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Characterization of mRNA formulation and delivery by histidine-modified peptides

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Characterization of mRNA formulation and delivery by histidine-modified peptides

Abstract:

The growing interest in gene therapy has resulted in the development and application of many novel therapeutic approaches, which can offer potential cure or treatment to previously incurable diseases, by the delivery of genetic materials inside the cells. Consequently restoring, replacing, or inhibiting gene expression.

The effector molecules deployed in gene therapy are based on nucleic acids. However, physicochemical properties of these molecules, such as high negative charge and large size, limit their effective uptake by target cells and this has been identified as the key obstacle impeding the wider use of gene therapy. To improve the bioavailability, various drug delivery systems (DDSs) have been developed. One group of such DDSs are the cell-penetrating peptides (CPPs). CPPs are short peptides (consisting of 3-40 amino acids) usually with a positive charge and possess high transport capability for biomolecules across the cell membrane. The working mechanism of CPPs is based on the formation of non-covalent complexes/nanoparticles through electrostatic interactions with the negatively charged nucleic acids, such as mRNA.

In this thesis, a number of histidine-modified PepFect14 peptide CPP analogs were studied with the aim of tailoring pH-sensitivity with varying degrees of ionizability and cationic charge. It was found that replacing up to 3 out of 5 cationic amino acids in the PF14 peptide with histidines didn't affect its ability to form nanoparticles or reduce its mRNA delivery efficacy. Peptides with only 1 or no cationic charge were able to form complexes with mRNA but with sizes in the low micrometer range. Their inability to form nanoparticles together with their low cationic charge correlated with their low mRNA delivery efficacy in cell culture. Conclusively, these findings show that PepFect14 requires at the least 2 positive charges or more to be able to encapsulate mRNA into stable nanoparticles without losing efficacy.

Keywords:

Gene therapy, therapeutic oligonucleotides, mRNA delivery, histidine-modified peptides, nanoparticles, CPP, PepFect14

CERCS: T490 Biotechnology

mRNA formuleerimise ja transpordi karakteriseerimine histidiinidega modifitseeritud peptiididega

Lühikokkuvõte:

Kasvav huvi geeniteraapia vastu on viinud paljude uute avastusteni geenitehnoloogias, mis võimalvad potensiaalselt ravida mitmeid siiani ravimatuid geneetilisi haigusi. Vastavad lahendused põhinevad peamiselt erinevatel sünteetilistel oligonukleotiididel (ON), mis võimaldavad rakku sisenemisel taastada, asendada või reguleerida geenikspressiooni.

Tulenevalt oma füsikokeemilistest omadustest nagu kõrge negatiivne üldlaeng ning suur molekulmass pole oligonukleotiidid võimelised rakumembraane läbima ning jõudma oma rakusiseseste sihtmärkideni ning seda peetakse geeniteraapia laialdasemal rakendamisel suurimaks takistuseks. ON-de biosaadavuse parandamiseks on välja töötatud mitmeid ravimkandursüsteeme (RKS), mis põhinevad peamiselt lipiididel, polümeeridel või peptiididel. Antud töös keskendutakse rakku sisenvatel peptiididele (RSP), mis on enamasti lühikesed katioonsed 3-40 aminohappest koosnevad molekulid, mis on võimelised kõrge efektiivusega transportima erinevaid ravimmolekule läbi rakumembraanide. ON-d seotakse RSP-de külge kas kovalentselt või nagu käesolevas töös läbi elektrostaatiliste interaktsioonide peptiidi positiivsete ning ON negatiivsete laengute vahel kompleksidesse/nanoosakestesse, mida stabiliseerivad lisaks veel hüdrofoobsed interaktsioonid.

Käesolevas uurimuses töötati välja hulk peptiide, kus modifitseeriti hästi uuritud RSP-d, PepFect14, histidiinidega, et parandada nende pH tundlikust muutes nende ioniseeritavust ning katioonset laengut. Katsete tulemused näitavad, et kuni 3 katioonse aminohappe histidiini vastu vahetamine 5-st ei vähendanud peptiidide võimet efektiivselt formuleerida mRNA-d nanoosakestesse ning vahendada geenikspressiooni rakkudes. Samas kui 4 ja 5 katioonse aminohappe välja vahetamine pärssis neid protsesse pea täielikult. Kokkuvõttes näitavad käesoleva töö tulemused, et PepFect14 puhul on vaja vähemalt 2 positiivset laengut, et formuleerida mRNA-d stabiilsetesse nanoosakestesse ning vahendada efektiivset mRNA transporti rakkudesse.

Võtmesõnad:

Geeniteraapia, terapeutilised oligonukleotiidid, mRNA transport, histidiinidega modifitseeritud peptiidid, nanoosakesed, RSP, PepFect14

CERCS: T490 Biotehnoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

ASO: antisense oligonucleotide

CPP: Cell-penetrating peptide

CR: Charge ratio

DDS: Drug delivery system

DLS: Dynamic light scattering

DMSO: Dimethyl sulfoxide

Fluc mRNA: Firefly luciferase messenger RNA

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

LNP: Lipid nanoparticle

MQ: MilliQ water

mRNA: Messenger RNA

MTT: 3-(4,5-dimethyl-2thiazoly)-2,5-diphenyl-2h-tetrazolium bromide

PDI: polydispersity index

pDNA: plasmid DNA

PF14: PepFect14

RFU: Relative fluorescence unit

RLU: Relative light unit

SDS: Sodium dodecyl sulfate

SCO: Splice correcting oligonucleotides

siRNA: small interfering RNA

SSO: splice switching oligonucleotide

Z-ave: Z-average size

INTRODUCTION

Recent advances in gene technology have resulted in the development of a variety of methods for treating or even curing genetic diseases that were previously believed to be incurable with traditional drugs. Such methods are mostly based on nucleic acids, such as mRNA, small interfering RNA (siRNA), antisense oligonucleotides (ASO), or a more recent addition CRISPR/Cas9. These therapeutic modalities must be able to cross the cell membrane to reach their target inside the cell. Unfortunately, due to their large size and usually high negative net charge, these molecules have very limited ability to cross the cell membrane. To improve delivery to the cells, various drug delivery systems (DDSs) have been devised that can encapsulate nucleic acids into nanoparticles and ensure their effective delivery release inside the cells. DDSs are based mainly on cationic and/or ionizable lipids, polymers, or peptides.

Cell-penetrating peptides (CPPs) are one class of DDSs that have demonstrated a strong potential when it comes to the delivery of nucleic acids (genetic materials) across the cell membrane. CPPs are short peptides that are usually cationic and/or amphipathic in their nature and come with the ability to form nanoparticles with various nucleic acids and synthetic oligonucleotides. Nanoparticle formation is mediated by electrostatic interactions and further stabilized by hydrophilic/hydrophobic interactions. One example of such peptides are the PepFects.

Many researchers have been exploring ways to enhance the delivery properties of CPPs through investigating various modifications, such as lipidation, PEGylation, adding targeting ligands or modulating pH-sensitivity, with the aim of augmenting their ability to form more stable nanoparticles with nucleic acids, improving cellular uptake, endosomal escape and delivery. The addition of pH-sensitivity has worked really well for lipid nanoparticles (LNPs), where the employment of ionizable lipids (pKa~6.5), which would be neutral under physiological pH and become cationic at endosomal pH, increased the delivery efficacy of LNPs up to 8000-fold (Jayaraman et al., 2012). Consequently, this led to the approval of the first LNP-based oligonucleotide drug, Patisiran, in 2018. The goal of this thesis is to enhance the endosomal escape and delivery efficacy of PepFect14 for mRNA delivery. This will be achieved by using the rationale form of ionizable lipids whereby cationic amino acids in the PepFect14 sequence will be exchanged to histidines to introduce pH-sensitivity/ionizability, enhance endosomal escape and increase delivery efficacy.

1 LITERATURE REVIEW

1.1 Gene-based therapy

Gene therapy is a therapeutic strategy that aims at altering a person's genes or gene expression to treat diseases. Gene therapy can offer a potential treatment option for many genetic disorders where presently no treatments are available. Gene therapeutic strategies can be designed to replace a disease-causing gene with a healthy copy of the gene, inactivate a disease-causing gene that is no longer functioning, or introduce a new or modified gene into the body (https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/what-gene-therapy). With gene therapy having such enormous potential, numerous ongoing studies are being conducted to improve and optimize the delivery of various biomolecules inside cells, such as messenger mRNAs, siRNAs, ASO, SSO (De et al., 2021; Dowdy, 2017). The focus of this work will be on the mRNA.

1.2 mRNA therapeutics

There has been increased interest over the years in applying mRNA as a therapeutic for vaccination, treatment of genetic diseases, and protein replacement therapies (Kowalski et al., 2019). Translational efficiency, intracellular stability, as well as immunostimulatory activity of mRNA are the critical parameters to be optimized specifically for each therapeutic application (Granot & Peer, 2017; Kowalski et al., 2019). For mature eukaryotic mRNAs, the 5' cap and 3' poly(A) tail contribute to the efficient translation as well as prolonged half-life. For example, anti-reverse cap analogs (APCA) and poly(A) tail of 120-150 bp incorporated into in vitro transcribed (IVT) mRNAs have been shown to improve the expression of encoded proteins and mRNA stability (Mockey et al., 2006). Another major breakthrough was the reduction of the immunostimulatory activity of mRNAs by chemically modifying the RNA bases (Svitkin et al., 2017). Furthermore, lack of effective means for intracellular delivery is considered one of the most significant obstacles faced by the broader application of mRNA as a therapeutic modality (Kaczmarek et al., 2017). The high anionic charge density and hydrophilicity of nucleic acids restrict the passive diffusion of mRNA across the cell membranes. Despite the wide variety of DDSs studied for different types of RNA drugs, mRNA molecules are significantly larger (600–10,000 kDa) than siRNAs (~14 kDa) and ASOs (4–10 kDa) adding to the difficulty of mRNA delivery (Dowdy, 2017). Large and charged mRNAs can be encapsulated inside nanoparticles. Their successful

delivery of mRNA involves the enhancement of present formulations as well as the development of a new generation of biomaterials with greater potency (Fenton et al., 2016; Lokugamage et al., 2021).

1.3 Non-viral vectors

Drug delivery systems (DDSs) are essential components in gene therapy and the most common drug delivery approaches rely on viral or non-viral vectors. The mechanism of viral delivery, or transduction, involves encapsulating DNA (or, in some cases, RNA) into a virus particle. By using the normal viral infection pathway, genes are efficiently and selectively delivered to cells (Robbins & Ghivizzani, 1998). The main drawbacks of using viral vectors are their inherent immunogenicity and cytotoxicity, while for certain types of viral vectors another cause of concern is the phenomenon known as insertional mutagenesis. Therefore, non-viral vectors have gained significant attention due to their lower immunotoxicity. In a survey looking at the use of viral and non-viral vectors in clinical trials between the years 2004 and 2013, it was noted that there had been a major increase in the use of non-viral vectors as opposed to the viral. Recently, non-viral vector products have entered clinical trials in ever-increasing numbers as efficiency, specificity, gene expression duration, and safety have improved (Ramamoorth & Narvekar, 2015). Usually, non-viral vectors are composed of various lipids, polymers or peptides. Lipid-based drug delivery systems employment has shown to be successful, most notably by their recent use with vaccines for the COVID-19; mRNA-based vaccines (Baden et al., 2021; Understanding mRNA COVID-19 Vaccines | CDC) and siRNA-based drugs for genetic disorders (Medeiros et al., 2021). Polymers and peptides are currently in similar phases of development and are extensively studied in the pre-clinical and clinical trials as gene therapy drug delivery systems.

1.4 Cell-penetrating peptides (CPPs)

Some 30 years ago, several research groups discovered that certain short peptide sequences were capable of crossing the cell membrane. As a group, such peptides with cellular delivery capacity have become known as CPPs, also known as protein transduction domains. CPPs are typically composed of less than 30 amino acids and have proved to be capable of carrying a variety of therapeutic agents into cells, including small molecules, nucleic acids, synthetic oligonucleotides (main focus of this thesis), therapeutic proteins, viruses and imaging agents

(Ramsey & Flynn, 2015). Over the decades the discovery of many CPPs has led to their classification into three categories according to physicochemical characteristics, CPPs are split into three categories: hydrophobic, cationic, and amphipathic (Jafari et al., 2015). Another classification of CPPs is based on the origin of the peptide in question: nature-derived, synthetic or chimeric (Jafari et al., 2015).

In general, CPP-mediated delivery of oligonucleotides can occur following two main vectorization strategies: 1) direct coupling, where the CPP is covalently bonded to the oligonucleotide; and 2) non-covalent complex formation between the peptide and the oligonucleotide (Kole et al., 2012). Non-covalent complexes/nanoparticles are formed primarily through electrostatic interactions between the positive charges of peptides and the negative charges of oligonucleotides, hydrophobic interactions and hydrogen bonding. After formation, the complexes are further stabilized by hydrophobic interactions along with the charge interactions (Mäe et al., 2009; Reischl & Zimmer, 2009) and later taken up by cells.

The cellular uptake mechanism of the complexes formed between the CPPs and nucleic acids (their translocation mechanism) may differ depending on the nature of the CPP and the experimental conditions. However, CPPs have been reported to usually be taken up by cells through the following pathways: by various types of endocytosis or in certain cases by direct penetration through the cellular membrane (Cerrato et al., 2014; Gräslund et al., 2011) (Figure 1). It should be noted that there is a general consensus in the field that almost all nanoparticles/complexes with CPP and nucleic acid-based cargo are primarily taken up by endocytic pathways.

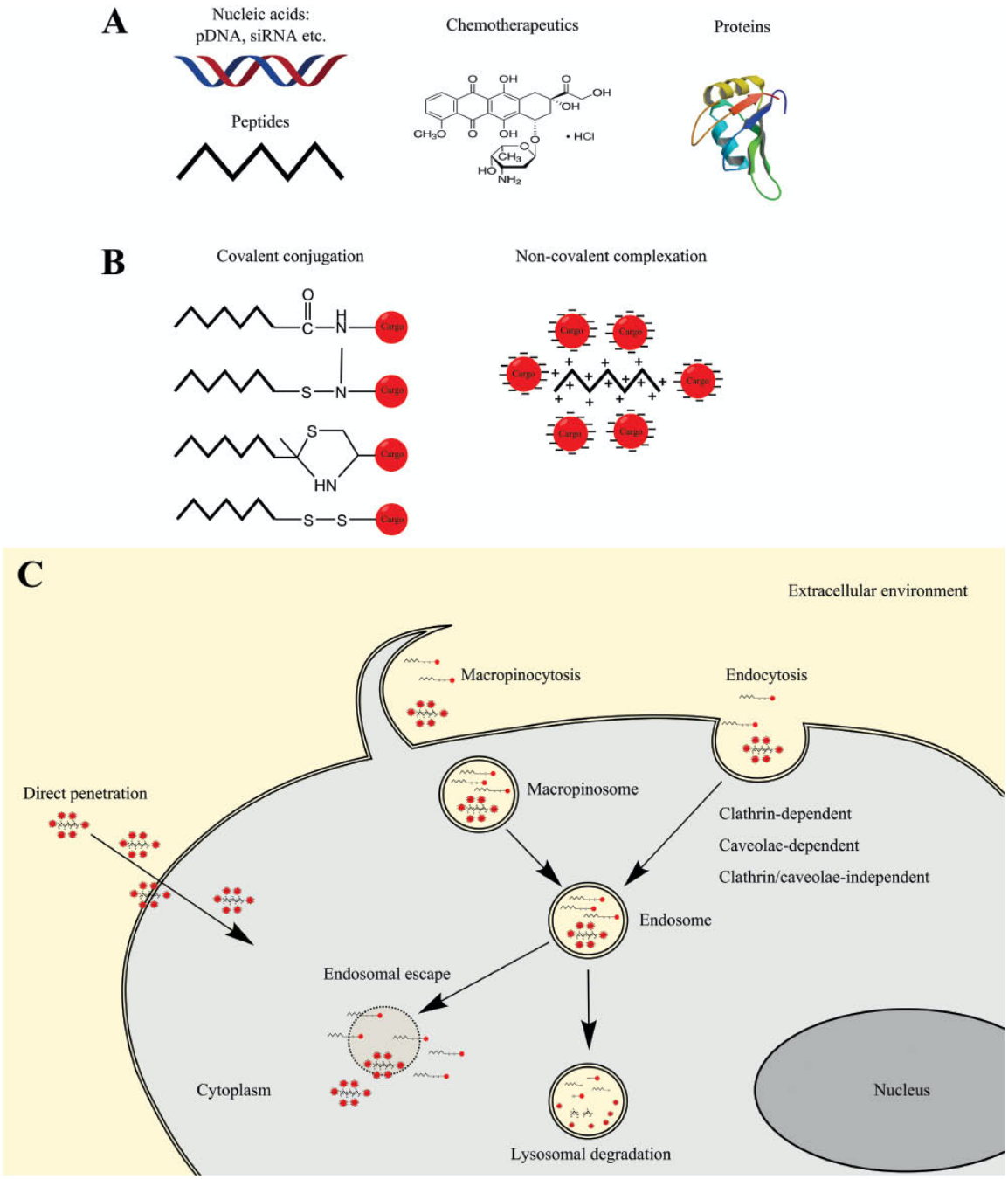

Figure 1. Different strategies for drug delivery with CPPs and uptake mechanisms. *Different therapeutics agents (A). Covalent conjugation and non-covalent complexation of therapeutic cargo (B). The different cellular uptake mechanisms of CPPs (C)* (Cerrato et al., 2014).

1.5 Endosomal Escape

Endosomal escape is a major bottleneck in using non-viral vectors for the delivery of nucleic acids. Complexes formed between oligonucleotides and CPPs are taken up by cells mostly

via the endocytic pathway (Figure 1). In this process after internalization by the endocytic cellular transport mechanisms whereby after internalization they are incorporated into endocytic vesicles and follow the maturation into early and late endosomes, and ultimately accumulate in the lysosome (Iversen et al., 2011). Thus, the escape of CPPs from the endosomal system is necessary for successful transfection (Varkouhi et al., 2011) and this can be considered the rate-limiting step for effective delivery. Consequently, enhancing the escape from the endosomes (and the release of the oligonucleotides/nucleic acids) by the slightest factor can have a great effect on improving cellular delivery. The fact that endosomal escape efficiency is generally poor complicates our understanding of how CPP-cargos escape from the endocytic route (Erazo-Oliveras et al., 2012). With that being said, many strategies and mechanisms have been proposed and researched to improve the endosomal escape efficiency of the CPP-oligonucleotide complexes, including for example strategies like the use of multivalent CPPs and PH dependent membrane-active peptides (Erazo-Oliveras et al., 2012).

As high amounts of these cationic CPPs are required for efficient encapsulation of oligonucleotides, as a consequence, The complexes formed will carry a positive surface charge. This positive surface charge has been sometimes also related to the potential side-effects emanating from the CPPs, as cationic peptides are known to be able to interact with the negatively charged cell membrane. Thus, a strategy that has shown to be successful in addressing this issue when using cationic lipids, has been developed and will, in this thesis, be implemented for CPPs. In the context of ionizable cationic lipids, the aim was to develop lipids with pKa values ranging from 5.6 to 6.9, that remain neutral under physiological pH while acquiring cationic charge when in the acidic environment of the endosomes. Therefore, the use of these ionizable lipids should have a neutral charge in blood circulation reducing immunostimulatory effects while enabling endosomal disruption and drug release in acidic endosomes. Scanning of multiple of these ionizable LNPs, such as MC3 (pKa~6.5), in vivo, has shown to improve delivery by a significant factor over previously used LNPs and their formulations (Jayaraman et al., 2012).

1.6 Modifications improving CPPs

Since the discovery of CPPs, various types of modifications have been applied to improve the stability and resistance of peptide/nucleic acid complexes against enzymatic degradation and ensure the release of nucleic acids/oligonucleotides from the complexes. Such methods

include the coupling of fatty acids to the N-terminus of peptides to increase the stability of the complexes and resistance to enzymatic degradation (Lehto et al., 2017).

Modification by cyclisation of CPPs has also been shown to improve the uptake efficiency (Lättig-Tünnemann et al., 2011), although whether this modification increases the endosomal escape or not is still debated (Patel et al., 2019).

Another line of modifications that have been shown to improve the delivery properties is the N-terminal acylation of the CPPs. For example, the addition of stearic acid to the N-terminus of the peptide has been shown to improve the delivery efficiency of the complexes by about 100-fold, increasing their uptake and also allowing for further stabilization of the complexes formed between the peptide and oligonucleotides (Futaki et al., 2001; Khalil et al., 2004).

1.7 Histidine modified cell-penetrating peptides

The peptide used in this thesis PepFect14, is of a stearic acid-modified peptide family known as the PepFects and will be used as a parent CPP to study the effect of histidine modifications on the PepFect14 peptide structure. It has been demonstrated that histidine-containing carriers can transport different oligonucleotides into the cytosol. Histidine (symbol His or H and also known as 2-amino-3-(1*H*-imidazole-4-yl) propanoic acid) is an essential a-amino acid that is used in protein synthesis. It has an a-amino group (protonated at physiological pH 7.4 R–NH_3^+), a carboxylic acid group (deprotonated at physiological pH 7.4 R–COO^-), and a partly protonated imidazole side chain, making it a positively charged amino acid with pKa ~6.0. This essential amino acid characteristics are perhaps the most diverse biochemical characteristics, which include, hydrogen bonding, aromaticity, coordination bonds with transitional metals, alkylation of the ring, which modifies the hydrophobicity of the imidazole ring, and pH-buffering. Many researchers have made use of these various properties of histidine to improve the already established use of non-viral drug delivery systems as carriers of nucleic acids for improving their endosomal escape efficiency. Imidazoles/histidines incorporated into carriers possess a range of functions, including increasing the stability of polyplexes in the extracellular environment, disrupting polyplexes within acidic endosomes, and enhancing endosomal lysis by osmotic swelling (He et al., 2020).

Overall, it has been reported that the incorporation of histidine lead to the development of pH-responsive peptides with improved endosomal escape efficiency (Chu et al., 2015; Lo &

Wang, 2008). This pH sensitivity is attributed to the fact that histidine's pKa is around 6, thus they are deprotonated at physiological pH (7.4), but when histidine-modified CPPs are taken up by cells, where the pH gradually decreases in the endosomal system, they become increasingly ionized and thus should have the capacity for increased endosomal escape by proton-sponge-type of mechanism.

Different studies have incorporated histidine to a number of CPPs. For example, histidine modification along with the fatty acid chain of different lengths on NickFects peptides significantly increased the potency of nanoparticles formed between CPP/oligonucleotide (Porosk et al., 2019). In another report, histidine modification on PepFect3 peptide showed increased bioactivity. Since the incorporation of histidines seems to decrease the bioactivity of PepFect14-based analogs (Regberg et al., 2016), further investigation along these lines is needed.

1.8 PepFects

PepFects are a family of peptides developed by professor Ülo Langel's laboratories in Stockholm and Tartu. This family of peptides is derived from the CPP called transportan, which is a chimeric combination of fragments from the neuropeptide galanin in the N-terminal end, and a fragment of mastoparan, the wasp venom peptide toxin, in the C-terminal end (Pooga et al., 1998). Later, this transportan sequence was optimized and modified for the purpose of increasing the delivery efficiency, creating an N-terminally shortened analog. The shortened analog of transpotan, TP10 (Soomets et al., 2000) was N-terminally modified with stearic acid, which led to the development of the first PepFect peptide, PepFect3 (Futaki et al., 2001; Lehto et al., 2017; Mäe et al., 2009). Various other analogs have been developed over the years, notably, the PepFect14 (Ezzat et al., 2011), which will be discussed in more detail below as it is used as a basis for the peptides developed in this thesis.

Table 1. The origin of PepFect14

Peptide	sequence	Ref
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-NH_2	(Pooga et al., 1998)
Transportan 10	GYLLGKINLKALAALAKKIL-NH_2	(Soomets et al., 2000)
PepFect3 (PF3)	Stearyl-AGYLLGKINLKALAALAKKIL-NH_2	(Mäe et al., 2009)
PepFect14 (PF14)	Stearyl- AGYLLGKLLOOLAAAALOOLL-NH_2	(Ezzat et al., 2011)

1.9 PepFect14

PepFect14 is one of the most studied peptides among the PepFect family of CPPs. Over the years, this universal transfection agent has been shown to successfully deliver various types of cargo molecules, such as splice correcting oligonucleotides (Ezzat et al., 2011), mRNA (van den Brand et al., 2019), and pDNA (Veiman et al., 2015). The goal of nucleic acid delivery vectors is to neutralize their high negative charge and condense them into stable nanoparticles/complexes, limiting degradation while enabling efficient delivery. Due to positive charge, this peptide is able to form complexes with various negatively charged nucleic acids as a result of the electrostatic interactions. The complexes are further stabilized by the hydrophobic interactions caused by the steric acid and the hydrophobic amino acids. It has been reported that the longer the fatty acid chain length and the consequent increase in hydrophobicity results in increased efficiency of functional delivery of oligonucleotides (Lehto et al., 2017).

2 THE AIMS OF THE THESIS

The aim of the thesis is to study pH-sensitive peptide analogs of PepFect14 with different degrees of ionizability/cationic charge and evaluate their ability to formulate and deliver mRNA in cell culture models. The individual aims are as follows:

i) To evaluate the ability of histidinylated PepFect14 peptide analogs to encapsulate mRNA into peptide/mRNA complexes.
ii) To characterize peptide/mRNA complex/nanoparticle formation.
iii) To study the mRNA delivery efficacy in cell culture.
iv) To investigate the effect of these peptide/mRNA nanoparticles on cell viability.

3 MATERIALS AND METHODS

3.1.1 Used peptides

To have an analytical understanding of the effects of modifying peptides with histidine, peptides of different numbers of histidine integrated into them were used in this thesis. The peptides were named according to their histidine chain length (His2-His4) and their peptide backbone origin (14 according to the PepFect14). These peptides were synthesized by solid-phase peptide synthesis (SPPS), a method of peptide synthesis developed by Robert Bruce Merrifield in 1963, and for which he was awarded a Nobel prize in chemistry 1984 (Merrifield, 1963). SPPS is the main method of synthesis for proteins and peptides that are not expressed in bacteria. In this thesis, PepFect14 peptide was used as a parent control in the experiments to study the effect of the inclusion of the histidine-modification (His2-14, His3-14, His4-14, His5-14).

Table 2. **Peptides used in this thesis**

Peptide	Sequence	MW	Charge at pH 7.4	Charge at pH 6.5
PF14	Stearoyl-AGYLLGKLLOOLAAAALOOLL-NH_2	2407.3	+5	+5
His2-14	Stearoyl-AGYLLG**H**LL**H**OLAAAALOOLL-NH_2	2438.3	+3	+5
His3-14	Stearoyl-AGYLLG**H**LL**HH**LAAAALOOLL-NH_2	2461.6	+2	+5
His4-14	Stearoyl-AGYLLG**H**LL**HH**LAAAAL**H**OLL-NH_2	2484.3	+1	+5
His5-14	Stearoyl-AGYLLG**H**LL**HH**LAAAAL**HH**LL-NH_2	2507.3	+1	+5

3.1.2 Preparation of non-covalent complexes

The non-covalent complexation has been broadly applied to various non-viral vectors, such as cationic lipids, and polymers, but this concept was not applied to CPPs till the end of the 90's. As previously described, CPPs form complexes with nucleic acids by electrostatic interaction between the positive charges of the peptide and negative charges of nucleic acids, and particles are further stabilized by the hydrophobic interactions (Morris et al., 1997).

Thus, the complex preparation was carried out by mixing the peptide with the mRNA allowing the formation of the complexes by electrostatic interactions (Figure 2).

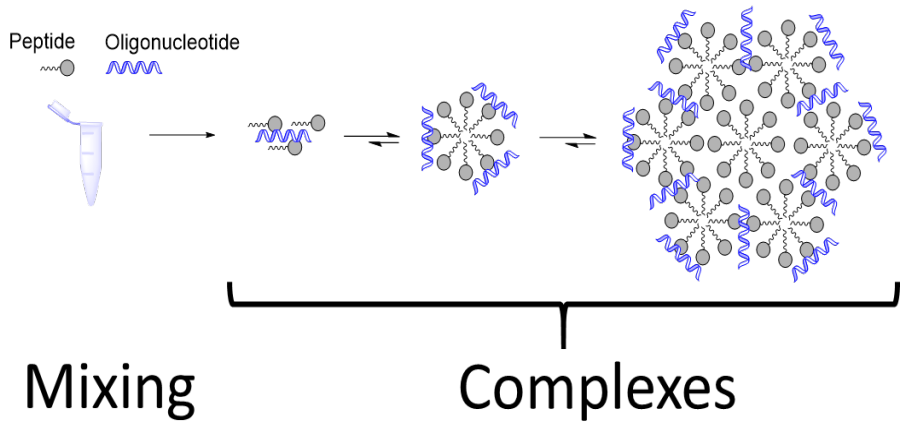

Figure 2. Non-covalent complexes formation. *The peptide and the mRNA are mixed together. The complexes are formed due to the electrostatic interactions and are further stabilized by hydrophobic interaction.*

Since the peptides studied were modified with varying numbers of histidine, meaning the charges of each peptide were differently charged at pH 7.4, different protocols for each peptide/mRNA complex formation were used, as well as different concentrations of peptides for purpose of optimizing the assays. How the complexes were prepared in detail will be explained separately for each method.

3.1.3 SyBr Gold quenching assay

After the formation of complexes, the amount of free accessible mRNA was determined using the SyBr Gold assay. SyBr Gold is an intercalator and this dye molecule can bind between two planar bases/base pairs of DNA. In this thesis, it is used on the basis that SyBr gold can only interact with the free mRNA that has not been complexed by the peptides, so the loss of fluorescent signal indicates the formulation of peptide/mRNA complexes, as the free mRNA is not any more accessible for the dye (Kolbeck et al., 2021). The mRNA quantity was detected using a spectrofluorometer. Results obtained from this experiment were expressed as encapsulation efficiency.

The experiment was carried out at different charge ratios (CR) to determine a CR where all the mRNA is complexed by the peptides as well as to evaluate the effects of histidine on the complexation of mRNA.

For this experiment, the complexes between the Fluc mRNA and peptides were formed in 10 mM HEPES buffer at pH 7.4, and at different charge ratios (0-4.0). The materials used for this experiment were, firstly, the 1 µg/µl mRNA stock solution that was diluted with Milli-Q water to 0.1µg/µl. All peptides had an initial concentration of 1mM. Peptide solutions of PepFect14 (PF14), His2-14 and His3-14 were diluted to 0.1 mM, and peptides His4-14 and His5-14 were diluted to 0.2 mM. Following the different protocols of complex formation for each peptide according to their charge (with a final dose of mRNA constant for all) complexes were prepared for each peptide at different CRs (0-4.0), with a final volume of 100µl, by mixing the mRNA solution with the HEPES buffer, followed by adding the peptide solution (for all peptides), mixing and incubation of the complexes for 15 minutes at room temperature. The 100x SyBr Gold solution was diluted with 10 mM HEPES buffer at pH 7.4 to a 1x solution. In a 96-well black plate, 180 µl of 1X SyBr Gold was added and 20 µl of complexes were added. The plate was left for 15 minutes at room temperature away from light. The fluorescence measurement was done at an excitation wavelength of 495nm, an emission wavelength of 537nm, and at room temperature. A mRNA solution without any peptide was used as a positive control and all the results were normalized to that value and converted to encapsulation efficiency percentage (EE%= 100%-$100\% \times RFU_{sample}/RFU_{mRNA}$).

3.1.4 Dynamic light scattering (DLS)

For determining the particle size, size distribution, and zeta potential of the nanoparticles, all of which are important parameters that impact the fate of the nanoparticles in vitro and in vivo, the method of dynamic light scattering (DLS) was applied. DLS measures the scattering intensity over a period of time (Carvalho et al., 2018). The solution is exposed to a beam of light and this method utilizes the Brownian motion of nanoparticles in a solution to determine their size; small-sized particles move faster meaning they scatter light at a higher rate, while the larger-sized particles move slower and thus scattering the light at a slower rate. The DLS machine will measure the fluctuations in the scattering intensity and use it to calculate the size as well as the size distribution (Carvalho et al., 2018; Philo, 2006) (Figure 3). Although this technique only assumes that the particles are spherical in shape

meaning the data yielded is not fully accurate, a second limitation is larger particles overshadowing smaller ones.

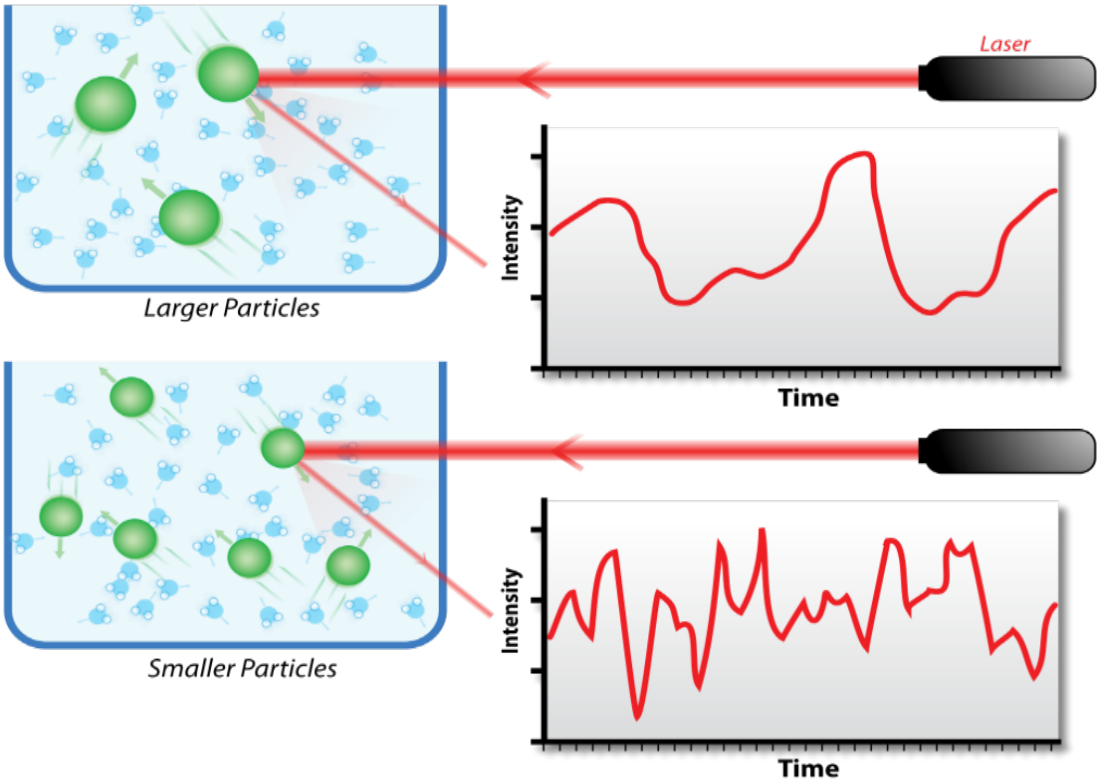

Figure 3. Dynamic light scattering. *A hypothetical graph showing the scattering of the laser light by the larger molecules gives slower fluctuation of reflected light intensity (on the top), in the bottom the smaller particles scatter light at higher frequency with faster fluctuations.* (*https://en.wikipedia.org/wiki/File:DLS.svg)*

DLS is also able to measure the zeta potential of the nanoparticles. In simple words, the zeta potential is the surface charge of nanoparticles. When a nanoparticle is dispersed in a solvent an electrical double layer of ions is formed on its surface with two regions, an inner region (stern layer) where the ions are strongly bonded to the particle with a negative charge and an outer region where they are loosely bound (Figure 4). When an electric field is applied the colloidal particle starts moving based on its charge and its potential at the slipping plane- which is the limit of the electrical double layer, is where the zeta potential is measured

(Bhattacharjee, 2016). Meaning that the zeta potential is calculated on the surface of the slipping plane.

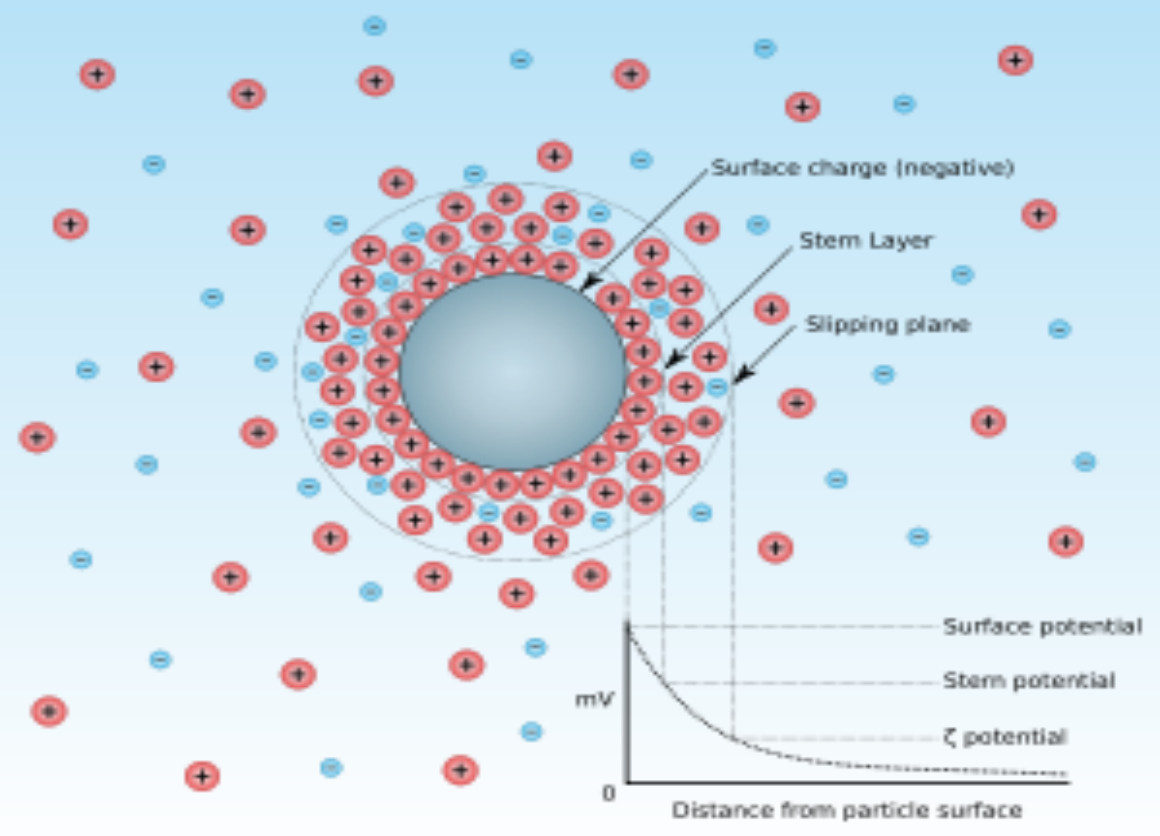

Figure 4. Electrical double layer of a nanoparticle dispersed in solvent. *The zeta potential is the potential which is measured at the slipping plane of the dispersed nanoparticle when known electric field is applied (https://en.wikipedia.org/wiki/Zeta_potential).*

And this movement of the charged particles is measured based on the Doppler velocimetry and the charge on the liquid as well as the surface charge of the particle is calculated (Lehto T, 2018). Particles with zeta potentials between -30 and +30 are considered to have low colloidal stability and are more likely to aggregate.

Complexes between the FLuc mRNA and the peptides were formed in 10 mM HEPES buffer at a pH of 7.4 and at a fixed charge ratio of 4.0 within a final volume of 100 µl. The stock solutions of peptides (varies according to peptide) and the mRNA were diluted to the same concentrations as the SyBr Gold assay experiment and in the same order of preparation. For the use of these complexes for DLS, a 10X dilution in HEPES buffer was made. For size and

size distribution measurements, cuvettes with stoppers were used, and for ZP measurements disposable folded capillary zeta potential cuvettes were used (Malvern).

3.1.5 Cell culture

The wild-type A549 cells are widely utilized lung carcinoma epithelial cell line and it was used to carry out all the cell experiments of this thesis... These included the transfection studies to evaluate the delivery efficacy and the cell viability assays to evaluate the potential toxicity profile.

A549 cells were grown in a 75cm^2 cell culture flask with a filter ventilation cap, in Dulbecco's Modified Eagle Medium (DMEM) containing 100 U/ml penicillin and 0.1mg/ml streptomycin and 10% fetal bovine serum. The cells were split every other day and kept at a temperature of 37ºC and 5% CO_2 in a humidified atmosphere.

3.1.6 In vitro mRNA transfection

For transfection experiments, A549 cells were seeded in a 96-well plate (10 000 cells/well) one day before each mRNA transfection experiment in 100µl of cell media. Then 10µl of peptide-mRNA complexes were added to the 96-well plate and left for another 24 hours. Two parameters were investigated *in vitro*, the delivery efficiency of the complexes as well as their toxicity. All experiments were carried out in serum containing media.

3.1.7 Luciferase assay

Firefly luciferase reporter mRNA was used to determine the delivery/transfection efficiency of the CPPs. Luciferase is an enzyme that catalyses the oxidization of the substrate luciferin into oxyluciferin and a photon is emitted per each conversion. This bioluminescence can be measured using a luminometric analysis. The A549 is a mammalian cell line which does not normally express luciferase enzyme. Therefore, FLuc mRNA encoding for the luciferase protein was used as a readout to measure the effective delivery of mRNA to the cytosol of cells.

For this experiment, the complexes between the FLuc mRNA and peptides were formed in 10 mM HEPES buffer at pH 7.4, and at a fixed charge ratio of 4. The mRNA and PepFect14

stock solutions were diluted to the same concentration as the SyBr Gold assay. His2-14 and His3-14 stock solutions were diluted with buffer HEPES to 200 µM, and peptides His4-14 and His5-14 were diluted with buffer HEPES to 400 µM. Preparation of Complexes of peptides His4-14 and His5-14 followed the same protocol of formulation. The complexes formed had an mRNA dose of 1µg in a total volume of 50 µl, and preparation took place following the same order as SyBr Gold assay followed the dilution series. to create a dose curve. 10 000 A549 cells per well were seeded in 100 µl of DMEM media in a 96-well plate, 24 hours before the experiment. The cells were treated with 10 µl of the complexes or left untreated (control) and PepFect14 was used as a positive control. The plate was incubated at 37ºC for 24 hours before each luciferase assay measurement. The media was removed carefully from the wells and the cells were lysed with 50 µl of 0.1% Triton-100/DPBS lysis solution. 40µl of freshly prepared luciferase reaction buffer (prepared in-house) was added to the wells and 80 µl of the content was transferred into a white plate. The luminescence was read using a GloMax Luminometer machine and measured in RLU (relative light unit).

3.1.8 Viability assay

Cell viability assay gives information about the potential toxicity of the formulations that were used for transfection. In addition, it gives us an insight on the optimal dosing of the formulations. For evaluating the effects of the complexes on the viability of cells MTT (3-(4,5-dimethyl-2thiazoly)-2,5-diphenyl-2h-tatrazoluim bromide) assay was used. This assay is able to detect the metabolic activity of the cells in terms of cell proliferation/viability.

The cells were seeded in a 96-well plate 24 hours before treatment with complexes. The same formulations and doses as for the luciferase assay were used and the cells were incubated for 24 hours at 37 ºC. DMSO was used as a positive control as it is toxic to cells. On the next day, the cells were treated with 10µl of MTT reagent followed by incubation at 37 ºC for 3 hours. During this time, the nicotinamide adenine dinucleotide in viable cells reduces the MTT reagent into formazan crystals, which allows a downstream spectrophotometric evaluation of the metabolic activity of the cells. These formed crystals were dissolved by adding 100µl of acidified SDS into the wells before incubating overnight. The absorbance was measured on Multiskan 60 Microplate spectrophotometer (TECAN) at a wavelength of 570 nm and a reference wavelength of 690 nm.

3.1.9 Data analysis

All experiments took place in the Institute of Technology. The only exception was DLS experiments, which were carried out in the Estonian University of Life Sciences.

All experiments were carried out in triplicates.

Data analysis was carried out in the MS Excel or GraphPad Prism 9 programs. Data are represented as a mean of at least 3 separate experiments.

3.2 RESULTS

3.2.1 Analysis of complex formation

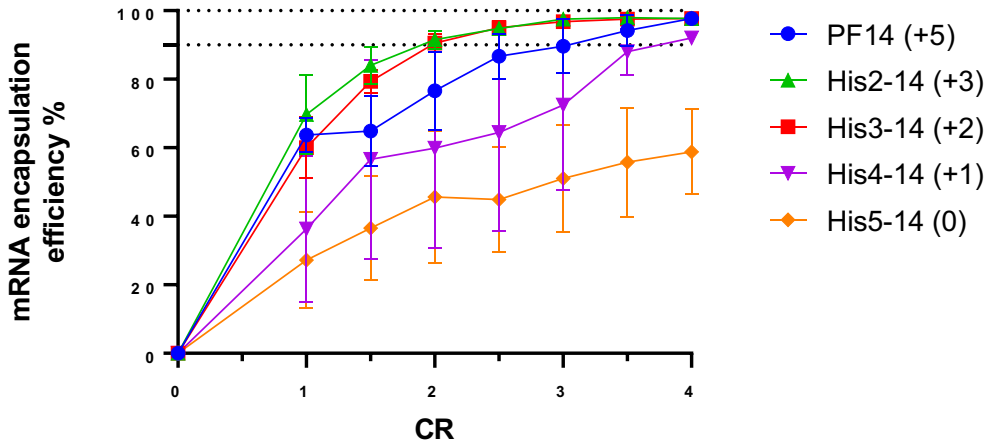

Figure 5. Characterization of histidine modified peptide/mRNA complexes. *SyBr Gold assay was used to study the capacity of each histidine modified CPP to complex mRNA at different charge ratios (CRs) (0, 1, 1.5, 2, 2.5, 3, 3.5, 4.0) and a fixed mRNA dose of 0.1µg, at pH 7.4 (using HEPES buffer for formulation). The quenching of SyBr Gold due to mRNA complexation was measured and results were normalized to mRNA values and from that converted to the percentage of encapsulation efficiency (EE%). n=3.*

In order for the peptides to be effective in mRNA delivery, they have to be able to form complexes with mRNA. Measurement of fluorescence generated by the addition of intercalator SyBr Gold, which binds to free mRNA that did not form complexes with peptides was carried out using a spectrofluorometer. The data generated was first normalized to free accessible mRNA, encapsulation efficiency refers to the percentage of mRNA that have successfully been entrapped by peptides consequently forming nanoparticles. EE% is calculated by (the subtraction of free mRNA from the total quantity of mRNA added) divided by the total mRNA added.

Complete complexation will be referenced as ≥95% encapsulation efficiency of mRNA. The results showed that peptides, His2-14 and His3-14 showed the highest efficiency in mRNA encapsulation at all the different charge ratios of the formed complexes. mRNA is

completely complexed at CR3.0 for peptides His2-14 and His3-14 meaning that the complexation is stable and reproducible at this CR. The control, PF14, completely complexed the mRNA at CR4.0. Peptide His4-14 with a charge of +1 encapsulated approximately 90% of the mRNA, meaning 10% of the mRNA was free for the dye to intercalate. Surprisingly, peptide His5-14, with zero positive charges showed the ability to encapsulate approximately 55% of mRNA at CR4 (Figure 5).

3.2.2 Nanoparticle characterization:

Size, size distribution, and zeta potential (ZP), all of which are important physicochemical characteristics that play an important role in determining the fate of the complexes formed in vitro as well as in vivo. The effects of histidine modification on these physicochemical characteristics were investigated at a pH of 7.4 and at CR4.0. Complexes were formulated in the same way as in the previous experiment (SyBr Gold) and the total amount of mRNA per sample was 1.0 µg. After complexes formation, the solutions of the complexes were diluted 10 times for both size and zeta potential measurements.

Complex size formed between mRNA and peptides PF14, His2-14, and His3-14 were 125, 139 and 118 nm, respectively. Peptides His4-14 and His5-14 formed larger aggregates with sizes over 5000 nm and 2000 nm, respectively, indicating that the positive charges are still important for efficient nanoparticle formation. Size distribution of the mRNA complexes formed by PF14 and His2-14 had a polydispersity index (PDI) of ~ 0.4 and His3-14 a PDI of ~ 0.45. The size distribution of complexes formed between mRNA and peptides His4-14 and His5-14 were of PDI of ~0.4 and more than 0.6 respectively (Figure 6A). The zeta potential of the nanoparticles formed between the mRNA and the peptides. ZP of nanoparticles formed by peptides PF14, His2-14, and His3-14 is around +30 mV, and a much lower ZP of around -30 mV for complexes formed between mRNA and peptides His4-14, His5-14 (Figure 6B). Conclusively, these result shows that modification of PF14 with histidines has no significant effects on the size, size distribution, or on the zeta potential of the complexes when the charge of PF14 is reduced from +5 to +2 (His3-14).

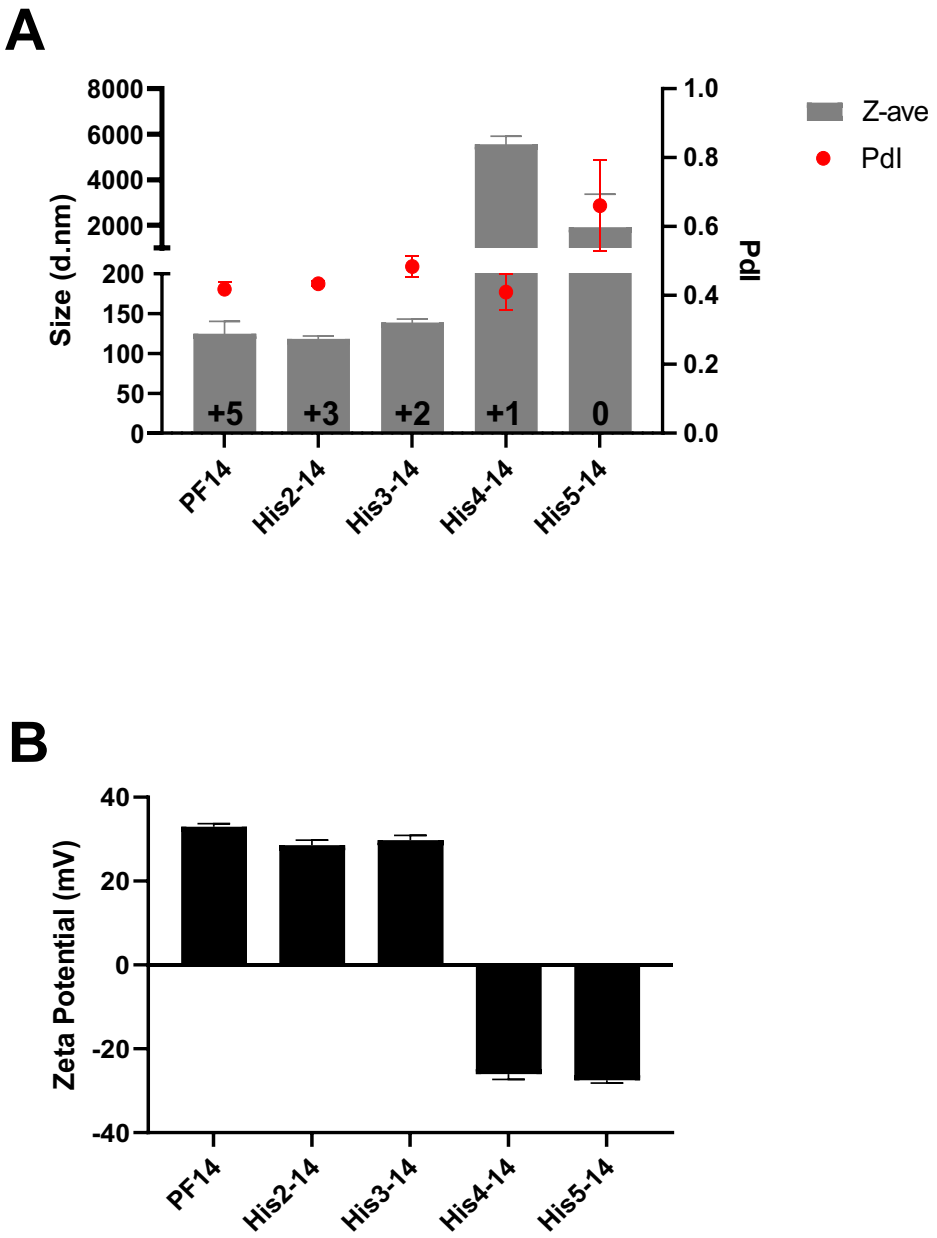

Figure 6. Physicochemical characterization of complexes. *DLS analysis was carried out to analyze (A) size and size distribution, and (B) the zeta potential of the complexes. Complexes were formed at a fixed charge ratio of 4.0 and mRNA dose of 1.0 µg at pH 7.4 in HEPES buffer. The complexes were diluted prior to the experiment. The number on bars in panel A refers to the net charge of the peptides at pH7.4 (n=3). Z-ave: zeta average size, PdI: polydispersity index.*

3.2.3 Effect of histidine substitutions on mRNA transfection:

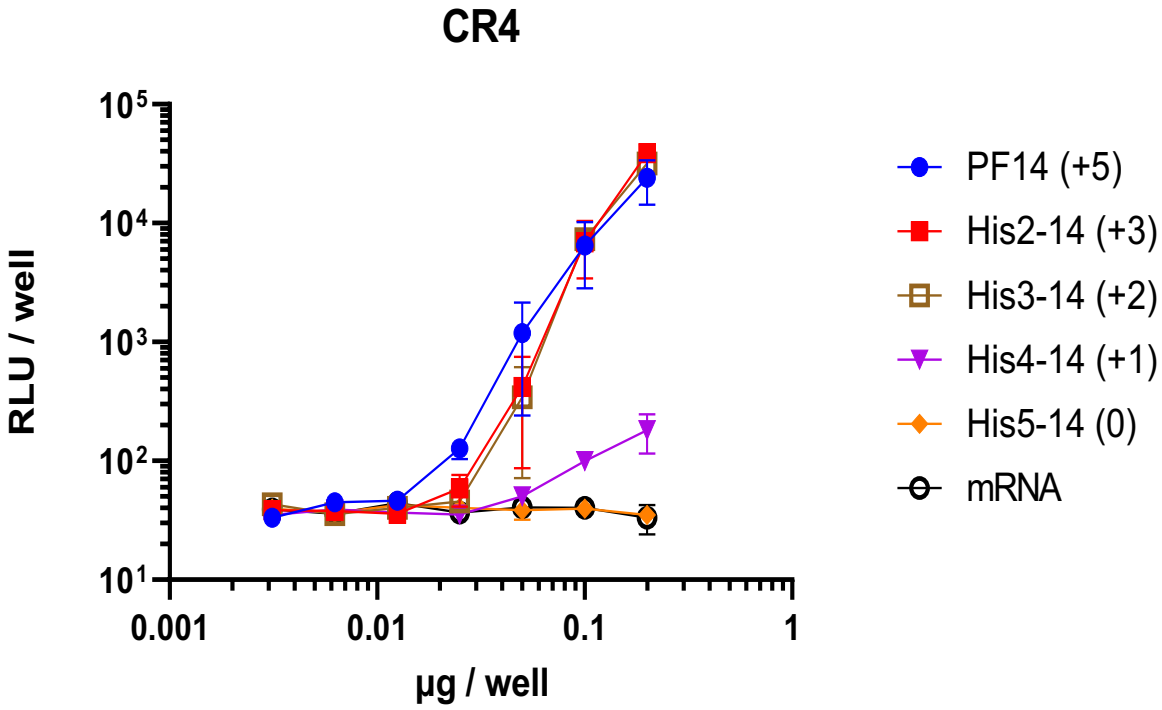

Figure 7. mRNA transfection in A549 cells. *Luciferase assay was used to investigate the transfection efficiency of the peptides at different doses of mRNA and fixed charge ratio 4.0. A549 cells were treated with the complexes for 24 hours at different doses. The luminescence emitted by luciferase catalysing the added substrate is measured in relative light units (RLU). PF14 and mRNA alone were used as positive and negative controls, respectively (n=3).*

Luciferase reporter gene assay was used to determine the transfection efficiency of the nanoparticles in vitro. In this assay, luciferase encoding mRNA is used for the delivery and once inside the cells and released to the cytosol of the cells it will be translated into the luciferase protein. The activity of this enzyme can be measured by using luciferase assay and luminometric analysis. The cells were treated at different doses with complexes formulated at CR4.0 with firefly luciferase encoding mRNA. Upon treatment of A549 cells for 24h the luciferase expression was quantified from the cell lysate.

Significant changes in the delivery efficiency of peptides PF14, His2-14, and His3-14 as opposed to His4-14 and His5-14 at higher doses of mRNA. Peptides His2-14 and His3-14 gave similar expression values and followed the same trend at all doses, with the same efficiency as the positive control PF14. Peptides with the poorest ability to transfect cells were His4-14 and His5-14. His4-14 showed luciferase expression approximately one order of magnitude higher than His5-14 which showed the same luciferase expression as negative control mRNA alone (Figure 7) In conclusion, this data shows that the net charge of the PF14 peptide can be reduced from 5 for PF14 to 2 positive charges for His3-14 without significantly losing luciferase activity in A549 cells.

3.2.4 Tested peptides showed negligible effect on cell viability

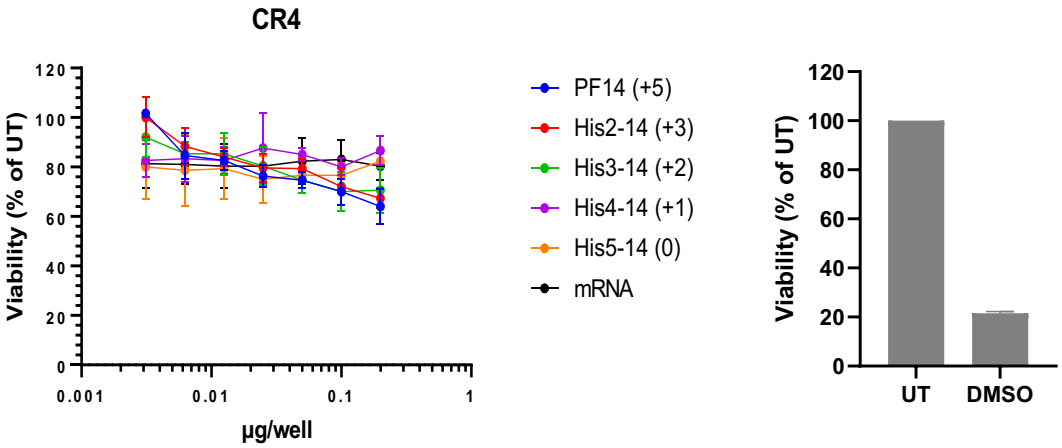

Figure 8. Viability evaluation. *For measuring the viability of the cells MTT reagent was used and such study gives us information regarding the potential toxicity of the formulations used. The complexes between mRNA and peptides were prepared same as the luciferase assay. A549 cells were treated with the complexes for 24 hours at different doses and results were normalized to untreated cells (n=3). mRNA was used as negative control and DMSO as a positive control.*

Cell viability was measured by evaluating the metabolic activity of the cells after treatment with MTT and dissolving the formazan crystals with SDS. Such cell viability studies support the nanoparticle formulation studies by giving first line information on the potential toxicity

of the formulations and their optimal dosing. The cells were at different doses with complexes formulated at pH 7.4 and CR4.0 with firefly luciferase encoding mRNA.

Peptides His2-14 and His3-14 show a gradual decrease of viable cells as the dose increases following the pattern of viability of the control, PF14. The viability of cells is not as affected by using peptides His4-14 and His5-14 (Figure 8). Overall, the peptides of the study show no major adverse effects of toxicity on the metabolic activity of the cells.

3.3 DISCUSSION

3.3.1 Characterization of peptide/mRNA complexes

mRNA encapsulation efficiency

In general, the non-covalent complexation relies on the charge differences between the cationic peptides and the highly negative mRNA that allows their association via electrostatic interactions while the complexes are further stabilized by hydrophobic interactions provided by the amphipathic nature of the peptides, all of which allow peptides to condense the mRNA into stable nanoparticles. Hence, to formulate the complexes we used peptide/mRNA charge ratio which is calculated based on positively charged amino acid groups in the peptide and the mRNA negative charges in the phosphate backbone. On the other hand, it should be noted that when PF14 and His4-14 with net charge of +5 and +1 respectively are formulated at CR4.0, the actual peptide/mRNA molar ratio for His4-14 is 5-fold higher than for PF14.

Due to the ionizable nature of histidines (pKa~6.0) in these peptides around and below pH6.0 and lower the complexes were formed in HEPES buffer at pH 7.4 in order to guarantee that the ionizability would not affect the actual charge ratio. As peptide His5-14 does not have any charge at pH7.4, CR could not be calculated for these formulations and peptide was formulated in the same way as with His4-14.

The data shows that the amount of accessible mRNA decreases as the charge ratio increases for all the tested peptides (Figure 5). His2-14 and His3-14 showed higher affinity toward mRNA than PF14 at lower charge ratios while complete complexation (above 95% EE%) was achieved already at CR3.0 and at CR4.0 for PF14. The reason for PF14 to be less efficient at lower CRs in complex formation than His2-14 and His3-14, which have net charges of +3 and +2, could be that there is around 1.7-fold to 2.5-fold higher amount of peptide in these complexes compared to PF14. Hence, this molar excess drives the complexation "reaction" equilibrium toward the more effective product/complexes formation. At CR4.0, peptides PF14, His2-14, His3-14 showed complete complexation of the mRNA, meaning that the complexes are stable and reproducible at this CR (Figure 5). Surprisingly even peptide His4-14 with only +1 charge is able to reach almost complete complexation (90%) at CR4.0, while His5-14 is able to encapsulate over 55% of the mRNA, indicating that the cationic net charge of the peptides might be less important than thought before for complex formation.

Although, it should be taken into consideration that peptides with lower charges are in higher concentrations, driving the equilibrium toward more effective product (complex) formation.

In summary, CR4.0 was chosen for further studies because it yielded the highest possible encapsulation efficiency for all the peptides and gave the most consistent conditions for head-to-head comparison in the following studies.

Physicochemical characterization of peptide/mRNA complexes

Physicochemical properties such as size, surface charge and size distribution are known to affect the fate of nanoparticles in vivo. For example, size is known to drastically affect the biodistribution of nanoparticles in vivo (Alexis et al., 2008; Tang et al., 2014) while too high and too low surface charge can lead to opsonization and clearance by mononuclear phagocyte system (Roser et al., 1998).

The effects of histidine modification on these physicochemical properties of the nanoparticles were studied at a fixed CR4.0 and fixed dose of mRNA in HEPES buffer at pH7.4 as most peptides showed the highest encapsulation efficiency at that CR.

Peptides, His2-14, His3-14 and PF14 are all able to form NPs with sizes under 150 nm with a positive surface charge around +30 mV (Figure 6). This is in good accordance with findings from other studies where PF14 has shown to be able to form NPs with similar properties also with pDNA (Veiman et al., 2015), SCO (Ezzat et al., 2011) and siRNA (Ezzat et al., 2012). The formation of large aggregates by His4-14 (+1) and His5-14 (0) shows that the removal of the cationic charges abolishes the ability of these peptides to encapsulate mRNA into nanoparticles. The inability of these peptides to form NPs also shows that the histidines in these peptides are not positively charged to a significant degree at pH7.4 which is important because it is known that pKa values proteins can shift (Tynan-Connolly & Nielsen, 2007). A similar inability to form NPs with oligonucleotides has been previously shown for PF14 analogs with very short fatty acid analogs in the N-terminus instead of the stearic acid where their ability to form stable NPs was abolished when the peptides could not provide enough hydrophobic interactions for the stabilization of the complexes (Lehto et al., 2017).

Overall, here we have identified here another limitation in the chemical properties of PF14 in terms of charge. For being able to encapsulate mRNA into stable NPs PepFect14 requires at least 2 or more positive charges in the peptide sequence. The extent at which charge interactions can be replaced by hydrophobic interactions and vice versa should be studied in

the future in order to understand the roles of underlying interactions in the peptide/oligonucleotide complexes.

3.3.2 Characterization of the mRNA delivery in cell culture

mRNA transfection efficiency

For the purpose of validating if these novel histidine-modified peptides are able to mediate mRNA delivery and mediate efficient mRNA expression in cell cultures, the cells were treated overnight at different doses with complexes formulated at CR4.0 with firefly luciferase encoding mRNA. Transfection experiments in the A549 cellular mode showed that all peptides were able to induce dose-dependent luciferase expression, while His2-14 and His3-14 showed very similar activity to PF14 (Figure 7). His4-14 showed significantly lower luciferase expression but surprisingly, with only one positive charge, it was still able to induce some luciferase expression. It can be expected that the low activity of His4-14 is mainly due to its inability to form NPs with mRNA which could be endocytosed by the cells but at the same time, the large size of these aggregates can enhance the sedimentation of these complexes on top of the cells. Hence, it is plausible that it causes the local effective concentration on the cell membranes to be much higher than the NPs which maintain their size in cell media. Though it should be studied in the future if the cellular uptake of peptides correlates with the luciferase expression data to explain the role of peptide net charge in oligonucleotide delivery in more detail. His5-14 peptide was shown to be completely inactive as similarly to the negative control (free mRNA) group, transfection levels remained on the background level.

As the idea for the design of these peptides came from ionizable cationic lipids, the first histidinylated PF14 analogs were not as effective as the optimized LNP formulations. The reasons for that can be many-fold. First, the complexes have to be formulated at lower pH and then stabilized at neutral pH as is the case for LNPs. Secondly, ionizable peptides require other lipids (incl. helper lipids, cholesterol and PEG-lipids) in the formulation to be active, as is the case with ionizable lipids. Thirdly, it has been shown by Quantitative structure-activity relationship studies that the ideal pKa for ionizable lipids is very close to 6.7 and hence the pKa of histidines, which is around 6.0, could be a bit low. If that is the case, it would require the design and synthesis of completely new amino acid analogs with these

desired properties. Nevertheless, further data from our group indicates that when these peptides were studied for membrane activity on erythrocytes at pHs 7.4, 6.5 and 5.5, only His4-14 and His5-14 showed pH-dependent hemolytic activity at pH5.5 and 6.4, providing proof that ionizability is quite close to the desired range (data not shown).

Toxicity is dose dependent

For measuring the viability of the cells MTT viability assay was used. We can conclude that the toxic effects of these peptides were negligible overall (Figure 8). Although complexes formed between mRNA and peptides His2-14 and His3-14 showed an increase in toxicity as the mRNA dose increased these histidine-modified peptides showed slightly less toxicity than the parent peptide PF14. Peptides His4-14 and His5-14 showed the lowest toxicity which can partially be explained by their physicochemical characteristics, as these complexes were large in size and had a negative zeta potential. In conclusion, tested peptide/mRNA did not show outstanding toxic effects affecting the cell viability and could be considered safe for further evaluation in vivo, except His4-14 and His5-14 which are not able to form nanoparticles.

Overall, it can be concluded from the in vitro data that by reducing the charge of the peptide, which could decrease the activity and possible side-effects of the CPP, the total amount of peptide in the complexes can be increased, which in turn could improve the tolerability of these complexes.

SUMMARY

In order to characterize the novel histidine-modified peptides for their potential to effectively deliver mRNA in cell cultures, different experimental studies were carried out to validate their properties. Firstly, with the mRNA encapsulation studies it was confirmed that all the designed peptides were able to form peptide/mRNA complexes at the highest tested charge ratio 4.0. Further studies on the physicochemical properties of these complexes by DLS showed that only PF14 (+5), His2-14 (+3) and His3-14 (+2), peptides with net charge of +2 or above are able to efficiently encapsulated mRNA into stable NPs with positive surface charge at pH7.4.

The cell culture studies of these complexes were in good accordance with their physicochemical properties where peptides which were able to form NPs were also able to efficiently delivery mRNA. Interestingly, PF14 analogs with 2 and 3 histidine substitutions (His2-14 and His3-14) were both able to induce comparable mRNA delivery and luciferase expression to PF14 in cells. Furthermore, cell viability studies showed that all the tested peptides were well tolerated by the cells and could be studied further in vivo.

Future studies should aim to understand the roles of the underlying interactions better in the peptide/oligonucleotide complexes by studying the extent at which charge interactions can be replaced by hydrophobic interactions and vice versa. Furthermore, peptides His2-14 and His3-14 showed very promising results for mRNA encapsulation and delivery efficacy and should be investigated further in vivo. Peptides His4-15 and His5-14 which have higher degree of ionizability should be investigated further in more complex formulation incorporating cholesterol, helper lipids and PEG-lipids (similar composition as LNPs) in order to reveal their true potential.

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