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HPV L2 protein derived endosomal escape enhancers

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Abstract:

Delivery of macromolecular therapeutics, and specifically their endosomal escape is one of the challenges in the medicine. Although there are several delivery methods developed, there is still a need to overcome the challenges associated with endosomal entrapment. In this work HPV16 L2 protein and peptides derived from this protein were applied for increasing transfection efficacy of CPP mediated delivery of nucleic acid. For this, peptides derived from HPV L2 protein specific regions, that are responsible for L2 mediated endosomal trafficking of viral DNA were tested. Both L2 protein and new synthesised peptides showed promising results for applying them as additives to the CPP/NA nanoparticles for therapeutic use and protein production in the future.

Keywords: protein, endosomal escape, transfection, cell-penetrating peptides

CERCS: T490 Biotechnology

HPV L2 valgu ja selle põhjal disainitud peptiidide rakendamine efektiivsemaks endosomaalseks vabanemiseks

Lühikokkuvõte:

Makromolekulaarsete terapeutikumide rakendamine terapeutilistel eesmärkidel on limiteeritud eelkõige nende piiratud rakku jõudmise tõttu. Kuigi terapeutikumide rakku toimetamiseks on välja töötatud mitmeid meetodeid, on nende peamiseks puuduseks molekulide lõksustumine endosoomidesse. Käesolevas töös kasutati HPV16 L2 valku ning selle põhjal disainitud peptiide suurendamaks rakku sisenevate peptiidide poolt vahendatud nukleiinhapete transfektsiooni efektiivsust. Peptiidide disain põhines HPV16 L2 valgu spetsiifilistel järjestustel, mis vastutavad viraalse DNA L2 vahendatud endosomaalse suunamise eest. Nii L2 valgu kui ka disainitud peptiidide lisamine rakku siseneva peptiidi ja nukleiinhappe vahel moodustunud nanopartiklitele andis paljulubavaid tulemusi nende kasutamiseks tulevikus nii terapeutilistel kui ka valgutootmise eesmärkidel.

Võtmesõnad: valk, endosomaalne vabanemine, transfektsioon, rakku sisenevad peptiidid

CERCS: T490 Biotehnoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

CPP – cell-penetrating peptide

HPV – human papillomavirus

PV – papillomavirus

L2 – minor capsid protein of human papillomavirus

CR – charge ratio, the ratio between the positive charges from peptide to negative charges from plasmid phosphate backbone

NA – nucleic acid molecules, depending on the context it may refer to plasmids, siRNA, etc.

pDNA – plasmid DNA

TP10 – cell-penetrating peptide transportan 10

NF51 – cell-penetrating peptide NickFect51

NP – nanoparticle

TLR – Toll-like receptor

FA – fatty acid attached to the N-terminus of the synthesised peptide. In this case saturated fatty acid stearic acid, which has 18 carbons in its chain (C18).

INTRODUCTION

Limited delivery of macromolecular therapeutics is one of the main challenges in today's medicine. Lots of potential therapeutics fail in pre-clinical stage because of this reason. Finding a way to increase their cellular uptake would be beneficial as many macromolecular drugs, such as proteins, nucleic acids (NA) and peptides would open new therapeutic possibilities. Different delivery methods have been developed for increasing cellular uptake of these therapeutics in both in vivo and in vitro experiments. Delivery of such macromolecules into mammalian cells is termed as transfection, which is an important biotechnological tool that aims to introduce foreign genetic materials into cells.

Depending on aim and cell line, several biological, chemical and physical approaches of the transfection have been developed. One of the chemical approaches are cell-penetrating peptides (CPP). Since the end of the last century, CPPs have become one of the effective tools for cellular delivery. They are positively charged short peptides which usually contain approximately 5 to 40 amino acids. There are several CPPs that are based on sequences from viral proteins. Viruses have had millions of years to refine their internalisation and escape mechanisms to reach intracellular targets. It should be noted that the first discovery of such peptides was also based on viral protein. In 1988 two different research groups simultaneously discovered the trans-acting activator of transcription (TAT) peptide from human immunodeficiency virus 1 (HIV-1).

There are several mechanisms of entry that are proposed for CPPs, including direct penetration or using endocytosis pathways. Today it is generally accepted that CPP/cargo nanoparticles use mainly endocytosis pathways for entering into cells. The nanoparticles are then entrapped in endosomal compartments, and if endosomal escape is not efficient enough, the particles are degraded in lysosomes. Endosomal entrapment is one of the main limiting barriers for not only CPP based delivery, but also for other delivery approaches, such as polyplex or liposome based delivery. To overcome this issue, different strategies have been applied.

The aim of the current work is to enhance the transfection efficacy of previously developed CPP-based delivery vectors, by harnessing the mechanisms used by viruses. For this, strategies were based on the second late protein (L2) of human papillomavirus (HPV). It was discovered that L2 together with viral DNA (vDNA) can escape the late endosome. Effect of HPV L2 protein was tested as an additive component in transfection. A series of

peptides were designed based on HPV L2 protein. These peptides were synthesized and their effect as an additive component in transfection was tested.

1 LITERATURE REVIEW

1.1 Therapeutic nucleic acids

Usually when talking about genetic diseases many people think about disorders, such as albinism, thalassaemia, cystic fibrosis, haemophilia, etc., which are quite rare. However, everyone's genetic basis plays a role in the development and severity of all diseases (Jackson et al. 2018) Aberrations in the DNA, its splicing or expression, may lead to different disease states. Instead of using small molecule drugs to reduce the symptoms, NA based therapy offers an opportunity to prevent the development of the disease or alleviate the disease state at the genetic level (Weng et al. 2020).

Although nucleic acid based therapy's history starts from 1978 with the inhibition of the RNA translation of Rous sarcoma virus by a specific tridecamer oligodeoxynucleotide (Stephenson and Zamecnik 1978), the first gene therapeutic in the world Gendicine, developed for head and neck cancer treatment, was approved only after 25 years in China (Zhang et al. 2018). Developing NA based drugs is still a priority for biopharmaceutical companies. Different NAs offer immense and versatile opportunities as therapeutics. They can be used to express a gene (e.g. plasmid, messenger RNA (mRNA)), silence aberrant expression through RNA interference (e.g. small interfering RNA (siRNA), microRNA (miRNA)) (Cullen 2017), alter splicing (e.g. splice correction oligonucleotides) and they can also be applied as biotechnological tools (e.g. plasmids for protein production) or vaccines in clinical use (e.g. mRNA) (Table 1). Usage of an unmodified extracellular NA molecules is limited due to their native properties such as, high density of negative charges, size, inadequate permeability through the cell membrane and high susceptibility to degradation in both intra- and extra-cellular environments (Ren et al. 2012).

Additional challenges also arise depending on the NA type. For example, mRNA molecules must be modified to avoid immune detection through pattern recognition receptors, such as Toll-like receptors (TLR), more specifically TLR-3, TLR-7, TLR-8, and retinoic acid-inducible gene I (RIG-I) (Kormann et al. 2011). RNA vectors typically have lower physical stability, as they are much more susceptible to enzymatic degradation than DNA and thus require additional protecting measures, such as end-blocking, base modification, vehicle choice, etc. These protection measures, however, may introduce their own confounding issues (Yin et al. 2014).

Table 1. Nucleic acid based molecule classes, their advantages and limitations.

NAs	Description	Advantages	Limitations	References
pDNA	Plasmid DNA – circular and double-stranded DNA. Used for expression of a gene.	Relatively stable, incorporation of large genes, expression levels can be regulated by different components in the sequence	Circular DNA limits the possibilities of modifications, covalent linkage	(Hardee et al. 2017)
vDNA	Viral DNA	Natural cell internalization and genetic payload delivery ability	Immunogenicity and absence of possibility for repetitive delivery	(Hardee et al. 2017)
mRNA	Messenger RNA – protein expression modality	Does not have to reach nucleus and absence of the genomic integration risk	High production cost, poor stability	(Weng et al. 2020)
siRNA	Small-interfering RNA – double stranded RNA, 21-25 bp, terminal overhangs. Target in cytoplasm (RISC). Uses intrinsic RNA interference.	Cytoplasmic delivery is sufficient. Effective post-translational tool for gene silencing	It can be degraded by enzymes in the blood stream. For crossing cell membrane independently they are too large	(Porosk et al. 2019) (Mirzaei et al. 2021) (Subhan, Attia and Torchilin 2021) (Cullen 2017)
miRNA	MicroRNA – non-coding, small, endogenous RNA. Regulates gene expression in more than 60% of human genes.	Potential therapeutic targets for many diseases	Tissue-specific delivery, kidney clearance, stability, cellular uptake and inhibitory ability of various chemical composition	(Ha and Kim 2014) (Carreras-Badosa et al. 2020) (Duygu et al. 2019)
ASO	Antisense oligonucleotides – small-sized, single-stranded NA	Targeting both, nuclear and cytoplasmic located lncRNA	Difficult cellular uptake and reaching target tissues	(Rinaldi and Wood 2018) (Bennett et al. 2017)

In recent years one of the NA applications, gene editing, became a popular topic. The newest gene editing technique clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) especially brought resonance in comparison with previously known methods like zinc finger nuclease (ZFN) technology and transcription activator-like effector nuclease technology (TALEN). It is simpler and easier to perform and also more effective than others.

1.1.1 Plasmid DNA gene vectors

Plasmids are small, circular, dsDNA molecules naturally found in bacteria. In the bacterial cell, they are separate from bacterial chromosomal DNA and replicate independently from it. Plasmids vary significantly in their size (~1 Kbp – 1000 Kbp). They can be used as tools to clone, transfer and manipulate genes, this kind of plasmids termed as vectors.

DNA fragments can be instructed into the plasmids, creating recombinant plasmid. Plasmids can be expressed in bacteria and also in mammalian cells. Their utility and safety gives opportunities for their clinical use as therapeutics for several mono and polygenic, as well as infectious diseases. Cancer, diabetes, hepatitis and influenza are just some of these diseases (Hardee et al. 2017). It is easier and cheaper to design, manipulate for therapeutic use and produce plasmids than vDNA and RNA-based NAs. Unwanted and unnecessary sequences can be removed (Hardee et al. 2017). They are significantly less prone to integrate unlike viral vectors (Izsvák et al. 2009) and can be delivered repeatedly.

Despite all advantages plasmids have several limitations as well. Variety of pDNA preparations consist of several topological variants of the pDNA: linear and open circular forms are the unwanted ones. Plasmids require specialized modifications, physical forces or vehicles for delivering their payloads to the cell and nucleus. Depending on the delivery method there can be breakage of the plasmid leading to reduced expression (Stenler, Blomberg and Smith 2014). Bacterial sequences of plasmids can cause immune response, gene silencing and replication of antibiotic resistance genes.

1.2 Nucleic acid transfection methods

As stated above, NA do not cross cell membranes by themselves and the use of exogenous NA requires transport or delivery methods of some sort. To overcome delivery challenges of NA, different approaches have been used over the years. Chemical modifications of NA backbone, like methylation (Chen, Zhao and He 2016), increases the stability to degrada-

tion, however, do not really help deliver them effectively into the cell. Alternatively, instead of altering the NA, different delivery methods have been developed.

Transfection is a process by which foreign NA is transferred to a host cells. This process includes any method for gene delivery, but is usually used in the context of non-viral methods. Transfection can result in transient or stable genetic changes to the host (transformation). The transfection methods are broadly classified into three groups; biological, chemical, and physical.

Physical transfection approaches include electroporation, direct injection, laser-based transfection, biolistic particle delivery (Mehier-Humbert and Guy 2005), magnetic field based (magnetofection) (Scherer et al. 2002), ultrasound based (sonoporation) (Kim et al. 1996) etc. The physical methods directly influence the cell membrane in order to increase the NA internalisation. The main limitations of physical transfection methods are related to this. They are usually damaging cell membranes, leading to the loss of viability, require expensive equipment and extensive technical optimization. In addition, as they need to physically disrupt the membrane, the equipment requires close proximity of the host cells and DNA. This limits their bio distribution and application for in vivo transfection.

Viral vector based approach is considered biological delivery method. They method are widely used because of their natural ability to invade cells and deliver a manipulated genetic payload for therapeutic use. They have been considered as the most efficient (Verma and Somia 1997), however as several biosafety (genome integration) and immunogenicity (repeated delivery is limited, possible host rejection) issues have been related to these, alternative delivery methods have been developed (Kim and Eberwine 2010).

New nanotechnology based systems improve NA therapy from different aspects. They increase biosafety, transfection efficacy, targeting particular tissues and organs and also degradation time of NA nanoparticles. Generally for production of NA therapeutics in large amounts non-viral vectors are easier and cheaper (Mali 2013). The mostly used methods are chemical transfection methods. General idea of chemical methods is identical: cationic delivery vectors form positively charged nanoparticles with their cargo and negatively charged cell membranes attract them. One of the widely used and effective chemical methods is cell penetrating peptides (Lindgren et al. 2000).

1.2.1 Cell-penetrating peptides

Today there are over 1700 confirmed CPP sequences, and many more predicted (CPPsite 2.0). The CPPs can be categorized based on their origin, length, charge, hydrophobicity, application, cargoes which they deliver to cells and some other features (Milletti 2012), (Reissmann 2014). Their primary classification was based on their origin (Lindgren et al. 2000), dividing CPPs into protein derived (Milletti 2012), synthetic and chimeric CPPs. Protein derived CPPs are for example Tat (48-60), which is derived from HIV-1, and penetratin. Synthetic CPPs include sequences that are not naturally found in protein sequences, such as polyarginine and model amphipathic peptide. Chimeric CPPs consist of different parts, both protein derived and synthetic, or combination of any (Pooga and Langel 2015). One of the widely known chimeric peptide is transportan, which consists of sequences from two naturally found proteins, wasp venom and mastoparan (Lindgren et al. 2000)

The main nominator for all CPPs is that they are able to penetrate cell membranes, including mammalian, plant as well as bacterial cells. Additionally, CPPs are able to deliver associated cargo, such as nanoparticles, small molecules, peptides, proteins, NAs and other cargoes into the cell with them. CPPs significantly increase cellular uptake of NAs and the peptide can be attached to their cargo via covalent linkage or associated into a nanoparticle non-covalently. Although the covalent linkage is more commonly used and produces homogenous nanoparticles, the main advantages of non-covalent association strategy include its versatility, meaning that different NA to CPP ratios can be used and additional components can be added, lower production costs compared to covalent linkage, as it does not require modified monomers or complicated chemical reactions used for covalent linking, and the NA does not require modifications, which helps to maintain the bioactivity of NA. The non-covalent strategy is easy to preform, as it requires only mixing of the components, opposed to the synthesis of CPP-conjugate in the covalent linking strategy. The main disadvantage of the method is that it is not easy to create and distinguish homogeneous nanoparticles (Arukuusk et al. 2015). The non-covalent complex formation strategy was proposed and patented by Dr. Gilles Divita and his research group (Gros et al. 2006).

In order to enter into the cells, CPPs harness different entry mechanisms, such as direct penetration, endocytosis-mediated entry and translocation through the formation of a transitory structure. Penetration mechanisms of CPPs depend on many factors, such as environmental conditions (temperature, pH and ionic strength), concentration of peptides, conditions of cells, like viability, confluence, type and etc. (Bechara and Sagan 2013). De-

pending on these factors the internalization mechanism can change from endocytosis to direct penetration (Duchardt et al. 2007), (Fretz et al. 2007), which shows that CPPs can adapt to different conditions. For average peptide concentrations and culture conditions, endocytosis is the main internalization mechanism in many cargo delivery experiments (Pooga and Langel 2015).

Nanoparticles' size is highly important for in vitro and in vivo applications. Besides affecting specificity and mechanisms of cellular uptake it also affects passive accumulation of tumors (Lehto, Kurrikoff and Langel 2012), (Scholz and Wagner 2012). For successful transfection the size of nanoparticles should be adjusted carefully. Particles bigger than 1 μm settle and rise concentration of the bottom part of the cell culture (Arukuusk et al. 2015). At physiological conditions a significant number of CPPs are positively charged due to the number of cationic amino acids in the sequence (Milletti 2012).

Another critical point for the successful transfection of the nanoparticles is their optimal stability. Interaction of nanoparticles with serum proteins of the blood can end up with early release of nucleic acids from the nanoparticle (van Asbeck et al. 2013). However, as it should be released after entering into the cell for providing its function, interaction of CPP with NA must be appropriately balanced (Viola et al. 2010), (Kwok and Hart 2011). Different methods have been developed for keeping suitable stability, like stearylation (Mäe et al. 2009).

Deletion analogue of transportan, transportan 10 (TP10) is efficient for the delivery of NAs. By adding N-terminal fatty acid modification, non-covalently formed nanoparticles are more stable (Futaki et al. 2001). PepFect (PF) (Lehto et al. 2012) and NickFect (NF) (Arukuusk et al. 2013) family CPPs are further modifications of TP10. These agents are mainly used for forming nanoparticle complexes with NA for transfection (Margus, Padari and Pooga 2012). Such complexes are suitable for use in both in vitro and in vivo experiments (Andaloussi et al. 2011). NF and PF complexes also show less toxicity for cells in the media, than liposome-based transfection complexes (Lehto et al. 2012). NickFect51 (NF51), used in this work, is a third generation CPP which shows high transfection efficacy without cytotoxicity for a wide range of cell lines. It is also significantly effective CPP for protein expression and production (Arukuusk et al. 2013).

1.3 Internalization of nanoparticles and endosomal entrapment

Endocytosis is a cellular internalization process. Cells use it for taking up substances from outside to the inside of the cell. The process is energy dependent, receptor mediated or receptor-independent, and can be divided into two categories: phagocytosis and pinocytosis. Pinocytosis refers to the cellular uptake of fluids, Pinocytosis is also divided into four categories: macropinocytosis, clathrin-dependent and caveolin-dependent endocytosis, and lipid-raft endocytosis. Endocytosis is a vital process for cell survival, and is also noted as the main entry pathway for nanoparticles (NP). The successful entry of NA based therapeutics into the cells is one of the main limitations of these kind of therapeutics today.

NPs can use different endocytosis pathways, often simultaneously. The study of endosomal trafficking of nanoparticles is therefore complicated, as inhibition of one pathway may lead to the activation of an alternative endocytosis entry path. Additionally, the entry and consecutive trafficking is affected by the used cell line, experimental setup, NP structure (LeCher, Nowak and McMurry 2017), additives in the media (Ezzat et al. 2012) etc.

In the endocytosis process (Figure 1), a small membranous organelle, endosome, is formed and further trafficked in the cell. The first formed compartments are called early endosomes (EE). As a result of acidification EE matures into late endosomes (LE) and then into a lysosome, where NA which entered into the cell are degraded (Pei and Buyanova 2019). EEs are characterised by 6.3 pH. Further sorting and trafficking takes place from these endosomes. From EE, the contents are sorted to multivesicular bodies or trafficked to LE. LEs are characterised by 5.5 pH. In the LE, first degradation steps begin. From LE the contents are usually addressed to lysosomes for degradation. In the lysosomes the low pH leads to activation of degrading by proteolytic enzymes, cathepsins.

The entrapment of NPs in the endosomal pathway followed by degradation in lysosomes, is a major limiting factor for their therapeutic application. In order to reveal their full therapeutic potential, the NPs must be able to escape endosomes and reach their intracellular targets (Lundin et al. 2008).

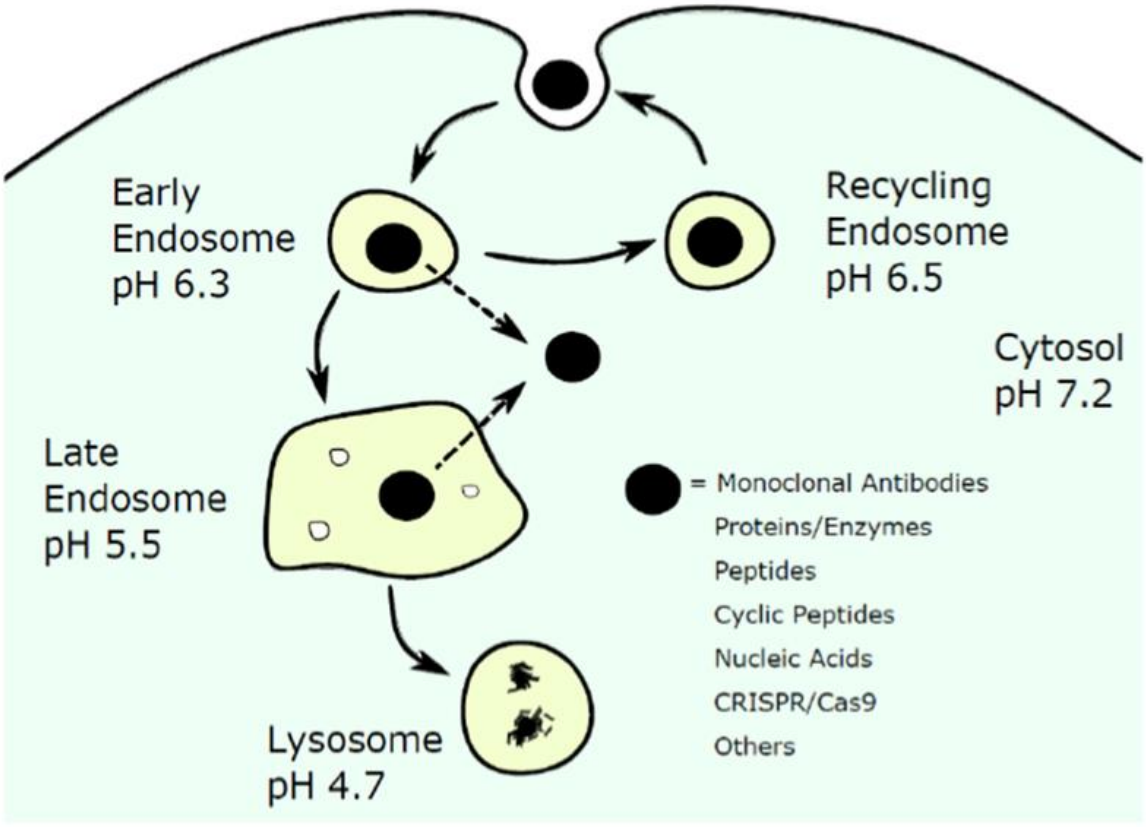


Figure 1. Illustration of endocytosis and endosomal entrapment. Foreign nanoparticles delivered into the cell being degraded in the lysosome as a result of acidification. (Pei and Buyanova 2019)

1.3.1 Nanoparticle endosomal release strategies

As mentioned previously, the endosomal release after entering the cells, is crucial for successful NA delivery. The exact escape mechanism is still unknown and several theories of the escape mechanism have been proposed for different delivery strategies. Among these hypotheses, pore formation, membrane disruption and vesicle budding and collapse can be applied to CPP based delivery (Pei and Buyanova 2019).

Depending on the interactions between the CPP and the phospholipid bilayer, two different types of pores can form on the cell membrane surface – barrel-stave pores and toroidal pores. The barrel-stave barrel-shaped pores form when peptides are interacting with the lipids in the membranes so they are located parallel to them (Shai 2002). The toroidal pores form as a result of perpendicular binding of peptides to the membrane surface (Jenssen, Hamill and Hancock 2006). Nevertheless, as the lipid bilayer's thickness is similar to macromolecules' average diameter, this hypothesis does not explain how macromolecules can

translocate through the endosomal membrane without blocking the pore (Pei and Buyanova 2019).

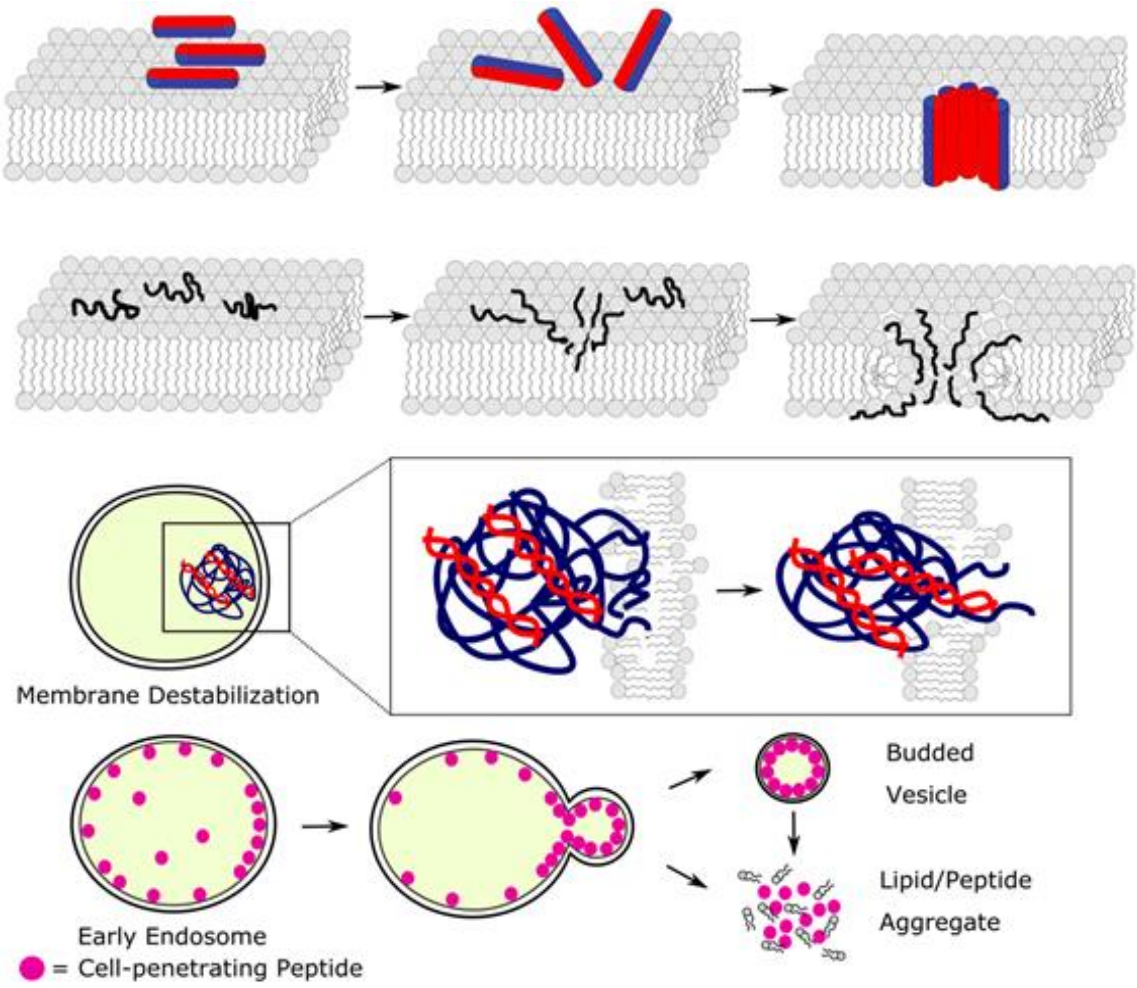


Figure 2. Proposed endosomal escape theories for CPP/cargo nanoparticles: Barrel-stave pore formation; Toroidal pore formation; Membrane destabilization / disruption; Vesicle budding and collapse theory. Adapted from (Pei and Buyanova 2019)

According to the membrane destabilization or disruption theory, either CPP alone or the NP interacts with the endosomal membrane through hydrophobic or charge-charge interactions. This leads to the disruption of cell membrane in the site of interaction. Because of the locality of the membrane disruption/destabilization, the majority of the NP cannot escape the endosome. The disruption requires physical interaction of the CPP or NP with the lipid bilayer, like in the pore formation. Limited by this, it offers little explanation how the escape is possible without complete disruption of the endosome (Pei and Buyanova 2019).

The vesicle budding and collapse hypothesis unlike other previous hypothesis, explains how macromolecular cargos escape endosomes. By this theory, NPs do not interact physi-

cally with lipid bilayers. Instead carriers, such as CPPs, attach to the endosomal membrane's luminal leaflet, causing membrane deformation and lipid domains enriched with CPP that form small vesicles. These vesicles subsequently break into lipid/peptide aggregates and the vesicular contents are released into the cytosol. At this time point at the budding neck CPPs are highly concentrated, which gives high potential energy because of membrane deformation. Endosomal acidification probably contributes to budding event with increasing binding likeliness of cationic CPPs. During this endosomal escape process endosomal membrane is not damaged and several vesicle budding and collapse events can happen simultaneously or sequentially on the same endosome (Pei and Buyanova 2019).

1.4 Human papillomavirus (HPV)

Papillomaviruses (PV) are very common, small, non-enveloped, icosahedral DNA viruses that infect skin and mucosa of most mammals, birds and reptiles. They are highly tissue-specific, with the exception of bovine papillomavirus 1 and 2, and although more than 200 different types of PV have been identified, the infection mechanisms are fundamentally similar (Aksoy, Gottschalk and Meneses 2017). Human papillomavirus (HPV) family with over 150 members are able to infect humans, and HPVs with transforming potential, especially HPV16 and HPV18, have been linked to development of cancers. They are the cause of almost all cases of cervical, and majority of head and neck cancers (Doorbar et al. 2012).

HPV16 8 bp DNA encodes six early proteins (E1-6) and two late proteins (L1 and L2). The L1 (major capsid protein) and L2 (minor capsid protein) self-assemble into icosahedral capsid, 55-60 nm in diameter. The capsid consists of 72 L1 pentamers, and L2 proteins buried in the inner surface of the pentamers, with the exception of a portion of the N-terminus of L2 (Buck et al. 2008), (Kondo et al. 2007). Although the L1 can assemble into VLPs, making L2 dispensable for capsid formation, the L2 dramatically increases the efficacy of DNA encapsidation (Darshan et al. 2004), (Buck et al. 2008)

The capsid proteins mediate the binding, internalization and trafficking of the virus. These processes additionally require host cellular components and mechanisms. PV as non-enveloped viruses have to cross or disrupt membranes during virus entry, whereas enveloped viruses can fuse with host membrane to enter. For most non-enveloped viruses, the passage of the capsid to the cytoplasm is mediated by lytic peptide released by the proteolytic cleavage of capsid protein (in our case the L2). This lytic peptide (with several simi-

larities to a CPP) physically disrupts the membrane and forms large pore (Zhang et al. 2018), enabling the escape.

During the entry the PV has evolved a unique mode of intracellular trafficking by harnessing membrane-bound retrograde transport vesicles. PV is, after interactions with cell surface receptors, subsequently internalized via a non-traditional endocytic pathway, which is clathrin-, caveolin-, flotillin-, dynamin- and cholesterol-independent. This novel pathway is related to macropinocytosis, as it involves actin dynamics, Na^+/H^+ exchangers, tyrosine kinase, p21-activated kinase and protein kinase C signalling; yet it is distinct from macropinocytosis due to lack of membrane protrusions and presence of small, evenly shaped vesicles rather than large and irregularly shaped vacuoles upon HPV16 endocytosis (Schelhaas et al. 2012)

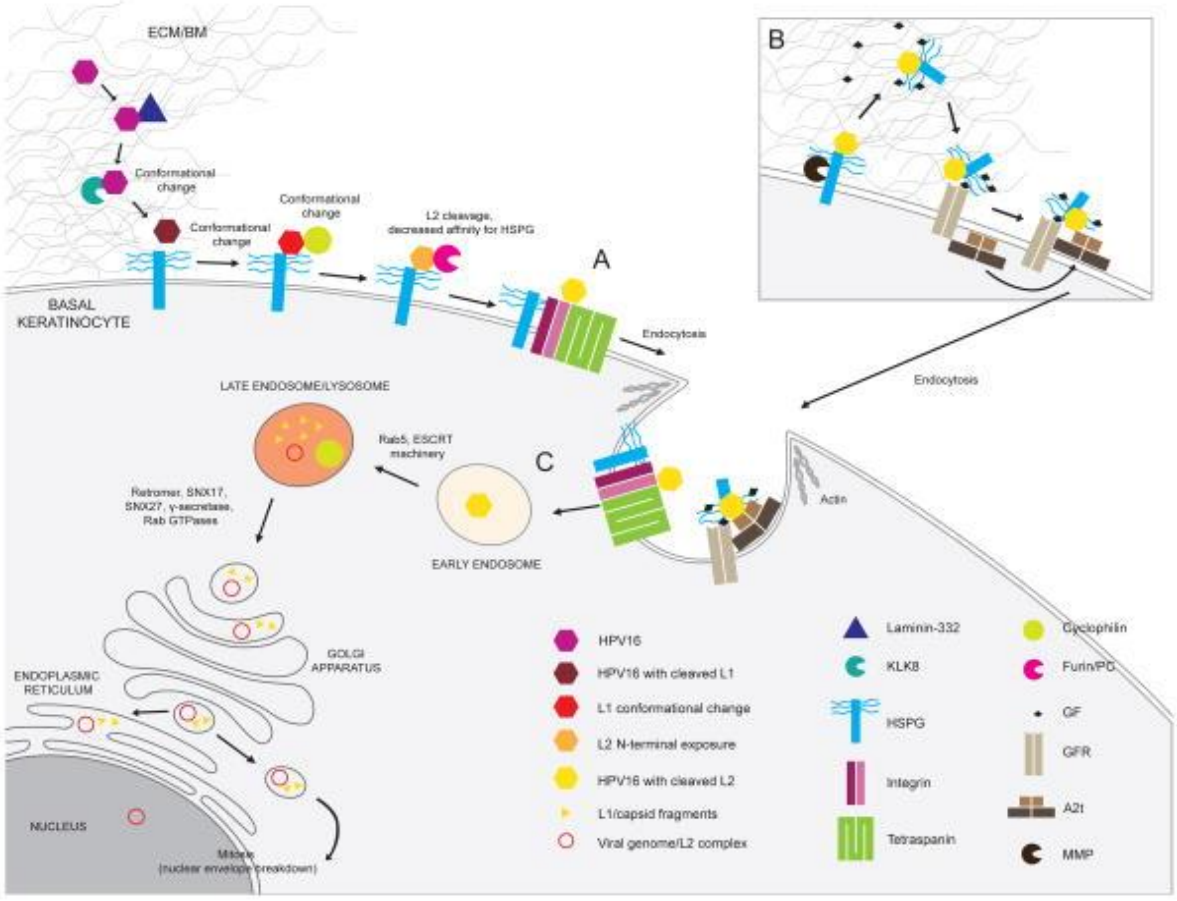


Figure 3. HPV16 binding, internalization and trafficking during host cell infection (Aksoy et al. 2017)

The entry and trafficking of HPV (Figure 3) starts with binding to Laminin-332 in the extracellular matrix (ECM)/basement membrane (BM). Kallikrein-8 (KLK8) splits the L1 protein which changes the viral capsid. On the cell surface HPV16 attaches to heparin sul-

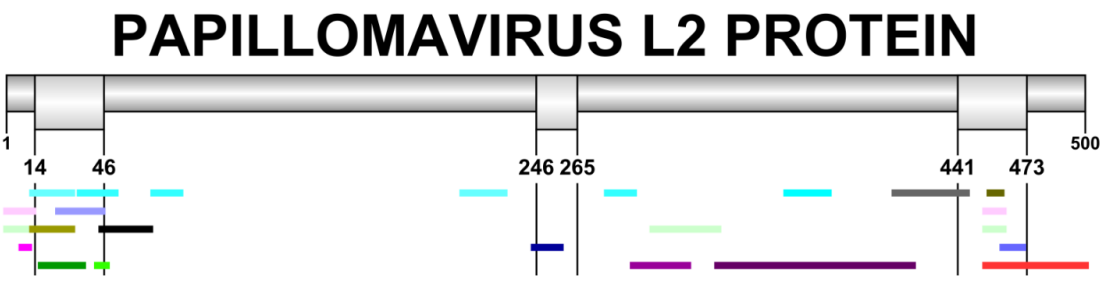
fate proteoglycans (HSPGs). Conformational changes reveal the N-terminal region of the L2 protein in the viral capsid and can be facilitated by cyclophilins. Furin/proprotein convertases (PCs) split the unprotected N-terminus of L2, causing reduced affinity of HPV for the HSPGs. It allows the virus to move to an uptake receptor system, which is not identified. The uptake receptor complex candidates are integrins and tetraspanins (Figure 3, A). Alternatively, during usual cell surface HSPG turnover matrix metalloproteases (MMPs) can cleave HSPGs with bound virus, as a result virus-HSPG complexes end up in the ECM (Figure 3, B). A growth factor of (GF)/HSPG/HPV16 system and growth factor receptors (GFRs) can afterwards interact with each other on the cell surface. GFRs activation can lead Annexin A2 tetramer (A2t) translocation from the inner leaflet to the outer leaflet of the plasma membrane. The HPV16/HSPG/GFR/A2t system may later be uptaken by the cell. Endocytosis happens via a mechanism similar to macropinocytosis involving actin (Figure 3, C). The virus moves through the endo-lysosomal system and L1 mostly disconnects from the viral DNA/L2 complex here. The viral DNA/L2 complex moves through the Golgi and can enter the endoplasmic reticulum (ER). Alternatively, before getting access to the nucleus it can stay in an ER cisternae during mitosis.

1.4.1 PV L2 protein and its functions in the virus entry, trafficking

HPV16 L2 protein contains 473 amino acids with regions for different functions and interactions (Figure 4). Although some of these functions/regions are the same for all PVs, others are more specific for human papillomavirus. The L2 protein is important for the virus entry, DNA trafficking into the nucleus and in the productive phases (Darshan et al. 2004).

As mentioned above the L2 protein has several functions which are important for the HPV:

- Import of viral DNA into the nucleus via binding to host importins.
- Localisation of viral DNA to host PML-bodies in the nucleus for activating early gene expression (infection).
- Interacting with the E2 early protein in order to promote late gene expression and inhibit its transcriptional activation (Okoye et al. 2005).
- Interacts with the viral genome and encapsides the genome during virion assembly



- surface exposed - 13-31 (Yang et al. 2003b); 35-51; 69-81; 212-231; 279-291; 362-381) - (Kawana et al. 1998)

- DNA binding (1-13; 454-462) - (Bousarghin et al. 2003)

- L1 binding domain – found in HPV11, but form sequence homology HPV16 also, 412-455

- NLS - 300-330 - (Florin et al. 2006) and 440-445 - (Finnen et al. 2003); 1-13 ($_{1}$MRHKRSAKRTKRA$_{13}$) and 454-462 ($_{455}$LRKRRKRL$_{462}$)- (Darshan et al. 2004), (Wang and Roden 2013)

-Receptor binding site - 13-31 - (Yang et al. 2003a)

- Actin binding site -25-45 - (Yang et al. 2003b)

- Sortin Nexin 17 binding site 245-257 (Bergant Marušič et al. 2012), virion trafficking (Pim et al. 2015), late endosome escape (Bergant Marušič et al. 2012)

- Dynein interacting domain 456-461 (Florin et al. 2006) is involved in interactions with host dynein in the intracellular microtubule-dependent transport of viral capsid towards the nucleus (Schneider et al. 2011)

- NES 462 - 472 (Mamoor et al. 2012)

- furin cleavage site 8-11 is required for viral trafficking after the endosome (localisation in TGN (Pereira, Hitzeroth and Rybicki 2009)

- membrane destabilising peptide 454-500 (Kämper et al. 2006), includes a CPP sequence (Zhang et al. 2018). It possesses a CPP sequence that protrudes through the host cell endosome, interacts with cytoplasmic retromer cargo and mediates the capsid delivery to the TGN (Popa et al. 2015).

- ND10 localisation signal 330-420 (Pereira et al. 2009)

- tSNARE syntaxin 18 protein interaction site 43-47 mediates vesicular transportation to and from ER (Bossis et al. 2005)

- central nuclear retention sequence in the HPV16 L2 (amino acids 291–316) is important for L2 retention in the nucleus (Pim et al. 2015), (DiGiuseppe et al. 2014)

-N-terminal transmembrane domain (Bronnimann et al. 2013)

Figure 4. A) PV L2 protein domains with their known functions – adapted from (Pereira et al. 2009) and (Wang and Roden 2013).

The pH reduction and different cellular host proteins contribute the degradation and/or disassembly of the viral capsid, that is an important step for successful internalization of the L2/pseudogenome complex into the nucleus (Kämper et al. 2006). However, it is not clear if low pH is a direct reason for capsid degradation or plays an indirect role, for example, activation of cellular proteases responsible for viral capsid degradation.

Trafficking from the endosomal to the trans-Golgi network (TGN)/Golgi system and subsequent compartments has been found to be facilitated by L2 protein and a different host cellular factors, such as Sorting Nexin 27 (SNX27), Sorting Nexin 17 (SNX17), γ-secretase and retromer complex components (Aksoy et al. 2017). The pseudogenome without L2 is not independently trafficked to TGN (DiGiuseppe et al. 2014). It has been hypothesised that L2 mediates the L2/pseudogenome complex trafficking out of endosomal compartments by the capacity of transmembrane domains at L2 N- and C-termini to destabilize membranes (Bronnimann et al. 2013, Day et al. 2013).

SNX27 interacts with HPV16 L2 via the central region of L2 and a PDZ domain. SNX17 is involved in the trafficking of the L2/pseudogneome complex from late endosome/lysosomal compartments (Bergant Marušič et al. 2012). SNX17 interacts with L2 protein through NPxY binding motif and viral escape from LE is facilitated by the SNX17/L2 interaction. It was hypothesised that the L2/SNX17 interaction prevents the virus from trafficking to the lysosome. SNX17 increases the L2 protein stability by delaying the trafficking via the endosomal compartments. These studies proposed that the L2/SNX17 interaction is needed for the L2/pseudogenome complex trafficking from the late endosome/lysosome.

The activity of the γ-secretase, cellular membrane-associated protease, is important for HPV infection, exit from endosome and entry to Golgi and ER (Zhang et al. 2014). HPV16 L2 binding to the retromer complex is crucial for HPV trafficking from the endosomal pathway and localization to the TGN (Popa et al. 2015). It was proposed that L2 interacts with the retromer complex via the endosomal membrane and the hypothesis is supported by a study which demonstrates the L2 C-terminal transmembrane site has membrane penetrating ability. The mutations to the HPV16 L2 C-terminus (Kämper et al. 2006) are located downstream from the identified retromer recognition sites (Popa et al. 2015). The C-terminal deletion residues from L2 protein disrupted the ability of the protein to penetrate membranes. It could have prevented exposure of retromer binding domains from the endosomal membrane (Day et al. 2013).

HPV16 L2 interacts with dynein, a motor protein which drives retrograde transport along microtubules, particularly with DYNLT1 and DYNLT3, the dynein light chains. The L2 dynein interaction domain was depicted to the C-terminal 40 domains, which is a transmembrane domain able to penetrate membranes, such as endosomes. The close contact of these two domains shows the possibility of L2 to interact with dynein via a vesicular membrane while translocation to following compartments during infection (Kämper et al. 2006, Florin et al. 2006, Schneider et al. 2011)

HPV16 L2 N-terminal residues 25–45 interacts with actin (Yang et al. 2003b). N-terminal transmembrane site is located downstream (45–67 residues) from the actin-binding domain, providing the possibility that L2 N-terminus could interact with β-actin across an endosomal membrane while translocating to subsequent compartments (Bronnimann et al. 2013).

2 THE AIMS OF THE THESIS

The aim of this research work is to increase the efficacy of existing CPP/pDNA nanoparticle transfection method by applying mechanisms used by viruses – in this case HPV.

First HPV16 L2 protein was tested for increasing transfection efficacy.

As the next step different short peptides were designed based on known functions of the L2 protein.

Later new peptides were designed by incorporating further CPP-like peptide elements.

After the design of the peptides they were synthesized and tested in order to find out their effect on transfection efficacy of CPP/pDNA nanoparticles.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Materials

Materials used in this work are provided on supplementary.

3.1.2 Cell culture maintenance

Chinese hamster ovary cell subpopulation K1 (CHO-K1) was chosen for experiments in this work. The CHO cell line is widely used mammalian cell line. The cells were maintained on 100 mm tissue culture dishes at 37 °C and 5% CO_2 in Dulbecco´s Modified Eagle´s Medium (DMEM). DMEM is a widely used basal medium suitable for a range of cell lines. To support the cell proliferation and growth, media was supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% foetal bovine serum (FBS). 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin antibiotics were added to the media to limit the growth of gram-positive and gram-negative bacteria. Cells were grown as a monolayer and passaged regularly by the time reaching ~90% cell confluency. Except where marked differently, serum containing media was used for all experiments.

1 x PBS (w/o Ca and Mg) was used for washing cells between steps, to remove cell debris and traces of media. For detachment of cells for passaging or prior seeding, cells were washed with 1 x PBS and 0.25% trypsin-EDTA was added and further incubated. The wash is needed, as the components in the media can reduce the enzymatic activity of trypsin. Trypsin is a serine protease that cuts peptide chains. When added to the cells the adhesion proteins by which the cells attach to the plate, are cut and the cells detach from the plate. DMEM media was used to wash the cells of the plate, inactivate trypsin, and dilute the cells to the required cell density. The number of cells was detected from a small sample mixed 1:1 with 0.4% trypan blue. The trypan blue dye helps to create contrast between viable cells and background, and additionally, as it is able to cross damaged or dead cell membranes, the dead cells can also be distinguished. For counting and viability measurement CytoSMART cell counter (The Netherlands) was used.

3.1.3 Peptide synthesis

Peptides were synthesized manually or by an automated peptide synthesizer (Biotage® Initiator+ Alstra™), using fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis technique with Fmoc-Rink-amide ChemMatrix resin (0.4 mmol g^{-1} loading) for obtaining C-terminally amidated peptides. Following de-protection with 20% piperidine/DMF (2 x 20 min), Fmoc-protected amino acids (5 eq) were coupled using 5 eq. TBTU and 5 eq. HOBt as activators and 10 eq. of DIEA as a base (1 h, RT). Cycles of de-protection and coupling were repeated until desired sequence was obtained. The fatty acid (5 eq.) was coupled manually to the unprotected N-terminus of P1-C18, P2-C18, P3-C18, P4-C18 and P5-C18 peptides and the reaction was allowed to proceed overnight at the room temperature.

Table 2. Peptides synthesized for this study. FA – fatty acid (stearic acid, C18) coupled to the N-terminus of the peptide sequence. O* - synthesis is continued from the sidechain amino group of ornithine.

Peptide	Sequence (C-terminally amidated)	Synthesis mode
NF51	Stearoyl-AGYLLGO*INLKALAALAKKIL	Machine synthesis, manual addition of FA
P1	ADAGDFYLHPSYYMLRKRRKRLPYFFSDVSLAA	Machine synthesis
P1-C18	Stearoyl-ADAGDFYLHPSYYMLRKRRKRLPYFFSDVSLAA	Machine synthesis, manual coupling of FA
P2	PTKLITYDNPAYEGIDVDNT	Machine synthesis
P2-C18	Stearoyl-PTKLITYDNPAYEGIDVDNT	Machine synthesis, manual coupling of FA
P3	ASATQLYKTCKQAGTCPPDIIPKVEGKTIAEQIL	Machine synthesis
P3-C18	ASATQLYKTCKQAGTCPPDIIPKVEGKTIAEQIL	Machine synthesis, manual coupling of FA
P4	FLYALALAALRKRRKLAA	Machine synthesis
P4-C18	Stearoyl-FLYALALAALRKRRKLAA	Machine synthesis, manual coupling of FA
P5	YYLALALAALKKRKRLAA	Machine synthesis
P5-C18	Stearoyl-YYLALALAALKKRKRLAA	Machine synthesis, manual coupling of FA
P6	LITYDNPAYEFYLHPSYYMLRKRRKR	Manual synthesis
P7	NPAYEGIDFYYMLRKRRKR	Manual synthesis

Cleaving peptides from the resin was achieved by adding a mixture of trifluoroacetic acid, 2.5% triisopropylsilane and 2.5% water (2 h, RT) to resin-bound peptides. A gradient of acetonitrile/water containing 0.1% TFA was used for peptide purification on a C4 column (Phenomenex Jupiter C4, 5 µm, 300 Å, 250 × 10 mm) by reversed-phase high-performance liquid chromatography (HPLC). Matrix-assisted laser desorption-ionization (MALDI)/time of flight mass spectrometry (Bruker Microflex LT/SH, USA) was used for analysing the molecular weight (MW) of the synthesised peptides. Dilutions of accurately weighed substances and absorption of tyrosine, where applicable, were basis for determination of the peptide concentrations (Arukuusk et al. 2013).

3.1.4 Formation of nanoparticles between CPPs and plasmid

Transfection is mediated by so-called complexes. Complex is a mixture of CPP (NF51), pDNA and water with addition of HPV16 L2 protein or peptides derived from this protein (PX: P1 – P7). Complexes were formed by applying different mixing strategies of added components (Figure 6).

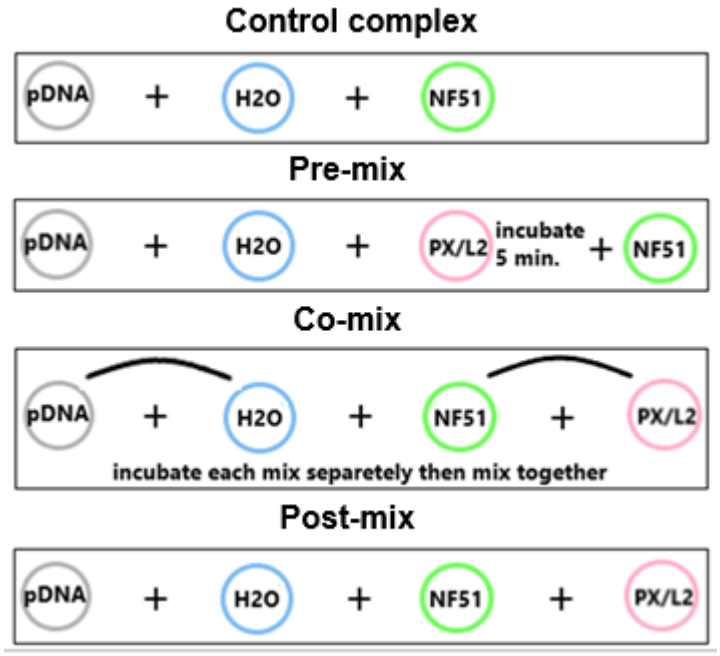


Figure 6. Mixing strategies for CPP/pDNA and peptide or protein (L2) complexes. NF51 is a CPP and PX is any of peptides (P1 – P7) derived from HPV L2 protein.

Complexes were formed using a non-covalent complex formation strategy, where plasmid and CPP form stable nanoparticles in the water environment spontaneously due to electrostatic and hydrophobic interactions. This complex formation strategy was chosen as it is very versatile, conditions can be varied without the need to synthesise new peptides or

without the need to modify the cargo. In addition, this complex formation strategy allows adding different components, such as other peptides or even protein, to the solution. Complexes formed between CPP, NF51 and pDNA were made based on charge ratio (CR), which takes into account the positive charges on the peptide surface and negative charges in the plasmid DNA phosphate backbone

All the synthesised peptides have different charges and some of them even have negative charge. Therefore, the addition of HPV peptides was done based on their final concentration on the cells. Different concentrations of L2 protein and peptides were tested to find optimal concentrations which would increase transfection efficacy and not affect viability of cells. As a result, 0.25, 0.5 and 1 uM final concentrations of HPV derived peptides were used for screening and 0.5 uM final concentration was used for complexation assessment.

In order to evaluate complex formation, PicoGreen® DNA quantitation assay was used for measuring DNA in complex mixtures. 90 ul of MQ solution was added to black well-plate (w/p). Pre-incubated complexes were added to the wells and mixed by pipetting. Subsequently, 10-20 ul of PicoGreen working solution (25 ul master and 2975 ul MQ) was added to each well. Results were read by Sunrise™ absorbance microplate reader (Tecan Austria GmbH).

3.1.5 Transfection efficacy measurement – total reporter protein levels detection from cell lysate

24 h prior experiment 10,000 cells per well were seeded on flat-bottom transparent 96 well-plates in serum containing media and incubated at 37 °C and 5% CO_2 overnight.

For the detection of total reporter protein levels transfection was performed with pMC.BESPX.GauFluc2. Plasmid dose per well was 0.1 ug. Complexes were added in 1/10th of the final volume to the cells. After transfection, cells were further incubated for ~24 h, at 37 °C and 5% CO_2. Following this, media was aspirated, cells were washed with 1xPBS and 0.1% Triton X-100 solution was added for cell lysis. Cells were kept at +4 °C for 20 min for lysis.

During incubation time of cells, for screening the intracellular delivery efficacy fresh luciferase detection solution, luciferin was mixed using 25 mM DTT, 1 mM D-luciferin, 1mM ATP, 25 mM Coenzyme A, 1mM EDTA, 20 mM Tricine, 1 mM $MgCO_3$, 5 mM $MgSO_4$ and ultrapure water. 20 ul of incubated cell lysate was transferred to black frame white

well 96-well plates and 100 ul of luciferin solution was added for reaction. Luminescence was detected using GLOMAX™ 96 luminometer (Promega, Sweden) (Helmfors, Eriksson and Langel 2015).

The remaining cell lysate on transparent 96 w/p was used for total protein quantification in wells. BCA Protein Assay Kit was used for the quantification and standard dilution series was used as a reference. 100 ul of the protein assay kit solution was added to the remaining 10 ul of cell lysate. After 30 min incubation at room temperature absorption was measured at 562 nm wavelength by microplate reader.

3.1.6 Transfection efficacy measurement – population of transfected cells assessed by flow cytometry

Fluorescence-activated cell sorting (FACS) analysis was used for measuring transfected cell population. For this 50,000 cells per well were seeded on 24 well-plate and transfected with plasmid (pEGFP-C1) encoding green fluorescent protein.

Complexes were mixed with 0.5 ug pDNA per well. Media on cells was changed to 500 ul of fresh serum containing media (untreated cells) or 450 ul of fresh media and 50 ul of complex and 4 h after adding complexes the media was changed and cells further incubated for 20 h.

After incubation media was aspirated, cells were washed with 1 x PBS to get rid of media components and 100 ul of 0.25% trypsin-EDTA was added for detachment of cells from the plate. After cells' detachment, the cells were washed off the plate with 400 ul of 1 x PBS supplemented with 1% FBS. The FBS was added to prevent non-specific binding. The cells' suspension was transferred to clean 1.5 ml tube and samples were analysed on Attune™ NxT Flow Cytometer (Thermo Fisher Scientific). GFP positive cell population was determined based on two parameters: side scattered light (SSC) and forward scattered light (FSC). Gating 0.1%, cells with SSC and FSC according to untreated cells.

3.1.7 Lysosomal disruption/endosomal escape – neutral red assay

For the lysosomal disruption/endosomal escape neutral red assay was used, which is based on lysosomal uptake of neutral red dye. For the experiment 15,000 cells per well were seeded on 96 well-plate.

Complexes were prepared as described above. The media was changed to fresh phenol red free DMEM media and 10 ul of complexes (0.1 ug pDNA dose per well) and 20 ul of neutral red solution (0.33% in DPBS). Cells were incubated at 37 °C and 5% CO_2 for 5.5 h after which cells were washed with 1 x PBS, aspirated dry and 100 ul of neutral red detection mix was added (50% ethanol, 1% acetic acid in ultrapure water) and cells incubated in dark and on the shaker at room temperature for 10 min. Absorption was measured with the Sunrise™ (540 nm). Background of cell free wells with the same treatments (media, neutral red, PBS, detection mix) was deducted and untreated cells (cells w/o complexes) were taken as 100% (Repetto, del Peso and Zurita 2008).

3.1.8 Statistics

Statistical analysis was performed in GraphPad Prism software. T-test was chosen for transfection efficacy of peptides and Bonferroni was used for endosomal escape evaluation.

3.2 RESULTS AND DISCUSSION

3.2.1 Transfection with HPV L2 protein

Previously different approaches have been developed to increase cellular delivery and endosomal escape of macromolecular therapeutics, which are important challenges in today's medicine. As one of these approaches, cell penetrating peptides are broadly used. It has been shown that stearylated CPPs, like NickFect family, when associated with cargo enter into the cells by endocytosis. Particularly NF51 is taken up by cells rapidly, and escapes endosomal pathway (Pärnaste et al. 2016).

In this work CPP NickFect51 was used to transfect reporter plasmid into CHO cells. For this complexes were formed at different CRs to find the optimal conditions for this peptide, and for these conditions (seeded cell density, media, incubation time, plasmid dose). As a pre-screening experiment for this work 4 different CRs of NF51/pDNA complexes were tested in CHO cells with serum containing media in order to find optimal CRs for further experiments. Transfection was done with luciferase encoding plasmid and luminescence measurement was done 24 h after the transfection. From tested CRs 2:1 and 3:1 were found to be the efficient having significant difference from CR1 and CR4 (Figure 7). Cells were treated only with pDNA, w/o addition of NF51, and untreated cells were included in experimental setup as controls.

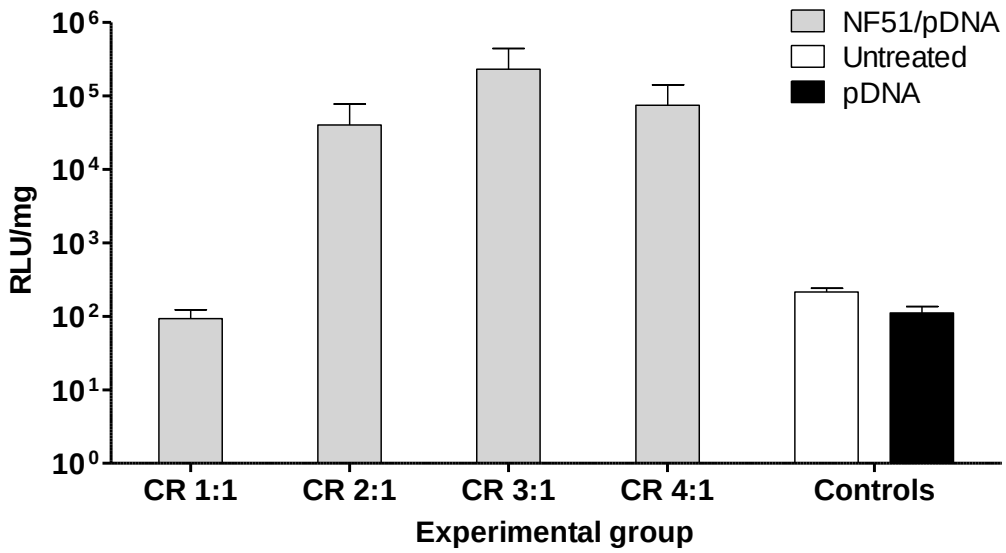


Figure 7. Transfection of CHO cells with non-covalently formed NF51/plasmid NPs. The NPs were formed at different CRs and as a negative control plasmid and untreated cells were used. Transfection efficacy was measured with luciferase assay and luminescence measurements were normalised to absorbance of reporter protein based.

It has been found that L2 late protein of HPV 16 together with viral genome escapes late endosome (Bergant Marušič et al. 2012), which lead to hypothesis that transfection of cells with presence of L2 increases cellular internalization and endosomal escape of CPP/NA nanoparticles. Based on this hypothesis, L2 protein was added to the NF51/pDNA nanoparticle complexes for treatment of CHO cells in serum containing media, as in previous experiment, with the aim to increase their transfection efficacy. In order to perform transfection of NF51/pDNA complexes with L2 protein/peptide addition, experiments were done with CR2 of NF51 using post-mix complexation strategy with different concentrations of L2 protein. For transfection of the luciferase encoding plasmid w/o NF51, 3 different concentrations of L2, 0.1, 0.5 and 1 uM were tested and it was found that L2 alone (w/o CPP) does not increase transfection efficacy irrespective of it concentration. Although for transfection of NF51/pDNA complexes with significantly lower concentration of L2 results were positive: L2 increases transfection efficacy of NF51/pDNA complex 10 fold with 0.13 uM concentration (Figure 8).

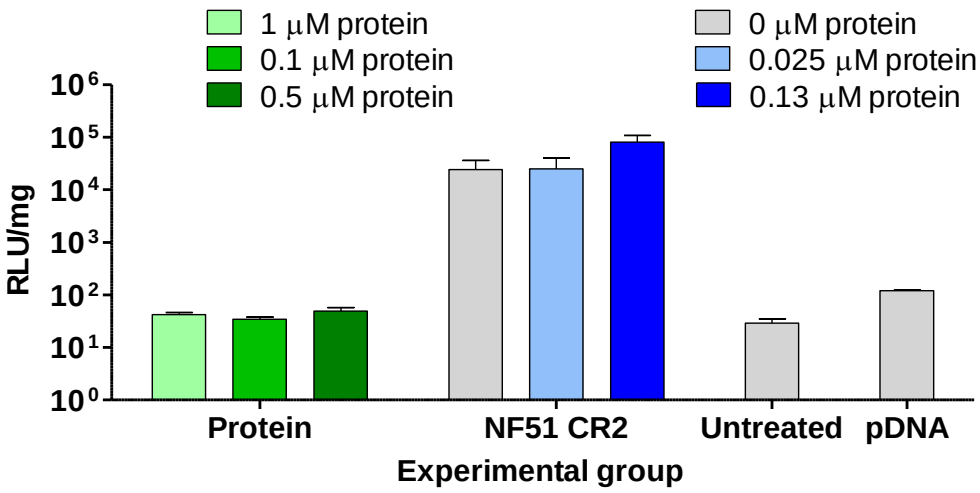


Figure 8. Transfection of CHO cells with non-covalently formed NF51/pDNA NPs and HPV L2 protein. Plasmid and untreated cells were used as negative controls. Transfection efficacy was measured with luciferase assay and luminescence measurements were normalised to absorbance of reporter protein. L2 protein increases transfection efficacy of NF51/pDNA NPs.

Next, FACS analysis were performed according to the described protocol (chapter 3.1.6) for identifying percentage of transfected cell population with the same experimental conditions as in previous experiments (Figure 9).

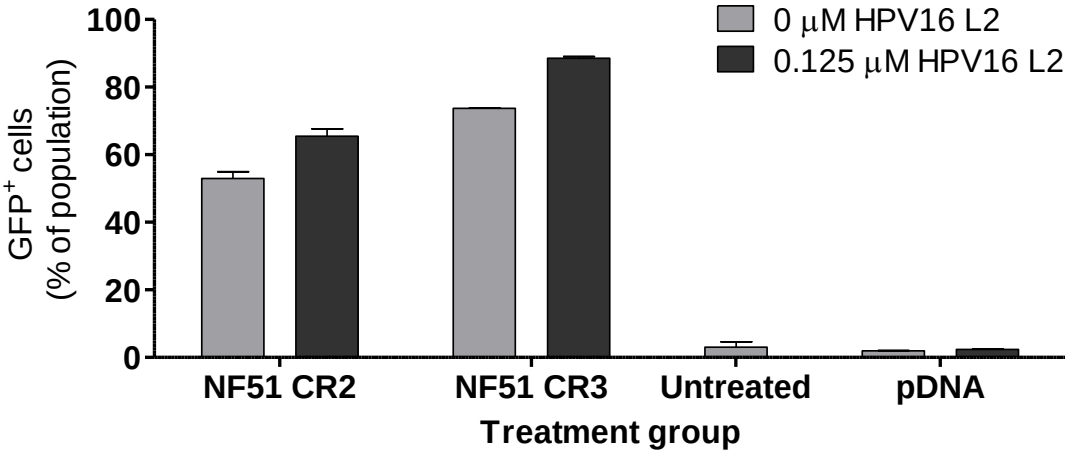


Figure 9. FACS analysis – transfected cell population percentage measurement. CHO cells were transfected with non-covalently formed NF51/pDNA NPs and HPV L2 protein. Plasmid and untreated cells were used as negative controls. Addition of HPV L2 protein to NF51/pDNA complexes increases population of transfected cells.

Cells were transfected with pEGFP-C1 and addition of similar concentration of L2 protein to the complexes. It was found, that HPV L2 protein increased transfected cell population by approximately 20% in both, CR2 and CR3 of NF51 and reaching 90% in case of CR3 (Figure 9). This result is especially impressive for applying L2 protein in therapeutic protein production.

One of the important parameters for biological applications, cytotoxicity of the L2 protein, was measured after transfection. Results show that L2 protein is not toxic for cells and can be an efficient tool for in vivo applications, as well as for therapeutic protein production (Figure 10).

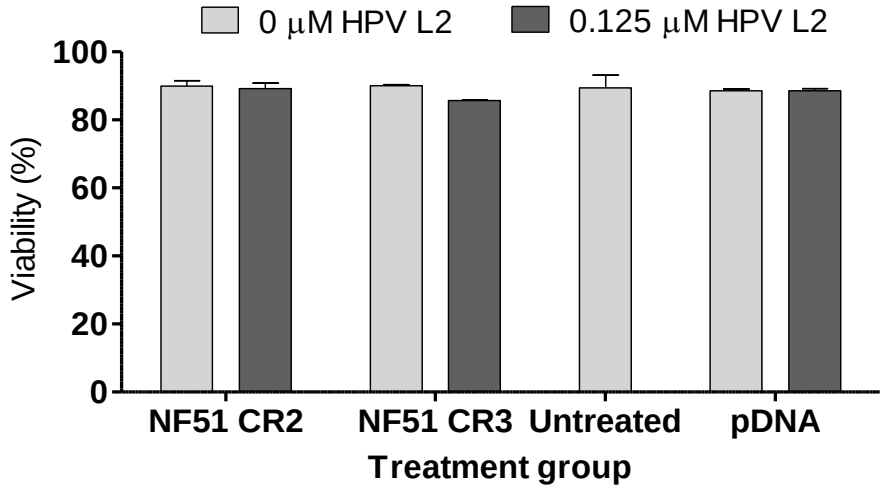


Figure 10. Viability of transfected cells after L2 protein treatment. CHO cells were transfected with non-covalently formed NF51/pDNA NPs and HPV L2 protein. Plasmid and untreated cells were used as negative controls. L2 protein does not affect morphology and granularity of cells.

3.2.2 Design basis of the peptides

The promising results with L2 protein inspired the design of shorter peptides derived from L2 protein. As explained in the literature review section of the thesis, L2 has different domains with functions responsible for cellular uptake and endosomal escape (Pereira et al. 2009). Three different approaches were applied for peptide design.

For the first screening of peptide design, specific regions from the HPV 16 L2 protein were chosen that have been related to either cellular contacts or endosomal escape functions (Figure 11).

MRHKRSAKRTKRASATQLYKTCKQAGTCPPDIIPKVEGK-
TIAEQILQYGSMGVFFGGLGIGTGSGTGGRTGYIPLGTRPPTATDTLAPVRP-
PLTVDPVGPSDPSIVSLVEETSFIDAGAPTSVPSIPPDVSGFSITTSTDTTPAILD-
INNTVTTVTTHNNPTFTDPSVLQPPTPAETGGHFTLSSSTISTHNYEEIPMDTFIVST-
NPNTVTSSTPIPGSRPVARLGLYSRTTQQVKVVD-
PAFVTTPTKLITYDNPAYEGIDVDNTLYFSSNDNSINIAPDPDFLDI-
VALHRPALTSRRTGIRYSRIGNKQTLRTRSGKSIGAKVHYYYDLSTIDPAEEIEL-
QTITPSTYTTTSHAASPTSINNGLYDIYADD-
FITDTSTTPVPSVPSTSLSGYIPANTTIPFGGAYNIPLVSGPDIPINITDQAPSLIPIVPG-
SPQYTIIADAGDFYLHPSYYMLRKRRKRLPYFFSDVSLAA

Figure 11. HPV16 L2 sequence used for peptide selection. With marked HPV16 L2 14-46, HPV16 L2 246-265 and HPV16 L2 441-473 chosen for this work. >sp|P03107|VL2_HPV16 Minor capsid protein L2 OS=Human papillomavirus type 16 OX=333760 GN=L2 PE=1 SV=1.

The HPV16 $L2_{441-473}$ peptide (P1) is derived from the L2 region w/o modifications. In the L2 protein sequence aa 456-462 is a nuclear localisation signal (NLS) (Wang and Roden 2013), aa 456-461 covers a dynein interacting domain (Florin et al. 2006), DNA binding site in aa 454-462 (Bousarghin et al. 2003), part of L1 binding site in aa 412-455 (Finnen et al. 2003), and aa 462-472 is a NES (nuclear export signal) (Mamoor et al. 2012). This also includes a part of a membrane destabilising peptide in aa 454-500 found in PV (Kämper et al. 2006). The arginine-rich part was discovered to possess similarities with CPPs and it probably protrudes through endosomal membrane allowing interactions with cytoplasmic retromer cargo, which mediates the trans-Golgi delivery of PV capsid (Popa et al. 2015). In the sequence, the dynein interaction site and the transmembrane domain capable of penetrating membranes are in close contact, and it has been suggested that possibly L2 could interact with dynein through a vesicular membrane while trafficking to subsequent compartments during infection (Schneider et al. 2011), (Florin et al. 2006, Kämper et al. 2006). The aim of including this sequence was to increase cellular uptake of CPP/pDNA complexes.

The HPV16 $L2_{246-265}$ peptide (P2) is derived from L2 region, which has been shown to include Sortin Nexin 17 (SNX17) binding site at aa 245-257 (Bergant Marušič et al. 2012) and has been related to both virion trafficking (Pim et al. 2015) and trafficking

L2/pseudogenome complex from late endosome/lysosome (Bergant Marušič et al. 2012). The SNX17 is binding to a highly conserved binding motif (NPAY) and when SNX17/NPAY interact, the trafficking through endosomal compartments might be delayed and SNX17 mediates the endosomal escape from late endosomes preventing the degradation in lysosomes. SNX17 was also found to enhance the stability of L2 by delaying the trafficking in the endosomal pathway (Bergant Marušič et al. 2012). The aim of including this part of the sequence was to alter the trafficking pathway used by NF51/pDNA complexes.

HPV16 $L2_{14-46}$ peptide (P3) is derived from L2 N-terminal part and from a multifunctional domain. In the L2 protein aa 13-31 is a receptor binding site able to bind to the cell surface (Roden et al. 2000, Yang et al. 2003a), aa 25-45 is actin binding site (Yang et al. 2003b), (Bronnimann et al. 2013) and aa 13-31 and 35-51 are exposed on the surface (Yang et al. 2003a), (Kawana et al. 1998). The peptide is downstream from two consensus furin cleavage sites, Arg5 (2RHKR5) and Arg12 (9RTKR12), and for efficient infection the cleavage at Arg12 is crucial (Bronnimann et al. 2016).

For the second round of the peptide design, two strategies were chosen: a) predicted CPP, based on the C-terminal region of L2 (P1), and b) predicted CPP, based on fused parts from P1 and P2. For the first, predictions were based on HPV16 or HPV18 L2 protein C-terminal region, and screened these for predicted CPPs. The P4 and P5 peptide sequences were designed based on HPV16 or HPV18 L2 protein C-terminal region. While designing peptides, program developed for cell-penetrating peptide prediction (Hällbrink) was used to predict if designed peptide sequence was a potential CPP. The peptides include a CPP sequence (Zhang et al. 2018).

Secondly, fusing sections from HPV16 $L2_{246-265}$ and HPV16 $L2_{441-473}$, predicted as CPP. The P6 and P7 were designed based on sections from HPV16 $L2_{246-265}$ (NPAY region needed for SNX17 binding and trafficking) and HPV16 $L2_{441-473}$ (NLS, DNA interacting site, dynein interacting domain, CPP), fused and predicted as CPP.

The stability of the nanoparticles, especially when non-covalent complexation strategy is used, increases with the addition of fatty acid moieties to the peptides (Pärnaste et al. 2017). P1 – P5 peptides, were also synthesised with fatty acid modification. As there was no significant increase of efficacy or observed decrease, P6 and P7 peptides with C18 were not included (Figure 12).

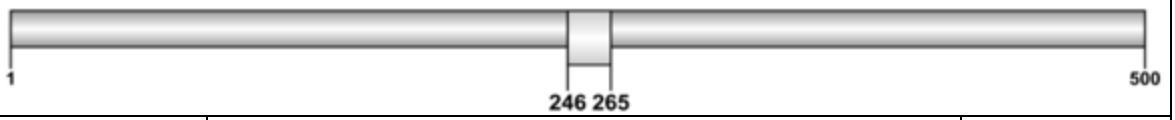
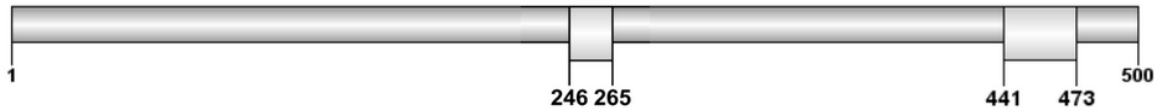
Peptide	Sequence (C-terminally amidated)	Origin
Peptides derived from the protein sequence without extensive modifications		
P1	ADAGDFYLHPSYYMLRKRRKRLPYFFSDVSLAA	HPV16 L2
		
P2	PTKLITYDNPAYEGIDVDNT	HPV16 L2
		
P3	ASATQLYKTCKQAGTCPPDIIPKVEGKTIAEQIL	HPV16 L2
		
Peptides derived from L2 protein sequence C-terminal region, predicted as CPP, modified		
		
P4	FLYALALAALRKRRKLAA	HPV16 L2
P5	YYLALALAALKKRKRLAA	HPV18 L2
Peptides derived from fusing sections of HPV16 $L2_{246-265}$ and HPV16 $L2_{441-473}$, predicted as CPP		
		
P6	LITYDNPAYEFYLHPSYYMLRKRRKR	HPV16 L2
P7	NPAYEGIDFYYMLRKRRKR	HPV16 L2

Figure 12. Peptide sequences synthesised from HPV16 L2 protein.

3.2.3 Synthesis of peptides

After the design, all peptides were synthesised either by automated peptide synthesiser, or manually, purified by reversed-phase HPLC, MW were calculated by MALDI and all peptides except P3-C18 were tested in cell-culture experiments with CHO cells. The P3-C18 was hard to purify and the final yield from synthesis was non-existent and therefore it was not possible to test the peptide in vitro (Table 3).

Table 3. Synthesised HPV L2 derived peptides with their MW and yield.

Peptide	MW	Mass (mg)	Peptide	MW	Mass (mg)
HPV16 P1	3952	**40.5**	HPV16 P1-C18	4235	**7**
HPV16 P2	2236	**19**	HPV16 P2-C18	2519	**1.9**
HPV16 P3	3655	**5.9**	HPV16 P3-C18	3938	**0**
HPV16 P4	2042	**11.9**	HPV16 P4-C18	2324	**4.7**
HPV18 P5	2030	**20.7**	HPV18 P5-C18	2312	**28**
HPV16 P6	3391	**6.6**	HPV16 P7	2492	**2.6**

The C-terminal region of P1 has poor solubility, which complicates its synthesis and purification. It is also one of the reasons why for last peptides the C-terminal part after K/R-rich domain was left out. The K/R-rich C-terminal part also makes the peptide a CPP, so placing this at the terminal would be more rational (Zhang et al. 2018).

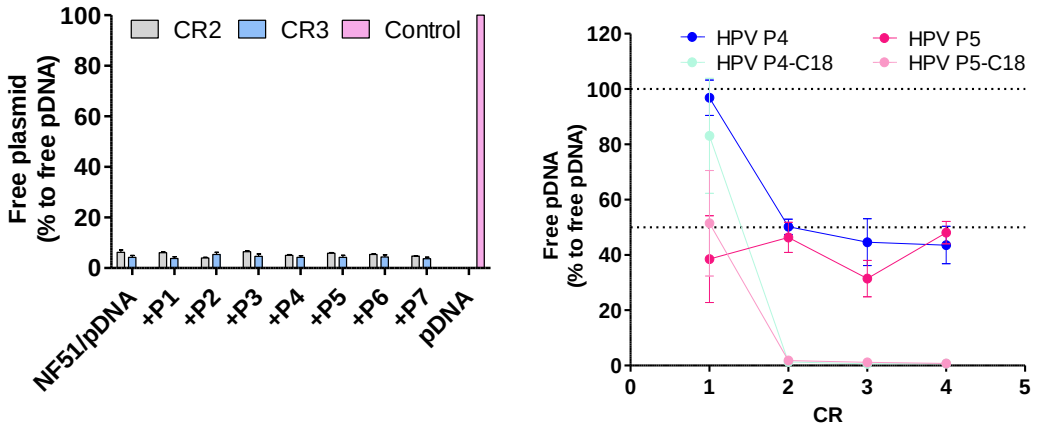


Figure 13. Formation of NF51/pDNA complexes with HPV L2 derived peptides. The addition of HPV L2 derived peptides do not affect the stability and the peptides are able to form complexes with the DNA. The ones with C18 fatty acid group are more effective in complex formation. Fluorescence normalised to free plasmid.

Experiments with PicoGreen assay showed that all peptides interact with CPP/pDNA complexes and form nanoparticles. P1 peptide is able to bind pDNA but not fully condense it. As it was expected based on previous studies, N-terminally stearylated peptides are more effective in binding DNA and forming nanoparticles (Figure 13).

3.2.4 Transfection with HPV L2 protein derived peptides

As the results with the HPV L2 protein experiments showed increase in transfection efficacy of CPP/pDNA nanoparticles, the second main aim was to investigate if the increased transfection efficacy could be achieved with the addition of peptides derived from the protein. The main part of the work contains transfection experiments of synthesised peptides with different concentrations and mixing strategies. The advantage of using peptides over protein is that it is easier and cheaper to synthesise short peptide sequences from the protein using main functional domains, rather than to use the whole protein.

Transfection of peptides was done with the same protocol and conditions as for protein. Separate graphs for each peptide with all used concentrations and mixing strategies, as well as graphs of peptides with fatty acid group are provided in supplementary material.

Transfection results with the addition of peptides were normalized to NF51/pDNA control group (Figure 14). Based on the results of conducted experiments, 0.5 uM concentration of the peptides and post-mix mixing protocol for complex formation were chosen as optimal conditions for the most of the peptides.

In CR2 of NF51, all peptides except P2 increase transfection efficacy of NF51/pDNA nanoparticles to some extent. P1 increases transfection efficacy of CPP/NA nanoparticles more than 10 fold, compared to control group. However, P6 peptide, which was derived by fusing regions of L2 followed by slight modifications, showed the most impressive result. Difference from control group is 75 fold, which looks as a promising tool for CPP/NA delivery applications.

In general, effect of peptides on transfection of NF51/pDNA complexes in CR3 of NF51 is lower than in CR2 (Figure 14). Nevertheless, P6 peptide still is the one with the highest effect, but P7 also has similar effect, approximately 10 fold.

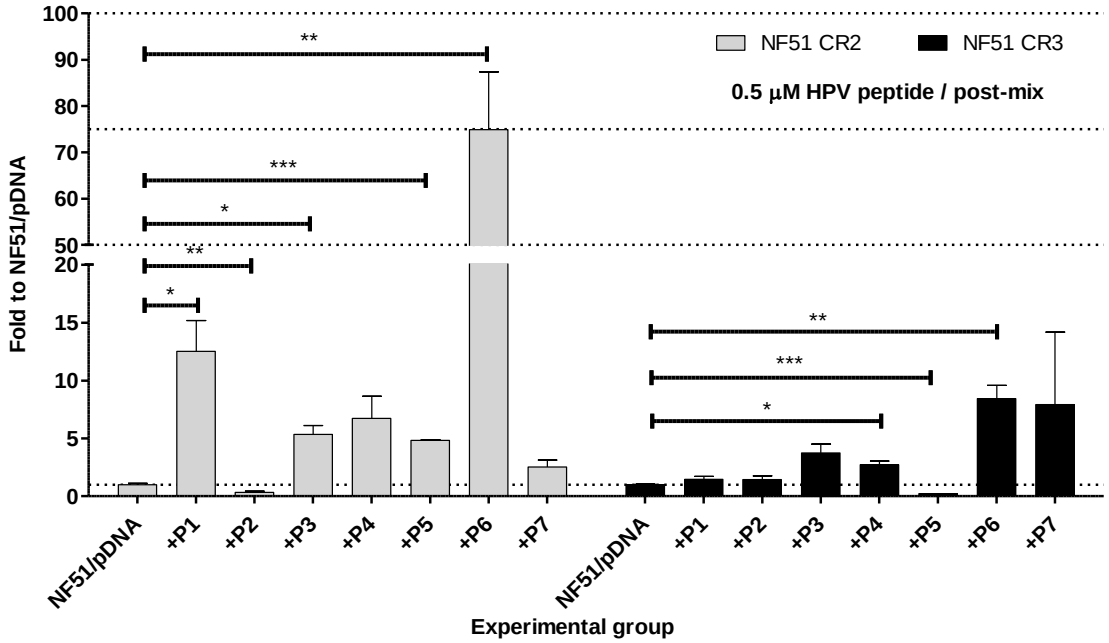


Figure 14. Transfection of CHO cells with non-covalently formed NF51/pDNA NPs and HPV L2 protein derived peptides. Transfection efficacy was measured with luciferase assay and luminescence measurements were normalised to absorbance of reporter protein. Peptides increase transfection efficacy of NF51/pDNA NPs. For the statistical analysis t-test was performed. p<0.05*, p<0.01, p<0.001*****

It should be noted that chosen concentration and the mixing strategy was not optimal for all of the peptides. Some peptides showed better results with other concentrations and mixing protocols. However, this effect was considered non-significant. Therefore, for overall comparison, all peptides were included with the same concentrations and conditions.

N-terminally stearylated peptides also left out from the main graph. Although they generally were not considered as effective as the ones without fatty acid group, some of them also have some positive effect in transfection efficacy.

In all experiments untreated cells and cells treated with only plasmid were included as additional controls. It was already shown in protein experiment that L2 alone without CPP does not increase cellular uptake of plasmid. Similarly, peptides also do not show positive results for delivering plasmid without CPP addition. Except P6, all other peptides even decrease plasmid transfection to the cells. Probably the high effect of P6 on transfection of the CPP/pDNA nanoparticles is the reason why P6 at least does not decrease the transfection, even though it does not increase too (Figure 15).

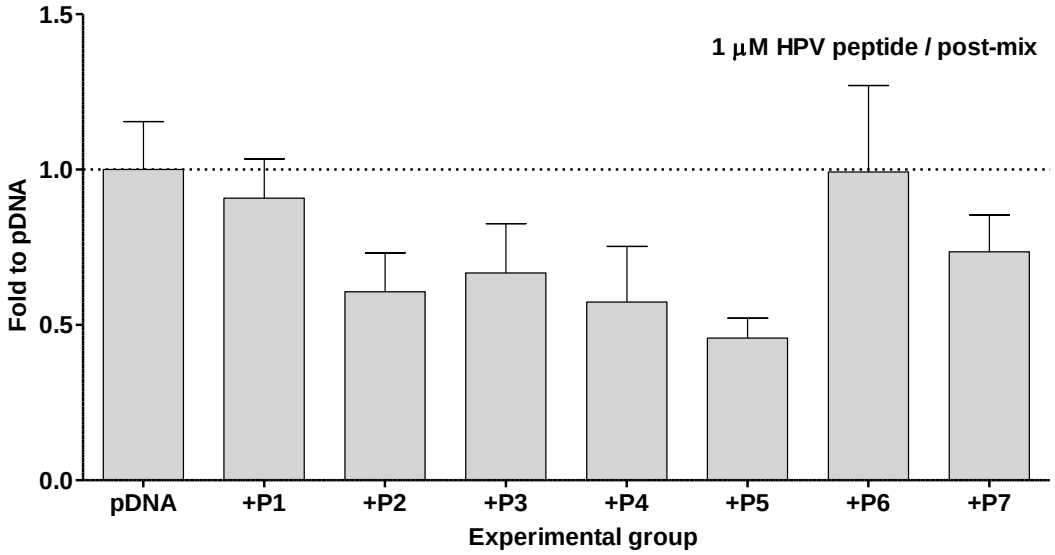


Figure 15. Transfection of CHO cells with pDNA and HPV L2 protein derived peptides. Transfection efficacy was measured with luciferase assay and luminescence measurements were normalised to absorbance of reporter protein. Peptides without CPPs do not increase transfection efficacy of plasmid.

3.2.5 Lysosomal disruption/endosomal escape – neutral red assay

There are several different methods available for evaluating endosomal escape. However, it is still challenging to monitor this specific process. In this work, neutral red uptake assay was chosen for the final experiment. Assay is based on lysosomal uptake of neutral red dye by cells. Neutral red dye has zero charge at physiological pH. Because of that dye cannot penetrate into the cell. Lysosome has a proton gradient in order to maintain a lower pH. Therefore, the dye becomes charged inside the lysosome. However, when the lysosomal membrane is damaged pH gradient is reduced and it cannot retain the dye. Results were normalized to untreated cells and all peptides show difference in the neutral red levels compared to untreated cells and cells transfected with plasmid. The addition of peptides leads to disruption of lysosome and this could be due to endosomal escape property of peptides (Figure 16).

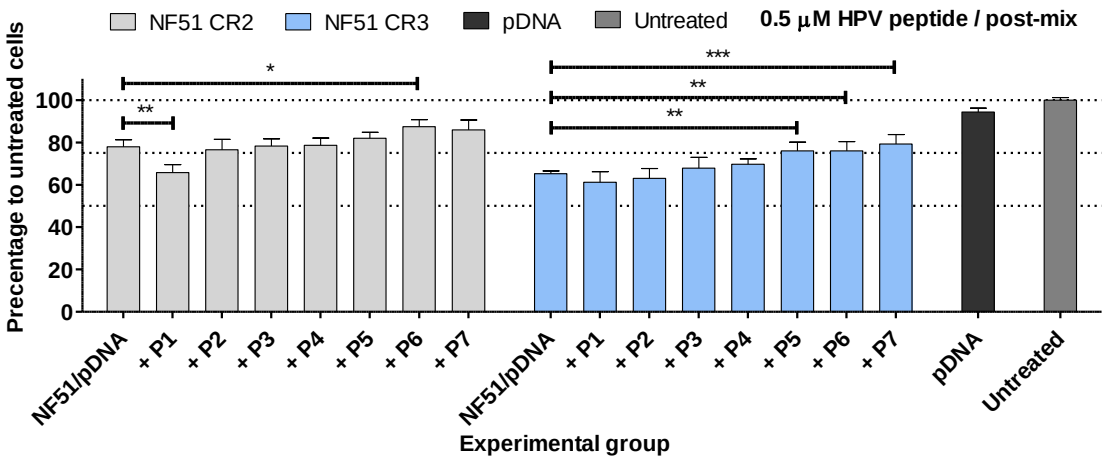


Figure 16. Lysosomal disruption of transfected cells. CHO cells were transfected with non-covalently formed NF51/pDNA NPs and HPV L2 protein derived peptides. NR added shortly after complexes to media. Background reduced from the absorbance and normalised to untreated cells (100%). Statistical analysis: 2wayANOVA with Bonferroni post-test. p<0.05*, p<0.01, p<0.001***.**

SUMMARY

During this research work new peptides derived from L2 protein of HPV16 were designed, synthesised and tested as additive components to CPP/pDNA nanoparticle complexes for increasing their transfection efficacy and endosomal escape.

The addition of L2 protein to the CPP/pDNA nanoparticle complexes showed an increase in transfection efficacy, both in the total reporter protein levels and in the transfected cell population. The peptides derived from L2 protein affected the transfection efficacy and intracellular trafficking. P2 peptide addition led to the decrease of transfection efficacy, whereas P6, similarly to the protein, lead to an increase of transfection efficacy. Method can be developed further by testing other conditions as well, introducing further modifications in the peptide and combining peptides with other CPPs.

Synthesised peptides can be tested for in vivo experiments in the future.

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Appendix

Materials

BioSite – HPV L2 protein

Cell line – Chinese hamster ovarian cells (CHO K1)

Reporter plasmid for luciferase measurement – reporter plasmid expressing gaussian luciferase and firefly luciferase pMC.BESPX.GauFluc2 (made by our research group)

Clontech – pEGFP-C1 - Reporter plasmid for green fluorescent protein (GFP) measurement mammalian expression plasmid expressing green fluorescent protein

Corning – PBS, Trypsin-EDTA 25%, Sodium pyruvate, DPBS, Dulbecco's Modified Eagle's Medium (DMEM, 10-013CV)

Thermo Scientific – Trypan blue, Pierce™ BCA Protein Assay kit

Sigma Aldrich – DTT, ATP, Fetal bovine serum (FBS)**,** Coenzyme A**,** Acetic acid**,** Piperidine

Perkin Elmer – D-luciferin

Gibco – Penicillin-Streptomycin mix

Boster – Neutral red solution

Applichem – Triton X, EDTA

Fisher chemical – DMC Dimethylformamide

Honeyvell – DCM – Dichloromethane

Biotage – Rink amide ChemMatrix

Iris Biotech GmbH – amino acids

Fmoc-Ala-OH

Fmoc-Arg(Pbf)-OH

Fmoc-Asn (Trt)-OH

Fmoc-Asp(otBu)-OH

Fmoc-Cys(Trt)-OH

Fmoc-Gln(trt)-OH

Fmoc-Glu(otBu)-OH

Fmoc-glycine

Fmoc-His(Trt)-OH

Fmoc-Ile-OH

Fmoc-Len-OH

Fmoc-Lys (Boc)-OH

Fmoc-Met-OH

Fmoc-Phe-OH

Fmoc-Pro-OH

Fmoc-Ser(otBu)-OH

Fmoc-Thr(tBu)-OH

Fmoc-Trp(Boc)-OH

Fmoc-Tyr(tBu)-OH

Fmoc-L-valine

Fmoc-Lys(MH)-OH

Fmoc-L-Om(BOC)-OH

Fmoc-L-Thx-OH

Results. Graphs of HPV L2 protein derived peptide experiments

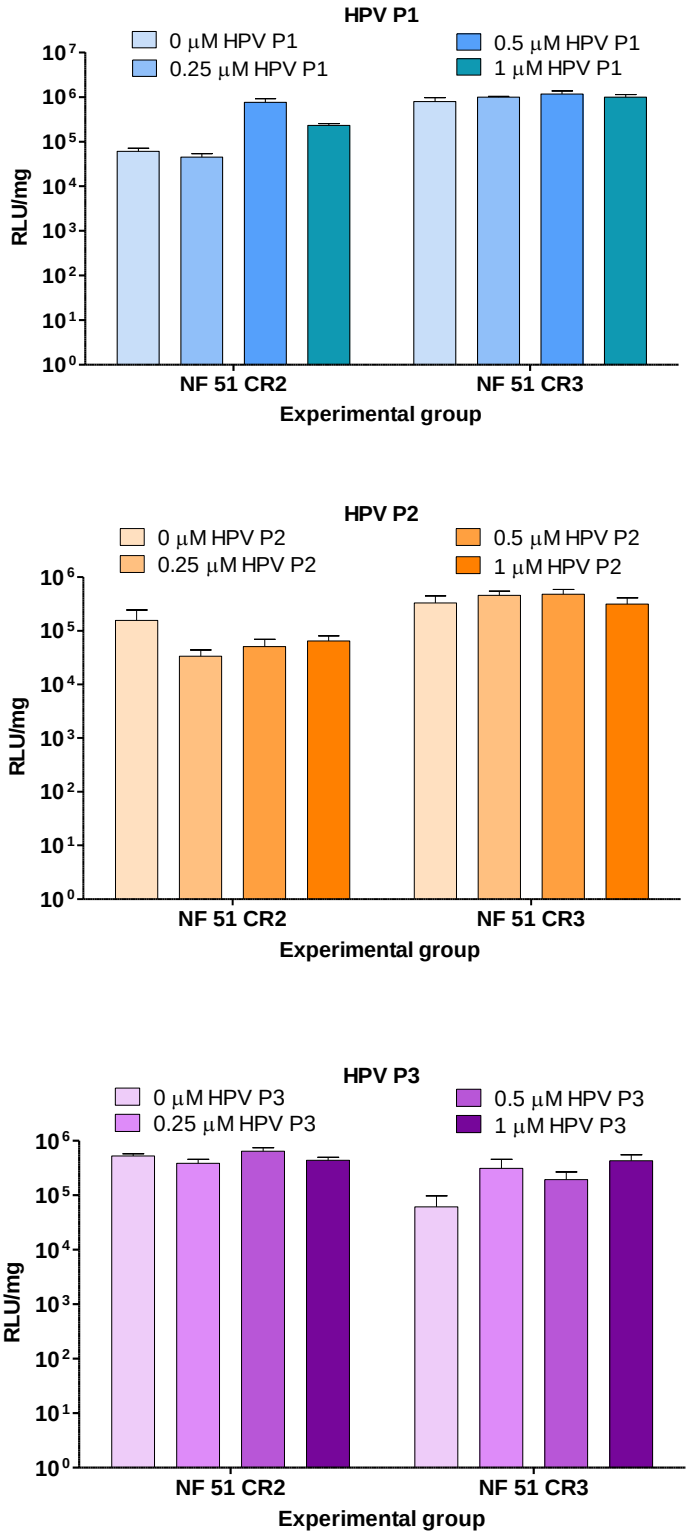


Figure 17. Transfection of CHO cells with NF51/pDNA NPs and HPV L2 protein derived peptides. Transfection efficacy was measured with luciferase assay and luminescence measurements were normalised to absorbance of reporter protein.

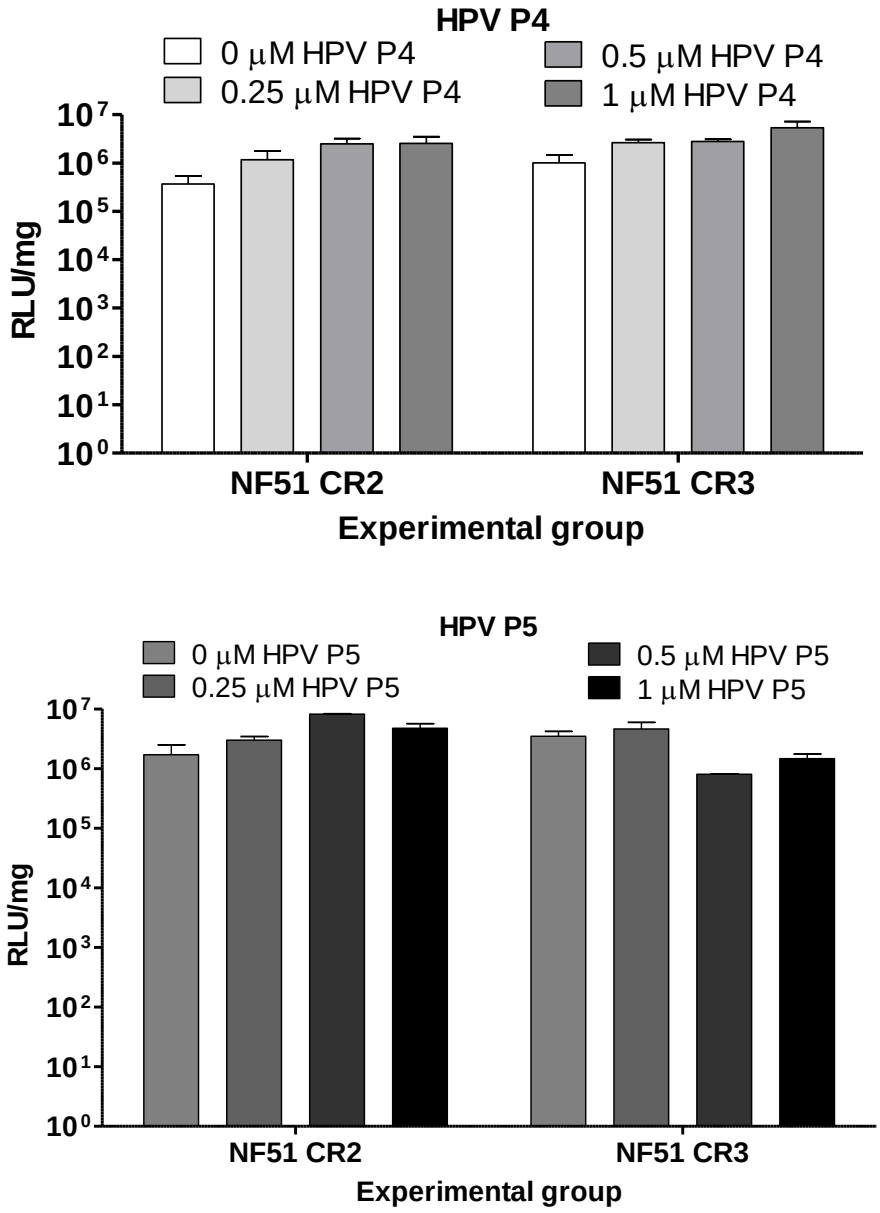


Figure 18. Transfection of CHO cells with NF51/pDNA NPs and HPV L2 protein derived and modified peptides. Transfection efficacy was measured with luciferase assay and luminescence measurements were normalised to absorbance of reporter protein. P4 slightly increases transfection efficacy in both CRs and with all concentrations.

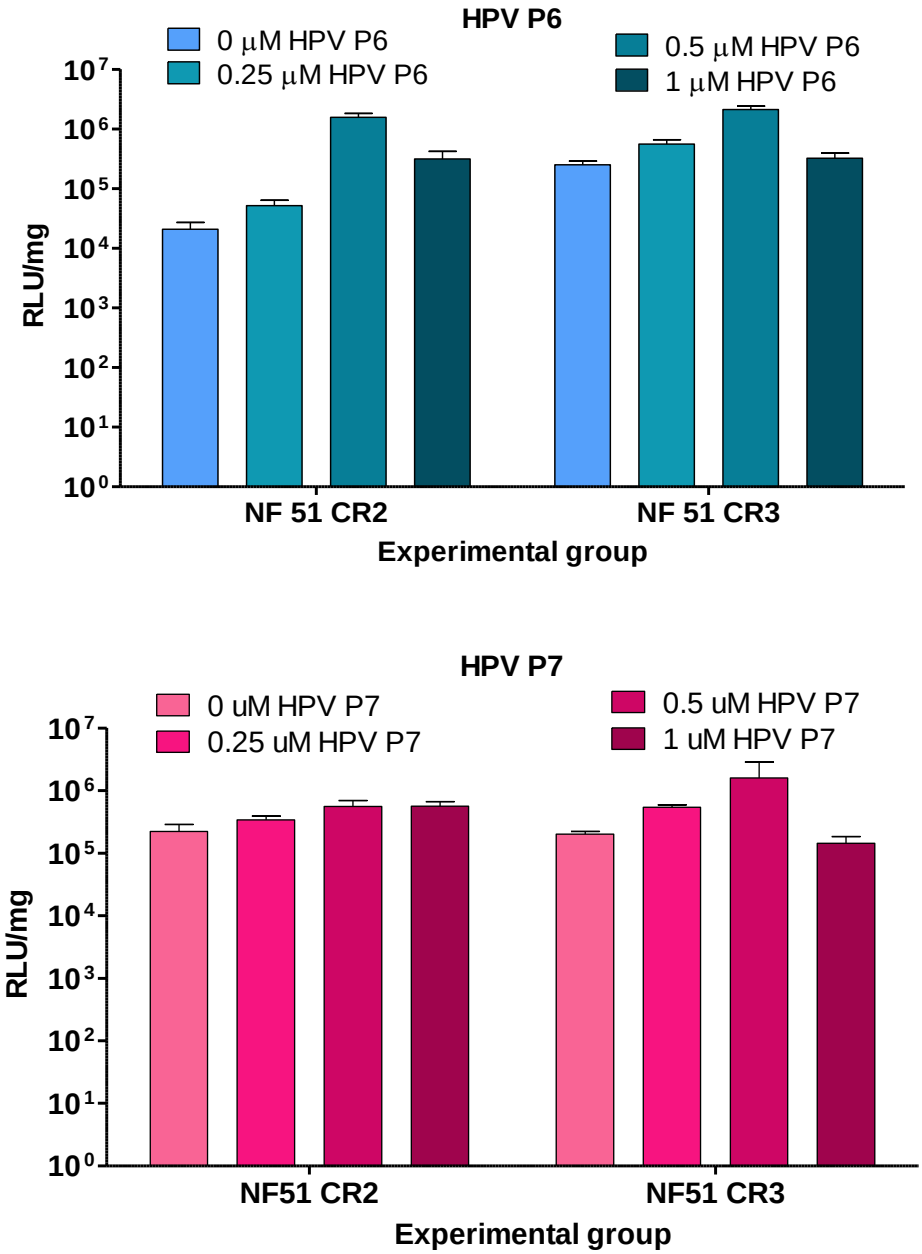
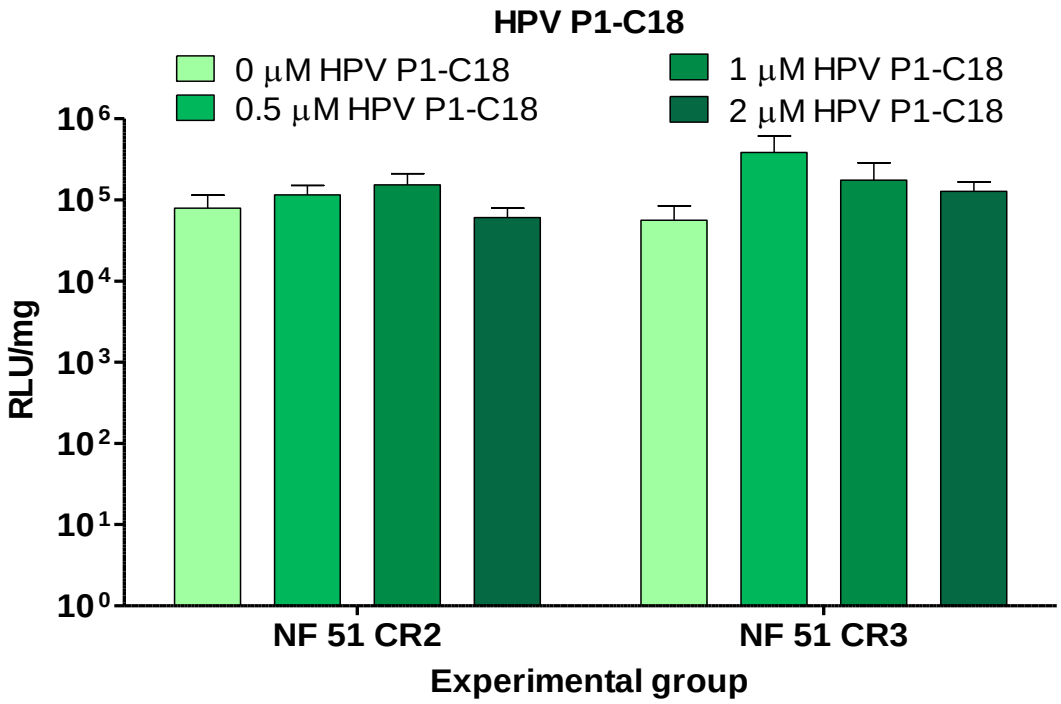
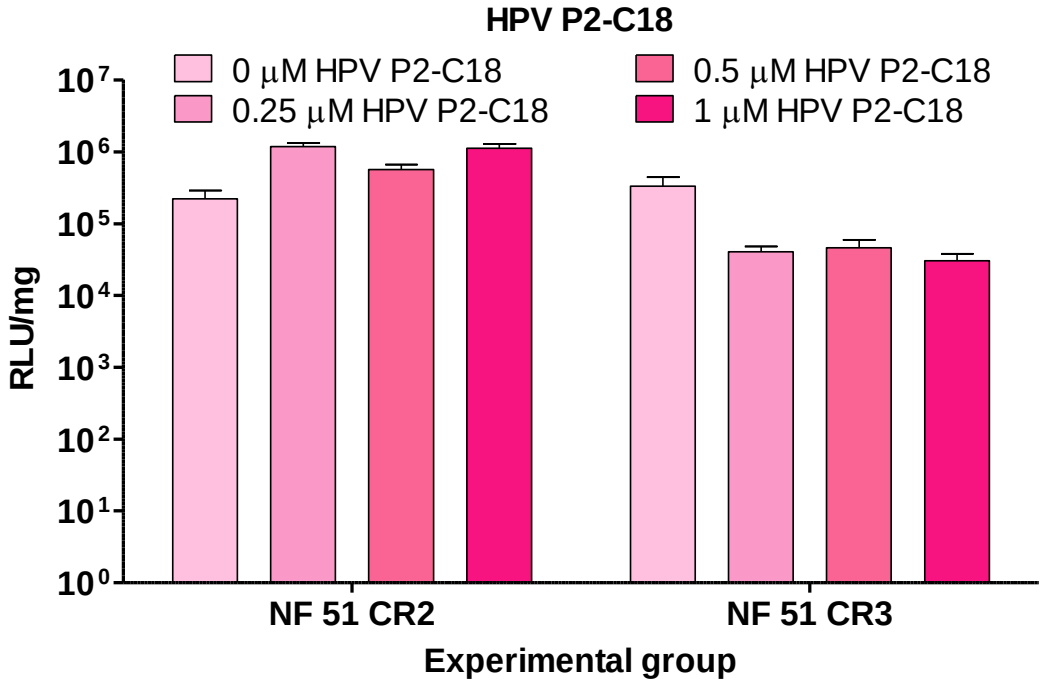


Figure 19. Transfection of CHO cells with NF51/pDNA NPs and peptides derived and modified from fusing sections of HPV16 L2 protein. Transfection efficacy was measured with luciferase assay and luminescence measurements were normalised to absorbance of reporter protein. P6 in both CRs and P7 in CR3 with 0.5 uM concentration have a significant effect on transfection.


HPV P1-C18
0 μM HPV P1-C18
0.5 μM HPV P1-C18
1 μM HPV P1-C18
2 μM HPV P1-C18
RLU/mg
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
NF 51 CR2
NF 51 CR3
Experimental group


HPV P2-C18
0 μM HPV P2-C18
0.25 μM HPV P2-C18
0.5 μM HPV P2-C18
1 μM HPV P2-C18
RLU/mg
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
NF 51 CR2
NF 51 CR3
Experimental group

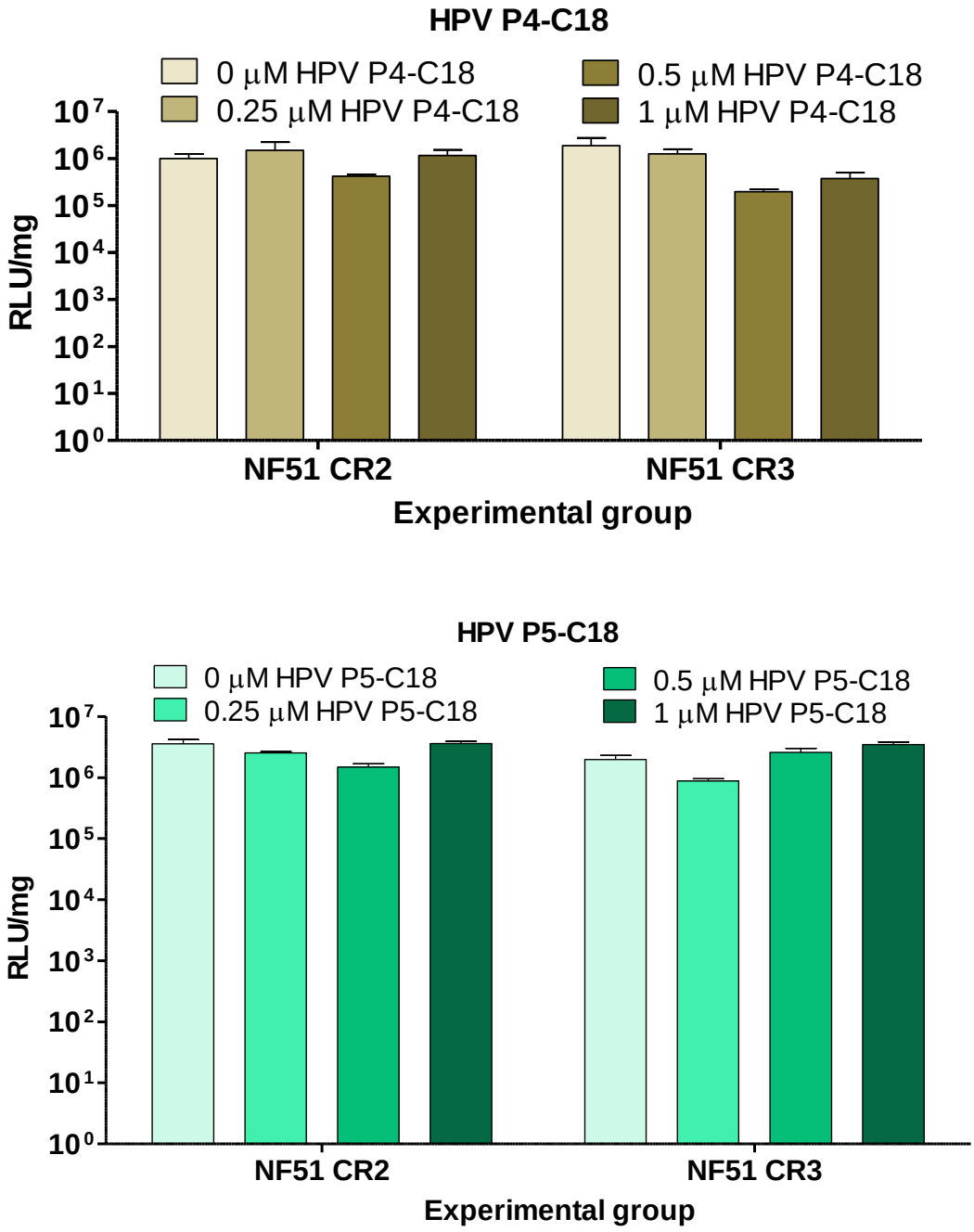
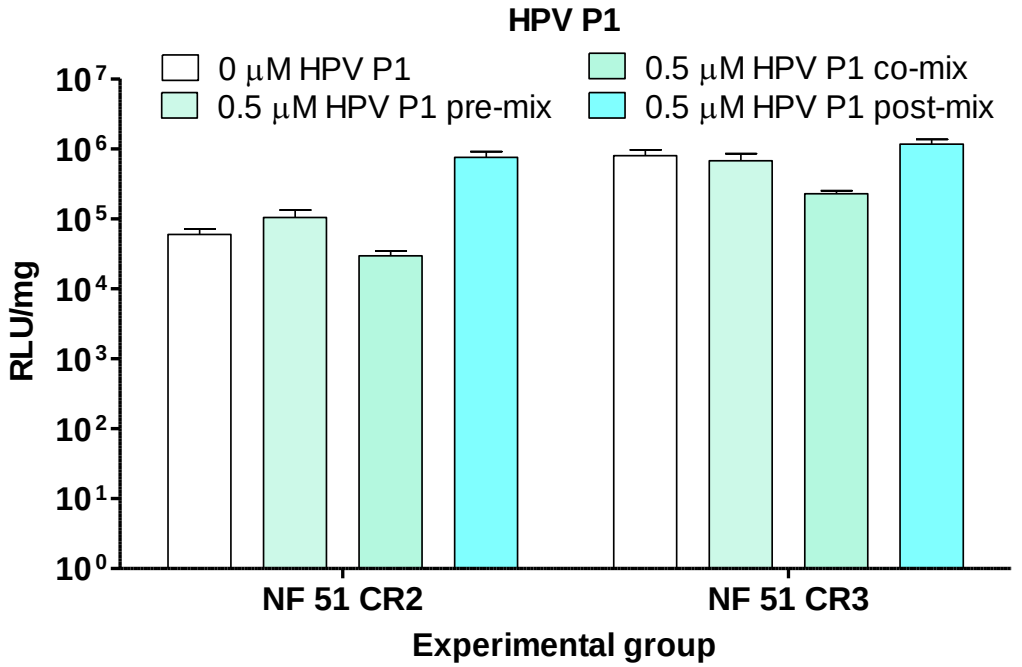
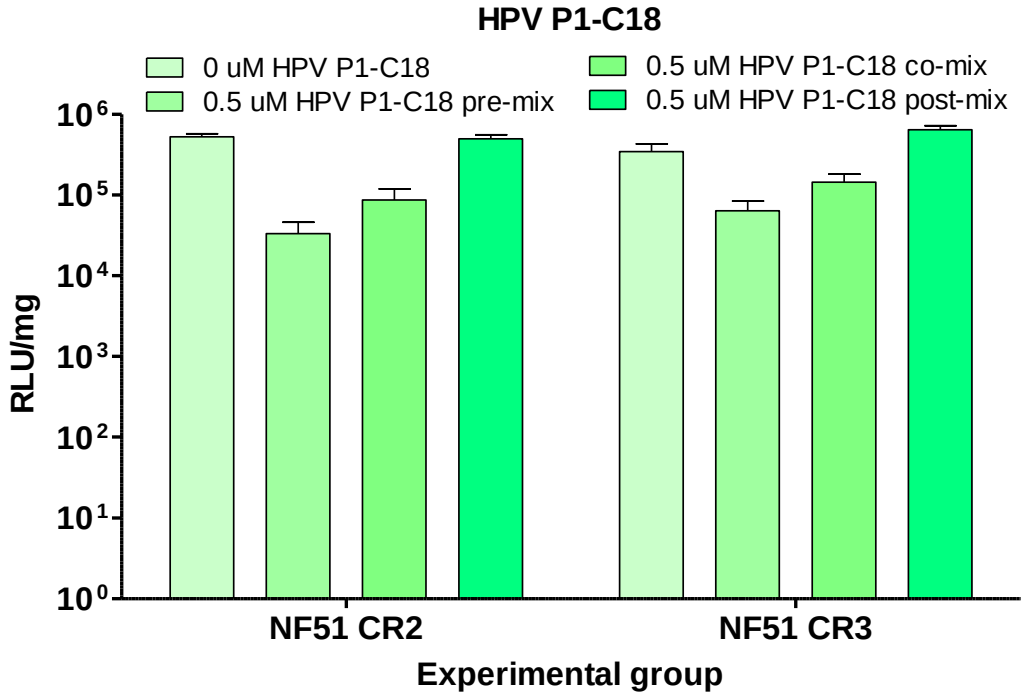


Figure 20. Transfection of CHO cells with NF51/pDNA NPs and N-terminally steary-lated HPV16 L2 protein derived peptides. Transfection efficacy was measured with luciferase assay and luminescence measurements were normalised to absorbance of reporter protein. P1-C18 in CR3 and P2-C18 in CR2 increase transfection efficacy. In general, these peptides do not have significant effect on transfection of CPP/pDNA complexes.


HPV P1
0 μM HPV P1
0.5 μM HPV P1 pre-mix
0.5 μM HPV P1 co-mix
0.5 μM HPV P1 post-mix
RLU/mg
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
NF 51 CR2
NF 51 CR3
Experimental group


HPV P1-C18
0 uM HPV P1-C18
0.5 uM HPV P1-C18 pre-mix
0.5 uM HPV P1-C18 co-mix
0.5 uM HPV P1-C18 post-mix
RLU/mg
10^6
10^5
10^4
10^3
10^2
10^1
10^0
NF51 CR2
NF51 CR3
Experimental group

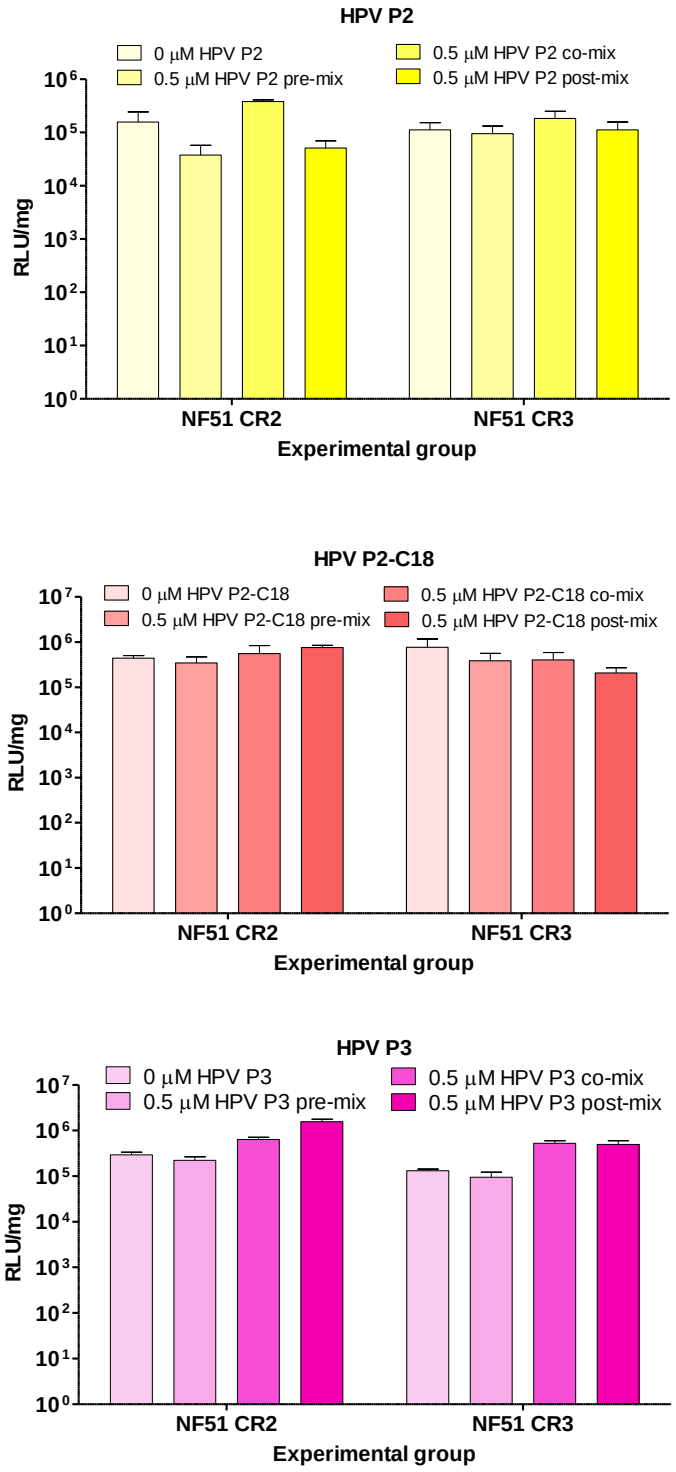


Figure 21. Transfection of CHO cells with NF51/pDNA NPs and HPV L2 protein derived peptides by applying different mixing strategies. Transfection efficacy was measured with luciferase assay and luminescence measurements were normalised to absorbance of reporter protein. For P1, P1-C18 and P3 post-mix is the best mixing strategy. For P2 co-mix is the best and P2-C18 with any mixing strategy does not affect transfection.

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20/05/2021