iGEM Team Heidelberg 2022

NOSE-TO-BRAIN DELIVERED **ANTIVIRAL** RNAS AGAINST NEUROINFECTIONS

picture: igem.org

CONTENTS

What is iGEM?

International Genetically Engineered Machine Competition

The iGEM organization is the world's biggest competition in synthetic biology. Here, students from all over the world can engage in research trying to solve real and pressing problems of society at large and push the boundaries of our understanding of synthetic biology, with diverse projects ranging from fields like environment protection to disease therapeutics.

Every year, over three hundred teams worldwide work in dry and wetlabs on a project idea they have designed themselves. Later, students gather to present their work to some of the greatest living minds in the life sciences and fellow students. While competing for the grand prize of iGEM - the BioBrick trophy, young biotechnology enthusiasts from all around the world get to blend with each other.

The iGEM organization is more than just a competition, it is a platform where young scientists learn crucial aspects of research early on their careers and the beneficial uses of synthetic biology is shown to the society.

PIs

PROF. DR.
STEFAN WÖLFL

PROF. DR.
GERT FRICKER

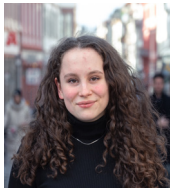
MEMBERS

ANASTASIYA
VLADIMIROVA

ANOUK
DUPE

BIANCA
GREUL

BINNUR
ÖZAY



CLARISSA
SCHÄFER

DARIUS
SZABLOWSKI

FRANZISKA
LAM

HEINRIKE
GILLES

JOHANN SORA
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LAURA
EBERT

LINDA
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MICHEL
TARNOW

NINA
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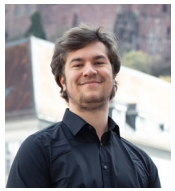
PATRICK
FUCHS



PAUL
BRUNNER

SEBASTIAN
RICKERT

Team Heidelberg

We are a young group of Master's and Bachelor's students at the University of Heidelberg from different areas of study such as biochemistry, molecular biotechnology, pharmacy, and medicine. Due to our broad heterogeneity in education and expertise as well as our combined passion for synthetic biology, we were able to assemble into a strong, interdisciplinary team of eighteen talented and motivated students eager to contribute to the rapidly developing world of synthetic and computational biology.

As an independent research project, besides our scientific tasks in the wetlab and drylab, we also work in various areas of responsibility like human practices, finances, safety, design, wiki and social media. Most students engage in multiple tasks in different areas. The team and its members are depicted on the left with their respective areas of responsibility.

Our advisors, students from former iGEM teams of Heidelberg, who successfully participated in last years' competition winning a gold medal, continue to contribute to iGEM by constructively critiquing our work and guiding the team throughout our iGEM journey.

The heads of our project are Prof. Dr. Stefan Wölfl and Prof. Dr. Gert Fricker, who support us with the implementation of our project and are always on hand with advice and encouragement.

ADVISORS

DUC THIEN
BUI

ELIZAVETA
BOBKOVA

JONATHAN
HERTH

LAURA
SANCHIS PLA

MARILENA
WITTMAACK

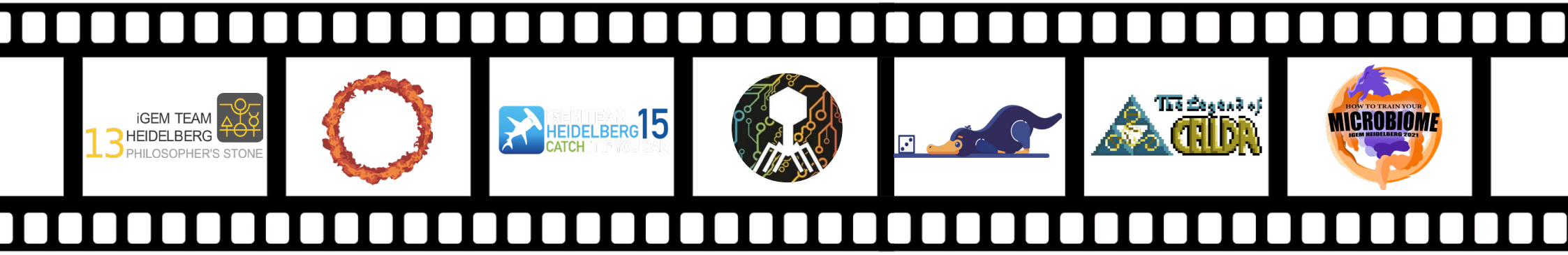
MICHELLE
EMMERT

Background

Students from the University of Heidelberg have established an iGEM tradition and have contributed to many successful projects in the competition in the last decade. Among numerous side prizes, the Heidelberg iGEM team has won the grand prize in the years 2013 & 2014; and in 2015 & 2017, the teams were able to reach the third place in the contest. With diverse projects like “The Ring of Fire”, a project about circular proteins, or “The Phage and the Furious”, a project around phage assisted evolution, Heidelberg has earned a reputation for innovative projects especially in the foundational advance sector. Last year, on top of winning a gold medal for their project on cloning with natural transformation, the Heidelberg team was also nominated for best therapeutic project, best software tool, and best new composite part.

A successful past which we strive to continue once more this year. We are the iGEM Team Heidelberg 2022; eighteen students from the University of Heidelberg with a shared passion for synthetic biology and a common ambition to participate in and win the iGEM 2022 competition. Consisting of members with diverse academic backgrounds, and students from former iGEM Heidelberg teams to advise us, our team is well equipped to navigate through the highly interdisciplinary landscape of modern biology all the way to the top of the grand prize mountain.

Our research group is completed by veterans of synthetic biology: our advisors Prof. Dr. Wölfl and Prof. Dr. Fricker, reliable partners who can help us to find new paths if we get stuck in dead ends. Together we make up a strong team that is well prepared for the fierce challenges of iGEM.



2013 PHILOSOPHER’S STONE

2014 THE RING OF FIRE

2015 CATCH IT IF YOU CAN

2017 THE PHAGE AND THE FURIOUS

2019 FANTASTIC YEASTS AND HOW TO EVOLVE THEM

2020 THE LEGEND OF CELLDA

2021 HOW TO TRAIN YOUR MICROBIOME

Project Description

Overcoming the blood brain barrier by delivering siRNAs packed in lipid nanoparticles through the nasal route to treat viral neuroinfections, in this case the herpes simplex virus.

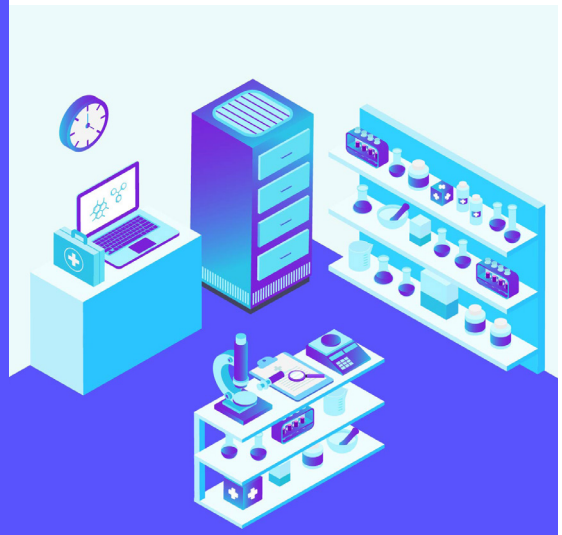
The properties of the blood brain barrier enable it to protect the central nervous system from pathogens, but also restricts drug delivery and therapeutic efficacy, thereby causing hardships in drug development for neurodegenerative diseases (Ding *et al.*, 2020). This is why research on how to overcome these difficulties in drug delivery to the brain is of great importance. Due to the nose's direct connection to the brain via the olfactory nerve, the so-called 'nose to brain' delivery route bypassing the blood brain barrier is of great interest in the scientific world (Hanson *et al.*, 2008). A higher drug availability in the brain, a reduced degradation through the presence of the drug in the olfactory bulb, and the prevention of the unnecessary dispersion of drugs through systematic clearances are the most important advantages of using intranasal delivery methods. However, mucociliary clearance and poor drug permeation through nasal mucosa present limitations. Novel delivery methods, such as nano drug carrier systems, are adapted to overcome these limitations (Agrawal *et al.*, 2018). Between these, lipid nanoparticles (LNPs) are most suitable as a delivery platform for RNA therapeutics (Shepherd *et al.*, 2021).

Our goal is to design a highly efficient LNP-based system that delivers different siRNA into the brain via the nose-to-brain route, in order to neutralize potential neurological threats. This way, the blood brain barrier is circumvented, and with siRNA production and subsequent transportation remaining the same, numerous neurological diseases could be targeted with this treatment.

Being a highly prevalent virus with a potential of causing significant harm to the human brain, we decided on the herpes simplex virus as the neurological disease we want to focus on in our project.

Herpes viruses are double-stranded DNA viruses which rapidly spread between humans through close contact to contagious individuals, and infect their new hosts through mucous membranes or damaged skin, causing oral and perioral (HSV-1) or genital (HSV-2) infection and establishing a persisting, lifelong infection (Jiang *et al.*, 2016; Bradshaw *et al.*, 2016). Today, HSV-1 infections are highly prevalent with a seropositivity among older adults estimated to 60-90% worldwide (Bradshaw *et al.*, 2016).

By retrograde transportation through the olfactory nerve (Bradshaw *et al.*, 2016), the virus can furthermore reach the central nervous system, where it triggers an acute inflammatory response causing meningitis and encephalitis (Marcocci *et al.*, 2020). The mortality rate of this disease exceeds 70% without any treatment (Small *et al.*, 2019), and herpes simplex encephalitis is the most common cause of fatal sporadic encephalitis (Small *et al.*, 2019). Furthermore, most survivors do not return to their baseline functions (Small *et al.*, 2019), and markers for HSV-1 reactivation have also been positively correlated with an increased risk of Alzheimer's disease, making HSV a pressing matter of drug development from various perspectives (Marcocci *et al.*, 2020).



The mortality rate can be reduced significantly when treated with Acyclovir (ACV), a nucleoside analogon, which has proven to be effective for prophylaxis and stabilizing herpetic infections (Small *et al.*, 2019). However, a variety of different side effects are very common when applied, and long term administration of this drug, especially in immunocompromised patients, has shown to lead to drug resistance, making the disease even more dangerous (Piret *et al.*, 2011).

Therefore, the development of novel strategies to treat HSV is urgently needed. This is where we come in with our project idea. In our project, we plan to import siRNA into affected cells, where it will be integrated into the cell's own RNA-induced silencing complex (RISC) and eliminate the pathogen after binding to the targeted mRNA and cleaving it (Whitehead *et al.*, 2011). Thereby, the siRNA would facilitate an effective and moreover highly specific treatment of neural infections, while no significant resistance is developed when administered periodically and/or over a long period of time, making it a possible treatment for immunosuppressed patients as well (Manda *et al.*, 2019).

The main focus will be the production, modification and transportation of siRNA into the neurons, pursuing an even distribution, high stability of the siRNA in the LNP, and a great efficacy in targeting the virus.

The production of siRNA will take

place in E. coli using an siRNA expression plasmid. Furthermore, a variety of complex stabilizing systems as well as chemical modifications on the RNA will be tested in order to accomplish the highest possible stability and therefore longevity of the siRNA during transport.

Considering that the herpes simplex virus reaches the brain through a primary infection in the nasopharyngeal region ascending into the olfactory nerve, we decided to focus our project on the nose-to-brain route, being furthermore an efficient, promising way of drug delivery bypassing the blood brain barrier in general (Goldman *et al.*, 2012).

Finding a suitable delivery system for the siRNAs into neurons is a complex, yet essential aspect. Lipid nanoparticles (LNPs) seem to be the optimal candidate for the RNA delivery system and therefore the system we will use, providing beneficial properties such as structural flexibility, low toxicity and biocompatibility (Yonezawa *et al.*, 2020) and therefore being suitable for our plan to use the nose-to-brain route to deliver therapeutics (Rassu *et al.*, 2017). Moreover, we plan to optimize the delivery via LNPs by simulating the effect LNP composition has on vesicle fusion and release capability.

After establishing the successful production and transportation of siRNA, we will shift our focus to developing a benchtop two component testing system. It should be able to produce LNPs on a rapid scale as well as allow testing on a multi-organoid system. We plan to integrate a novel platform which allows both the analysis of LNP quality and quantity, and adequate adjustment of the flow rate for LNP production. The delivery efficacy of potential therapeutics will be judged by detection of an established reporter system.

Lastly, in order to test the efficacy of the nose-to-brain route and to achieve reliable results without resorting to animal testing, we plan to utilize an organoid system comprised of epithelial stem cells as a natural barrier and olfactory neurons stably expressing a viral protein of HSV via transformation, in order to evaluate knockdown efficiency.

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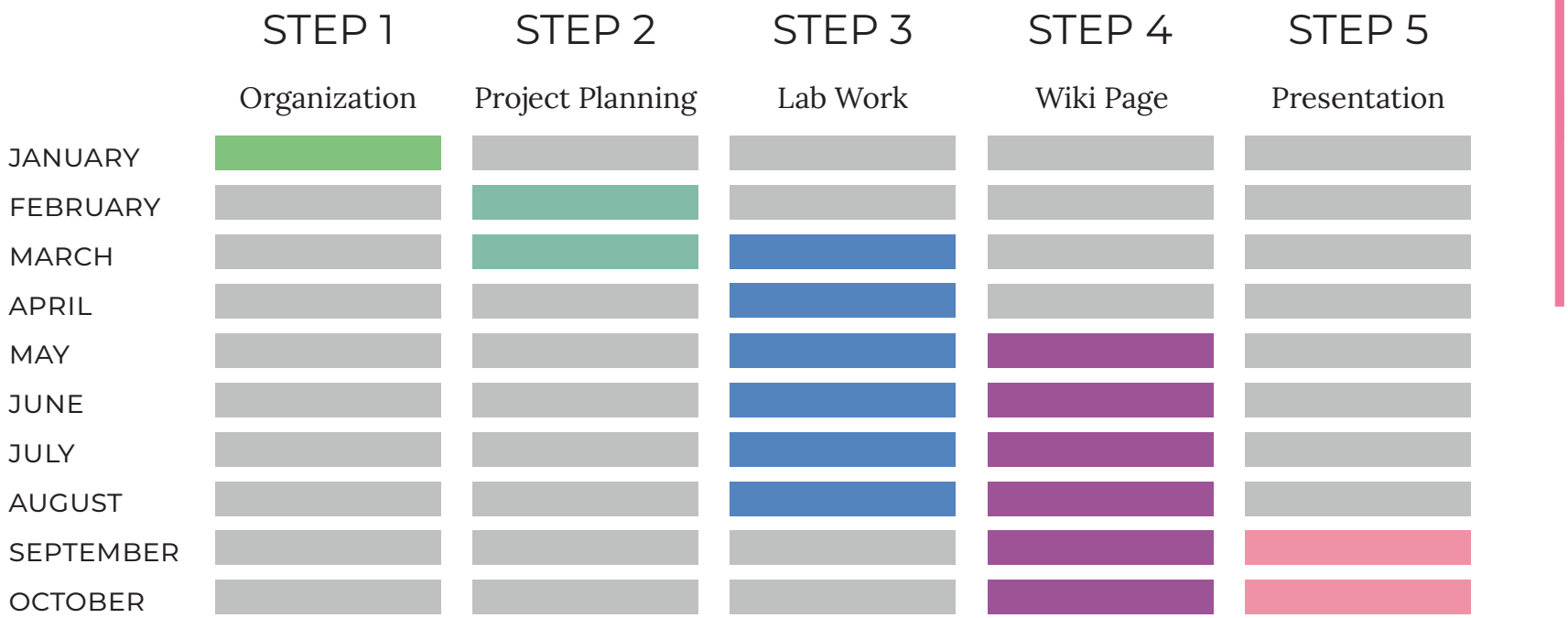
Project **Timeline**



Labwork

In cells, RNA interference leads to gene silencing at the messenger RNA (mRNA) level. If foreign double-stranded RNA is introduced into the cell, it is cleaved into small interfering RNAs (siRNA) by the Dicer enzyme, which then get incorporated into the RNA-induced silencing complex (RISC). This complex unwinds the siRNA duplex and cleaves its sense strand. The activated RISC-siRNA complex binds and degrades the foreign mRNA in the cell complementary to the antisense siRNA strand. It is also possible to introduce synthetic siRNA directly into the cytoplasm, which, in the form of a RISC-siRNA complex, binds to the mRNA of a specific target gene and thus silences it, inhibiting the translation of its encoded proteins. (Robbins *et al.*, 2009; Whitehead *et al.*, 2011)

There are different methods of how to produce siRNA, depending on the goal of the experiment. For our aim, it is most suitable to express stable and highly potent siRNA from an siRNA expression plasmid in *E. coli*. The siRNAs are generated from long dsRNA strands and contain multiple siRNA sequences. The method uses the discovery that p19, a plant viral siRNA-binding protein, stabilizes siRNA-like small RNAs in *E. coli* in selectively binding to it without sequence specificity.

A glutathione S-transferase (GST) fusion protein expressing system is used to express His-tagged p19 from a tac promoter together with hairpin target sequence RNA, which is controlled by a T7 promoter. Both are induced with IPTG. To produce the hairpin plasmid, a two step cloning approach is necessary. At the beginning, a copy of the target DNA is inserted into a set of two restriction sites and then the second copy of the target DNA is inserted in reverse orientation. The siRNA plasmid is transformed into an *E. coli* host strain that expresses T7 RNA polymerase. For purification, the siRNAs are isolated from the bacterial lysate with nickel beads to capture the His-tagged p19. After that, the siRNAs are eluted with 0,5% (wt/vol) SDS and are further cleaned by anion-exchange HPLC. The final siRNA product can then be transfected into mammalian cells and is as efficient as commercially available synthetic siRNAs. (Huang and Lieberman, 2013)

In order to counteract the inherent instability of RNA in physiological conditions, we want to test different stabilizing systems for siRNA packaged in LNPs. One method which has proven successful in vesicle-mediated delivery is the asymmetric linking of a cholesterol molecule to a 3'-end of the siRNA (Haraszti *et al.*, 2018). We also consider testing the addition of an Ago2-protein to the solution (see Fig. 1), meaning the siRNA would be stabilized as a protein-siRNA-complex in the LNP, and its specificity would furthermore be enhanced (Li *et al.*, 2018). Another option would be the introduction of chemically modified RNA nucleotides (such as 2'-O-methyl or 2'-F) to the E.-coli-based siRNA production culture, which would then be incorporated into the synthesized siRNA, enhancing its longevity in physiological conditions (Watts *et al.*, 2008).

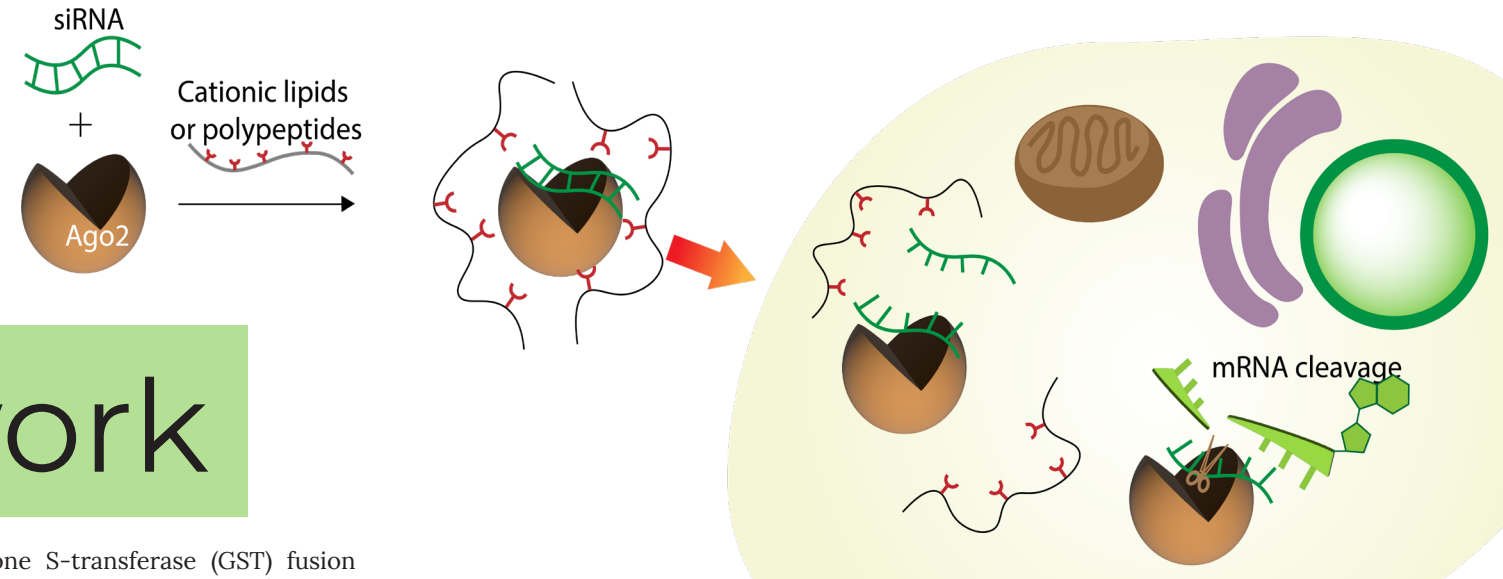


Fig. 1: Schematic overview of the co-delivery of siRNA and Ago2 into cells

The use of nanotechnology for transport via the blood-brain barrier is a promising option for our project. The advantages include biocompatibility, protection of the drug from degradation, extracellular transport (Battaglia *et al.*, 2018), and reduced side effects by limiting the drug distribution to non-targeted areas (Lee and Minko, 2021). Lipid nanoparticles (LNPs) have shown to possess many benefits for nose-to-brain drug delivery in comparison to other available nanoparticles (Rassu *et al.*, 2017). They have an excellent biocompatibility profile, show low toxicity and immunity, and can be easily functionalized and prepared on a large scale (Yonezawa *et al.*, 2020), which is why we chose to use LNPs for packaging and delivering our siRNA. Effective LNP-packaged siRNA drugs such as Patisiran and Givosiran are examples that have already been approved by the European Union (Verband Forschender Arzneimittelhersteller e.V., 2021), which shows that our idea to use siRNA in LNPs as a therapeutic has a great potential of success as well.

A therapeutic lipid nanoparticle for RNAi generally consists of a hydrophobic core homogeneously coated with polyethylene glycol (PEG) lipids (Zhang *et al.*, 2020). Inside the hydrophobic core, ionizable lipids aggregate into inverted micelles around the encapsulated RNA molecules (Zhang *et al.*). These lipids have a pKa value between 6.2 and 6.5 (Yan *et al.*, 2022). Under physiological conditions (pH=7), the uncharged ionizable lipids are responsible for the biocompatibility and stability of the lipid nanoparticle (Yan *et al.*, 2022). Under acidic conditions, the lipids are positively charged and interact with the negatively charged RNA molecule (Zhang *et al.*, 2021), showing

a high encapsulation efficiency on RNAs (Yan *et al.*, 2022). Furthermore, the lipids are also positively charged in the acidic environment of endosomes and can therefore bind to negatively charged endosomal membranes. The interaction leads to the disruption of the endosome, finally resulting in enhanced endosomal escape (Zhang *et al.*, 2021). Among the ionizable lipids, LNPs based on diole methyl-4-dimethylamino butyrate (DLin-M3C-DMA, Patent NO. US8158601B2) are one of the most effective systems delivering siRNA (Yan *et al.*, 2022).

The introduction of PEG lipids such as DMG-PEG2000 can help to avoid particle aggregation and therefore increase storage stabilization (Yan *et al.*, 2022). In the case of the LNP delivered siRNA drug Patisiran, the PEG lipids also support the stability of the nanoparticle after administration, enabling uptake into its target organ (Zhang *et al.*, 2020). For further stabilization of the LNP, cholesterol and 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) can be introduced (Yan *et al.*, 2022).

For further optimization of the nose-to-brain delivery, the surface of lipid nanoparticles can also be linked with targeting ligands. These include wheat germ agglutinin (WGA), a lectin with specific binding to N-acetyl-D-glucosamine and silicic acid, which is presented amongst others on the surface of the epithelial cells of the olfactory mucosa. Another lectin that can be used as targeting ligand is solanum tubers lectin (STL), a glycoprotein that binds to N-Acetyl-D-glucosamine of the nasal cavity epithelium. Concerning that lectins can show immunotoxicity, small lectin-like peptides have been discovered as an alternative. Amongst them are Odorranalectin (OL), a small peptide initially derived from frog skin that binds to L-fucose expressed in the olfactory epithelium, and lactoferrin (Lf), a glycoprotein and ligand of the lactoferrin receptor (LfR) that is highly expressed in brain endothelial cells and neurons. Another reported targeting ligand exhibits a part of the rabies virus glycopeptide (RVG29) responsible for cellular entry and virus fusion. It efficiently interacts with the nicotinic acetylcholine receptor (NAchR) present in CNS cells. (Borrajo and Alonso, 2021)

For the delivery of siRNA, we want to test two nanocarrier formulations that have been established for the nose-to-brain pathway. One of them is a Tat-modified PEG-poly (ε-caprolactone), or PEG-PCL, nanomicelle, which effectively transports siRNA into the olfactory and trigeminal nerves and was used during in vivo experiments for the treatment of Ischemia. The other formulation comprises chitosan-modified nanoparticles tested to treat glioblastoma and Huntington's disease. (Borrajo and Alonso, 2021)

Aside from the development of a lipid nanoparticle (LNP)-based siRNA therapeutic for the treatment of Herpes simplex infections, the development of a benchtop two component system is planned, which will allow the rapid and easy production of LNPs and testing on an multi-organoid system, as well as evaluation of both processes.

With the rise of LNPs in research and therapy, microfluidic high-throughput production platforms have gained considerable attention. Microfluidic devices enable a scalable, precise and reproducible production of LNPs compared to bulk production (Shepherd *et al.*, 2021).

Several key factors have to be considered during the design of a device suitable for LNP production (Roces *et al.*, 2020). Due to the randomness of LNP formation, bulk production is not suitable. This is mirrored by the fact that bulk production experiments show low reproducibility, large LNPs (> 100 nm) and a poor loading efficiency (Shepherd *et al.*, 2021). A problem with microfluidic approaches is the existence of laminar flow, which makes the mixing of the aqueous phase and the lipid phase only possible through diffusion at the phase interface. To overcome this problem micromixers have been developed. These can be classified as either active or passive micromixers. Active micromixers need external force (e.g. pressure, temperature etc.) to create turbulence which enhances LNP formation. To create these structures in microfluidics is often complicated and time consuming. Contrary to that, passive micromixers are created by the creation of structures inside the microfluidic devices which inherent turbulence formation (Nady *et al.*, 2021). Therefore, passive micromixers were chosen because of their relative ease of use and cost efficiency. One specific structure, the staggered herringbone micromixer (SHM), has proven to be a very effective way of LNP production and has been further refined in the last years (Nady *et al.*, 2021; Roces *et al.*, 2020; Shepherd *et al.*, 2021; Whulanza *et al.*, 2018).

Polydimethylsiloxane (PDMS) has proven to be one of the most widespread used materials for realization of such devices due to its low cost, durability and compatibility with the used reagents (Nady *et al.*, 2021; Shepherd *et al.*, 2021; Torino *et al.*, 2018; Whulanza *et al.*, 2018). Several means of PDMS device production have been developed (Martínez-López *et al.*, 2017; Torino *et al.*, 2018; Whulanza *et al.*, 2018). For the production of our devices we decided to use proton beam lithography and conventional UV-lithography as described in (Nady *et al.*, 2021).

Our aim is to develop a PDMS based single channel SHM device for LNP production. The produced LNPs should be smaller than 100 nm in diameter and the desired mixing capability of the device should be at least 90 %. Analysis of LNP quality and quantity as well as control of the flow rate is provided by the usage of a self-designed platform based on an ArduinoNano (see Fig. 2), which controls the flow-rate of both liquids

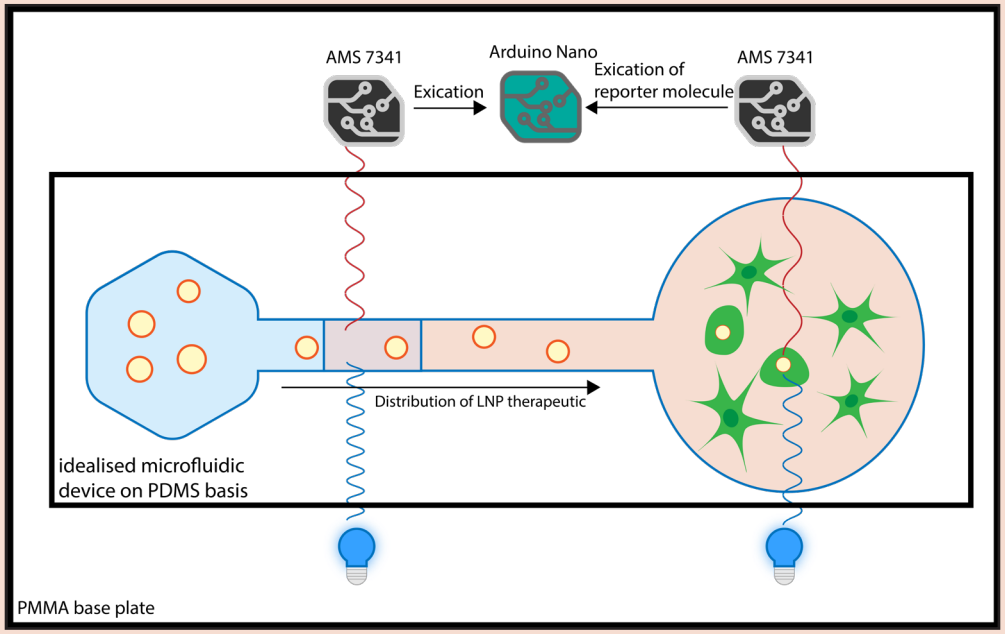


Fig. 2: Conceptual design of our "lab on a chip" device.

and measures the LNP size with dynamic light scattering.

As mentioned above, the technology should also allow for the testing of LNP-based therapeutics on a microfluidic device. This device can be either based on PDMS or Polystyrene, which is commonly used for cell culture flasks. For the analysis of delivery efficiency, detection of established reporter systems such as eGFP, Luciferase etc. will be used. This will be achieved by a sensor -photodiode using the Luciferase system or an AMS7341 for eGFP detection- connected to the ArduinoNano. For the eGFP, a light source in the range of 470 ± 20 nm will also be necessary. All analytical components will be fixed on a Polymethylmethacrylate plate.

After being developed, the siRNAs and the LNPs have to be assessed. For the proof-of-concept, they will be applied to a cell culture of an accessible cell line (e.g. HEK-293 or HeLa cells). This will provide us with a first insight into the success of siRNA delivery, knockdown efficiency, and off-target effects on the transcriptome, which will be evaluated using RNA sequencing. For the estimation of the siRNA knockout performance, the cell line will express a protein from the Herpes simplex virus. A possible target for silencing could be the UL10 gene which encodes the envelope protein, glycoprotein M (gM) (Manda *et al.*, 2019).

In the drylab, we will try to simulate the effect the LNP's composition will have on its size, as well as vesicle fusion and thus its release capability (Pink *et al.*, 2019; Fernandez-Luengo *et al.*, 2017). This will be done by using a multitude of existing approaches such as "NAMD Scalable Molecular Dynamics" (Phillips *et al.*, 2005) and "LAMMPS" (Thompson *et al.*, 2022) for simulation of large biomolecular systems, e.g. to simulate the molecular flow and distribution of the involved therapeutic agents in the nasal mucosal barrier or the cellular bilayer. Another approach is "COMSOL" (Dickinson *et al.*, 2014) with a special focus on the importance of viral load as well as analysis of optimal LNP sizes and lipid composition for optimal cargo delivery. (Fernandez-Luengo *et al.*, 2017; Humphrey *et al.*, 1996)

A further important aspect is the evaluation of properties an LNP would need to have in order to effectively release its content, so we can make sure our LNP is able to release the contained siRNA into the target cells. Combining this information with the transcriptomic analysis (Lowe *et al.*, 2017), either from our wetlab work or from existing transcriptomic datasets (see Fig. 3), we will try to calculate the best compromise between the size of LNPs directly influencing its loading capacity, its capability to penetrate the nasal mucosal layer, and its vesicle fusion capability (Lin *et al.*, 2012).

To further test the viability and efficiency of our nose-to-brain delivery system, we will use an organoid system, which presents many benefits. Organoids are three dimensional cell culture systems that represent human physiology more closely than two dimensional cell cultures (Jensen and Teng, 2020). Additionally, organoids constitute an ethical and effective complementary system to animal testing. The organoid system we propose for the preliminary testing will consist of olfactory neuronal and epithelial stem cells which will grow on an extracellular matrix scaffold containing Matrigel. Different mediums will be added consecutively to differentiate them to the olfactory epithelium. We will generate organoids-on-chip with microfluidic technology, which provides us with the possibility to regulate many parameters more precisely than with organoids from standard plate assays. These parameters include flow conditions, shear stress and nutrient delivery, which we can optimize in order to increase the accuracy of our organoids (Duzagac *et al.*, 2021).

With regard to the aforementioned organoids-on-a-chip device, we will create a layer made of epithelial cells like nasal epithelial cells. This epithelial layer will be the outer layer of a multi-organoid system which can also contain other cell types like neuronal cells. Thus the delivery will be tested in a system modeling the nose with its neurons, which the Herpes simplex virus uses to enter the nervous system (Shivkumar *et al.*, 2013).

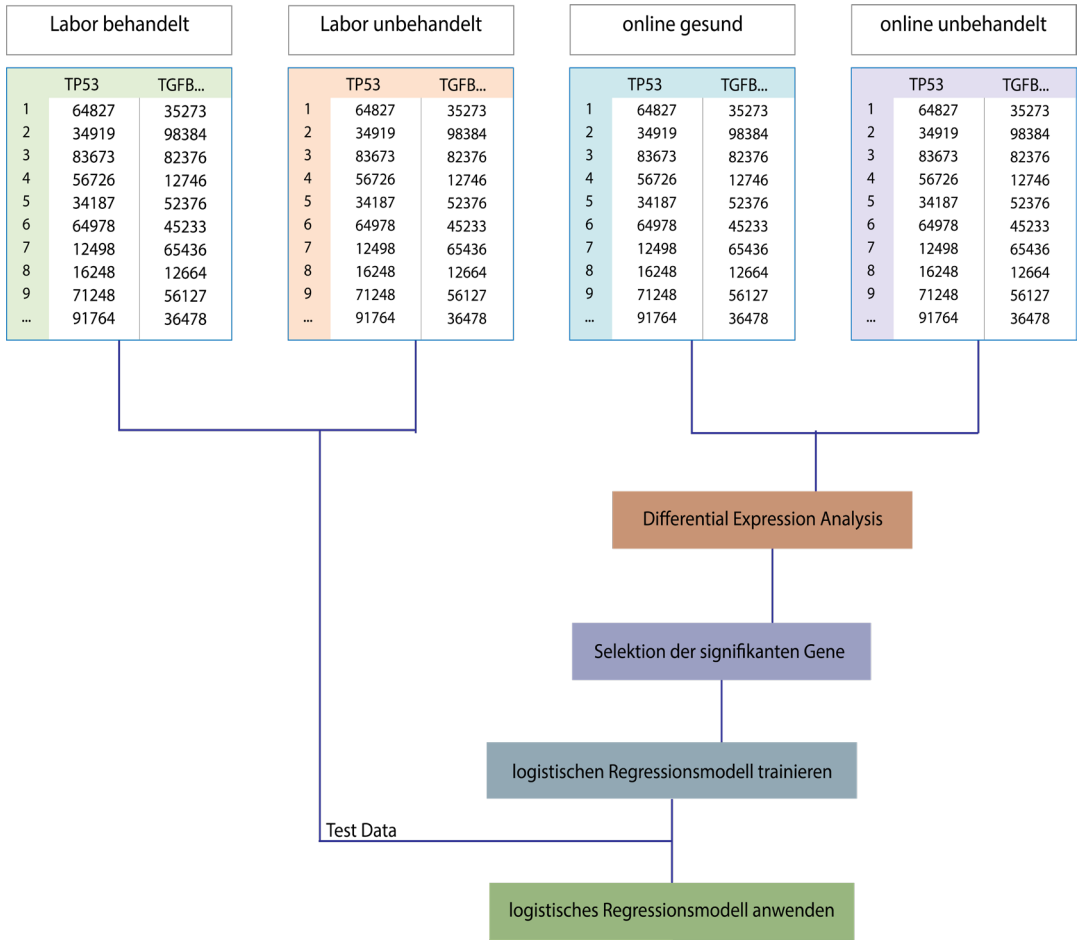


Fig. 3: Evaluation of wetlab data by use of transcriptomic analysis.

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Services	**1.000 €**

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Contact Us!

contact@igem-heidelberg.com

Universität Heidelberg
c/o Prof. Stefan Wölfl
& Prof. Gert Fricker
Im Neuenheimer Feld 364 & 329
69120 Heidelberg