WP3.4. Development of Nanoparticles for targeted gene editing (Konvalinka)

Research aims

- Design and characterization of membrane anchored T7 RNA polymerase and production of stable regulatory Tet-off HEK293 cells expressing modified T7 RNA polymerase
- Design and characterization of an adaptor protein that shuttles specific mRNA towards the cell membrane, anchors in the membrane and displays targeting moiety
- Development of cell-based methodology, including modified T7 RNA polymerase and adaptor protein, that enables production of cell-derived functionalized nanoparticles carrying mRNA
- Design of CRISPR/Cas9 for degradation of HBV cccDNA
- Development of a synthetic approach of targeted mRNA-nanoparticles production including large production of IVT-mRNA encoding CRISPR/Cas9 and its subsequent coating with liposome-based nanoparticles with specific targeting moiety
- Testing of both cell-based and synthetic nanoparticles in tissue cultures

Research plan and methodology

Building on our previous achievements in the development of modular synthetic antibody mimetics based on functionalized polymers, we propose a multidisciplinary approach that combines state-of-the-art molecular biology techniques, eukaryotic cell biology with polymeric synthetic chemistry (Dr. Petr Cígler, Dr. Pavel Majer) to develop an advanced strategy enabling mRNA encoding programmable nuclease to be targeted into specific cells. The undruggable target of our high interest represents the eradication of HBV cccDNA, which will also complement the overall scientific concept of this proposal.

The outcomes of our effort will also enable a specific delivery of functionalized DNA aptamers developed in Dr. Michal Hocek's and Dr. Edward Curtis's laboratories. This project will thus support Dr. Hocek's and Dr. Curtis's research in DNA aptamers.

In spite of substantial recent improvements in mRNA-based therapeutics, especially in cancer immunotherapy, mRNA as a drug in non-immunotherapy-related in vivo applications still remains in the preclinical stage of basic research. The major pending challenge represents the obstacle how to accurately deliver the IVT-mRNA into the target cell type. Moreover, the combination of IVT-mRNA-based technologies with recent advances in the field of programmable nucleases enabling gene editing of more precise yet still immense off-target effect, requires a well designed targeting approach. In this respect, we propose to develop cell-based methodology for production of cell-derived functionalized nanoparticles carrying mRNA. The mRNA will encode CRISPR/Cas9 nuclease that enables HBV cccDNA destruction in order to minimize or even eradicate the latent HBV.

To test our approach, we will first produce the stable regulatory Tet-off HEK293 cells expressing T7 RNA polymerase (Fig. 1.). The polymerase, once translated, will associate with the cell membrane through the membrane-tagging mechanism. Subsequently, the cell will be transfected with the plasmid encoding fluorescent protein mKATEII under T7 promoter. Transcribed mRNA will accumulate near the membrane supported also through an adaptor protein with an extracellular tagging moiety. The adaptor will combine a specific RNA rich binding motif (such as MS2) with a membrane functionalized targeting structure (myristoylated eGFP). Huge accumulation of mRNA will endorse exosome formation. Functionalized exosome carrying mKATEII mRNA will be subsequently released into the extracellular space and harvested. Next, the exosomes will be used for specific targeting of the non-transfected HEK293 cell line and "transfection" efficacy will be evaluated by flow cytometry and confocal microscopy. These initial studies will pave the way for experiments with mRNA encoding CRISPR/Cas9 nuclease that will degrade cccDNA of HBV infected cells[1] (collaborative effort with Dr. Jan Weber).

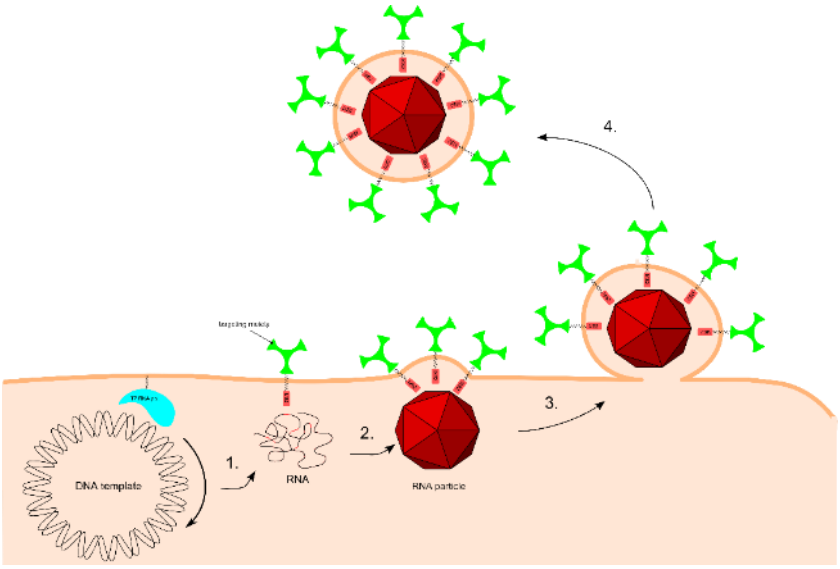

Fig.1. Schematic representation of cell-based methodology of mRNA particles production. 1. Step represents mRNA transcription near cell membrane. 2. Step shows mRNA accumulation and anchoring with the cell membrane through an adaptor protein. Subsequent exosome formation (Step 3) and its release (Step 4).

As a second approach of targeted mRNA-nanoparticle production, we will use and modify recent liposome-based "synthetic" strategies. Synthetic liposomes, as the closest relatives to natural exosomes, could be characterized by minimal immunogenicity (in the case of stealth liposomes) and also by their directed "uncoating" within the target cell enabling endosomal escape and subsequent drug release. Thus, an inverse-type of liposomal micelles containing IVT-mRNA will be created in vitro by mixing IVT-mRNA with cationic lipid DOSPA (2,3-Dioleyloxy-N-[2-(sperminecarboxamido)-ethyl]-*N*,*N*-dimethyl-1-propylammonium chloride) and the helper lipid DOPE (1,2-Dioleoyl-*sn*-glycerophosphatidylethanolamine) (Fig. 2A.)[2]. Next, asymmetric liposome production from an inverse emulsion will be performed: the inverse micelles will be accelerated through the "outer" lipid interphase containing neutral lipids (i.e. DOPE) and lipids conjugated to the targeting moiety (modified DOPE) (Fig. 2B)[2]. The aqueous phase is where the final asymmetric liposomal particles containing IVT-mRNA and functionalized targeting moiety are produced. The aforementioned process can be hastened by centrifugation.

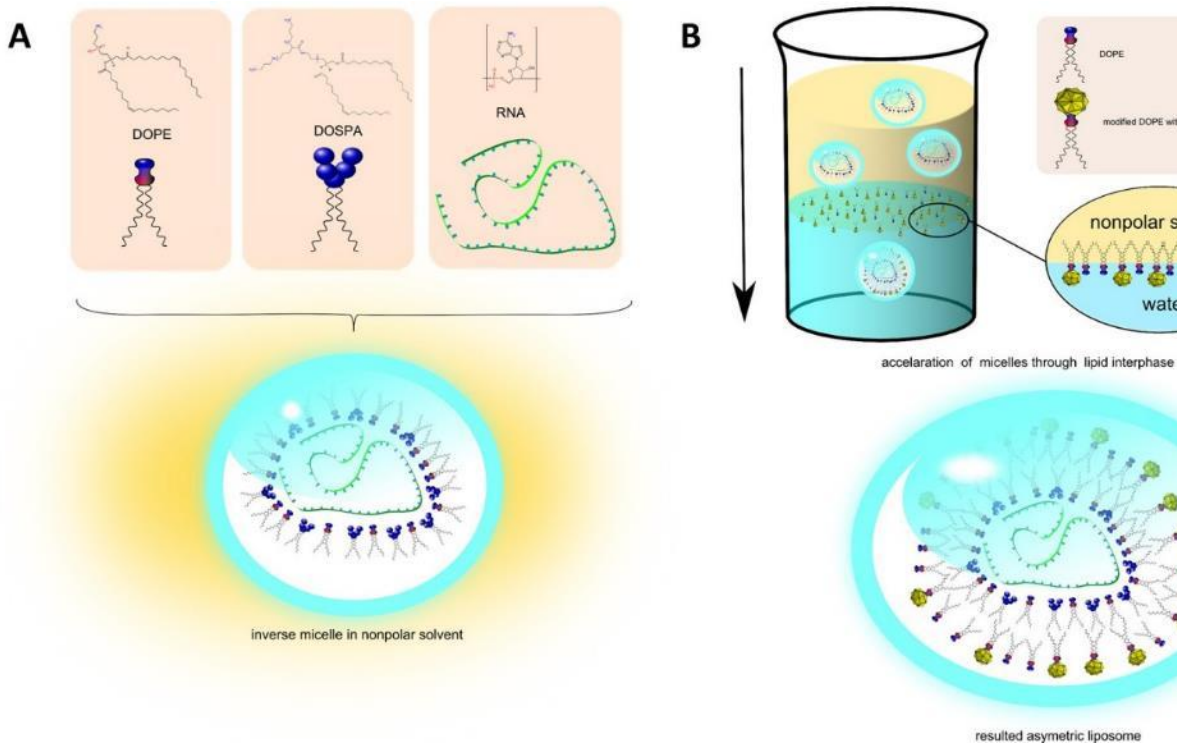

Fig.2. *Sketch of production of liposome containing IVT-mRNA with targeting moiety. A) Mixing of cationic lipid DOSPA with IVT-mRNA and the helper neutral lipid DOPE resulting in production of inverse micelles. B) Acceleration procedure of asymmetric water-soluble liposome formation with functionalized DOPE for efficient targeting.*

Meanwhile, an alternative "synthetic" approach using liposomal particles also formed by IVT-mRNA, DOSPA and DOPE lipids, but creating multilamellar liposomes in buffer solution will be conducted (Fig.3.)3. Multilamellar liposomes will be extruded and washed in order to produce large unilamellar vesicles (LUV). Those will be conjugated with functionalized HPMA (*N*-(2-Hydroxypropyl)methacrylamide) copolymer with amino-reactive groups and targeting moieties. This outer layer of functionalized HPMA will improve the stability of the LUVs and enable cell targeting.

Both of the "synthetic" strategies producing liposome-like functionalized nanoparticles will also enable a specific delivery of DNA aptamers developed in Dr. Michal Hocek's and Dr. Edward Curtis's laboratories and thus translate various expertise to achieve a collaborative goal.

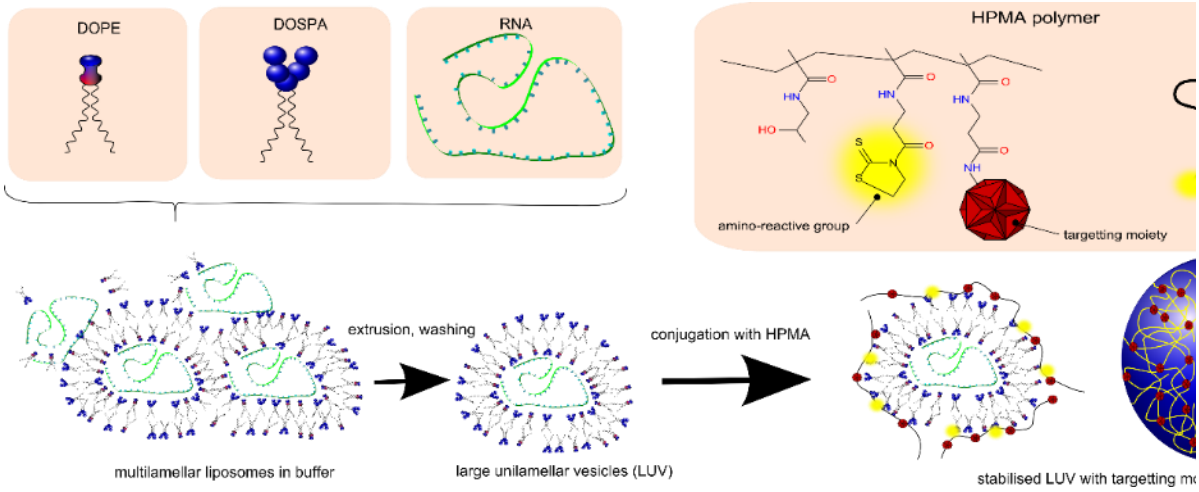

Fig.3. *Sketch of an alternative approach to produce liposomes containing IVT-mRNA with targeting moiety. First, a multilamellar liposomes will through extrusion and washing procedures form LUVs that will be*

subsequently conjugated with HPMA copolymer by an amino-reactive groups. Stabilized LUVs with mRNA cargo and targeting moiety will be produced.

As a relevant clinical target for our approach of "Targeted Gene Editing", we chose Hepatitis B virus infection. HBV infection is a global public health problem that affects millions of people worldwide who are at a high risk of developing chronic liver diseases such as hepatitis, cirrhosis, or hepatocellular carcinoma. Current therapies for chronic hepatitis B that utilize nucleot(s)ide analogues control but do not eliminate HBV infection. The key factor to this persistence is an intracellular HBV replication intermediate – covalently closed circular DNA, which resides in the nucleus of infected cells as a non-integrated plasmid-like molecule and serves as a transcriptional template for HBV. Despite intensive HBV research, the strategies for cccDNA degradation and elimination are limited. Thus our ultimate goal is to apply our targetable liposome-based nanoparticles combined with Cas9 mRNA and gRNAs to degrade cccDNA and therefore pave the way to cure HBV infection

References

[1] Kennedy, E. M., Kornepati, A. V. R. & Cullen, B. R. Targeting hepatitis B virus cccDNA using CRISPR/Cas9. *Antivir Res* **123**, 188-192, (2015).

[2] Whittenton, J. *et al.* Evaluation of asymmetric liposomal nanoparticles for encapsulation of polynucleotides. *Langmuir* **24**, 8533-8540, (2008).

[3] Fenske, D. B. & Cullis, P. R. Entrapment of small molecules and nucleic acid-based drugs in liposomes. *Liposomes, Pt E* **391**, 7-40, (2005).

Research schedule

2018-2021

- Design and characterization of membrane anchored T7 RNA polymerase and production of stable regulatory Tet-off HEK293 cells expressing modified T7 RNA polymerase
- Design and characterization of adaptor protein
- Development of cell-based methodology, including abovementioned modified T7 RNA polymerase and adaptor protein, that enables production of cell-derived functionalized nanoparticles carrying mRNA
- Design of CRISPR/Cas9 for degradation of HBV cccDNA
- Large production of IVT-mRNA encoding specific CRISPR/Cas9
- Development of synthetic approach of targeted mRNA-nanoparticles production including large production of IVT-mRNA encoding CRISPR/Cas9 and its subsequent coating with liposome-based nanoparticles with specific targeting moiety

2019-2022

- Testing of biological and activity properties of our nanoparticles in tissue cultures. Optimization of the most efficient procedure for carrying CRISPR/Cas9 mRNA or DNA aptamers

Publications and patents

Publications (Jimp)

	Jimp
2017	1
2018	1
2019	2
2020	2
2021	2
2022	2
Total	10

Nature Communications
PNAS
Cell Reports
Scientific Reports
Nucleic Acids Research
Angewandte Chemie International Edition
Journal of Medicinal Chemistry
Nanotoday
Nanoscale, international journal of nanomedicine

Patents and patent applications

	Patents (granted)	International patent applications (filed)
2017	0	0
2018	0	0
2019	0	0
2020	1	1
2021	0	0
2022	0	0
Total	1	1

We expect IP protection in the following areas:
Cell-based vesicles enabling specific targeting.

Cooperation with foreign institutions

- Ecole Polytechnique Lausanne, Switzerland, (laboratory of Christian Heinis) on the development of cell-based nanoparticles enabling targeting
- Gilead Sciences, development of cell-based nanoparticles enabling targeting