Chapter 6

Summary and Outlook

Nowadays, countless patients are struggling with genetic dysregulation-based diseases, such as cancer,[1] peripheral arterial disease,[2] hyperlipoproteinemia type I,[3] beta-thalassemia,[4, 5] adenosine deaminase-deficiency,[6] spinal muscular atrophy,[7] optic atrophy,[8] and polyneuropathy of hereditary transthyretin-mediated amyloidosis.[9] Furthermore, viruses are the cause of infectious diseases such as Zika,[10, 11] MERS,[12] Ebola,[13] influenzas,[14, 15] and SARS-CoV-2.[16] Multidisciplinary efforts between biologists, clinicians, engineers, and physical and chemical scientists are required to develop novel therapeutic strategies to treat these diseases at the transcriptional and translational level.[17]

To date, small molecule and recombinant protein-based drugs have been the focus of translational research with much successful medicine in clinical use. However, they also exhibit their own benefits and limitations. The inherent limitation of small molecule drugs is that they require a high systemic exposure to ensure sufficient therapeutic efficacy with the risk of potential off-target side effects.[18, 19] Recombinant protein-based drugs are investigated in protein replacement therapy (*e.g.* insulin to manage diabetes) and chemotherapeutic antibodies (*e.g.* checkpoint inhibitors to treat cancer). However, due to the high molecular weight and polarity of recombinant proteins, typically they cannot enter cells limiting their therapeutic effect.[20]

Nucleic acid-based therapeutics offer the opportunity to address a wide range of diseases at the transcriptional and translational level, and potentially to address the root cause of disease at the genetic level.[21-23] Nucleic acid-based therapeutics include short interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotides (ASO), and messenger RNA (mRNA). Depending on the type of RNA used, the therapeutic outcome ranges from gene knockdown to induced expression of a selected target protein with minimal adverse effects.[23] Among these RNA-based drugs, mRNA has become a promising therapeutic for many applications, including vaccine development for infectious disease,[10, 11, 14, 16] HIV,[24] and cancer,[25] and tissue regeneration to enhance wound healing or repair damaged organs and tissue.[26-29] Significant research has been devoted to the development of nanocarriers to overcome the delivery problem of mRNA. To date, lipid nanoparticles (LNPs) represent the most successful mRNA delivery vector, as evidenced by the clinical approvals of two LNP formulations, Pfizer's BNT162b2 and Moderna's mRNA-1273.[30-32] Their success is partly due to their unique properties, such as simple chemical synthesis of lipid components, scalable manufacturing processes of LNPs, and wide packaging capability.[33] However, their transfection performance is still hampered by endo/lysosomal escape efficiency, as only a small fraction of mRNA (<5%) was reported to reach the cytoplasm resulting in protein expression.[34]

In this thesis strategies to enhance the delivery efficiency of mRNA and drugs using LNPs and liposomes as the nanocarrier are described. Fusogenic coiled-coil peptides were introduced in LNPs and liposomes and the effect on mRNA/drug delivery in different cell lines was studied.

Inspired by SNARE proteins, we have previously shown that the coiled-coil peptide pair K4/E4 triggers efficient membrane fusion between liposomes and cells, facilitating efficient delivery of drugs into cells.[35-37] In **Chapter 2**, the fusogenic coiled-coil peptides were introduced into common LNP formulations to enhance mRNA transfection efficacy. The Onpattro LNP formulation was modified with lipopeptide CPE4, and the addition of CPE4 did not change the physicochemical characteristics of the nanoparticles nor the mRNA encapsulation efficiency. By employing confocal imaging and flow cytometry analysis, the cellular internalization efficiency was measured. It was shown that the coiled-coil peptides enhanced LNP uptake by 63-fold resulting in enhanced protein expression. Furthermore, mechanistic studies revealed that the major pathway for cell uptake was via membrane fusion thereby omitting the less efficient endocytosis pathways. This substantial transfection efficiency improvement after modification of the LNP with coiled-coil peptides can be applied to other cell types, including hard to transfect cell lines (*e.g.* T cells) required for T-cell therapy.

Our group has shown that efficient liposomal delivery could be achieved using coiled-coil peptides.[35-37] In **Chapter 3** the effect of peptide K dimerization on membrane fusion was investigated. Three different dimer designs were synthesized and their structural differences were characterized. Confocal microscopy and flow cytometry measurements showed that PK4 induced the highest binding affinity for cells pretreated with CPE4. Cellular uptake efficiency and the pharmacological effect of the antitumor drug doxorubicin was studied next. Liposome-cell fusion was efficient for this dimer as compared to the linear dimer designs and the benchmark peptide monomer. Thus the novel peptide dimer design is able to deliver drugs into cells more efficiently and will be tested in an *in vivo* setting in the future.

We have shown that fusogenic coiled-coil peptide modified LNPs deliver RNA more efficiently to cells as compared to LNPs. In **Chapter 4** the delivery of mRNA into cardiomyocytes was explored. Myocardial infarction (MI) has been the leading death cause in heart diseases since the human heart cardiomyocytes have a very limited regenerative capacity after MI, the injured cardiac cells only rely on scar tissue replacement to maintain organ integrity.[38] Cardiomyocytes derived from induced pluripotent stem cells (iPSC-CMs) represent the best cell source for human cardiac disorders and cardiac regeneration but require efficient transfection. A novel incubation protocol was developed to transfect these cells. mRNA transfection efficiency of different incubation protocols was compared, and the 1-step incubation protocol achieved improved mRNA transfection with an optimal CPK4:CPE4 ratio of 1:1. The mRNA transfection enhancement using 1-step incubation was compared for three clinically approved LNP formulations and observed that transfection was independent of LNP composition. In all cases the introduction of the fusogenic coiled-coil peptides significantly improved mRNA expression in iPSC-CMs. This optimized mRNA delivery platform could be very promising for further *in vivo* cardiomyocyte research towards the treatment of MI.

mRNA-LNPs are the current state-of-the-art in mRNA vaccination approach since the approval of the Covid-19 mRNA vaccines. In **Chapter 5**, we evaluated the influence of LNP lipid composition on the T cell immune response towards the development of cancer vaccines. In this study we varied the exact lipid composition by varying the ionizable lipid (IL), cholesterol (derivative) and the percentage of the fusogenic helper lipid DOPE. A small library of LNPs was evaluated on the ability to transfect bone marrow-derived dendritic cells, antigen presentation, and T cell stimulation responses. We studied whether replacing cholesterol by β-sitosterol and/or DOPE would boost mRNA

transfection resulting in an enhanced immune response. It was shown that the introduction of β-sitosterol only exerted enhanced transfection in LNPs when MC3 was used as the IL, while exhibiting varied transfection efficiency effects on different cell lines when C12-200 and cKK-E12 were used as the IL. Replacing cholesterol with DOPE resulted in mixed mRNA transfection efficiencies in different cell types. We demonstrated that the LNP-mRNA vaccine candidates can generate significant activation of BMDCs as evidenced by the upregulation of the co-stimulatory receptors (CD40 and CD86] and IL-12 expression, robust T cell proliferation, and enhanced cytokine production *ex vivo*.

We have shown fusogenic coiled-coil peptides enhance mRNA delivery using LNPs in multiple cell lines, including hard to transfect Jurkat cells and cardiomyocytes *in vitro*. However, sometimes the *in vitro* results do not translate to *in vivo* performance, further *in vivo* investigations are therefore required to validate the presented findings. Currently, *in vivo* studies using local injection of mice cardiomyocytes are in progress and will give insight in the ability to treat MI. The second open question is that after delivering mRNA, will it have a relevant therapeutic effect in a mice model? The presented mRNA delivery system based on coiled-coil peptides and LNPs is most likely suitable for local administration, while systemic (intravenous) administration might be more complex. Dimerization of peptide K4 resulted in an enhanced drug delivery efficiency, but the used incubation protocol needs to be also studied in a relevant *in vivo* model to truly validate its usefulness.

In this thesis, we successfully used fusogenic coiled-coil peptides to deliver low molecular weight drugs (*e.g.* doxorubicin) and macromolecular mRNA. This resulted in an enhanced antitumor effect and significantly increased the mRNA transfection efficiency compared to state of the art and clinically approved liposome/LNP formulations. This work further simplified the incubation protocol of our coiled-coil peptide modified LNP system resulting in the successful transfection of cardiomyocytes, which holds great promise for heart regeneration therapy after myocardial infarction. Finally. LNP-mRNA candidates that elicit potent BMDC activation and T cell proliferation were identified and can be used in the development of future candidate cancer vaccines. I hope this work will contribute to the mRNA delivery technology with enhanced *in vitro* and *in vivo* therapeutic performance and potent protective immunity against cancer.

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