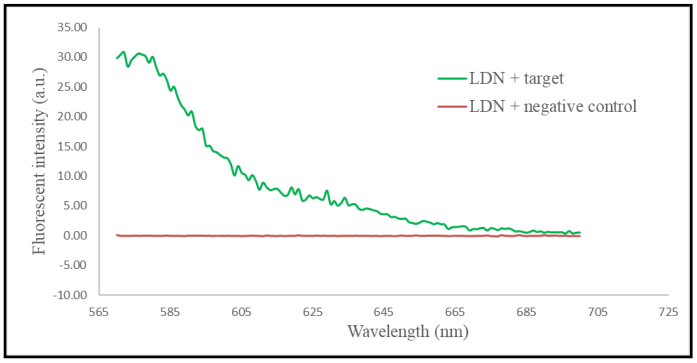


FIGURE 3.
The fluorescence emission spectrum of the LDN system in the presence or the absence of the target BSJ site (100 nM).

IV. DISCUSSION

This innovative lung cancer diagnostic tool provides early detection of malignancy based on liquid biopsy, using circRNAs as specific biomarkers. The main feature of liquid biopsies, apart from offering rapid results, is the ease of sampling compared to traditional histological biopsies, which mainly require surgery, which reasonably explains the ever-increasing research interest of the medical community regarding this method. Regarding the detection of circRNAs, Linear DNA Nanostructure, based on Rolling Circle Amplification, proves to be a promising and superior target technique compared to others. First of all, there is a detection limit at 1 pM, thus increasing the method's accuracy since the biomarker can be distinguished even in minimal quantities. Quantitative detection is also enabled; hence just a single circRNA molecule can catalyze multiple LDNs, leading to signal amplification. Despite the low throughput of the technique and the time-consuming creation of the probes needed -in order to reach the appropriate distance between them so that the binding sequence is not obstructed, LDN seems to be preferable related to hybridization-based methods (e.g., Northern blotting, RNA sequencing) because of their low sensitivity and the usually high amount of RNA necessary, as well as the time needed for the results.

Furthermore, PCR types that can be used for circRNAs detection, such as qRT-PCR and RT-ddPCR, are way costlier than LDN, and ligation-based PCR has the disadvantage of one circRNA molecule detected at a time. As for the Stem-loop primer-induced double exponential amplification-based circRNA detection, where Loop-mediated isothermal amplification (LAMP) is included, an elaborated and costly design of primers is required. Finally, enzyme-based methods provide lower amplification efficiency than RCA and PCR. Although it has many advantages over other techniques, Linear DNA Nanostructure has still not been excessively applied in the

diagnosis of cancer due to the limited research conducted on it (Mi et al., 2022), (Zhang et al., 2020)

V. CONCLUSION

Overall, combining the examination of blood samples, also known as liquid biopsy, and the detection and quantification of certain overexpressed circular RNAs as specific biomarkers using Linear DNA Nanostructure as the target method can lead to the production of a new screening test for lung cancer. It is important to highlight the need for more research to be done so that this diagnostic tool is approved as a regular and safe examination. However, such an easily and quickly performed technique that does not require specialized equipment can evolve into a widely used method in the future, aiming to successfully diagnose lung cancer and other types of cancer at early stages, where the disease is still curable.

APPENDIX

Supplementary Information S1:
https://docs.google.com/document/d/1bxK8p6EoGrHzu2PJiBdNH7FCvMPQRxgFhdJxnP-z1ws/edit?usp=sharing

Supplementary Information S2:
https://docs.google.com/document/d/168s3RfsqdBMwfxp6rqLInnSIVCvtomaSRVDBlXRRmiU/edit?usp=sharing

ACKNOWLEDGEMENT

We would like to thank our Principal Investigators: Assistant Professors Kalogianni Despoina and Kallergi Galatea for their help throughout the project and for providing us with the necessary laboratory equipment to conduct our research. Also, we would like to thank R.G.C.C S. A for funding this work.

REFERENCES

Lung Cancer incidence rates. (2020). World Health Organization. https://gco.iarc.fr/today/online-analysis-multi-bars?v=2020&mode=cancer&mode_population=countries&population=900&populations=900&key=asr&sex=0&cancer=39&type=0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=10&group_cancer=1&include_nmsc=0&include_nmsc_other=1&type_multiple=%257B%2522inc%2522%253Atrue%252C%2522mort%2522%253Afalse%252C%2522prev%2522%253Afalse%257D&orientation=horizontal&type_sort=0&type_nb_items=%257B%2522top%2522%253Atrue%252C%2522bottom%2522%253Afalse%257D

Lung Cancer mortality rates. (2020b). World Health Organization. https://gco.iarc.fr/today/online-analysis-multi-bars?v=2020&mode=cancer&mode_population=countries&population=900&populations=900&key=asr&sex=0&cancer=39&type=0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=10&group_cancer=1&include_nmsc=0&include_nmsc_other=1&type_multiple=%257B%2522inc%2522%253Afalse%252C%2522mort%2522%253Atrue%252C%2522prev%2522%253Afalse%257D&orientation=horizontal&type_sort=0&type_nb_items=%257B%2522top%2522%253Atrue%252C%2522bottom%2522%253Afalse%257D

Nasim, F., Sabath, B. F., & Eapen, G. A. (2019). Lung Cancer. Medical Clinics of North America, 103(3), 463–473. https://doi.org/10.1016/j.mcna.2018.12.006

Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2022). Cancer statistics, 2022. CA: A Cancer Journal for Clinicians, 72(1), 7–33. https://doi.org/10.3322/caac.21708

Kristensen, L. S., Andersen, M. S., Stagsted, L. V. W., Ebbesen, K. K., Hansen, T. B., & Kjems, J. (2019). The biogenesis, biology and characterization of circular RNAs. Nature Reviews Genetics, 20(11), 675–691. https://doi.org/10.1038/s41576-019-0158-7

Wang, C., Tan, S., Li, J., Liu, W. R., Peng, Y., & Li, W. (2020). CircRNAs in lung cancer - Biogenesis, function and clinical implication. Cancer Letters, 492, 106–115. https://doi.org/10.1016/j.canlet.2020.08.013

Li, W., Liu, J. B., Hou, L. K., Yu, F., Zhang, J., Wu, W., Tang, X. M., Sun, F., Lu, H. M., Deng, J., Bai, J., Li, J., Wu, C. Y., Lin, Q. L., Lv, Z. W., Wang, G. R., Jiang, G. X., Ma, Y. S., & Fu, D. (2022). Liquid biopsy in lung cancer: significance in diagnostics, prediction, and treatment monitoring. Molecular Cancer, 21(1). https://doi.org/10.1186/s12943-022-01505-z

Nooreldeen, R., & Bach, H. (2021). Current and Future Development in Lung Cancer Diagnosis. International Journal of Molecular Sciences, 22(16), 8661. https://doi.org/10.3390/ijms22168661

Huang, Y., Qin, S., Gu, X., Zheng, M., Zhang, Q., Liu, Y., Cheng, C., Huang, K., Peng, C., & Ju, S. (2022). Comprehensive Assessment of Serum hsa_circ_0070354 as a Novel Diagnostic and Predictive Biomarker in Non-small Cell Lung Cancer. Frontiers in Genetics, 12. https://doi.org/10.3389/fgene.2021.796776

Mi, Z., Zhongqiang, C., Caiyun, J., Yanan, L., Jianhua, W., & Liang, L. (2022). Circular RNA detection methods: A minireview. Talanta, 238, 123066. https://doi.org/10.1016/j.talanta.2021.123066

Jiao, J., Xiang, Y., Duan, C., Liu, Y., Li, C., & Li, G. (2020). Lighting Up CircRNA Using a Linear DNA Nanostructure. Analytical Chemistry, 92(18), 12394–12399. https://doi.org/10.1021/acs.analchem.0c02146

Zhang, P., Guo, N., Gao, K., Su, F., Wang, F., & Li, Z. (2020). Direct recognition and sensitive detection of circular RNA with ligation-based PCR. Organic & Biomolecular Chemistry, 18(17), 3269–3273. https://doi.org/10.1039/d0ob00625d

Pinsky, P. F. (2014). Assessing the benefits and harms of low-dose computed tomography screening for lung cancer. Lung Cancer Management, 3(6), 491–498. https://doi.org/10.2217/lmt.14.41

Glažar, P., Papavasileiou, P., & Rajewsky, N. (2014). circBase: a database for circular RNAs. RNA, 20(11), 1666–1670. https://doi.org/10.1261/rna.043687.113

Zadeh, J. N., Steenberg, C. D., Bois, J. S., Wolfe, B. R., Pierce, M. B., Khan, A. R., Dirks, R. M., & Pierce, N. A. (2010). NUPACK: Analysis and design of nucleic acid systems. Journal of Computational Chemistry, 32(1), 170–173. https://doi.org/10.1002/jcc.21596

Lorenz, R., Bernhart, S. H., Höner Zu Siederdissen, C., Tafer, H., Flamm, C., Stadler, P. F., & Hofacker, I. L. (2011). ViennaRNA Package 2.0. Algorithms for Molecular Biology, 6(1). https://doi.org/10.1186/1748-7188-6-26

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Research, 31(13), 3406–3415. https://doi.org/10.1093/nar/gkg595

AUTHORS

First Author – Bochalis Eleftherios, Undergraduate Pharmacy Student, University of Patras, left.bochalis@gmail.com

Second Author – Konidi Garifalia, Undergraduate Pharmacy Student, University of Patras, garifaliakon@gmail.com

Third Author – Dereki Irene, Undergraduate Pharmacy Student, University of Patras, irenenter@gmail.com

Correspondence Author – Bochalis Eleftherios, Undergraduate Pharmacy Student, University of Patras, left.bochalis@gmail.com .

Project PAGGAIA: An innovative approach for Precision Agriculture, using Metagenomics and Machine Learning

Marina Dimitriou[1], Agisilaos Kounelis[2], Christoforos Kasimatis[3], Panagiotis C. Bantounas[1], Despoina Chrysanthou – Tsakiridou[1], Dimitra Dedousi[1], Marina V. Fronimou[1], Maria Panagiota Georgopoulou[4], Kalliopi Giantsi[1], Evaggelia Katri[1], Nikolaos Katsenios[7], Evgenia Kostimpa[1], Elvira Koutsouki[1], Dimitrios Leonidakis[10], Yannis N. Manoussopoulos[6], Ioanna Nika[5], Eliana Rodrigues – Christodoulide[1], Violetta Papadimitriou[5], Katerina L. Pramandioti[1], Georgios Raptis[8], Stavroula Siamoglou[1], Ioannis Sina[2], Stamatis Tsantos[1], Christina Tsianava[1], Lamprini Vlachogianni[9], Aspasia Efthimiadou[7], Vasilis Kostopoulos[4], George P. Patrinos[1]

[1] iGEM Patras, University of Patras, Department of Pharmacy
[2] iGEM Patras, University of Patras, Department of Computer Engineering and Informatics
[3] iGEM Patras, University of Piraeus, Department of Informatics
[4] iGEM Patras, University of Patras, Department of Mechanical Engineering and Aeronautics
[5] iGEM Patras, University of Patras, Department of Medicine
[6] iGEM Patras, Laboratory of Virology, ELGO - Demeter, Patras
[7] iGEM Patras, Department of Soil Science of Athens, Institute of Soil and Water Resources, Hellenic Agricultural Organization - Demeter
[8] iGEM Patras, National and Kapodistrian University of Athens, Department of Biology
[9] iGEM Patras, University of Patras, Department of Biology
[10] iGEM Patras, University of Thessaly, Department of Agriculture Crop Production and Rural Environment

***Abstract*- The interactions between the various soil microorganisms are challenging to study, and thus remain unknown. A major drawback in the field of Agriculture is the inability to collect significant data, regarding soil quality for cultivation purposes, in large and isolated areas. Precision Agriculture is an innovative farming management concept, aiming to revolutionize agriculture and soil improvement, both by increasing crops yields and at the same time preserving natural resources. Hence, Data Analysis and Machine Learning models are considered the future of Agriculture, providing advice to farmers about the best possible cultivation practices. The goal of the project, named Precision AGriculture using Genomics, Artificial Intelligence and Aero-transportable equipment or in short PAGGAIA, is to create a protocol that includes molecular, physicochemical, and agronomical characteristics and analyzes them through Artificial Intelligence and more specifically Machine Learning, in order to propose tailor-made interventions to farmers and specialists. Since the project is underway there are no available results and the following research paper describes the procedures followed.**

***Index Terms*-** Drone, Machine learning, Next-generation sequencing, Precision agriculture, Portable equipment

I. INTRODUCTION

Tomato (Solanum lycopersicum L.) was selected for our project since it is considered as one of the most significant vegetables worldwide, ranked second in production as well as in consumption (Migliori et al., 2017).

A determining factor in agriculture is the lack of important data regarding soil physical and chemical properties in large and isolated areas. Moreover, the increasing demand of crops due to the increasing population has made it mandatory for farmers to increase the yield as well as improving the quality of the crops. Precision agriculture is an innovative farming management concept aiming to revolutionize agriculture, by increasing crops yields while preserving natural resources. In recent years, precision agriculture has been enabled by the advent of the Global Positioning System (GPS) that facilitates the farmers' ability to locate their precise position in the field. This system allows the mapping of soil attributes, such as crop yield, topography features, soil organic matter, electrical conductivity, moisture levels, nitrogen levels, pH, and minerals (Stafford, 2000).

Precision agriculture has also been enabled by unmanned aerial vehicles that can be easily operated. These drones can be equipped with cameras and other equipment and can provide additional features and geographic data, also known as geodata. Recently, the field of Genomics has emerged in order to determine the soil's microflora. This has been made possible by performing Metagenomic analysis, in order to identify bacterial species with Next-Generation Sequencing (Brown et al., 2017). Therefore, this information can be taken into consideration when making recommendations for soil improvement, using integrated artificial intelligence-based decision support tools.

Machine Learning (ML) is a field of computer science that uses computers to learn from data. Past studies have pointed out the need for such data-driven methods in Precision Agriculture. In a past experiment, data being gathered from tomato crops do not seem to have linear connection, using both environmental conditions and current and previous crop yields (Qaddoum et al., 2013). In another research, the Total Soluble Solids of tomato (◦Brix) were predicted using raw data, including weather data, soil analysis, and previous quality features. In this model, 12 different algorithms were used, in order to find the best one. The Decision Support System (DSS)

that was created, predicted scoring less than 0.085 (Kasimatis et al., 2022). The optimal amount of irrigation was predicted using a Bayesian-Ridge based DSS in cotton cultivation without affecting the expected yield. The results increased the yield by reducing the times of irrigation (Leonidakis et al., 2021).

In this project, at the experimental field area where tomato plants were cultivated, the soil was impregnated with bacteria, which belong to the species *B. subtilis, B.amyloliquefaciens* and *B.thuringiensis*. Therefore, these bacteria are used in this case as Plant Growth Promoting Bacteria (PGPB). More specifically, they are used as biostimulants, to help promote the growth, yield and quality of crops (Glick, 2012). Such effects of bacteria have been proven to increase the yield of sweet corn in Greece, when used to impregnate the soil before cultivation. The results showed that B. mojavensis increased yield by 16% and B. subtilis by 13.8% (Katsenios et al., 2022). Moreover, in a study conducted in the same area, the application of bacteria increased the yield, the dry weight as well as total soluble solids in the tomato fruits (Katsenios et al., 2021).

The goal of the project is to create a protocol that includes molecular, physicochemical, and agronomical characteristics and analyzes them through Artificial Intelligence and more specifically Machine Learning, in order to propose tailor-made interventions in terms of field improvement to farmers and specialists.

II. MATERIALS AND METHODS

SAMPLING

Sampling was performed at different locations of an experimental field area that has been cultivated with tomatoes and where selected bacterial strains were added to the soil near the transplanted tomato plants. Since only soil samples were analyzed, the field size does not have any importance. Strict protocol was followed, as the sample collectors changed gloves for each sample, in order to avoid cross contamination of the samples. This process can also be automated by using aerotransportable equipment, such as specially designed soil-sampling drones. For this purpose a custom-made soil sampling mechanism, attachable to a DJI Phantom 3 Drone, was designed according to the report of Blake Rolfing et al.. The soil sampling mechanism is based around a single-acting, spring-return pneumatic piston. Further research needs to be done in order to meet all of the design metrics, especially the weight metric, so as to achieve the desired weight value.

NGS

Metagenomic analysis will be performed for these samples, specifically through Next Generation Sequencing (NGS), which allows rapid, real-time sequencing of nucleic acids. With the use of NGS techniques, it is possible to characterize microbes that are found in low abundance or that cannot be grown with the use of traditional culturing methods. In addition to this advantage, NGS does not require special sample preparation and contamination is reduced to the minimum. Furthermore, with NGS it is also possible to perform parallel sequencing of different samples. Therefore, with the application of this technology, the bacterial composition of the soil will be identified (Wooley et al., 2010). This metagenomic analysis can also be performed by using a portable sequencing device, which can operate anywhere, even at the sample source, which in this case is the crop field (Brown et al., 2017). Due to its simple workflow, it can provide immediate data streaming for rapid, actionable results. Furthermore, this type of device can generate short to ultra-long reads for ultimate experimental flexibility and also has low equipment cost. Additionally, the real time data streaming that this type of device provides, allows to stop sequencing, when the wanted data is obtained, while the on-site analysis approach also minimizes the possibility of sample degradation. Data derived from the metagenomic analysis will be utilized in the development of an innovative protocol, in combination with physicochemical, and agronomical data and with the use of a Machine Learning model. High quantity of reliable data is often a requirement for many machine learning models in order to provide accurate predictions.

WEATHER DATA

The weather data that was used came from weather stations from the National Observatory of Athens which gather data, which includes temperature and rain and wind measurements, every 10 minutes (Lagouvardos et al., 2017). Weather data consists of Average, Minimum, Maximum Temperatures and the total rainfall (in mm) for each month from April to September. The reason for not using weather data of the whole year is that tomatoes are cultivated in April and gathered in September, so having more data would be redundant and would only complicate our model. These data are used to find the correlation between quality features and weather conditions.

SOIL TEXTURE

The soil texture of the fields was measured using the method of Bouyoucos (1962) and the soil taxonomy of USDA (1999).

The Field Sensors that were used in the field were the SMT 12 and ECH2O EC-5 (MeterGroup, Pessl Instruments), measuring temperature and moisture of soil at 27 cm inside the ground.

MACHINE LEARNING MODEL

In order to test the predictive value of the dataset, multiple regressors are evaluated using K-fold cross validation. The first part of the algorithm is model selection, in which the best regressor both in terms of time and accuracy is selected. Then, the correlation between the available variables is extracted and the results are compared with the coefficients derived from purely statistical methods. This ensures that the model has found the important variables and is trained accordingly. Moreover, feature elimination methods are applied to improve the score and make the model faster. After the selection of the best model using the default parameters, hyperparameter tuning is applied iteratively and leads to the repetition of the design and build phase of the model. This part of the algorithm aims at finding the combination of hyperparameters that maximizes the model's performance during the test phase while preventing overfitting.

The best model is used to predict ways to improve the conditions of the soil in a given field. That enables farmers to access valuable information such as better in season yield understanding, rigorous data collection related to soil testing, plot measurement, weather pattern and crop analysis. Initial advice is based on natural ways. In case no such ways can improve it, microorganisms are proposed. That bio-sustainable approach has a positive environmental impact, since it leads to reduction in the use of fertilizers, the best possible yield, and the consumers' protection.
The development of a Machine Learning model regarding the prediction of quality features and yield, was based on weather data, soil data from the fields, quality characteristics and historical yield data. Table1 contains the variables that will be included in the model.

TABLE 1: Variables contained in the dataset

Variable	Type
Average temperature (4 months)	Float
Minimum temperature (4 months)	Float
Maximum temperature (4 months)	Float
Precipitation (4 months)	Float
Ph	Float
Clay_Perc	Float
Silt_Perc	Float
Sand_Perc	Float
Ca2+	Float
Mg2+	Float
K+	Float
Zn2+	Float
Mn2+	Float
Cu+	Float
Fe3+	Float
B	Float
Nitrogen	Float
Organic_Matter	Float
Phosphorus	Float
Soil_Texture	String
Salts_Perc	Float
Electrical_Conductivity	Float
Microorganisms	String
Ratio	Float

III. RESULTS AND FINDINGS

Results will be published after the completion of the project.

IV. DISCUSSION

PAGGAIA combines the knowledge and state-of-the-art tools of Precision Agriculture, Synthetic Biology, Metagenomics and Machine Learning in order to provide critical information and advice to farmers. More specifically, they will be able to access valuable information such as better in season yield understanding, rigorous data collection related to soil testing, plot measurement, weather pattern and crop analysis. As an extension to the dataset, satellite data from the experimental areas can be collected from existing APIs. Therefore, based on the physicochemical data of the soil and using the Machine Learning algorithm, analysis for each crop can be performed. Yield prediction using DSSs is extensively used nowadays (Bhakta et al., 2019, Kasimatis et al., 2022). Based on previous experience, the model which will be created will be similar to a model used for yield prediction, but it will be soil oriented instead of plant oriented. In addition GIS technology has been widely used for the assessment of soil (Shit et al., 2016, , Denton et al., 2017). The combination of the above mentioned technologies, that are already developed, will be able to provide the DSS that will use the data to create the prediction.

Data derived from the metagenomic analysis on the soil samples, will lead to conclusions regarding which of the bacterial species that were used to impregnate the soil, survived, multiplied and potentially helped with the plant growth. Consequently, these results in combination with the physicochemical and agronomical data mentioned before, will help advise farmers on which additives should be used in the soil and help reduce the use of fertilizers.

V. CONCLUSION

The aim of this project is soil optimization and increase of agricultural yield, accessibility of agricultural data even in remote areas and thus to ensure profitability, sustainability and environmental protection. These goals will be achieved through an algorithm that predicts the biodiversity of the soil and provides information on which crops should be planted in each case. This innovative protocol is expected to be a business changing tool for the farmers, resulting in the enhancement of soil quality and crop yield. This approach also enables sustainability and has a positive environmental impact, as it leads to the reduction of fertilizers and consequently to the protection of consumers' health. Therefore, it can be considered as a useful tool for scientists and professionals in the field of Agriculture.

Future work aims at expanding the described protocol and implementing a DSS which will propose to the farmer the ideal crop type for the field that data provided, as well as propose ways of improving the field using natural ways where possible in order to reduce the environmental impact. Limitations only include obtaining data from farmers and following the advice from the DSS as they are afraid of following advice from a computer.

REFERENCES

Bouyoucos, G.J., 1962. Hydrometer Method Improved for Making Particle Size Analyses of Soils 1. Agron. J. 54, 464–465. doi:10.2134/agronj1962.00021962005400050028x

Carlson, T.N., Ripley, D.A., 1997. On the relation between NDVI, fractional vegetation cover, and leaf area index. Remote Sens. Environ. 62, 241–252. doi:10.1016/S0034-4257(97)00104-1

Chen, J., Jönsson, Per., Tamura, M., Gu, Z., Matsushita, B., Eklundh, L., 2004. A simple method for reconstructing a high-quality NDVI time-series data set based on the Savitzky–Golay filter. Remote Sens. Environ. 91, 332–344. doi:10.1016/j.rse.2004.03.014

Kasimatis, C.-N., Psomakelis, E., Katsenios, N., Katsenios, G., Papatheodorou, M., Vlachakis, D., Apostolou, D., Efthimiadou, A., 2022. Implementation of a decision support system for prediction of the total soluble solids of industrial tomato using machine learning models. Comput. Electron. Agric. 193, 106688. doi:10.1016/j.compag.2022.106688

Katsenios, N., Andreou, V., Sparangis, P., Djordjevic, N., Giannoglou, M., Chanioti, S., Stergiou, P., Xanthou, M.-Z., Kakabouki, I., Vlachakis, D., Djordjevic, S., Katsaros, G., Efthimiadou, A., 2021. Evaluation of Plant Growth Promoting Bacteria Strains on Growth, Yield and Quality of Industrial Tomato. Microorganisms 9, 2099. doi:10.3390/microorganisms9102099

Lagouvardos, K., Kotroni, V., Bezes, A., Koletsis, I., Kopania, T., Lykoudis, S., Mazarakis, N., Papagiannaki, K., Vougioukas, S., 2017. The automatic weather stations NOANN network of the National Observatory of Athens: operation and database. Geosci. Data J. 4, 4–16. doi:10.1002/gdj3.44

Leonidakis, D., Psomakelis, E., Kasimatis, C.N., Katsenios, N., Kakabouki, I., Roussis, I., Mavroeidis, A., Efthimiadou, A., 2021. Development of Decision Support System Based on the Bayes ARD Algorithm for Irrigation of Cotton. Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca Hortic. 78, 112. doi:10.15835/buasvmcn-hort:2021.0031

Mamatkulov, Z., Safarov, E., Oymatov, R., Abdurahmanov, I., Rajapbaev, M., 2021. Application of GIS and RS in real time crop monitoring and yield forecasting: a case study of cotton fields in low and high productive farmlands. E3S Web Conf. 227, 03001. doi:10.1051/e3sconf/202122703001

Qaddoum, K., Hines, E.L., Iliescu, D.D., 2013. Yield Prediction for Tomato Greenhouse Using EFuNN. ISRN Artif. Intell. 2013, 1–9. doi:10.1155/2013/430986

Singha, C., Swain, K.C., 2022. Evaluating the NDVI based Rice and Potato Yield Prediction map Using GIS Geostatistical Environment, in: 2022 Second International Conference on Advances in Electrical, Computing, Communication and Sustainable Technologies (ICAECT). pp. 1–7. doi:10.1109/ICAECT54875.2022.9807981

Brown BL, Watson M, Minot SS, Rivera MC, Franklin RB. MinION™ nanopore sequencing of environmental metagenomes: a synthetic approach. Gigascience. 2017 Mar 1;6(3):1-10. doi: 10.1093/gigascience/gix007

Bernard R. Glick, "Plant Growth-Promoting Bacteria: Mechanisms and Applications", Scientifica, vol. 2012, Article ID 963401, 15 pages, 2012. doi: 10.6064/2012/963401

Wooley, J.C., Ye, Y. Metagenomics: Facts and Artifacts, and Computational Challenges. J. Comput. Sci. Technol. 25, 71–81 (2010). doi:10.1007/s11390-010-9306-4

Shit, P.K., Bhunia, G.S. & Maiti, R. Spatial analysis of soil properties using GIS based geostatistics models. *Model. Earth Syst. Environ.* **2**, 107 (2016). doi:10.1007/s40808-016-0160-4

O.A. Denton, V.O. Aduramigba-Modupe, A.O. Ojo, O.D. Adeoyolanu, K.S. Are, A.O. Adelana, A.O. Oyedele, A.O. Adetayo & A.O. Oke | Manuel Tejada Moral (Reviewing Editor) (2017) Assessment of spatial variability and mapping of soil properties for sustainable agricultural production using geographic information system techniques (GIS), Cogent Food & Agriculture, 3:1, doi: 10.1080/23311932.2017.1279366

Migliori, C. A., Salvati, L., Di Cesare, L. F., Scalzo, R. L., & Parisi, M. (2017). Effects of preharvest applications of natural antimicrobial products on tomato fruit decay and quality during long-term storage. Scientia Horticulturae, 222, 193-202. doi.org/10.1016/j.scienta.2017.04.030

Stafford, J. V. (2000). Implementing precision agriculture in the 21st century. *Journal of agricultural engineering research*, *76*(3), 267-275. doi: 10.1006/jaer.2000.0577

Bhakta, I., Phadikar, S., & Majumder, K. (2019). State-of-the-art technologies in precision agriculture: a systematic review. Journal of the Science of Food and Agriculture, 99(11), 4878-4888. doi: 10.1002/jsfa.9693

AUTHORS

First Author – Marina Dimitriou, Undergraduate Student, University of Patras, Department of Pharmacy, marinadimitriou00@gmail.com

Second Author – Agisilaos Kounelis, Undergraduate Student, University of Patras, Department of Computer Engineering and Informatics, kounelisagis@gmail.com

Third Author – Christoforos Kasimatis, Masters Student, University of Piraeus, Department of Informatics, xristoforoskasimatis@gmail.com

Correspondence Author – George P. Patrinos, Professor, University of Patras, Department of Pharmacy, gpatrinos@upatras.gr

Bst DNA polymerase mutagenesis and fusion with DNA-binding proteins for improved thermostability, processivity, and strand displacement activity

Nicoletta de Maat*, Victor Di Donato*, Avery Mooring*, James Petropoulos*, Kody Klupt*, and John Allingham*

* Queens_Canada, Queen's University

***Abstract* - Loop-mediated isothermal amplification (LAMP) is a highly sensitive and specific method for point of care (POC) DNA amplification (Burbulis et al., 2015). Research has found *Bst* DNA polymerase useful in LAMP testing for various waterborne pathogens (Jin et al., 2021) due to its strand displacement (SD) and reverse transcriptase (RT) activities. However, its lack of 5' 3' exonuclease activity (New England Biolabs) leaves room for error in DNA amplification. As such, we predicted mutations to the thumb domain of *Bst* 2.0 DNA polymerase, a homologue of *Bacillus stearothermophilus* DNA polymerase I large fragment (New England Biolabs), with alternate residues would increase thermostability. Further improvement can be made via fusion with Sac7e, a double-stranded DNA-binding protein (dsDNA-BP) isolated from *Sulfolobus acidocaldarius* for greater SD activity and processivity (Kalichuk et al., 2016). Improved processivity, thermostability, and enzymatic stability of the DNA polymerase used in the LAMP reaction will increase the efficacy of POC DNA amplification. Results indicate alterations made upon *Bst* via mutagenesis and protein fusion produced significant enhancements to the LAMP reaction.**

***Index Terms* -** Bst DNA polymerase, fusion, LAMP, mutagenesis, Sac7e

I. INTRODUCTION

Thermostable DNA polymerases have become more readily available in recent years and play an important part in DNA amplification (Pavlov et al., 2004). Such polymerases are flexible enough to allow alterations to suit their target environment. The first known DNA polymerase was *Taq* polymerase, isolated from *Thermus aquaticus*, but several others have since been discovered (Pavlov et al., 2004). *Bst* DNA polymerase, a homologue of *Bacillus stearothermophilus* DNA polymerase I large fragment (New England Biolabs), is structurally homologous to *Taq* polymerase with the principal difference being *Bst*'s lack of N-terminal domain containing 5' 3' exonuclease activity (Ignatov et al., 2018).

DNA binding protein Sac7e is a mutant of Sso7d (Kalichuk et. al 2016), from the hyperthermophilic archaeon *Sulfolobus solfataricus* (Gera et. al, 2011). Members of this protein family have proven to maintain high thermostability, denaturation resistance, and secondary structure conservation (Gera et al., 2011). Therefore, this experiment fused Sac7e to a modified version of *Bst* polymerase with a $(GGGGS)_4$ linker for use in LAMP reactions of waterborne pathogens: *E. coli*, *Salmonella*, *Shigella*, and *Campylobacter*. LAMP for bacterial cell testing is well understood (Ahmad et al., 2017), however methods for improving the function of its primary polymerase are limited in previous research. Advances to the LAMP reaction make room for applications in on-site diagnostic testing in environmental, biochemical, and biotechnological fields. Through our research we aimed to answer if a re-engineered *Bst* DNA polymerase could enhance the overall reaction. We predicted greater thermostability in *Bst* through three single-point mutations at K549W, K582L, and Q584L and improved enzyme processivity with the addition of a dsDNA-BP such as Sac7e.

II. MATERIALS AND METHODS

Materials

WarmStart® LAMP Kit (DNA & RNA) including Bst 2.0 DNA polymerase was purchased from New England Biolabs to perform all LAMP reactions. Heat killed samples of waterborne pathogens *E. coli*, *Salmonella*, *Shigella*, and *Campylobacter* were used for all PCR and LAMP reactions. Fully modified *Bst* DNA polymerase and six LAMP primers designed to target conserved 16s rRNA regions between all listed pathogens from Integrated DNA Technologies (IDT) were used in LAMP amplification. Hydroxy naphthol blue (HNB) from New England Biolabs was added to LAMP trials for colorimetric detection and SpectraMax® QuickDrop™ Micro-Volume Spectrophotometer for DNA quantification.

PCR of E. coli K-12 genomic DNA

Genomic DNA was extracted from non-pathogenic *E. coli* K-12 and amplified using PCR, according to the standard amplification cycles shown in Table 1. Specific primers designed for the 16s rRNA region of *E. coli* K-12 were used for PCR. Products of PCR were run on an electrophoretic gel containing 0.5 g agarose, 50 mL TBE buffer solution, and 2.5 µL Red Safe DNA stain.

TABLE 1: PCR amplification cycles for E. coli genomic DNA

Cycle Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	98	5 min	1
Denaturation	98	10 sec	30
Annealing	63	30 sec	30
Extension	72	2 min	30
Final Extension	72	5 min	1

Termination	4	*Indefinite*	1

LAMP amplification using standard Bst DNA polymerase

Primers were prepared using New England Biolabs' WarmSmart® LAMP Kit 10X Primer Mix protocol, and the reaction setup was according to WarmSmart® LAMP Kit Reaction Setup (New England Biolabs), using HNB as the detection component and standard *Bst* DNA polymerase from the kit as the strand-displacing enzyme. LAMP was carried out at 65°C for 40 minutes using the primers displayed in Table 2.

TABLE 2: Optimized LAMP primer sequences

Primer	DNA Sequence
F3	TCTCGTAGAGGGGGGTAGA
B3	CGTTAGCTCCGGAAGCCA
FIP	TCAGTCTTCGTCCAGGGGGCCAGGTGTAGC GGTGAAATGC
BIP	CTCAGGTGCGAAAGCGTGGGACCTCCAAGT CGACATCGTT
LF	ACCGGTATTCCTCCAGATCTCT
LB	TAGATACCCTGGTAGTCCACGC

LAMP amplification using modified Bst DNA polymerase

Primers, reaction setup, and detection component were prepared and used as described above. Upon expression of fully modified *Bst* DNA polymerase, including point mutations at K549W, K582L, and Q584L and fusion of Sac7e through a $(GGGGS)_4$ linker, LAMP trials will be carried out using the modified *Bst* as the strand-displacing enzyme. The reaction will be carried out at 68°C for 40 minutes.

III. RESULTS AND FINDINGS

To test the efficacy of the forward and reverse primers, and provide a control to which we can compare all trials of LAMP, standard PCR was carried out for genomic DNA of *E. coli* K-12. Samples were amplified on an annealing temperature gradient from 58°C - 66°C. PCR products were run on an agarose gel for 40 minutes against a DNA ladder to quantify the products' molecular weight, confirming successful template amplification.

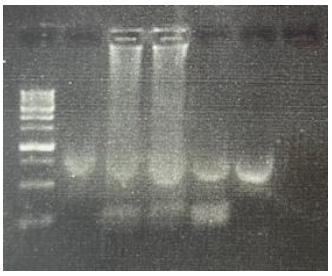


FIGURE 1.
Products of PCR for genomic DNA of E. coli K-12 displayed on agarose gel against DNA ladder. Samples 1 - 5 heated on a temperature gradient at 58°C, 60°C, 62°C, 64°C, and 66°C and amplified DNA shown at 175 bp.

All six LAMP primers were assessed in the first phase of LAMP reactions, including a trial on *E. coli*. Success of this reaction proved LAMP to be a superior method to PCR for waterborne pathogen on-site testing and served as a base to evaluate the effectiveness of modified *Bst* polymerase in future stages.

FIGURE 2.
LAMP for E. coli using standard Bst DNA polymerase. Negative control displayed on the left and positive sample of E. coli K-12 on the right. HNB used as the detection component and positive sample showed a colour change from violet to navy blue.

Quantifiable improvements made upon Bst are outlined in Table 3. YASARA protein modelling software (Version 22.8.22, 2022) with the FoldX extension were used to calculate Gibbs free energy (ΔΔG) of residues K549W, K582L, and Q584L before and after mutations. Greater thermostability will be further confirmed in the second phase of LAMP reactions using our modified *Bst* polymerase in the place of standard *Bst* in the first phase.

TABLE 3: In silico proof of thermostability improvement with K549W, K582L, and Q584L mutations

Residue	ΔΔG Prior to Mutation (kcal/mol)	ΔΔG Post Mutation (kcal/mol)
K549W	0	-0.126
K582L	0	-1.630
Q584L	0	-2.039
Overall Structure	**-150.13**	**-151.81**

DNA concentration of amplified *E. coli* using the standard polymerase was measured at 3084.0 ng/µL. Specificity of testing for bacterial cells still needs to be reduced by several orders of magnitude, but we aim to improve the reaction by at least one order of magnitude with our research. Biohazard precautions limited our testing of original versus modified *Bst* for *Salmonella*, *Shigella*, and *Campylobacter* samples.

IV. DISCUSSION

PCR for DNA amplification

PCR is well-developed and has been the most broadly used method for DNA amplification (Kai et al., 2000). This process involves repeated cycles of heating at varying temperatures to denature, anneal, and extend the template DNA for amplification (Liu et al., 2019). Two key components of the reaction include a set of forward and reverse primers for the addition of nucleotides, and *Taq* DNA polymerase to synthesize an amplified DNA strand (National Library of Medicine). PCR was used in the preliminary stages of this research to evaluate the annealing temperature of the primers and feasibility of amplifying *E. coli*, one of the four major pathogens being studied. The forward and reverse primers used in PCR of *E. coli* targeted a segment of 16s rRNA 175 bp long which matches the molecular weight observed by running PCR

products on an agarose gel against a DNA ladder (Figure 1). This confirms successful amplification of *E. coli* K-12 genomic DNA, and therefore effective PCR primers.

Limitations of PCR

While PCR can be advantageous due to its high sensitivity, a plateau of amplified product can be observed as pyrophosphate accumulates through each amplification cycle (National Library of Medicine). PCR is further limited as it takes several hours and cannot be carried out in a POC setting (Liu et al., 2019) as it requires an expensive thermocycler. PCR was helpful for proof-of-concept in this research but cannot always be applied practically in the field.

LAMP for DNA amplification

Conversely, a LAMP assay can accommodate for limitations in PCR, as it can be used in POC settings because the reaction can be carried out isothermally. LAMP involves six primers (Table 2): forward inner primer (FIP), backward inner primer (BIP), F3, B3, loop forward (LF), and loop backward (LB) for auto-cycling strand displacing DNA synthesis (Notomi et al., 2000). The first round of LAMP reactions (Figure 2) confirmed effective primers and provided a base concentration of 3084.0 ng/µL of amplified DNA using the WarmSmart® LAMP Kit including *Bst* 2.0 DNA polymerase (New England Biolabs) on *E. coli*.

The upcoming rounds of LAMP reactions on the same pathogenic strains as trial 1 are predicted to increase amplified DNA concentration by at least one order of magnitude using our fully modified *Bst* DNA polymerase. Such developments of DNA synthesis will suggest greater processivity via fusion with Sac7e and thermostability via K549W, K582L, and Q584L mutations to *Bst*. Our research shows potential to further improve the thermostability of *Bst* DNA polymerase via point mutations, as well as processivity and SD activity through DNA-BP fusion.

Mutagenesis of Bst Polymerase Using YASARA

In silico experimentation used YASARA (Version 22.8.22, 2022) protein modelling software to predict improvement of *Bst*'s thermostability before and after mutations were made (Table 3). Gibb's free energy indicated significant improvement from 0 to -0.126 kcal/mol, -1.63 kcal/mol, and -2.039 kcal/mol at K549W, K582L, and Q584L, respectively (Table 3). These specific residues were selected based on their positional similarity to residues in *Taq* polymerase that proved to increase thermostability in a 2009 patent study (Xi, 2009). We compared *Bst*'s structure to *Taq* and found high similarity, thus leading to the prediction that equivalent residues may exist in our protein of interest. From this, we found K549, K582, and Q584 located in *Bst*'s thumb domain, which further supported our hypothesis, as the thumb domain is understood to bend around and directly contact the template DNA (Ma et al., 2016). Overall, our chosen mutations led to an overall increase in stability of the enzyme from -150.13 kcal/mol before mutations to -151.81 kcal/mol (Table 3) and -152.05 kcal/mol with the fusion of Sac7e.

Detection of pathogens in LAMP reactions

HNB was used as the detection component in both phases of LAMP trials. HNB is a colorimetric indicator for alkaline earth metals (Goto et al., 2009). Addition of this reagent did not influence DNA amplification concentrations (Goto et al., 2009). Pyrophosphate ions (PPi) were released as a by-product of successful LAMP reactions that reacted with magnesium ions (Mg^{2+}) to form insoluble magnesium pyrophosphate (Goto et al., 2009). This reaction caused a depletion of free Mg^{2+} that could be detected by HNB to cause a colour change from violet to blue (Goto et al., 2009) as seen in Figure 2. This suggests a positive test for at least one of the listed pathogens.

V. CONCLUSION

The function of *Bst* DNA polymerase in DNA amplification is enhanced through mutations in its thumb domain and fusion with Sac7e to its N-terminus through a $(GGGGS)_4$ linker. We observed an improvement of thermostability from -150.13 kcal/mol to -152.05 kcal/mol and hope to see increase in amplified DNA as a result of K549W, K582L, and Q584L mutations and augmented SD activity via Sac7e. Our study was limited by potential biohazards, so future research may focus on validation of primers for *Salmonella*, *Shigella*, *Campylobacter*, and other waterborne bacteria. However, similar polymerase modifications allow for greater variety of LAMP applications in POC diagnostic settings (Burbulis et al., 2015). For example, modified *Bst* was designed with the intention of enhancing the LAMP reaction in a drinking water test and filter device for Indigenous communities in Canada. Greater thermostability allows for oscillation in temperature of LAMP to account for fluctuation seen in POC heaters, and better processivity or strand displacement activity leads to more accurate positive LAMP test results. Future research may explore other possible alterations that can be made in key enzymes like *Bst* to make them more suited for their target environment.

ACKNOWLEDGEMENTS

We thank Dr. Stephen Brown for his assistance and feedback throughout the experiment. We are also grateful to Dr. Bas Vriens, Dr. Thamali Kariyawasam, and Dr. Geoffrey Hall for their support in our research.

REFERENCES

Ahmad, F., Stedtfeld, R. D., Waseem, H., Williams, M. R., Cupples, A. M., Tiedje, J. M., & Hashsham, S. A. (2017). Most probable number - loop mediated isothermal amplification (MPN-LAMP) for quantifying waterborne pathogens in < 25 min. *Journal of Microbiological Methods, 132*, 27-33. https://doi.org/10.1016/j.mimet.2016.11.010

Burbulis, I. E., Yamaguchi, K., Nikolskaia, O. V., Prigge, S. T., Magez, S., Bisser, S., Reller, M. E., & Grab, D. J. (2015). Detection of pathogen-specific antibodies by loop-mediated isothermal amplification. *Clinical and Vaccine Immunology*, *22*(4), 374-380. https://doi.org/10.1128/cvi.00811-14

Gera, N., Hussain, M., Wright, R. C., & Roa, B. M. (2011). Highly Stable Binding Proteins Derived from the Hyperthermophilic Sso7d Scaffold. *Journal of Molecular Biology*, *409*(4), 601-616. https://doi.org/10.1016/j.jmb.2011.04.020

Goto, M., Honda, E., Ogura, A., Nomoto, A., & Hanaki, K. I. (2009). Colorimetric detection of loop- mediated isothermal amplification reaction by using hydroxy naphthol blue. *BioTechniques*, *46*(3), 167-172. https://doi.org/10.2144/000113072

Ignatov, K. B., Barsova, E. V., Fradkov, A. F., Blagodatskikh, K. A.,

Kramarova, T. V., & Kramarov, V. M. (2018). A strong strand

displacement activity of thermostable DNA polymerase markedly improves the results of DNA amplification. *BioTechniques*, *57*(2), 81-87. https://doi.org/10.2144/000114198

Jin, J., Duan, L., Fu, J., Chai, F., Zhou, Q., Wang, Y., Shao, X., Wang, L., Yan, M., Su, X., Zhang, Y., Pan, J., & Chen, J. (2021). A real-time lamp-based dual-sample microfluidic chip for rapid and simultaneous detection of multiple waterborne pathogenic bacteria from coastal waters. *Analytical Methods*, *13*(24), 2710-2721. https://doi.org/10.1039/d1ay00492a

Kai, E., Ikebukuro, K., Hoshina, S., Watanabe, H., & Karube, I. (2000). Detection of PCR products of *Escherichia coli* O157:H7 in human stool samples using surface plasmon resonance (SPR). *FEMS Immunology & Medical Microbiology*, *29*(4), 283-288. https://doi.org/10.1111/j.1574-695X.2000.tb01535.x

Kalichuck, V., Béhar, G., Renodon-Cornière, A., Davonski, G., Obal, G., Barbet, J., Mouratoi, B., & Pecorari, F. (2016). The archaeal "7 kDa DNA-binding" proteins: Extended characterization of an old gifted family. *Scientific Reports*, *6*(1), 1-10. https://doi.org/10.1038/srep37274

Liu, H. Y., Hopping, G. C., Vaidyanathan, U., Ronquillo, Y. C., Hoopes, P. C., & Moshirfar, M. (2019). Polymerase Chain Reaction and Its Application in the Diagnosis of Infectious Keratitis. *Medical Hypothesis, Discovery & Innovation Ophthalmology Journal*, *8*(3), 152-155.

Ma, Y., Zhang, B., Wang, M., Ou, Y., Wang, J., & Li, S. (2016). Enhancement of Polymerase Activity of the Large Fragment in DNA Polymerase I from *Geobacillus stearothermophilus* by Site-Directed Mutagenesis at the Active Site. *BioMed Research International*. https://doi.org/10.1155/2016/2906484

National Library of Medicine (n.d.). *Polymerase Chain Reaction (PCR)*. National Center for Biotechnology Information. Retrieved July 9, 2022, from https://www.ncbi.nlm.nih.gov/probe/docs/techpcr/

New England Biolabs. (n.d.). *Bst 2.0 DNA polymerase*. NEB. Retrieved June 9, 2022, from https://www.neb.com/products/m0537-bst-20-dna- polymerase

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, *28*(12), e63. https://doi.org/10.1093/nar/28.12.e63

Pavlov, A. R., Pavlova, N. V., Kozyavkin, S. A., & Slesarev, A. I. (2004). Recent developments in the optimization of thermostable DNA polymerases for efficient applications. *Trends in Biotechnology*, *22*(5), 253. https://doi.org/10.1016/j.tibtech.2004.02.011

Xi, L. (2009). Mutated and chemically modified thermally stable DNA polymerases (World Intellectual Property Organization, International Publication Number WO 2009/155464 A2). Patent Cooperation Treaty (PCT). https://patents.google.com/patent/WO2009155464A2/en#citedBy

YASARA [Computer software]. (2022). Retrieved from http://www.yasara.org/viewdl.htm

AUTHORS

First/ Correspondence Author - Nicoletta de Maat, Queen's University, 19nmdm@queensu.ca, 647-274-4564.

Second Author - Victor Di Donato, Queen's University, 19vzdd@queensu.ca.

Third Author - Avery Mooring, Queen's University, avery.mooring@queensu.ca.

Fourth Author - James Petropoulos, Queen's University, 19jwp7@queensu.ca.

Assisstant Authors - Kody Klupt (BScH), kody.klupt@queensu.ca and John Allingham (PhD), allinghj@queensu.ca.

Reviews

***Bst* DNA polymerase mutagenesis and fusion with DNA-binding proteins for improved thermostability, processivity, and strand displacement activity.**

by Nicoletta de Maat, Victor Di Donato, Avery Mooring, James Petropoulos, Kody Klupt, and JohnAllingham. (Queens_Canada, Queen's University)

"This is an important enzyme for a variety of amplification applications including LAMP. It is unclear why the abstract mentions 5' to 3' exonuclease even though the rest of the paper seems to not cover anything on this. Also, the phrase "leaves room for error in DNA amplification" usually refers to 3' to 5' exo, so this should be clarified too.
"We predicted greater thermostability in Bst through three single-point mutations at K549W, K582L, and Q584L". How many other amino acid substitutions were considered to arrive at these three specific substitutions? Please give more details on what process was used in the prediction (exactly how was Taq polymerase information used and prioritization of options from that?). How was YASARA chosen (Please give a peer-reviewed reference, e.g. onlinelibrary.wiley.com/doi/10.1002/prot.10104 and list at least one alternative method). In figure 1 (the key test) "amplified DNA shown at 175 bp." but where is 175bp on this gel (what ladder was used)? What are the other bands (and their significance)? Isn't the LAMP reaction quantitated by absorbance at 650 nm? Perhaps you could clarify that especially since the color change is a bit subtle in figure 2. "primers designed for the 16s rRNA region of E. coli": Are these listed explicitly? If so, make that clearer. If not, then include these explicitly. "Specificity of testing for bacterial cells still needs to be reduced by several orders of magnitude, but we aim to improve the reaction by at least one order of magnitude with our research". Why "reduced" rather than increased? How many is "several" and how did you arrive at those goals of "several orders" and "one order"?"

Dr. George Church

"de Maat et al. studied a thermostable DNA polymerase with the goal of finding variants with improved activity and stability for use in point-of-care DNA amplification reactions. This concise paper has a clear focus and reasonable goals. It is well drafted with good structure and attention to detail. This project has potential to spin off many more experimental results and the authors are in a good position to try new techniques to gain more insight into protein stability and activity. I have a few constructive comments below that I hope are helpful.
In the introduction, it is unclear what is the rationale or precedent for the specific manipulations planned—any prior work should be discussed and cited. Materials and methods are very brief and should be expanded to ensure reproducibility; specifically, how were samples heat killed? where did the bacterial samples come from? method for genomic DNA isolation should be noted; it would be useful to explain how the LAMP primers were chosen and to use meaningful names.
In Fig. 1, the ladder and bands need to be labeled and there need to be controls for authentic product and either inactivated polymerase or no polymerase.
Fig 2 needs to be discussed in the results. A better way to display the color change (it seems to be an intensity change more than hue) would be to measure spectra before and after amplification. If this assay is intended to produce binary results then it would be useful to run a DNA dilution series to establish a limit of detection.
For Table 3, as noted above, it is important to explain why these residues and specific mutations were chosen. If there is a molecular structure, a figure showing these residues might help explain to readers how these mutations would influence enzyme activity. A word of caution though: both computation and figure gazing can be misleading and significant work is necessary to establish true effects of mutations.
Table 3 caption should not say "proof"—these values are outputs of a calculation (a methods section should be provided for these results) which may have overwhelming systematic or random errors. It

may be useful for hypothesis generation, but does not prove the mutant proteins are more or less stable. Experimental validation of protein thermostability by circular dichroism or a similar technique would provide further support. The authors should also comment on why thermostability is the appropriate goal for this project. Many enzymes exhibit unpredictable changes in temperature dependency and increasing thermostability might paradoxically result in a less active polymerase."

Dr. Michael Funk

"de Maat et al. set out to improve LAMP assays for waterborne pathogens by modifying the polymerase used for this process, fusing the enzymes from two different microorganisms and making targeted mutations to improve their stability and processivity. The authors used protein modeling software to predict the effects of specific targeted mutations, then prepared to analyze their modified enzyme using LAMP with primers tested by PCR, though they only tested the standard enzyme so far. The use of multiple independent approaches and the validation of in silico findings using lab experiments are strengths of this work, and improving these assays is important for public health. This work is still in its early stages, however. For one thing, it would be important to compare the effectiveness of different versions of the polymerase, not just in silico but experimentally. In the ideal world, it would be good to do stepwise comparisons on different versions of the enzyme (with and without the enzyme fusion and each mutation). In the real world, that might be cost-prohibitive, but at minimum it would be important to show that the mutant version is better than the current standard and that the enzyme fusion is beneficial. It will also be critical to show that the approach works on clinically-relevant bacterial samples, as the authors plan to do, preferably starting not only in water or buffer but in biological fluids, i.e. testing it the way that one would analyze a sample obtained from a patient."

Dr. Yevgeniya Nusinovich

Redefining lung cancer diagnosis with the use of DNA nanostructures.

by Bochalis Eleftherios, Konidi Garifalia, Dereki Irene. (Patras Medicine, University of Patras.)

"Overall, this seems like a promising method with some experiments clearly described. It could be clearer whether this is limited to lung cancer and/or limited to circRNAs. If more broadly applicable, that topic should be explored. Also, what are the closest previous methods and their relative pros and cons. The term "molecular beacons" is used as a key point in the abstract, but never again in the main text or figures. The quencher is mentioned but not directly connected to the term "beacon". The key role of circRNAs is not clearly connected to the steps in Figure one (or even mentioned in it). There is no "Figure 1A". Figure 1 is barely explained (e.g. steps i, ii, ii). The authors might consider this paper as well: Dionis Minev, Christopher M. Wintersinger, Anastasia Ershova, William M. Shih (2021) Robust nucleation control via crisscross polymerization of highly coordinated DNA slats. Nat Commun. 2021; 12: 1741. PMCID: PMC7979912"

Dr. George Church

"Eleftherios et al. develop a preliminary technique for detection of circular RNA with the ultimate goal of use as a diagnostic for lung cancer in liquid biopsies. This paper has been carefully prepared and is clear and compelling in methods, results, and discussion. Although this work is still at a very early stage, the trajectory is clear, and the background helps explain the need and prior work leading up to this point. Whether this approach can be successful for circular RNA detection in real or simulated biopsy samples is an exciting question the authors are poised to answer in future work. There are just a few points I could make that might help presentation.

Fig 2 should ideally include a ladder for both gels. Fig 3, for "negative control", it might be more appropriate to specify the precise nature of the control (buffer) as in methods. A better control for these reactions might be target missing the primer sites. There is so far no work on specificity or tolerance to interference by non-target RNAs, which is an obvious direction for future work. The methods are generally clear and adequate, but additional detail for the protocols and equipment/kits would be appreciated to ensure reproducibility."

Dr. Michael Funk

"Eleftherios *et al*. designed a liquid biopsy method for lung cancer screening based on the detection of circular RNA in the blood. It's not clear how this approach would compare to other liquid biopsy methods that are gradually coming into clinical use, such as circulating tumor DNA detection, but as the authors make clear, circulating RNA has its benefits such as its stability as well as direct biological relevance. Another strength of the approach is pairing the detection of particular circular RNA (recognized by its back-splice junction site, which should be specific to this form of RNA) with emission of a fluorescent signal to facilitate convenient and accurate detection. Thus far, the authors have not been able to detect fully formed circular RNA, only a back-splice junction site associated with non-small cell lung cancer presented on linear RNA molecules. This raises the concern that there is something about the methodology that may not be compatible with detection of the circular RNA structure. Assuming that can be addressed, though, the approach sounds logical and potentially useful in the clinic if it proves to be at least as good as the analysis of circulating tumor DNA and hopefully also applicable to more than the one cancer type mentioned here."

Dr. Yevgeniya Nusinovich

Review on Phage Therapy.

by Ruchir Sahni (IISER-Pune-India, IISER Pune)

“Overall this is an exciting and well-researched topic, very clearly organized and described. A few small potential improvements below. "... ease in discovery of phages with very low cost ... in contrast to antibiotics, whose cost estimate for development at US$1.5 billion" This seems to be an unfair comparison of "discovery" vs "development". The latter typically includes human clinical trials for FDA approval. (but no phage therapy has achieved the efficacy of antibiotics (see: bacteriophage.news/fda-phage-therapy-for-covid19-patients -- highlighting Adaptive Phage Therapeutics (APT) a clinical-stage company) In the final paragraph, a few other examples on phage "harmful genes" would help, for example, botulism toxin, diphtheria toxin, cholera toxin, and Shiga toxin. Antibiotic resistance of bacteria is mentioned 10 times, but phage resistance is not mentioned once (nor is restriction enzymes, CRISPR, etc.) Also the prospects for engineering phages (which seems especially relevant to iGEM) is not mentioned (See for example, Sniprbiome.com/technology. The section on endotoxin release is a good start, but do antibiotics produce exactly the same release (given the same bacteria load)? and if not, why not? The conclusion is that "the regulatory framework remains the largest hurdle" , but it is not clear that this step has been (or should be) much different from regulation of antibiotics. Perhaps the biggest hurdles (and opportunities) have to do with engineering phage to thwart bacterial resistance and mammalian immunity.”

Dr. George Church

“Sahni reviews developments in phage therapy as an approach to treating bacterial infections. This article provides a competent summary of history and recent work in this area and is written clearly and with appropriate structure. Although I am open and sympathetic to the author's perspective, there are some issues that detract from the argument. An early, unreferenced statement is that phage history has been ignored in "the West" until recently. There is no evidence or discussion provided here either as to the lack of useful research in the mid-late 1900s or the geographic isolation, but it seems to follow many similar assertions in the literature. A critical examination of this paradigm might provide a more compelling introduction and rationale for the review. The summary of work in animals is competent and compelling, but there are only single paragraphs without specific references for case studies and clinical trials in humans, which leaves the reader unsure as to the efficacy of the treatments; only absence of adverse effects is noted in these sections. It could be the case that all of these trials were inconclusive or negative. The discussion of regulation is nuanced and interesting, although parts of this work would be better placed above when discussing trials. Ultimately, this article makes a compelling case that phage therapy is an active area of research currently but that more resources, attention, and positive results in state-of-the-art clinical trials are necessary to understand if/where it can be an effective part of antimicrobial therapy. The paper at times seems to pit phage therapy and chemical antimicrobial agents (and their proponents) against each other, which seems unnecessary and distracting. Despite these issues, the paper does provide a useful starting place for readers interested in the history and current state of research.”

Dr. Michael Funk

“This article by Ruchir Sahni is a review of the background knowledge and recent advances in the field of phage therapy, which is becoming increasingly important as antibiotic-resistant bacteria become more of a public health threat. The author thoughtfully sets the scene and touches on a lot of key topics with regard to both the underlying biology and the practical barriers interfering with the implementation of phage therapy. The background information and the overview sections are easy to follow and organized, but when it gets to the present situation and the challenges and opportunities, the article begins to feel more fragmented, with many short subsections. There are different possible ways to

address this, but what I might suggest is to group them by biology, so for example have a section talking about phages for respiratory infections all the way from animals to humans, including biological findings as well as benefits and drawbacks, another similar one for GI infections, etc., or whatever groupings make sense based on the types of infection and the advantages and disadvantages the phages present in a particular disease type. I would then reserve the last section for anything that may apply across different types of phage therapy, including the regulatory hurdles and lessons from other countries."

Dr. Yevgeniya Nusinovich

Afterword

We are now at the end of the third edition of the MSP-Vector, therefore we would like to conclude by extending our gratitude to everyone who allowed us to keep this tradition alive.

To the teams, we thank you for the dedication that you put into this project. For many teams, this was the first time participating in the MSP-Vector. We understand that writing an article adds a serious amount of work to the already extensive workload of running an iGEM project. Thank you for sticking with us throughout these last months, and for keeping up with all the deadlines. Your cooperation was essential in the making of the MSP-Vector. In the context of iGEM, teamwork and collaboration are essential skills. This is especially the case in a project like this, which groups parties from all over the world. These skills were evident not only in each team in the making of the article, but in the willingness you showed to work alongside other groups as well. A final thank you to all of you and congratulations on your work.

To our reviewers, Dr. George Church, Dr. Michael Funk and Dr. Yevgeniya Nusinovich. Your expertise added great value to the learning experience of the teams participating. Furthermore, your participation increased the journal's reputation, and will surely help future MSP iGEM teams to build a project bigger and better than this. We would like to thank you for accepting to help in this project, and hope that we will have the opportunity to work with you again.

Our personal thanks go to Dr. Roy Erkens and Dr. Kyle Jazwa, who were fundamental in improving our peer review process.

To Tim Davalan and Lotte Deneer, thank you for your mentoring and guidance, and for laying the blueprint for us.

Finally, to Dr. Erik Steen Redeker, thank you for bringing iGEM to Maastricht University and giving us the opportunity to embark on this journey.