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Development of viral particles for diagnostic and therapeutic applications and study of their interactions with cells

Vývoj virových částic pro diagnostické a terapeutické účely a studium jejich interakcí s buňkami

Doctoral thesis

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ABSTRACT

Polyomaviruses offer versatile platforms for biomedical applications as viral particles. This thesis is divided into two parts: First, it evaluates the potential of polyomaviral particles as antigens in serodiagnostics. We aimed to establish a method for detecting subtype-specific antibodies against polyomavirus BK, a significant pathogen in kidney transplant recipients. We investigated the seroprevalence of the Czech population and found that multiple subtypes of polyomavirus BK-derived antigens are needed to achieve higher sensitivity. We also analyzed the role of major capsid protein VP1 surface loops in antibody recognition and assessed the impact of mutations in these sites. Mutant particles with changes introduced into the DE and EF surface loops were evaluated as antigens for serological assays.

Second, this thesis investigates polyomaviral particles as carriers for intracellular delivery, addressing key challenges like protein corona and endosomal entrapment. We evaluated the impact of protein corona on polyomaviral particles and showed that it does not influence their retargeting in the serum environment.

Upon modifying viral particles with cell-penetrating peptides, we showed that these peptides, particularly when non-covalently associated with the capsid surface, can enhance the delivery efficiency. As we investigated the means of this enhancement, we especially sought to establish a method for endosomal escape detection with proximity ligation assay.

This thesis aims to contribute to a deeper understanding of viral particles' interactions with the extracellular as well as the inner cellular environment.

KEY WORDS: Polyomavirus, viral particles, cell-penetrating peptides, endosomal escape, protein corona, diagnostics, intracellular delivery

ABSTRAKT

Polyomavirové částice představují všestrannou platformu pro biomedicínské aplikace. Tato práce je rozdělena do dvou částí: První hodnotí potenciál virových částic jako antigenů v sérologické diagnostice. Klademe si za cíl zavést metodu pro detekci subtypově specifických protilátek proti polyomaviru BK, který je významným patogenem u pacientů po transplantaci ledvin. Zkoumali jsme seroprevalenci v české populaci a zjistili, že pro zvýšení senzitivity je nutné použít antigeny odvozené od vícero subtypů polyomaviru BK. Také jsme analyzovali roli povrchových smyček hlavního kapsidového proteinu VP1 v rozpoznávání protilátkami a hodnotili dopady mutací v těchto místech. Hodnotili jsme potenciál částic s mutacemi v DE a EF povrchových smyčkách jako antigenů pro sérologické testy.

Druhá část posuzuje virové částice jako nosiče pro vnitrobuněčnou dopravu, přičemž se zaměřuje na klíčové problémy, jako je proteinová korona a uvíznutí v endozomu. Hodnotili jsme vliv proteinové korony na polyomavirové částice a ukázali, že v prostředí se sérem neovlivňuje jejich přesměrování.

Modifikací částic pomocí peptidů penetrujících membrány jsme ukázali, že tyto peptidy (zejména pokud jsou nekovalentně připojeny k povrchu kapsidy) mohou zvýšit účinnost dopravy do buněk. Když jsme zkoumali mechanismy tohoto zvýšení účinnosti, zaměřili jsme se na ustavení metod pro detekci úniku z endosomu pomocí tzv. „proximity ligation assay“.

Práce si klade za cíl přispět k hlubšímu pochopení interakcí virových částic s extracelulárním prostředím i vnitřním prostředím buňky.

KLÍČOVÁ SLOVA: polyomaviry, virové částice, peptidy penetrující membrány, únik z endosomu, proteinová korona, diagnostika, vnitrobuněčná doprava

LIST OF ABBREVIATIONS

AA	amino acid
AdV5	adenovirus type 5
AF488	Alexa Fluor 488
BKPyV	polyomavirus BK
BKPyVAN	BK polyomavirus nephropathy
CCMV	Cowpea chlorotic mottle virus
CPPs	cell-penetrating peptides
CPMV	Cowpea mosaic virus
DLS	dynamic light scattering
EEA1	early endosome antigen 1
EIPA	5-(N-ethyl-N-isopropyl)amiloride
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
HaPyV	hamster polyomavirus
HBV	hepatitis B virus
HBsAg	HBV surface antigen
HIA	hemagglutination inhibition assay
HPV	human papillomavirus
GCPII	glutamate carboxypeptidase II
GST	glutathione S-transferase
IgG	immunoglobulin G
JCPyV	polyomavirus JC
KTx / KTR	kidney transplant / KTx recipients
MPyV	mouse polyomavirus
MRI	magnetic resonance imaging
NCCR	non-coding control region
PLA	proximity ligation assay
PSA	prostate-specific antigen
PsVs	pseudovirions
SV40	simian virus 40
TEM	transmission electron microscopy
Tf	transferrin
TfR	transferrin receptor
TMV	tobacco mosaic virus
VLPs	virus-like particles
VPs	virus-derived particles

1. INTRODUCTION

Polyomaviruses are small DNA viruses that infect humans and animals and are associated with various diseases, including cancer. They are interesting study subjects not only as human pathogens but also as tools for cell biology research. Their unique life cycle and interaction with host cells provide valuable insights into cellular processes.

Polyomavirus-based particles are indispensable as antigens for polyomavirus diagnostics due to their ability to mimic the structural and antigenic properties of the virus while being non-infectious. However, their versatility and unique properties also make them attractive for various biomedical applications. They can be engineered for gene therapy, vaccines, imaging, and drug delivery by exploiting their natural tropism towards specific cells. The high cargo capacity and stability of these particles make them a promising platform for these applications.

Moreover, polyomaviral virus-derived particles (polyomaviral VPs) offer several advantages over other nanocarriers: they can be produced in large quantities using recombinant expression systems like the baculovirus system, they lack pre-existing antibodies in humans when derived from animal polyomaviruses, and they can be modified with cell-penetrating peptides or other targeting molecules to enhance cellular uptake.

The current goals of polyomavirus-based particle research include improving their efficiency for gene delivery by optimizing endosomal escape mechanisms, optimizing their design for vaccine development through surface modifications that enhance immunogenicity, enhancing their drug delivery capabilities by encapsulating therapeutic agents within VPs and advancing our understanding of the biology of polyomaviruses through studies on serological cross-reactivity among different subtypes.

Overall, exploring the potential applications of polyomaviral VPs will contribute significantly to both diagnostic advancements in infectious diseases caused by these viruses and innovative therapeutic strategies across various medical fields.

1.1 Preface

In our laboratory, we study the replication cycle of polyomaviruses as well as their application potential. While the use of polyomavirus-derived antigens in serological testing is already quite common, their further modifications for this purpose were not yet fully explored. In intracellular delivery of various cargoes, viral particles stand out because of their unique properties: they are naturally designed to be effective in gene delivery while simultaneously supposed to overcome the obstacles posed by the extracellular environment and immune system (discussed in Chapter 2.1). They can serve both as a great experimental tool and inspiration for their design.

Polyomaviral particles are a great model as they are easy to produce (e.g., in a baculovirus expression system) and further manipulate (retarget, encapsulate cargo, or label), yet still offer some of the advantages of viruses, such as efficient cargo delivery. Additionally, in the case of animal polyomaviruses, an undoubtable advantage is the absence of preexisting antibodies in the human population (see Chapter 2.3.1.3). While easy production is especially important for their use for diagnostic purposes or vaccine manufacturing, their other advantages stand out in their application in intracellular delivery and gene therapy. The versatility of polyomaviruses and polyomaviral VPs enables us to explore all the areas mentioned. This thesis encompasses several projects focused on the above-mentioned topics, and consequently, the first part of this thesis is focused on the use of viral particles as an antigen in serodiagnostics of a human pathogen, polyomavirus BK, and the second part is focused on the intracellular delivery by viral particles derived from a model mouse polyomavirus (MPyV).

2. REVIEW OF LITERATURE

2.1 Viral particles in biomedicine

Current efforts in biomedical nanotechnology applications favor modifiable and biocompatible nanomaterials. Several nanomaterials (e.g., quantum dots, dendrimers, polymer vesicles, liposomes, lipidic nanoparticles, and protein-based nanostructures) are being evaluated as carriers for therapeutic and/or imaging reagents. The goal is to deliver such cargo *in vivo* in a safe yet effective and precisely targeted manner while efficiently shielding it until the desired location is reached. Thus, the cytotoxicity, immunogenicity, and production process must be considered. Virus-derived particles (VPs), an example of protein-based nanostructures, stand out as naturally biocompatible tools that were shaped by the evolution for this very task - to deliver their content into specific cells and intracellular location (reviewed in Steinmetz, 2010, 2013; Yildiz *et al.* 2011; Aljabali *et al.*, 2021).

The development of various expression systems enabled various uses for recombinant viral capsids or other structural parts, e.g., GST-fused viral protein monomers. Over the years, they were used in a variety of applications, often replacing the wild-type (live, attenuated, or inactivated) virus that was originally needed (i.e., in serodiagnostic tests or vaccines). These include biomedical approaches like gene therapy, immunotherapy, delivery of diagnostic or imaging agents, vaccines or serological tests, biocatalytic nanoreactors, material synthesis, and others.

The terminology used is rather inconsistent, and authors should always specify the composition of the particles used. Recombinant viral capsids can be called, for example, virus-like particles (VLPs) or pseudovirions (PsVs). VLPs usually denote capsid-like structures that assemble without the presence (i.e., subsequent encapsidation) of the viral genome. Thus, VLPs are, by definition, non-infectious. PsVs contain a reporter plasmid and are used to deliver nucleic acid into a cell. Often, these two terms are interchangeable or used inconsistently (the convention may also vary depending on the parent virus). Therefore, the overarching term VPs will be usually used in this thesis. The types of VPs used in this thesis, along with the specific modifications we used, are described in full in Chapter 4.

VPs can be produced by insect, bacterial, yeast or plant-based expression systems, and by purification of self-assembled particles as well as by *in vitro* assembly of purified capsid subunits (reviewed in Steinmetz, 2010; Machida and Imataka, 2015; Dashti and Sainsbury, 2020).

In general, VPs derived from bacterial and plant viruses are considered safer *in vivo* (reviewed in Steinmetz, 2010), but mammalian and even human viruses were also used in a variety of applications. For some (serodiagnostics and/or vaccines), it is actually necessary for the VPs to be derived from human viruses.

2.1.1 VPs in serodiagnostic and vaccines

VPs are often used as antigens in serological assays and vaccines as they have several advantages over other types of antigens. Being non-infectious and non-replicating makes them safer to use than live or inactivated viruses. Still, VPs can mimic the structural and antigenic properties of a virus, which makes them highly immunogenic and able to elicit a strong immune response. They can be produced in large quantities and with consistent properties, which makes them ideal and more cost-effective than other types of antigens. They have been used in many FDA-approved diagnostic assays for detecting infections such as human papillomavirus (HPV) and hepatitis B and C, among others.

A great example of the various properties of VPs is their use in serological assays. While VLPs are useful in ELISA-based assays, PsVs containing a reporter gene allow for the detection of neutralizing antibodies in neutralization-based assays. Neutralization-based assays with HPV VPs were found to be comparable in sensitivity but more specific than ELISA-based tests (Pastrana *et al.*, 2004). VPs are an ideal antigen for serodiagnostic tests in case the wild-type live virus is unavailable, e.g., it is not easily propagated. This was the case for, e.g., goose hemorrhagic polyomavirus (Zielonka *et al.*, 2006) or human papillomavirus 11 (HPV-11). HPV-11 VPs were shown to be comparable to virions both as antigens in serodiagnostic ELISA and in eliciting a neutralizing antibody response (Rose *et al.*, 1994). Indeed, HPV VPs are the first to be used in vaccines (reviewed in Schiller and Lowy, 1996; Roldão *et al.*, 2010). Even before that, an HBV vaccine containing recombinant HBV surface antigen (HBsAg) was developed. HBsAg assembles into particles that do not, however, resemble the parent virus (McAleer *et al.*, 1984). Since then, VPs have been the basis for vaccines not only against viral infections but even against diseases such as malaria: a vaccine containing chimeric HBsAg-based VPs carrying a *Plasmodium falciparum* epitope was developed (Regules *et al.*, 2011) and recently approved (Mohsen *et al.*, 2017). Similarly, a chimeric vaccine based on Q β VPs displaying a *Vibrio cholerae* polysaccharide elicits an antibody response in experimentally immunized mice and is a promising candidate for safe vaccination of young children for whom the currently available oral whole-cell vaccine is not suitable (Rashidijahanabad *et al.*, 2022). The use of VPs (instead of viruses) leads to safer preparation and administration (reviewed in Mohsen *et al.*, 2017; Zhang *et al.*, 2023). A detailed

overview of the fascinating advances in the field of VP-based and chimeric VP vaccines is beyond the scope of this thesis but has been extensively reviewed elsewhere (Plummer and Manchester, 2011; Mohsen *et al.*, 2017; Zhang *et al.*, 2023).

2.1.2 VPs in intracellular delivery

Viral particles naturally offer protection of nucleic acids or other cargo against nucleases and are predisposed to overcome intracellular barriers (see Chapter 2.1.3). When compared to synthetic carriers, viral vectors are considered more efficient but also may trigger immune responses and undesirable side effects, which limits their application potential (reviewed in Durymanov and Reineke, 2018). The potential for viral replication needs to be assessed and dealt with for every viral vector. The use of VPs also allows the exploitation of their natural tropism. High immunogenicity, which is advantageous for the use in vaccines, can be bypassed by an *ex vivo* approach in others (e.g., gene therapy). A detailed description of topics such as immunogenicity, biocompatibility, and biodistribution *in vivo* is reviewed elsewhere (Mellid-Carballal *et al.*, 2023a).

Besides their natural ability to deliver nucleic acids, VPs are also suitable as delivery agents for various therapeutics (reviewed in Aljabali *et al.*, 2021). Indeed, various compounds (typically cancer-cell-killing drugs or contrast agents) were shown to be efficiently delivered by VPs *in vitro* (reviewed in Yildiz *et al.*, 2011; Mellid-Carballal *et al.*, 2023b). By using the innate viral mechanisms intended for genome packaging, modified proteins can be incorporated within the VPs (Panthi *et al.*, 2021). One of the first VPs to be employed as a gene transfer vector were HBV-derived particles (Brandenburg *et al.*, 2005).

One of the most studied models of VPs is plant virus Cowpea chlorotic mottle virus (CCMV) derived particles. They allow for whole protein encapsidation (Minten *et al.*, 2009) and were also loaded with phthalocyanine (a light-absorbing organic molecule that enables photodynamic therapy) and subsequently entered macrophages and facilitated their cell death upon irradiation (Brasch *et al.*, 2011). They can also encapsidate gold nanoparticles (used, e.g., for medical imaging) and improve their biocompatibility and stability *in vivo* (Durán-Meza *et al.*, 2020). They were found to be suitable for gene delivery (Villagrana-Escareño *et al.*, 2019) and epitope presentation (Hassani-Mehraban *et al.*, 2015).

CCMV VPs were also used to deliver contrast agents for MRI, such as gadolinium. Using VPs for their delivery could enable specific tissue targeting (see Chapter 2.1.4) and significantly mitigate the side effects (Liepold *et al.*, 2007). Other plant viruses were also investigated for their ability to spontaneously encapsidate (Loo *et al.*, 2006, 2007; Huang *et al.*, 2007) or be loaded with

various cargoes (Loo *et al.*, 2008; Zeng *et al.*, 2013). Similarly, phages such as MS2 were found to be suitable nanocarriers as well (Wei *et al.*, 2009; Ashley *et al.*, 2011; Capehart *et al.*, 2013).

Phages and plant viruses were also used as a platform for the covalent attachment of various drugs (Ren *et al.*, 2007; Bar *et al.*, 2008) or drug-binding peptides (Oh *et al.*, 2015). Interestingly, even VPs derived from human viruses, such as rotavirus (Zhao *et al.*, 2011) or hepatitis E virus (Panda *et al.*, 2015), were investigated for this purpose.

2.1.3 Barriers of intracellular delivery

The use of nanoparticles *in vivo* poses some challenges. However, viruses are naturally predisposed to overcome barriers of delivery into cells, both extracellular and intracellular, and VPs exhibit overall higher efficiency than nonviral vectors (reviewed in Degors *et al.*, 2019).

Before even entering the cell, the first barrier that all nanoparticles encounter in *in vivo* applications is the occurrence of the protein corona (see Chapter 2.1.3.1). Then, the nanoparticles delivering their cargo into the cells need to deal with the cellular membrane. For successful cellular entry, VPs usually use the same receptor as their parent virus unless they are intentionally retargeted (see Chapter 2.1.4). Then, the endosomal vesicles containing VPs mature from early endosomes to recycling endosomes (some particles might be recycled back to the cellular membrane) or late endosomes and subsequently into lysosomes (reviewed in Durymanov and Reineke, 2018).

In the case of the delivery of nucleic acids, overcoming the nuclear envelope poses an additional challenge.

The use of viral particles benefits from their innate ability to overcome these barriers. However, viral evolution does not necessarily strive for the highest possible efficiency of infection, and cells also employ various defense strategies to intercept the ongoing infection (reviewed by Staring *et al.*, 2018). An additional and varying reduction in infectivity might even stem from the use of laboratory strains or artificial propagation methods. For example, the ratio of infectious to non-infectious particles in MPyV preparations was shown to be 1:50 to 1:1000 (Horníková *et al.*, 2015). Thus, these barriers are a limiting step worth studying, even for VPs.

2.1.3.1 Protein corona

When nanoparticles enter the bloodstream, they are met by the present serum proteins that tend to adsorb on the surface of these particles, forming the so-called protein corona. The protein corona phenomenon is a major drawback in current efforts in nanoparticle drug design as it interferes with nanoparticle targeting and/or uptake into cells and can influence the fate of particles *in vivo*. The

‘hard’ protein corona forms from proteins tightly bound to the particle surface. Further interaction with more dynamically exchanged proteins makes up a ‘soft’ corona (*Figure 1*, reviewed by Pareek *et al.*, 2018). Virus-based biomimetic nanotechnology is gaining popularity as a way to overcome the limitations of the synthetic nanoparticles that are currently the most used in biomedicine (reviewed in Chen *et al.*, 2022). Therefore, investigating the protein corona phenomenon in VPs is of great interest.

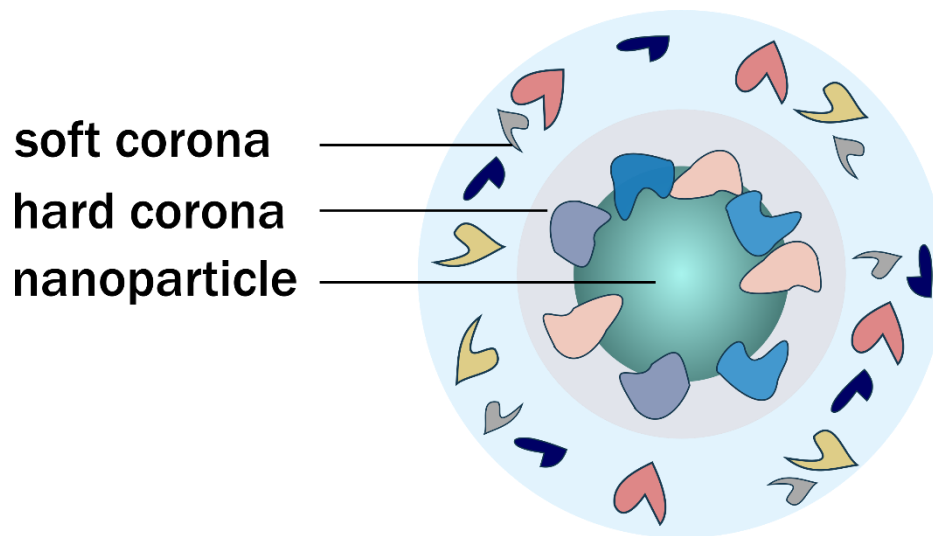


Figure 1: **Protein corona on a nanoparticle.** Proteins with high affinity are bound tightly to the surface of the nanoparticle, forming the hard protein corona, whereas the more dynamically exchanged proteins further from the particle make up the soft protein corona.

The potential advantage of VPs’ behavior in biological fluids was suggested by Berardi *et al.* (2019). However, the effect of the protein corona on VPs has not yet been thoroughly studied. Some pioneering work has already been done on plant virus-derived VPs. Indeed, TMV VPs were found to acquire less abundant protein corona upon incubation with human plasma than their synthetic counterparts. The impact on targeting and the *in vivo* behavior was also studied, and it was demonstrated that the choice of ligand is instrumental in the rate of particle clearance and biodistribution. Specifically, the protein corona enhances the interaction with some cell lines (e.g., HeLa). Modification with some targeting peptides (GPRPP) resulted in VPs’ aggregation, but others (CREKA) led to their rapid clearance (Pitek *et al.*, 2016).

Additionally, Cowpea mosaic virus (CPMV) was reported to remain stable and not form protein corona when exposed to gastrointestinal fluids, which is a known limitation of the oral administration of nanoparticles (Berardi *et al.*, 2018). An interesting insight into the VPs’ resistance to the protein corona formation offers a study that compares two VPs of different origins. Surprisingly, when comparing the properties of CPMV- (a plant virus) and Bluetongue virus-derived (animal virus) VPs, it was found that CPMV VPs resist the adhesion of biomolecules

in fluids, and this ability is not shared with the Bluetongue virus VPs (Berardi *et al.*, 2019). This is curious as animal viruses could be presumed to evade the bloodstream-posed barriers more efficiently.

The replication cycle of animal and human viruses relies on dissipation through the bloodstream and effective entry into the target tissue. The interaction with biological fluids is thus unavoidable and may even be beneficial. It is known that host factors binding the viral surface influence their tropism (Waddington *et al.*, 2008; Doronin *et al.*, 2012) or even be necessary for viral infectivity (Chang *et al.*, 2007). The respiratory syncytial virus and herpes simplex virus type 1 acquire protein corona that differs depending on the respective biological fluids and positively affects viral infectivity and interaction with the immune system (Ezzat *et al.*, 2019).

Adenoviruses (AdVs) readily interact with blood coagulation factors and other serum proteins. This may hinder the effectiveness of adenoviral vectors but also offers potential for purposeful manipulation (reviewed in Allen and Byrnes, 2019). The host factors binding of AdV influences its biodistribution profile and has severe implications for their use in gene therapy (reviewed in Lopez-Gordo *et al.*, 2014) and, by proxy, also for the use of other virus-based delivery systems. Recently, the addition of a synthetic corona was shown to protect AdV against protein corona formation and neutralization by antibodies *in vivo*. It also offers a way to improve transduction and influence AdV VP biodistribution (Wu *et al.*, 2019). Overall, the effect of protein corona on the use of specific VPs and their targeting remains to be thoroughly investigated. This thesis exclusively examines the protein corona on MPyV VPs (see results in Chapter 4.2.1.1, discussion in Chapter 5.2.1).

2.1.3.2 Endosomal entrapment

Viruses and virus-derived carriers usually utilize the endocytic pathway in order to reach their final intracellular destination. They need to avoid degradation in the lysosomal compartments as well as being directed back to the cell surface (reviewed in Grove and Marsh, 2011). While enveloped viruses typically use the fusion of the viral and endosomal membrane, non-enveloped viruses employ membrane-modifying proteins that help the virus escape the endosomal entrapment. Drug carriers of non-viral origin need to be modified in order to deliver their cargo successfully, and their sequestration in endosomes is considered a limiting step in intracellular delivery (reviewed in Shete *et al.*, 2014; Durymanov and Reineke, 2018; Simpson *et al.*, 2019).

2.1.3.2.1 Strategies to improve endosomal escape

For viral vectors, a conformational change of surface-exposed proteins triggered by receptor binding or acidification of endosomes is usually necessary in order to leave the endosome. Several strategies have been developed to improve endosomal escape of drugs and viruses and were reviewed in detail by Durymanov and Reineke (2018).

Briefly, cells can be treated *in vitro* with lysosomotropic agents. Their accumulation in endosomes induces osmotic swelling of the vesicles. Examples of such agents are chloroquine, sucrose and polyvinylpyrrolidone. Their use *in vivo* is complicated because of their toxicity. For *in vivo* applications, co-delivery with photosensitizers is more suitable. The disruption of the endosomal membrane is a result of light-induced oxidative damage. These compounds are widely used for photodynamic therapy and are considered safe.

Next, the modification with polyamines (e.g. polyethylenimine) or the incorporation of amphiphatic polycations can enhance endosomal release of nucleic acids.

For the purposes of this thesis, however, the most important approach is the use of cell-penetrating peptides (CPPs). It was reported that nonviral as well as viral vectors benefit from transduction enhancers, such as CPPs. These CPPs and protein transduction domains have been shown to be able to cross the endosomal membrane and deliver their cargo into the cytoplasm. We used the modification with CPPs in our research and their effects are reviewed in more detail in Chapter 2.1.5.

2.1.3.2.2 The detection of endosomal escape

The accurate assessment of endosomal escape efficiency is critical for the evaluation of cargo delivery systems. Endosomal sequestration poses a significant barrier to efficient intracellular delivery, making the development of precise measurement methods essential. Many commonly used methods reflect only the overall cellular uptake, which includes material that may be recycled back to the plasma membrane or degraded in the endolysosomal system (reviewed in Deprey *et al.*, 2019). While the delivery of biologically active cargo can provide indirect measurements through their effects (e.g., reporter gene expression) upon successful delivery and subsequent entry into the nucleus or cytoplasm, these methods do not solely reflect endosomal escape efficiency but also other steps in the delivery process (reviewed by Smith *et al.*, 2019).

Artificial membranes and various models, such as liposomes, are utilized to characterize the ability of nanocarriers or enhancers (e.g., CPPs) to cross the endosomal membrane (reviewed in Deprey *et al.*, 2019; Hausig-Punke *et al.*, 2022). Another approach involves measuring cargo

levels in cell lysates, specifically in subcellular fractions, to assess endosomal escape indirectly (reviewed by Deprey *et al.*, 2019).

Fluorescent markers like LysoTracker (which selectively stains acidic compartments) or pHrodo (which is pH-sensitive and becomes fluorescent upon acidification) can indicate endosomal disruption and cargo escape in live cells. Leakage assays track the cytosolic diffusion of the fluorescently labeled material after the endosomal disruption. Despite their utility, they face challenges due to low detection sensitivity, as noted by Smith *et al.* (2019). Conversely, calcein (fluorescent dye with self-quenching properties at higher concentrations) leakage experiments depend on its dilution upon its release from vesicles and subsequent restoration of its fluorescence (reviewed in Hausig-Punke *et al.*, 2022).

Advanced microscopy techniques such as super-resolution nanoscopy enable precise spatial mapping of nanoparticle behavior during endosomal escape by overcoming the diffraction limit of conventional microscopy. These methods provide qualitative insights into vesicle rupture dynamics, cargo release patterns (e.g., burst-like vs. gradual), and nanoparticle-endosome interactions at <20 nm resolution. While technically demanding due to requirements for specialized fluorophores and complex data analysis, nanoscopy uniquely distinguishes between membrane-adherent versus truly cytosolic nanoparticles – resolving key ambiguities in traditional fluorescence assays (reviewed in Andrian *et al.*, 2021).

Artifacts from immunofluorescence experiments can complicate their interpretation. The most discussed but often eventually overlooked in experimental design is the effect of fixation of cells. The fixation process often fails to immobilize particles effectively, leading to their diffusion and simulating endosomal escape (Johnston, 2017). This limitation is particularly significant in research involving CPPs, where distribution differences between fixed and non-fixed samples have been noted (Richard *et al.*, 2003).

Galectin assay relies on the labeling of galectins (often galectin-3, Maier *et al.*, 2012), a family of β -galactoside-binding animal lectins. Under normal circumstances, galectin-3 is distributed evenly throughout the cytosol but accumulates at sites of intracellular membrane damage. It has been utilized as a marker of endosomal rupture by adenovirus, with distinct spots indicating that galectin was recruited to the endosome following adenoviral escape (Maier *et al.*, 2012). Recently, a new assay utilized a mCherry-Galectin9 reporter cell line for the image-based quantification of endosomal escape (Munson *et al.*, 2021).

The escape from endosomes can be also detected in cells expressing cytosolic reporter protein SNAP-tag. This protein forms covalent bond with a small lysine reactive compound benzylguanine that can be conjugated on e.g. VPs. When these VPs enter cytosol, the formed

complexes can be detected on a gel mobility shift assay with anti-SNAP antibody (Keppler *et al.*, 2003). The SNAP-tag assay was previously used to map the trafficking of SV40 and adenovirus (Geiger *et al.*, 2011, 2013).

Researchers overall agree that a reliable assay for endosomal escape quantification is essential for the further development of nanoparticle delivery systems (Martens *et al.*, 2014; Smith *et al.*, 2019). Recent efforts to establish such assay led to the use of SNAP-switch: a fluorophore and a quencher are attached to benzylguanine and when it interacts with the SNAP-tag, the quencher is transferred to the SNAP-tag so the fluorophore becomes fluorescent and measurable on FACS (FitzGerald *et al.*, 2020).

Similarly, an upgraded version of the SNAP-tag assay, the Cyto-SNAP assay, was developed for the quantification of cytosolic delivery. Here, the cytosolic capture protein is assembled from SNAP-tag and mNeonGreen (mNeonGreen-SNAP-tag). SNAP-tag reacts with benzylguanine-biotin (biotin is later used during the quantification step) on the investigated compound (e.g. cell-penetrating peptide). The mNeonGreen is then used for high-affinity immunoprecipitation using magnetic beads coated with anti-mNeonGreen nanobodies, and the subsequent quantification is done by ELISA by streptavidin-horseradish peroxidase (Lucchino *et al.*, 2021).

Another recently developed assay uses a split reporter in the GFP complementation assay. The cargo (e.g., an antibody) fused with a GFP fragment enters the cytosol of the cell, expressing a complementing GFP part, and the resulting fluorescence signal can be measured (Kim *et al.*, 2015; Milech *et al.*, 2015; Schmidt *et al.*, 2015). The main limitation is the poor sensitivity, as quite a high cytosolic concentration of the reporter is required for reliable detection (reviewed in Teo *et al.*, 2021). Therefore, a much more sensitive system was developed using the same principle with split NanoLuciferase (Teo *et al.*, 2021). The Split Luciferase Endosomal Escape Quantification (SLEEQ) assay directly measures cytosolic delivery with high sensitivity. Importantly, it also allows for the distinction between the overall material delivered in the cell and the actual endosomal escape efficiency. The SLEEQ assay has demonstrated a detection limit of 5 pM, which is significantly more sensitive than previous methods like split GFP, which required concentrations above 0.3 μ M to produce measurable signals.

2.1.4 Modification of viral particles

Depending on the intended use, VPs can benefit from or even require further modification. Tailoring VPs for specific purposes can be achieved by attaching ligands to the capsid surface or through genetic engineering of structural viral proteins (*Figure 2*). A great advantage of genetic

modification is the high reproducibility of these changes when preparing large quantities or several batches of VPs (reviewed in Steinmetz, 2013). Ideally, the modification will enhance the stability of VPs (Manzenrieder *et al.*, 2011), allow their targeting or labeling, or increase their efficiency in the intended application.

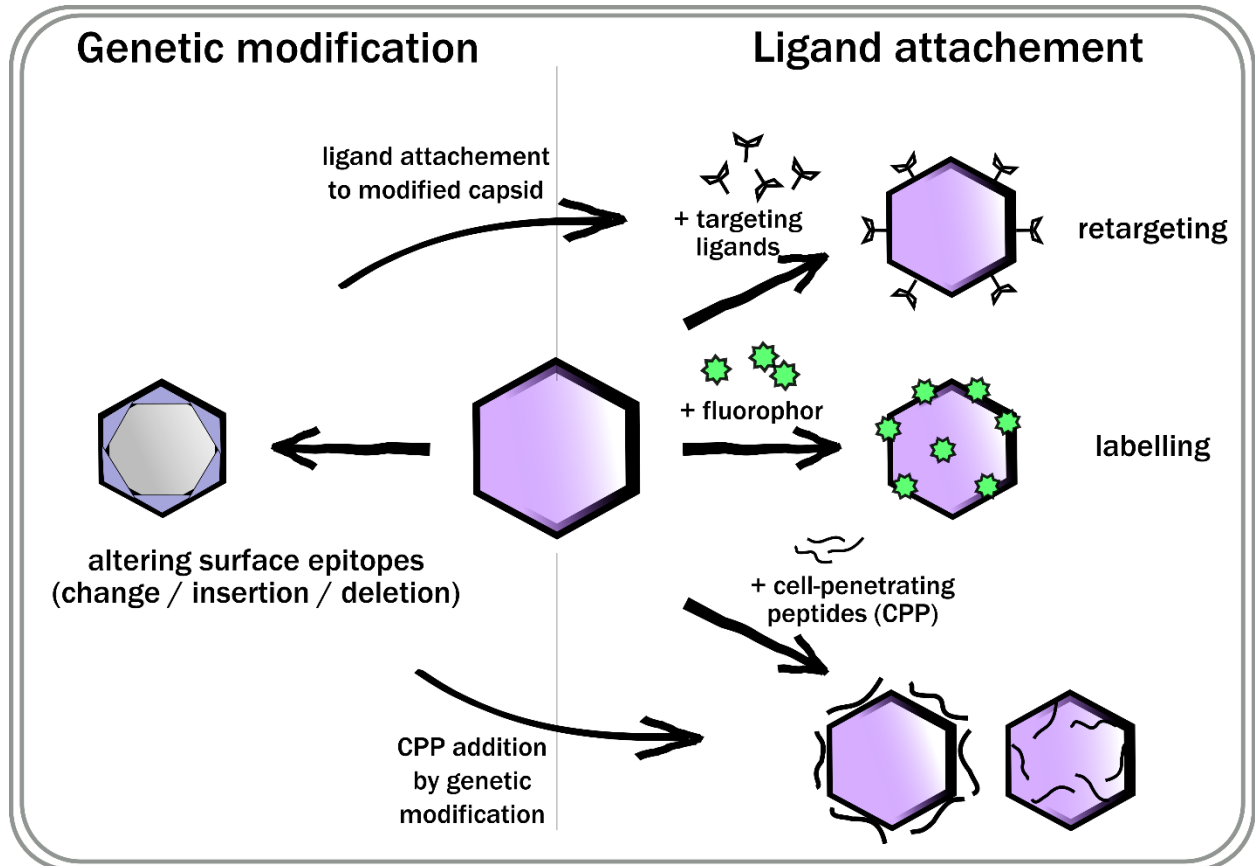


Figure 2: **Modifications of viral particles.**

In the case of genetic modifications, it is necessary to optimize the introduced changes to be functional, yet not too disruptive for the viral structure. For some viruses the ideal site in terms of stability or higher immunogenicity was already determined (Porta *et al.*, 1994; Takahashi *et al.*, 2008).

When VPs and their content are intended to reach specific tissue, an appropriate ligand (e.g., tumor-specific) can be added to their surface. Specific targeting enhances their efficiency and eliminates off-target effects.

Several *in vivo* studies show that ligands of cancer-cell-specific markers target VPs to tumor cells overexpressing the respective receptor. Examples of these ligands include transferrin or folic acid that were previously added to VPs of various origins (e.g. the MS2 phage or plant viruses, such as Hibiscus chlorotic ringspot virus, cucumber mosaic virus, TMV or CPMV or even adenovirus; (Destito *et al.*, 2007; Ren *et al.*, 2007; Zeng *et al.*, 2013, reviewed in Steinmetz, 2010). Similarly, displaying peptide F56 targets CPMV particles to tumors overexpressing vascular

endothelial growth factor receptor 1 (reviewed in Yildiz *et al.*, 2011). Interestingly, CPMV VPs were shown to be naturally internalized into mammalian endothelial cells (Lewis *et al.*, 2006).

Bacteriophage Q β VPs were targeted with the use of several molecules, such as epidermal growth factor (Pokorski *et al.*, 2011), transferrin (Banerjee *et al.*, 2010), or cyclic RGD (Hovlid *et al.*, 2014).

Chemical conjugation can add targeting molecules using residues naturally containing carboxyl, amine, or thiol groups on the VP surface. For example, lactobionic acid used to target rotavirus VPs carrying doxorubicin was added to the amine groups of the VPs. This effectively targets them to the hepatoma cell line (Zhao *et al.*, 2011). Chemical acylation of surface-exposed amines was also used to change the binding properties of Q β particles. Even single-point mutation in the surface-exposed lysines actually reduced the binding to the initial target cells (Martino *et al.*, 2021).

In case a favorable residue for chemical conjugation is not present, it can be added as was demonstrated on the VPs derived from CCMV (Gillitzer *et al.*, 2002; Vervoort *et al.*, 2021) or CPMV (Wang *et al.*, 2002; Chatterji *et al.*, 2004). CCMV mutant with introduced cysteines was found more advantageous (in the sense of favorable positioning on the surface) for attaching influenza M2E peptide than when the peptide was added to the wild-type particle using natural amines (Cantin *et al.*, 2011).

Another type of molecule that can be added to the capsid surface is a fluorescent label. Labeled VPs have found their use as a research tool as well as in novel diagnostic approaches. Fluorescently labeled VPs are considered a promising non-invasive imaging tool (reviewed in Steinmetz, 2010). As an example, CPMV VPs were used for vascular imaging of live mouse and chicken embryos (Lewis *et al.*, 2006). Fluorescent agents were also attached to canine parvovirus, which then still interacts with their natural receptor TfR, ultimately enabling the visualization of tumor cells by these modified particles (Singh *et al.*, 2006).

An interesting modification is the alteration or insertion of epitopes on the VP capsid. The repetitive and well-characterized capsid surface of many viruses offers a wide range of possibilities for further manipulation. This is used in the development of chimeric VPs for vaccines (see also Chapter 2.1.1) as well as for modifications of VPs for serodiagnostics. Their vast potential for vaccination purposes was well documented on papillomavirus-based VPs. Epitopes or polypeptides can be inserted in or fused with various parts of either major capsid protein L1 or minor capsid protein L2. This allows for harboring proteins/epitopes of varying lengths and will result in varying immunogenicity and antibody- or cell-mediated immune response (reviewed in

Xu *et al.*, 2006). Insertion of heterologous epitopes can increase the immunogenicity and induce a stronger antibody response against the foreign epitope (Peralta *et al.*, 2009).

Modifications of VPs are also useful for serological assays. Reduction of the serological cross-reactivity of flaviviruses was achieved by developing a novel antigen. VPs based on the St. Louis encephalitis virus and West Nile virus were mutated within the cross-reactive epitopes of the envelope protein. The antibody reactivity of resulting mutants was tested with various serum samples (including some from patients infected with other flaviviruses), and the mutant variants showed higher specificity than the wild-type VPs (Roberson *et al.*, 2007).

Later, the same approach, in combination with pre-incubation of serum with heterologous antigen, was used for the development of ELISA for differential serodiagnostic of other flaviviruses (Rockstroh *et al.*, 2017). A recent study further elaborated on this approach and achieved improved specificity with the use of the mutants and relative mutant to wild-type optical density (Tsai *et al.*, 2020).

2.1.5 Modification of VPs with cell-penetrating peptides

One of the modifications that has been used to customize viral nanoparticles for intracellular delivery is their association with cell-penetrating peptides. Cell-penetrating peptides are a class of short peptides (5–40 amino acids) that are able to penetrate through cellular membranes, delivering associated cargoes along with them. The association with cargoes can be achieved either covalently (be it chemical conjugation or genetic modifications) or by noncovalent bonds formed by the coincubation of cell-penetrating peptides and the cargo (often with an opposite electric charge). Both approaches were assessed using polyomavirus- and papillomavirus-derived VPs as a part of this thesis (Chapter 4.2.2). Cell-penetrating peptides can be designed artificially, but many are derived from naturally existing proteins that employ their membrane-penetrating properties in their original functions. An example of this is cell-penetrating peptides derived from viral proteins (reviewed in detail by *Hejtmánková et al.*, 2021).

Their limitation is potentially higher immunogenicity (which is also the case for bacteria-derived CPPs). Additionally, many CPPs used *in vitro* do not meet safety or biodegradability requirements for *in vivo* applications (reviewed in Durymanov and Reineke, 2018).

While the mode of action may vary (various mechanisms of increased uptake were proposed as well as facilitating the release from endocytic compartments), their ability to enhance the efficiency of intracellular delivery applies even for the already quite efficient adenoviral vectors and other VPs (Lehmusvaara *et al.*, 2006; Youn *et al.*, 2008; Eto *et al.*, 2009; Kida *et al.*, 2010; Park *et al.*, 2010; Chen *et al.*, 2011; Fenard *et al.*, 2013; Nigatu *et al.*, 2013; Liu *et al.*, 2014;

Majdoul *et al.*, 2016). They may also alter the tropism of VPs: HBV particles were successfully retargeted to human carcinoma cells (Gan *et al.*, 2018), adenovirus type 5 was shown to transduce cells non-permissive for wild-type adenovirus (Yu *et al.*, 2011).

CPPs can also aid in overcoming extracellular barriers such as protein corona (see Chapter 2.1.3.1), as the reduction of serum interaction with the surface of VPs modified with cationic polymers was documented (Kim *et al.*, 2010).

We reviewed the use of CPPs with VPs in a review (*Váňová et al., 2019*) and a book chapter (*Hejtmánková et al., 2021*) (see the summaries below or refer to the full publications in Attachment 1 and 2).

2.1.5.1 The Utilization of Cell-Penetrating Peptides in the Intracellular Delivery of Viral Nanoparticles

Jana Váňová, Alžběta Hejtmánková, Marie Hubálek Kalbáčová, and Hana Španielová
Materials, 2019, IF₂₀₁₉ = 3.057
see Attachment 1

In this review, we examine the potential of using cell-penetrating peptides as a means of transporting VPs of various origins into cells. The use of cell-penetrating peptides is a promising approach for the intracellular delivery of VPs in order to enhance the effectiveness of therapies based on these particles. We highlight the potential benefits of this approach, such as allowing for a lower dose of administered VPs to facilitate more efficient binding and/or internalization of these VPs on a broader spectrum of target cells.

Contribution

I participated in the writing and editing of the manuscript as well as figure design.

2.1.5.2 Cell-penetrating peptides in the intracellular delivery of viral nanoparticles

Alžběta Hejtmánková, Jana Váňová, and Hana Španielová
Vitamins and Hormones, 2021
see Attachment 2

This book chapter summarizes the current state of knowledge about cell-penetrating peptides derived from viral proteins and their use in viral nanoparticle delivery.

Contribution

I was the main contributor to the writing and editing of the manuscript and created all the figures.

Overall, the evidence suggests that the manipulation with VP surface offers great potential for precise tailoring to specific applications.

2.2 Polyomaviruses

The first discovered member of the *Polyomaviridae* family was MPyV in the 1950s. Since then, more than 100 members have been known, including viruses infecting mammals and birds (Moens *et al.*, 2017). There are currently 14 known species of PyVs infecting humans (Kamminga *et al.*, 2019). Originally studied for their tumorigenicity (“poly oma” comes from the Greek “many tumors”) as mammalian polyomaviruses have tumorigenic properties. Most are known to induce transforming changes *in vivo* or in cell cultures *in vitro*. During a productive infection cycle, the transformation of the cell is prevented by cellular lysis. In non-permissive cells, the production of tumor (T-) antigens leads to oncogenic changes, and transformation is not prevented as new virions are not assembled. *In vivo*, the immunosuppression of the host is considered to permit tumor development (reviewed in Dalianis and Hirsch, 2013). Since their discovery, simian virus 40 (SV40) and mouse polyomavirus (MPyV) have been intensively studied as model DNA tumor viruses. MPyV-derived particles were first used to carry heterologous DNA into cells in 1995 (Forstová *et al.*, 1995) and are the model VPs we use in our research. Additionally, we use VPs derived from a human pathogen polyomavirus BK (BKPyV) for applications in human serodiagnostics. Therefore, this chapter characterizes polyomaviruses with a focus on MPyV and describes the pathogenesis of BKPyV and the context of the importance of its serodiagnostics.

2.2.1 The structure and genome of polyomaviruses

The icosahedral capsid (*Figure 3*) of polyomaviruses is assembled from 72 pentamers of major capsid protein VP1 (Rayment *et al.*, 1982). In each pentamer, one of the minor capsid proteins (VP2 or VP3) is inserted (Barouch and Harrison, 1994). Their diameter of 45 nm makes them one of the smallest viruses and, along with the ability of VP1 to self-assemble (Salunke *et al.*, 1986), even in the absence of the minor capsid proteins (Montross *et al.*, 1991), also a promising candidate for biomedical applications. The VP1 protein contains four loops (BC, DE, EF, and HI) that make up the surface of the capsid (Stehle *et al.*, 1994). The C-terminus (apx. 60 amino acid residues) of VP1 facilitates the contact between individual pentamers (Stehle *et al.*, 1996), while the N-terminus contains a sequence-independent DNA binding site and binds the genome (Moreland *et al.*, 1991).

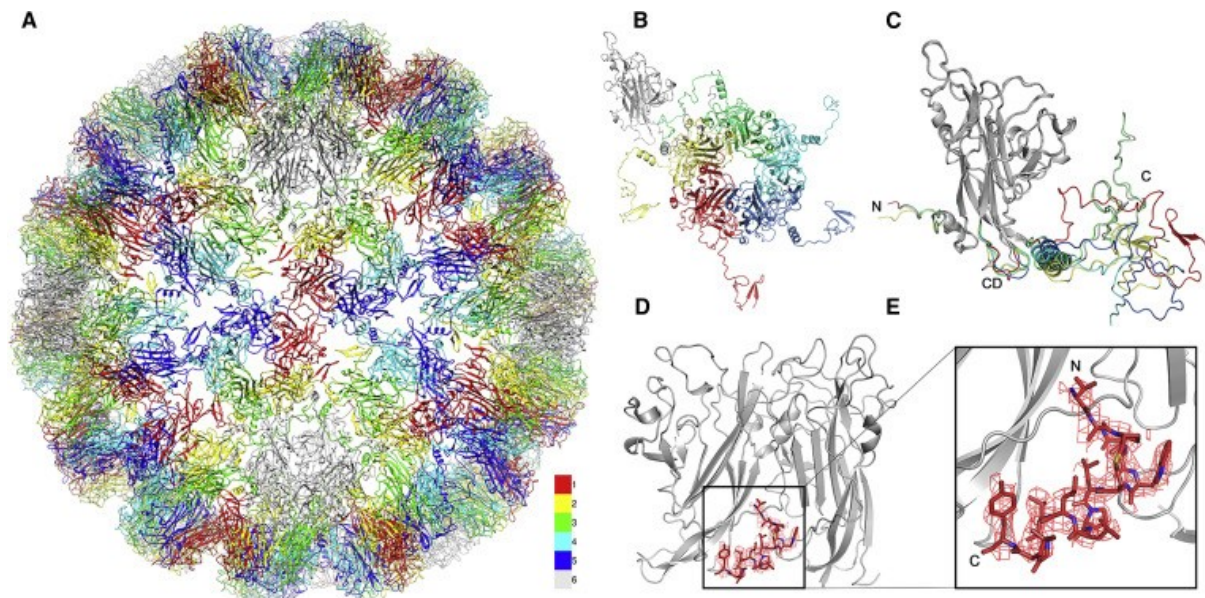


Figure 3: **Structure of BKPyV capsid.** The BKPyV capsid (A) is made up of 72 VP1 pentamers (B). Five VP1 monomers (C) form a pentamer in which the minor capsid protein VP2/3 (D, E) is buried. The image „Atomic Model for the BKV Capsid and Interaction with the Minor Capsid Proteins“ is reused from Hurdiss *et al.*, 2018, licensed under [CC BY 4.0](https://creativecommons.org/licenses/by/4.0/).

The genome of polyomaviruses is a 5 kbp long circular molecule of dsDNA. Generally, the genome consists of three regions: the non-coding control region (NCCR), which contains regulatory sequences; the early gene region, which codes T-antigens: small, large, and in some polyomaviruses (including MPyV) also middle T antigen; and late gene region coding the structural proteins VP1, VP2 and VP3, and in some polyomaviruses (including BKPyV) also non-structural protein called agnoprotein (Fields *et al.*, 2013).

The VP1 protein of MPyV forms the capsid and (mainly its BC loop region, Stehle *et al.*, 1994) is responsible for the contact with cellular receptors (gangliosides GD1a and GT1b) through interaction with sialic acid (Tsai *et al.*, 2003). The role of the hydrophobic minor capsid proteins VP2 and VP3, which are buried inside the pentameters, is not completely understood. Their absence renders the virus uninfecious (Mannová *et al.*, 2002), and because of their membrane activity, it is thought that they act as viroporins and facilitate the interaction with the ER membrane, thus playing a key role in the delivery of polyomaviral DNA into the nucleus (Rainey-Barger *et al.*, 2007; Huérfino *et al.*, 2017).

2.2.2 The passage of MPyV through the cell

The first step of infection is the interaction of MPyV VP1 with viral receptors, gangliosides GD1a and GT1b (Tsai *et al.*, 2003; Gilbert *et al.*, 2005), and co-receptor $\alpha 4 \beta 1$ integrin (Caruso *et al.*, 2003a; Caruso *et al.*, 2003b). Then, MPyV enters the cell via receptor-mediated clathrin-independent endocytosis (Richterová *et al.*, 2001; Mannová and Forstová, 2003). The virus is

guided into the ER through the endosomal-lysosomal system: the virus colocalizes with early endosome antigen 1 thirty minutes post-infection, and the acidification of these vesicles enables the infection to continue (Liebl *et al.*, 2006). MPyV can then be detected in vesicles containing Rab5 GTPases and then also late endosomes containing Rab7 (Zila *et al.*, 2014). The processes required for the transportation to the ER and eventual translocation into the nucleus are not well characterized, although evidence suggests that it includes the exit of the virus from the ER into the cytoplasm (reviewed in Mayberry *et al.*, 2021).

MPyV can be detected in the perinuclear area 3 hours after infection (Richterová *et al.*, 2001), but the genome is first detected in the nucleus after 6 hours (Mannová and Forstová, 2003). It is also clear that not all virions detected in the cell are successful at reaching the nucleus. In the nucleus, the infection commences with early transcription and continues with the replication of the viral genome and the transcription of late genes. After capsid assembly, the new virions exit the cells through lysis.

2.2.3 Polyomavirus BK

BKPyV was first isolated in 1971 (Gardner *et al.*, 1971). Most of the population (up to 90%) undergoes an asymptomatic infection in childhood (Stolt *et al.*, 2003), after which the virus persists in the kidneys (Heritage *et al.*, 1981; Chesters *et al.*, 1983). Although the persisting infection is usually silent, BKPyV is a known causative agent in a variety of complications in immunocompromised patients, specifically after transplantations (Gardner *et al.*, 1971; Bedi *et al.*, 1995; Purighalla *et al.*, 1995; Randhawa *et al.*, 1999). Also, there are some studies suggesting its potential role in bladder cancer (reviewed in Dalianis and Hirsch, 2013).

2.2.3.1 The four subtypes of BKPyV

Different subtypes (I-IV) of BKPyV were first identified in 1989 (Knowles *et al.*, 1989) and later established as four distinct serotypes based on neutralizing antibodies - antibodies raised against subtypes from one group fail to neutralize isolates from different groups. However, cross-reacting (but not cross-neutralizing) antibodies are also present in serum after infection, which complicates easy serological distinction since ELISA cannot discriminate between cross-reacting and neutralizing antibodies and differentiate antibodies against other subtypes (Pastrana *et al.*, 2012).

Similarly to varying tropism and pathogenic potential of different strains of MPyV (Mezes and Amati, 1994; Bauer *et al.*, 1995), the four BKPyV subtypes differ in tropism, replication rate, prevalence, and potentially even pathogenesis. In the European population, subtype I is the most

prevalent (60%), followed by subtype IV (30%). Subtypes II and III are considered rare (less than 10%) (Jin *et al.*, 1993a; Zheng *et al.*, 2007; Zhong *et al.*, 2009; Pastrana *et al.*, 2012). Subtype I was demonstrated to have a higher replication rate and/or efficiency than subtype IV in human proximal tubule epithelial and renal epithelial cells, which could be the reason why it is more frequent in the population (Nukuzuma *et al.*, 2006). Additionally, higher titers were observed for BKPyV-I than BKPyV-IV in patients with BK polyomavirus nephropathy (BKPyVAN, Krautkrämer *et al.*, 2009), even though the difference was not statistically significant in this particular study.

Interestingly, contrary to these findings, it is actually infection with BKPyV-IV that was reported to lead to BKPyVAN in kidney transplant recipients (KTR) more likely (Schwarz *et al.*, 2016). In some studies, BKPyV-I is more frequent in the urine of transplanted patients (Takasaka *et al.*, 2004; Krautkrämer *et al.*, 2009), which corresponds to its higher prevalence in the population. Some of the studies, however, might suffer from detection bias favoring BKPyV-I due to the use of suboptimal primers (Randhawa *et al.*, 2011).

The differences in the sequences of the subtypes do not lie only in the VP1-coding region. Each subtype has their own prototypical NCCR, and even intra-subtype differences were reported, even though the region is considered to be conserved during the evolution (Takasaka *et al.*, 2004). Some authors attribute the varying replication rate as well as the greater BKPyVAN-inducing potential of some isolates to the rearrangements in the NCCRs (Chen *et al.*, 2001; Azzi *et al.*, 2006), as rearrangements in the NCCR are associated with higher viruria and viremia and are also found in isolates from patients with BKPyVAN (Olsen *et al.*, 2006; Gosert *et al.*, 2008). Furthermore, the intra-subtype differences can also play a role. In the study of Wang *et al.*, the WW-strain (archetype subtype I strain) was found more frequently in the urine of immunosuppressed patients after liver transplantation (Wang *et al.*, 2016). The variations in the NCCR are thought to be the cause of the increased replication rate in comparison with the Dunlop strain¹ (also subtype I) (Wang *et al.*, 2016).

The sole role of rapid rearrangement of the NCCR was however not confirmed in an *in vitro* study by Tremolada *et al.*, who showed that other factors must be involved in the different replication rates of the subtypes (Tremolada *et al.*, 2010c).

The serological differences of the four groups were studied by Jin *et al.*, who identified a hypervariable region in the sequence of VP1 - AAs 61-83, which corresponds to the BC loop (Jin

¹ Dunlop strain is a prototypical BKPyV strain and it is derived from the original Gardner strain. The plasmid pBKV (34-2) that contains its genome (GenBank: V01108.1) is often used in research and diagnostics (reviewed in Henriksen *et al.*, 2015).

et al., 1993b). High intra-patient variability in the BC loop was also found in BKPyVAN patients (Luo *et al.*, 2012). The differences in the AA sequence of VP1 (and especially the hypervariable BC loop) can also contribute to different tropism of the BKPyV subtypes. The role of BC-loop AAs in interaction with the cellular receptors was already established for BKPyV as well as for other polyomaviruses (Freund *et al.*, 1991; Stehle *et al.*, 1994; Gee *et al.*, 2004; Neu *et al.*, 2013). Similarly, the production and subsequent testing of BKPyV VP1 mutants deficient in cell binding showed the importance of AAs in the BC and HI loop for receptor interaction (Dugan *et al.*, 2007). From varying hemagglutination properties, Pastrana *et al.* deduced that the subtypes differ in cell tropism. They confirmed this by transduction experiments and suggest it leads to different pathogenic potential *in vivo* (Pastrana *et al.*, 2013).

High polymorphism of BKPyV VP1 BC loop in kidney transplant (KTx) patients was previously detected (Baksh *et al.*, 2001; Tremolada *et al.*, 2010a), and it was shown that these changes accumulate over time as BKPyV progresses (Randhawa *et al.*, 2002). Mutations in the BC loop were shown not to be associated with a higher replication rate (Krautkrämer *et al.*, 2009). This was confirmed *in vitro*, as VP1 variants detected in KTx patients with BKPyVAN exhibited similar replication activity in Vero cells. Interestingly, the hemagglutination activity of these variants did not change when compared to the wild-type Dunlop strain, even though they caused more cytopathic effects. The authors conclude that the replication efficiency does not necessarily correlate with pathogenic potential (Tremolada *et al.*, 2010a). In another study, the same team of authors studied the progression of infection by different subtypes *in vitro*. Even though all viral subtypes initially exhibited the same growth rate, after 59 days post-infection, subtype III was clearly the least cytopathic with the slowest replication rate among the four subtypes, and the viral loads of subtype IV were lower than those of subtypes I and II. Furthermore, they introduced an amino acid specific for subtype III (Gln 68) into the sequence of VP1 of subtypes I, II, and IV (substituted 68 Leu to Gln) and tested whether this change would decrease viral growth. Surprisingly, the viral growth remained the same, suggesting that the difference in replication rate is not associated solely with this specific amino acid difference (Tremolada *et al.*, 2010c). Tremolada *et al.* also identified several amino acid changes in the VP1 sequence of BKPyV in the urine of patients with BKPyVAN, with most mutations detected in the BC and EF loop (Tremolada *et al.*, 2010b). Overall, the studies by this group indicate that the subtype-specific region in the BC loop is involved in the differences in the pathogenesis of BKPyV subtypes, but other regions, such as the EF loop, are also crucial. In a more recent study, the number of mutations in the BC loop in isolates from KTx recipients was associated with the duration of BKPyV viruria (McIlroy *et al.*,

2020). However, the different pathogenic potential was not confirmed by another recent report (Wunderink *et al.*, 2019a).

2.2.3.2 Posttransplant complications, their risk factors, and prevention

An active BKPyV infection after kidney transplantation is common, as BKPyV can be detected in the urine of 10-45% of kidney recipients (reviewed in Kwak *et al.*, 2002) and up to 10% of kidney recipients develop BKPyVAN, which can compromise the kidney function and can lead to loss of graft (reviewed in Hirsch *et al.*, 2005). Additionally, BKPyV causes hemorrhagic cystitis in patients after hematopoietic stem cell transplantation (Bedi *et al.*, 1995).

There are two possible sources of BKPyV causing BKPyVAN after kidney transplantation - reactivation of BKPyV persisting in the urinary tract of the recipient or *de novo* infection through the donated kidney. Initial reports discuss mainly the reactivation of the virus already present in the recipient due to the immunosuppression treatment following the transplantation (Binet *et al.*, 1999), and it was also observed in patients after the transplantation of hematopoietic cells and non-renal organs (Leung *et al.*, 2001; Iwamoto *et al.*, 2002; Stracke *et al.*, 2003; Wong *et al.*, 2007; O'Donnell *et al.*, 2009; Kuppachi *et al.*, 2016). Nonetheless, accumulating evidence supports the notion that it is the *de novo* infection transmitted from the donor that carries a high risk of BKPyVAN and associated adverse outcomes (Vera-Sempere *et al.*, 2006; Saundh *et al.*, 2013; Schmitt *et al.*, 2014; Schwarz *et al.*, 2016).

The first studies reported BKPyV as a cause of posttransplant complications in 1995 (Purighalla *et al.*, 1995; Randhawa *et al.*, 1999; Randhawa and Demetris, 2000; Hirsch, 2002). Since then, several risk factors of an active posttransplant BKPyV infection (which can lead to adverse outcomes) were identified. Even though, according to some studies, BKPyV viruria in donors does not necessarily correlate with the development of BKPyVAN in recipients (Verghese *et al.*, 2015), pretransplant BKPyV viruria in either donor or recipient was found to be a major risk factor by others and donor-derived transmission is thought to be an important source of infection in KTx (Schmitt *et al.*, 2014; Schwarz *et al.*, 2016). Donor seropositivity also carries a risk of BK viremia in the recipient after transplantation, even more so when the recipient was seronegative pre-transplant (Sood *et al.*, 2013), even pre-transplant seropositivity of the recipient is not protective against BK viremia (Bohl *et al.*, 2008). This might be due to the presence of antibodies that do not neutralize the acquired subtype, as recent studies showed that recipients without neutralizing antibodies are especially at risk of BKPyVAN due to donor-derived infections (Solis *et al.*, 2018; Lorentzen *et al.*, 2020). A recent study, however, claims that there is no association

of the clinical outcome with whether the BKPyV subtypes in the donor-recipient pair match (Wunderink *et al.*, 2019a).

Assessing the recipient's post-transplantation serostatus may be useful for monitoring the infection, as antibody response corresponds with the intensity of the infection but is not, however, indicative of BKPyV clearance (Bohl *et al.*, 2008). Similarly, the antibody titers in donors reflect the BKPyV replication levels and thus also indicate BKPyV levels in the donated kidney (Wunderink *et al.*, 2017a). Indeed, the antibody level in the donor is strongly predictive of BKPyVAN in the recipient (Wunderink *et al.*, 2017b). A recent study on 329 patients and 222 donors confirmed that patients at risk could be identified if the BKPyV seropositivity before transplantation and antibody titer measurements of both donor and recipient are known (Dakroub *et al.*, 2021).

Despite some reports of renal dysfunction following BKPyVAN, even after non-renal transplantations (reviewed in Kuppachi *et al.*, 2016), BKPyVAN is now thought to be mainly caused by the *de novo* infection contracted through the transplanted kidney. This is further supported by the notion that it is quite rare in patients after non-renal solid organ transplantation with similar immunosuppressive treatment (Pastrana *et al.*, 2012). BKPyV-IV seroconversion during the first year after transplantation was observed in 50% of initially seronegative kidney recipients. Wunderink *et al.* recently confirmed the correlation between the donor BKPyV serotype and the subtype replicating in the recipient (Wunderink *et al.*, 2019a).

The emergence of BKPyVAN in the 1990s is generally attributed to new immunosuppression protocols (reviewed in Hirsch, 2002). This was supported by more recent studies, which identified some immunosuppressive treatment combinations as risk factors (Egli *et al.*, 2009; Hirsch *et al.*, 2013a). Since early post-transplant BKPyV viruria and viremia in recipients is an indicator of potential later BKPyVAN (Schwarz *et al.* 2016), current strategies for BKPyVAN management include monitoring of BK viruria in patients and attenuation of immunosuppressive regime (Kasiske *et al.*, 2010). A reliable sign of an ongoing BKPyV infection is the appearance of so-called decoy cells (cells with viral inclusions) in urine. It was shown that in seropositive donor / seronegative recipient pairs, the subsequent early onset of decoy cells shedding in the recipient's urine with a significantly higher count of the cells (Hisadome *et al.*, 2020). However, their detection is not predictive of BKPyVAN (Binet *et al.*, 1999). The replication rate in patients with BKPyVAN was shown to be higher (Krautkrämer *et al.*, 2009), but the viral load detectable in urine does not differ between patients with or without BKPyVAN (Tremolada *et al.*, 2010b).

Changes in the immunosuppressive regime carry a risk of transplant rejection or graft-vs-host disease (Hirsch *et al.*, 2013b). The successful use of antibiotics was also reported in the treatment of BKPyVAN (reviewed in Hirsch, 2002) and BKPyV-associated hemorrhagic cystitis (Najafabadi *et al.*, 2020; Tooker *et al.*, 2020; Mehrvar *et al.*, 2021). Some authors emphasize the potential benefits of pre-transplant vaccinations or administration of intravenous immunoglobulins (Pastrana *et al.*, 2012; Wunderink *et al.*, 2017b), but this is not yet routinely done.

Another potential determinant of higher pathogenicity of some isolates is the aforementioned rearrangements in the NCCR, although their role in BKPyVAN has not been established. Anyway, the detection of BKPyV DNA in otherwise healthy, not immunosuppressed individuals is usually not possible even when persistent BKPyV infection is present. Therefore, the clinically more important predictor seems to be the presence or absence of neutralizing antibodies in both recipient and donor, even in the case of nonviremia and nonviruria.

2.2.3.3 Serological methods in BKPyV research

One of the current goals of BKPyV research is establishing a convenient serological test for pretransplant screening and stratification of at-risk patients (Sood *et al.*, 2013; Schmitt *et al.*, 2014; Schwarz *et al.*, 2016; Wunderink *et al.*, 2017a; Wunderink *et al.*, 2017b).

Detection of neutralizing antibodies is possible by neutralization-based assays, which test the ability of patient serum to inhibit (pseudo)infection by live virus or PsVs containing reporter gene. This method was used to differentiate the BKPyV serotypes (Pastrana *et al.*, 2012) and highlight the importance of neutralizing antibodies (Pastrana *et al.*, 2013; Solis *et al.*, 2018). However, it is quite inconvenient in clinical settings, as it takes at least 24 hours to reliably detect the neutralizing effect of serum and requires life cell cultures along with adequate equipment and dedicated space (Wunderink *et al.*, 2019a).

Enzyme-linked immunosorbent assay (ELISA) would be more convenient as it is a more rapid serological test with lesser equipment requirements. A coated antigen captures antibodies present in serum, and the subsequent binding of labeled secondary antibodies enables the detection of captured antibodies and even antibody titer measurement.

While neutralization-based assays require the use of whole virus or PsVs containing minor capsid proteins VP2 and VP3, in ELISA-based methods for BKPyV serology, the coated antigen are usually either VP1-derived VLPs or monomeric VP1 fused with glutathione S-transferase (GST-VP1). This further adds to their convenience as polyomaviral VP1 is easy to produce in, e.g., a baculovirus expression system, and naturally self-assembles into VLPs. The advantage of VLPs is displaying the conformational epitopes that were shown to be the main target for

antibodies (Randhawa *et al.*, 2009). Indeed, VLPs-based ELISA was shown to be more sensitive than ELISA with coated GST-VP1, while their reactivity was comparable when VLPs were denatured (Bodaghi *et al.*, 2009). That said, the use of such antigens enables the detection of a whole spectrum of antibodies that bind the VP1 protein but does not allow for the recognition of subtype-specific (neutralizing) antibodies.

Taken together, the most commonly used ELISA does not discriminate between the reactivity with subtype-specific and general antibodies which cross-react between the subtypes. For this distinction, neutralization-based assays are usually used. They are, however, inconvenient in clinical settings and the interpretation of their results was made more intricate by recent reports of cross-neutralizing antibodies (Lindner *et al.*, 2019).

An antigen competition assay (ACA, also called preadsorption assay, serum competition analysis, or competitive inhibition assay) was previously used to assess the serological cross-reactivity between polyomaviral species (Carter *et al.*, 2003; Viscidi and Clayman, 2006; Viscidi *et al.*, 2011). The method uses a pre-adsorption step, where soluble antigen captures the “unfavorable” (cross-reacting) antibodies, for example, given a BKPyV-positive serum sample that displays the reactivity with polyomavirus JC (JCPyV), while truly JCPyV-negative, adding JCPyV-derived antigen will result in binding all antibodies cross-reacting with JCPyV. The subsequent testing of such preincubated sera on BKPyV-coated antigen (e.g., on an ELISA) will reveal BKPyV-specific antibodies left in the sample. The same sample, when preincubated with BKPyV-derived antigen, should show no reactivity with coated BKPyV since all present antibodies (BKPyV-specific as well as JCPyV cross-reacting) should be captured by the soluble antigen. This way, the elimination of potential false positivity should be achieved.

With regard to polyomaviruses, this approach was also previously used for confirming species specificity of an FDA-approved JCPyV ELISA test (STRATIFY™ JCPyV Antibody ELISA) (Lee *et al.*, 2013). Recently, this principle was also used in conjunction with Luminex technology-based assay for BKPyV serotyping (Wunderink *et al.*, 2019b). This study addresses one of the aims of this thesis and was published simultaneously with our publication (Chapter 4.1.1.1, *Hejtmánková et al.*, 2019), which is also a part of this thesis, and it will be further addressed in the discussion (see Chapter 5.1.4). Briefly, the Luminex bead assay uses GST-VP1 bound to the surface of fluorescent beads. The beads contain two fluorescent dyes in various ratios, which results in 100 different combinations recognizable by the Luminex analyzer. Each bead type can then carry a different antigen. The present anti-VP1 human IgG interacts with the capture antigen and is detected through the interaction with biotinylated goat anti-human antibody and streptavidin–R Phycoerythrin as a reporter, which produces the fluorescence signal. The Luminex

analyzer lasers detect the reporter fluorescence as well as the bead type (i.e. antigen) specific fluorescence. It allows for fast serological testing against multiple agents at once (Waterboer *et al.*, 2005).

2.3 Polyomavirus-derived particles

The properties of the major structural protein of polyomaviruses, VP1, make them an ideal candidate for application in nanotechnology. Its production is relatively easy and possible in various expression systems, and upon expression, it self-assembles into capsid-like structures devoid of the viral genome and, therefore, noninfectious (Salunke *et al.*, 1986). The presence of minor capsid proteins, VP2 and VP3, is not essential for the assembly, but when they are coexpressed, it leads to their incorporation into the pentamers of VP1 (An *et al.*, 1999). Though they lack the viral genome, the incorporation of DNA into these VPs is possible through the sequence-independent interaction with VP1 (Gillock *et al.*, 1997; An *et al.*, 1999). The potential of polyomaviral VPs to carry genes into mammalian cells was proposed in the early 1970s (Osterman *et al.*, 1970; Qasba and Aposhian, 1971; Aposhian *et al.*, 1975).

Two types of polyomaviral particles consisting of VP1 are used: virus-like particles (VLPs) or pseudovirions (PsVs). VLPs are most commonly produced in insect cell lines using a baculovirus expression system, specifically baculovirus genetically engineered to express VP1 but, in some cases, also VP2 and/or VP3. PsVs are produced in a mammalian expression system and, besides VP1, usually contain also VP2, VP3, and a reporter gene. While VP1-VLPs can successfully enter the cell (Richterová *et al.*, 2001), only MPyV particles containing minor capsid proteins are able to transduce cells, as they are essential for successful infection of the wildtype MPyV (Mannová *et al.*, 2002). In the case of JCPyV, however, VP1-VLPs are able to deliver nucleic acids (Chou *et al.*, 2010; Fang *et al.*, 2015). As we mostly use MPyV-derived VPs for delivery (and SV40-VPs as control) and VPs derived from human polyomaviruses (mostly BKPyV), they will be the main focus of the following chapters.

2.3.1 Polyomaviral particles in biomedicine

MPyV (as well as SV40) is quite well characterized since it was used in the research of basic cellular mechanisms and tumorigenesis as well as the principles of viral capsid structure (Klug and Finch, 1965; Finch, 1974; Rayment *et al.*, 1982). This makes it an excellent model for further research.

2.3.1.1 The use of Polyomavirus VP in serodiagnostics

For serological purposes, human as well as animal polyomaviral VLPs are used. Over the years, they were used as antigens in ELISA (Stolt *et al.*, 2003; Viscidi *et al.*, 2003; Touzé *et al.*, 2010; Zielonka *et al.*, 2011; Nicol *et al.*, 2012, 2013), hemagglutination inhibition assay (Knowles *et al.*, 1989; Zielonka *et al.*, 2012) and neutralization assays (Pastrana *et al.*, 2009, 2012).

A known limitation of VLP use in serodiagnostics, especially in ELISA-based assays is the serological cross-reactivity between some polyomavirus species, which may result in a false positive signal. In case of BKPyV, this cross-reactivity applies also to known serotypes, which complicates diagnostic efforts for clinical purposes. Serological tests used for BKPyV diagnostics are reviewed in Chapter 2.2.3.3.

2.3.1.2 Polyomavirus VP-based vaccines

Although polyomaviral vaccines are not (yet) used to prevent polyomavirus infections, VLPs (in general) make an excellent basis for vaccines. Their use in vaccines is advantageous as they also function as an adjuvant due to their high immunogenicity (Kawano *et al.*, 2013; Kawano *et al.*, 2014). Another advantage is the possibility of preparing VLPs without the viral genome. We can utilize these properties to elicit immune responses against pathogens of non-polyoma viral origin through the preparation of chimeric vaccines. Foreign epitopes were inserted into the VP1 surface loops of polyomaviruses. Namely, parts of an HBV preS1 sequence were inserted into MPyV VLPs (Skrastina *et al.*, 2008) or hamster polyomavirus (Gedvilaite *et al.*, 2000) and, in both cases, used to successfully immunize mice. Vaccines based on pseudotyped HaPyV VPs as carriers for foreign epitopes were used to induce immune response in numerous studies (Zvirbliene *et al.*, 2006, 2014; Lasickienė *et al.*, 2012; Mazeike *et al.*, 2012) and it was shown that the choice of the carrier VP influences the immunogenicity depending on the properties of the inserted epitope (Gedvilaite *et al.*, 2015).

Minor capsid protein can be used in order to increase the size of inserted epitopes: immunization of mice induced humoral immunity against EGFP fused with the C-terminus of VP3 (Boura *et al.*, 2005) and cell-mediated immunity against a tumor marker Human Epidermal Growth Factor Receptor 2 (Her-2/neu) fused with the VP2 (Tegerstedt *et al.*, 2005a). On the other hand, this approach was not successful in all reported cases (Hrusková *et al.*, 2009).

Similarly to the above-mentioned chimeric malaria vaccine based on HBsAg VPs (see Chapter 2.1.1, Pattinson *et al.* prepared a vaccine containing chimeric MPyV VLPs carrying an epitope derived from *Plasmodium yoelii*. This vaccine effectively protects against this rodent malaria parasite (Pattinson *et al.*, 2019).

A recent study used HaPyV as a carrier for a murine prion protein and demonstrated successful immunization by the produced VPs. Moreover, they compared nine constructs (with various lengths and parts of the peptide fused with either VP1 or VP2 at different positions). One of these constructs (with the 37 aa long peptide inserted into the VP1) elicited a humoral immune response and significantly prolonged the survival of immunized mice after being challenged with the prions (Eiden *et al.*, 2021).

A rapid production of chimeric MPyV VLPs presenting foreign peptides (hemagglutinin-derived or M2e antigen from influenza and the J8 peptide from Group A Streptococcus) in *E. Coli* was developed to create convenient vaccines against these pathogens (Middelberg *et al.*, 2011; Anggraeni *et al.*, 2013; Rivera-Hernandez *et al.*, 2013; Seth *et al.*, 2016). These vaccines allow for the quick re-engineering for newly emerging strains (Wibowo *et al.*, 2014). It also enables fast preparation of high quantities of VLPs, presenting epitopes of more complex pathogens that are not as easy to produce in the form of harmless VLPs, such as rotavirus (Tekewe *et al.*, 2017). In the case of a veterinary MPyV-based vaccine against porcine circovirus, the VP1 protein carries the inserted epitope in the C-terminal part, and the resulting chimeric protein forms giant pentamers (Fraiberk *et al.*, 2017). The HaPyV VP-based chimeric vaccines have also proved useful for the generation of monoclonal antibodies (Zvirbliene *et al.*, 2006, 2014). Interestingly, the pre-existing immunity against the carrier VPs does not interfere with the immunization with these chimeric vaccines (Chuan *et al.*, 2013).

2.3.1.3 Polyomavirus VP-mediated gene therapy

As the interaction of VP1 with DNA is sequence-independent, DNA of nonviral origin can be encapsidated (Stokrová *et al.*, 1999). The packaging capacity of MPyV allows for the transfer of DNA fragments up to 3 kb (Forstová *et al.*, 1995). Moreover, JCPyV VLPs were found to accommodate DNA even larger, up to 9.4 kb (Fang *et al.*, 2012). In terms of safety, the virus-free preparation of polyomaviral VPs, in combination with the possibility to efficiently encapsidate DNA, gives them an advantage over other viral vectors, which keep their genome (even if usually modified or partially deleted; reviewed in Tegerstedt *et al.*, 2005b).

JCPyV VPs were extensively studied as a viral vector and were shown to deliver DNA as early as 1999 (Ou *et al.*, 1999). Since then, JCPyV VLPs have been reported to successfully deliver antisense oligodeoxynucleotides (Wang *et al.*, 2004), transduce bladder cancer cells, and inhibit the growth of bladder tumors in the xenograft mouse model through the delivery of a suicide gene thymidine kinase (Fang *et al.*, 2015; Lai *et al.*, 2021). Similarly, it was delivered into adenocarcinoma (Chen *et al.*, 2010; Chao *et al.*, 2016), lymphoma (Chao *et al.*, 2015), and prostate

cancer cells (Lin *et al.*, 2019a; Shen *et al.*, 2021). Short RNAs were also delivered by JCPyV VPs into adenocarcinomas (Lin *et al.*, 2019b) and macrophages (Chou *et al.*, 2010) and were also successful in *in vivo* experiments (Chou *et al.*, 2010; Hoffmann *et al.*, 2016). The natural neurotropism of JCPyV was exploited for the gene delivery into glioblastoma cells (Chao *et al.*, 2018).

The preexisting immunity to the parent virus can reduce the efficiency (reviewed in Shirley *et al.*, 2020), which makes JCPyV a less suitable candidate for gene therapy, as its seroprevalence reaches up to 91% in some healthy adult populations (reviewed in Wollebo *et al.*, 2015). However, some researchers reason that surface modifications (e.g., attachment of targeting moiety) might help solve the interference with an antibody-mediated immunity (Lai *et al.*, 2021).

MPyV VPs have the advantage of no pre-existing antibodies in the human population. *In vitro* transduction by MPyV VPs leads to either transient or stable gene expression (Forstová *et al.*, 1995; Soeda *et al.*, 1998; Krauzewicz *et al.*, 2000). Long-term expression was also achieved by MPyV VP-mediated delivery *in vivo* (Heidari *et al.*, 2000; Krauzewicz *et al.*, 2000; Clark *et al.*, 2001).

The portfolio of polyomavirus VPs for *in vivo* use in humans may be expanded by the use of other polyomaviruses, including hamster polyomavirus (Voronkova *et al.*, 2007), murine pneumotropic virus (Tegerstedt *et al.*, 2003), but also human polyomavirus BK (Touzé *et al.*, 2001) or SV40 (Kimchi-Sarfaty *et al.*, 2002). Besides pre-existing antibodies against the used polyomavirus, the serological cross-reactivity also needs to be taken into account.

2.3.1.4 The Polyomavirus VP-mediated delivery of other cargoes

Besides nucleic acids, various cargoes can be encapsidated through deliberate disassembly and reassembly of the VPs (Goldmann *et al.*, 2000). Alternatively, VP1 can be prepared in the form of pentamers, and then the VPs assembled *in vitro*, encapsidating the desired cargo, as was shown with, e.g., quantum dots (Li *et al.*, 2009). Cargo can also be attached to the inner VP surface through VP1. MPyV VP1 was modified to incorporate proline-rich ligands by the addition of a WW domain (which binds to short proline-rich sequences) (Schmidt *et al.*, 2001a). This approach allows the delivery of hydrophobic cargo and also the subsequent release of cargo in a controlled manner. A model hydrophobic anticancer drug was encapsidated in JCPyV VLPs through linkage with inner cysteine residues on VP1 and can be released by glutathione (Niikura *et al.*, 2013).

Several studies reported successful encapsidation and subsequent intracellular delivery of proteins through fusion with minor capsid proteins (Abbing *et al.*, 2004; Dashti *et al.*, 2018). Fusion with minor capsid proteins also allows the transportation of bigger molecules, such as

whole proteins. Similarly to the above-mentioned encapsidation of fused EGFP (Boura *et al.*, 2005), whole enzyme cytosine deaminase was transported by SV40 VLPs when fused with VP2 (Inoue *et al.*, 2008) and MPyV VLPs with VP2-fused prostate-specific antigen (PSA) successfully immunize mice against PSA-expressing tumors (Eriksson *et al.*, 2011).

2.3.2 Modifications of polyomaviral VPs

As with other VPs (see Chapter 2.1.4), there are several methods to modify polyomavirus-based VPs for various applications.

A peptide or protein can be attached to the surface of VLPs by chemical conjugation or genetic engineering and be used to target VLPs to specific cells or tissues or be displayed on the surface of the VLP. As was described above (see Chapter 2.1.1), immunization with chimeric vaccines leads to robust immune response against the displayed antigen.

A specific case of this modification is attaching an antibody to target either specific cells or antigens. This way, HaPyV VPs were used as a carrier for neutralizing antibodies against HBV. These VPs showed neutralizing properties *in vitro*. Interestingly, the surface-exposed antibody was actually fused with VP2 (Pleckaityte *et al.*, 2015).

Usually, VP1 is used for the surface modifications of polyomavirus VPs. It contains four surface loops, which can be altered to display an epitope, which is then exposed on the surface in 360 copies. The choice of the surface loop disrupted by the modification can influence the receptor-binding properties of the VPs. It has been demonstrated that modifications of the HI loop interfere with its role in binding sialic acid and thus effectively enable the retargeting of the VPs elsewhere (Gleiter *et al.*, 1999).

Zvirbliene *et al.* (2006) performed successful immunization with chimeric HaPyV VPs with an insert of up to 120 aa in the BC or HI loop. Interestingly, the formation of compact VPs was reportedly confirmed by electron microscopy. Others claim that inserting over 37 aa in length into the HI loop prevents the assembly of VPs (Schmidt *et al.*, 2001b; Shin and Folk, 2003). On the other hand, much bigger inserts were previously successfully added into the VP1 capsid (and specifically the HI loop) with the use of flexible linkers (Gleiter *et al.*, 1999; Gleiter and Lilie, 2001).

As with other VPs (see Chapter 2.1.4), the surface-exposed residues can be used for bioconjugation of targeting or labeling molecules. This way, lysines on MPyV VPs were used for the attachment of transferrin, which effectively retargets the VPs to transferrin-overexpressing cells (i.e., cancer cells; Zackova Suchanova *et al.*, 2017). In another study comparing various nanoparticles, the lysines exposed on MPyV VPs were again used to target them to glutamate

carboxypeptidase II (GCP II, a receptor overexpressed in prostate cancer tissue and cancer-associated neovasculature) by modification with a GCP II-inhibitor. (Neburkova *et al.*, 2018). The two studies also used surface lysines for fluorescent labeling of MPyV VPs with either rhodamine-NHS ester or NHS-Alexa Fluor 488.

Similarly to VPs of other origin (see Chapter 2.1.4), combining the targeting and labeling strategies was also used for polyomaviral VPs, e.g. SV40 VPs targeted by conjugating epidermal growth factor were labeled by encapsidation of mCherry fluorescent protein (Kitai *et al.*, 2011) or used as imaging agents for MRI when the modified VP1 was used to coat magnetic nanoparticles (Enomoto *et al.*, 2013).

Polyomavirus-based particles are highly versatile and can be engineered for various biomedical applications due to their unique properties. The major structural protein of polyomaviruses, VP1, is ideal for nanotechnology applications because its production is relatively easy, and it self-assembles into non-infectious capsid-like structures. The repetitive and well-characterized capsid surface of many viruses, including polyomaviruses, offers a wide range of possibilities for further manipulation. Polyomaviral VPs can be modified in several ways to be tailored for specific purposes. These modifications can increase the stability of VPs and allow for targeting or labeling or targeting to specific cells or tissues. Specific types of modification involve attaching an antibody to target specific cells or antigens or modifying the surface loops of VP1 to display an epitope. Additionally, polyomavirus VPs can encapsidate various cargoes, such as nucleic acids, proteins, or small molecules.

3. AIMS OF THE THESIS

This dissertation explores two main research directions in the field of VP modifications. First, it focuses on the use of BKPyV-derived VPs for serodiagnostics, which can be applied in seroprevalence studies and in the management of kidney transplant patients. Second, it investigates MPyV-derived VPs (and their modifications) as containers for efficient intracellular delivery into target cells (see *Figure 4*). The aims and the experimental section are divided accordingly into two parts.

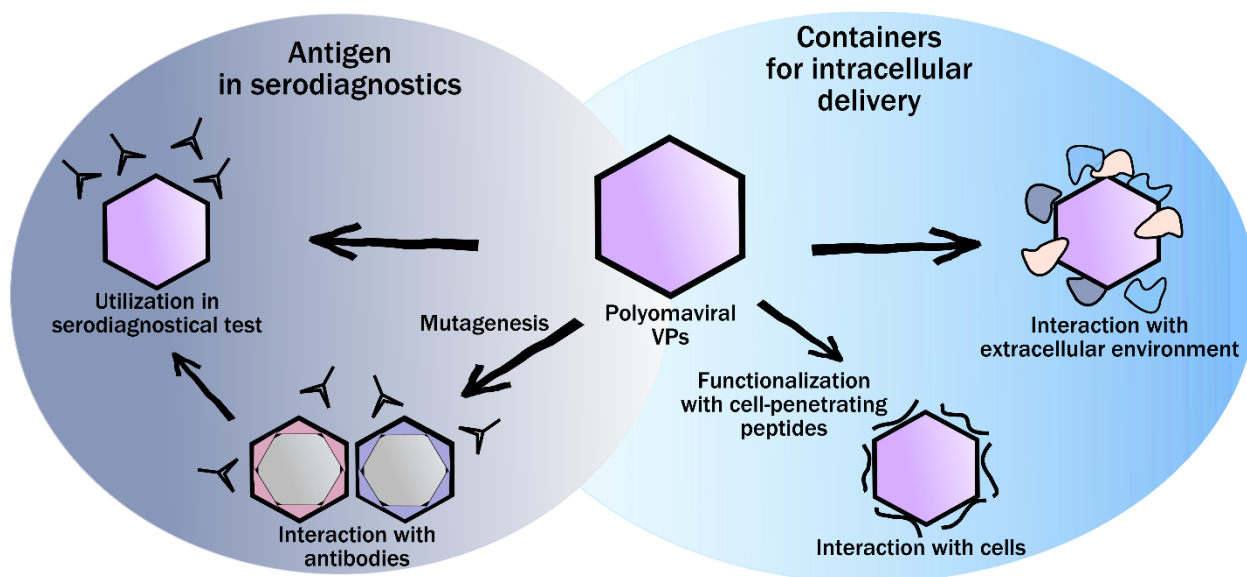


Figure 4: Overview of the topics explored in this thesis.

3.1 Part I - Viral particles as antigens in serodiagnostics

Polyomaviruses include several human pathogens. As a consequence, the use of polyomavirus-derived VPs as antigens in serodiagnostics and clinical research is already fairly established.

Out of the polyomaviruses infecting humans, BKPyV stands out as one of the clinically most important polyomaviruses, which causes severe complications in kidney transplant recipients. It was suggested (see Chapter 2.2.3.1) that the pathogenesis differs between the two most common subtypes (I and IV), and infection by one type does not protect the patient against potential *de novo* infection through the transplanted kidney. Therefore, a quick and easy serological assessment of patient-donor pairs would help to identify patients at risk and in need of further close monitoring and potential changes in the immunosuppression regime. However, the high seroprevalence and the existence of non-neutralizing antibodies that cross-react between subtypes complicate the design of such a test.

Aim 1.

We aimed to evaluate the BKPyV-derived VPs as a tool for a better understanding of BKPyV subtype cross-reactions in serological testing. Specifically, we wanted:

- **use them as antigens in the simple ELISA tests to determine whether the subtype-specific reactivity in serum samples from BKPyV-positive individuals can be detected**
- **use them as antigens in serological tests for differentiation of subtype-specific anti-BKPyV VP1 antibodies**
- **to characterize the epitopes responsible for antibody recognition by preparing mutant variants of BKPyV-derived VPs**

3.2 Part II - Viral particles as containers for intracellular delivery

Previously, the work of my colleagues showed that polyomaviral VPs could make a promising delivery system (Forstová *et al.*, 1995; Boura *et al.*, 2005; Spanielová *et al.*, 2014; Polidarová, 2016; Zackova Suchanova *et al.*, 2017; Žáčková Suchanová, 2018; Hustedová, 2020). However, similarly to other nanoparticles used for intracellular delivery, there are several areas in which polyomaviral VPs could also benefit from further enhancement.

3.2.1 Part II A - Characterization of the interaction of polyomaviral VPs with the extracellular environment

A major obstacle to *in vivo* nanoparticle use is the protein corona formed upon contact with serum proteins, which can hinder targeting to specific receptors. We hypothesized that this issue would not occur with virus-based particles. To test this, we utilized our model transferrin-modified MPyV VPs.

Aim 2.A

We aimed to characterize the interaction of polyomaviral VPs with serum and the effect of protein corona on their retargeting to transferrin receptors.

3.2.2. Part II B - Functionalization of polyomaviral VPs with cell-penetrating peptides and characterization of their interaction with cells

After overcoming the barriers of the extracellular environment, the VPs can also face several obstacles in the cells. Their entrapment in endocytic vesicles and subsequent degradation in lysosomes is often mentioned as the most limiting step for intracellular delivery of nanoparticles

of various origins. Even for viral particles, which are evolutionary tailored for this task, the necessary balance between high efficiency and avoiding rapid immune response and clearance often leads to surprisingly low delivery efficiency (it is estimated that only a few particles of mouse polyomavirus successfully reach the nucleus (Mannová and Forstová, 2003)). Our hypothesis was that candidate CPPs would enhance MPyV infection efficiency or pseudoviral VP transduction by facilitating particle release from the endolysosomal system. Given the well-characterized endocytic and intracellular trafficking of MPyV, it served as an ideal model to study CPP influence on endosomal escape and intracellular cargo transport.

Aim 2.B

We aimed to improve the efficiency of polyomaviral VPs by the cell-penetrating peptides and investigate the CPPs' effects on the endosomal escape of VPs and their interaction with cells. Therefore, our goal was to investigate the effect of CPP on the intracellular delivery of VPs via two types of modifications (non-covalent or covalent) of VPs by CPPs, with an emphasis on establishing a method for quantifying endosomal escape.

4. EXPERIMENTAL PART

This chapter is divided into two main parts in accordance with the aims. For a better understanding of the context of the projects and their interconnectedness with other results, the results presented in finished and/or published manuscripts are presented as summaries throughout this chapter. Please see the respective publications for further details and methodology (see Attachments). The materials and methods described in full in Chapter 4.2.2.1 were used to obtain the unpublished data presented in Chapter 4.2.2.2.

Our two main aims require two different polyomavirus-based VPs.

In the first part, the use of BKPyV-derived VPs in diagnostic tests is investigated. BKPyV is a ubiquitous human virus that causes severe complications in patients after kidney transplantation, and investigation of its proper diagnostics and seroprevalence is important for the prevention of these posttransplant complications. On the other hand, a non-human polyomavirus is needed for the delivery of therapeutic substances to avoid interactions with present antibodies in *in vivo* applications.

The second part is focused on the use of polyomaviral particles in intracellular delivery, specifically exploring the enhancement of delivery into cells by cell-penetrating peptides and the interaction of viral particles with the extracellular environment. Here, mouse polyomavirus (MPyV) is a suitable choice, as it is very well characterized as a model polyomavirus used for a long time to study polyomaviral replication cycle and structure. Therefore, for the second part, we use MPyV-derived VPs.

The main properties and various means of modifications are discussed in full elsewhere (see Chapter 2.1.4). In this thesis, we employed a variety of approaches: a wide range of modifications (genetic modifications on the surface and inside of the capsid, noncovalent and covalent attachment of peptides or fluorescent labels), different preparation methods, the use of wild-type virus as well as different VPs with or without minor capsid proteins or reporter genes. For a comprehensive summary of virus particle types and strategies of their use, see *Figure 5*.

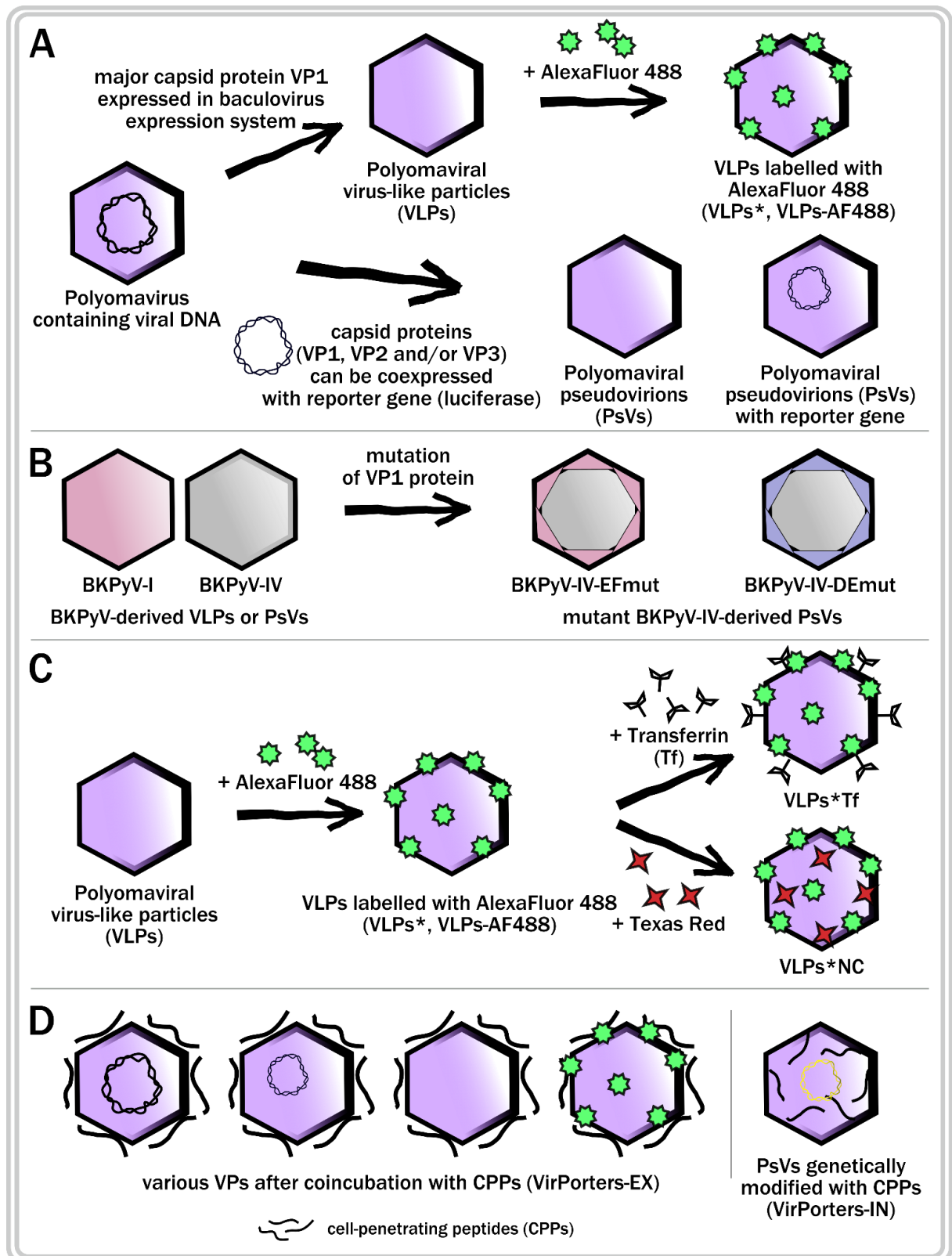


Figure 5: Schematic representation of VPs used in this thesis. A) VPs were derived from BKPvV or MPyV polyomaviruses. Virus-like particles (VLPs) were prepared in the baculovirus expression system and, in some cases, were further labeled with AlexaFluor 488 (VLPs*). Pseudovirions (PsVs) were produced in mammal cells, and in some cases, they were coexpressed with a reporter gene. B) In Part I, BKPvV-derived VLPs and PsVs were used in serological tests, and mutant PsVs derived from BKPvV-IV were used to characterize subtype-specific epitopes. C) In part II A, MPyV-derived VLPs* with added transferrin and control VLPs* with Texas Red were used to study the interaction of VPs in the extracellular

environment. D) In Part II B), VirPorters-EX, i.e., various VPs, were used after coincubation with cell-penetrating peptides (CPPs) to characterize the effects of CPP functionalization. VirPorters-IN, i.e., MPyV, derived PsVs with a reporter gene and internally attached CPPs, were used in several variants. In some cases (*Váňová et al., 2020*), the PsVs contained both minor capsid proteins (VP2 and VP3), while in other experiments (*Váňová et al., 2022*), VirPorters-IN contained only VP3 with the attached CPP.

4.1 Part I - Viral particles as antigens in serodiagnostics

One of the common applications of VPs is their use as antigens in serodiagnostic tests. While VLP-based ELISA is very commonly used for the investigation of BKPyV seroprevalence, the available data were usually obtained using only BKPyV-I VLPs as antigen despite the high prevalence of subtype BKPyV-IV in the European population (see Chapter 2.2.3.3). We capitalized on one of the greatest advantages that VLPs offer - their easy production, and compared their capacity for ELISA-based antibody detection with BKPyV-I VLPs and investigated the potential for subtype-specific antibody detection with these antigens on ELISA.

Our aim was to evaluate the use of both antigens derived from these subtypes of BKPyV in serological testing and to analyze the cross-reactivity of antibodies against two subtypes, BKPyV-I and BKPyV-IV.

The next aim was to prepare novel antigens that would enable us to distinguish anti-BKPyV-I and anti-BKPyV-IV VP1 antibodies. In the published literature, the role of the hypervariable BC loop in receptor interaction, pathogenesis, cross-reactivity, and viral growth was vastly explored (see Chapter 2.2.3.1). In our previous work, we constructed MPyV-derived VPs carrying a BKPyV BC loop. The BC loop of BKPyV VP1 contains a hypervariable sequence with amino acids characteristic for each subtype (Pastrana *et al.*, 2013). We replaced the MPyV BC loop with the corresponding sequence of BKPyV-I or BKPyV-IV in the hope that the context of the capsid of a related polyomavirus would enable the correct conformation for interaction with the subtype-specific antibodies while eliminating the reaction with cross-reacting antibodies, as MPyV is immunologically non-reactive with human sera. However, we showed that the BC loop on its own is not sufficient for the antibody interaction (Sekavova, 2017). Simultaneously, our colleagues from Vidia spol. s r.o. tried a different approach and prepared synthetic peptides derived from the BKPyV BC loop. However, their reactivity with antibodies was generally low, and the ability to distinguish BKPyV-I and BKPyV-IV seropositivity was negligible (unpublished results). Both outcomes of this project suggested that the subtype-specific epitope might contain the BC loop of VP1 but also engage another part of the capsid surface.

Therefore, we were further interested in the epitopes that bind the subtype-specific (neutralizing) antibodies. Out of the three remaining surface loops, the EF loop is a good candidate

as the distance between the EF and BC loops on the capsid surface corresponds with the distance between the two antigen binding sites of an IgG antibody (13.4 - 13.7 nm; Sosnick et al., 1992; Tan et al., 2008). We hypothesized that a particle derived from BKPyV-IV bearing the VP1 EF loop of BKPyV-I would create an unknown context for the neutralizing antibodies targeted into both the BC and EF loop of BKPyV-IV VP1. In this case, the reactivity with anti-BKPyV VP1 antibodies would drop.

Out of the four surface loops, the DE loop is considered the most immunogenic and contributes to the attachment to receptors of some polyomaviruses (Morávková, 2001; Caruso *et al.*, 2003b) and according to our predictions (*Figure 6*) is also immunodominant for BKPyV. We chose to employ a similar approach by mutating the BKPyV-IV VP1 DE loop to its BKPyV-I equivalent. We expect that due to its immunogenicity, the DE loop likely reacts with a wide range of antibodies, including those cross-reacting between the subtypes, and that changes in such an immunogenic epitope will indeed affect the reactivity with anti-BKPyV VP1 antibodies.

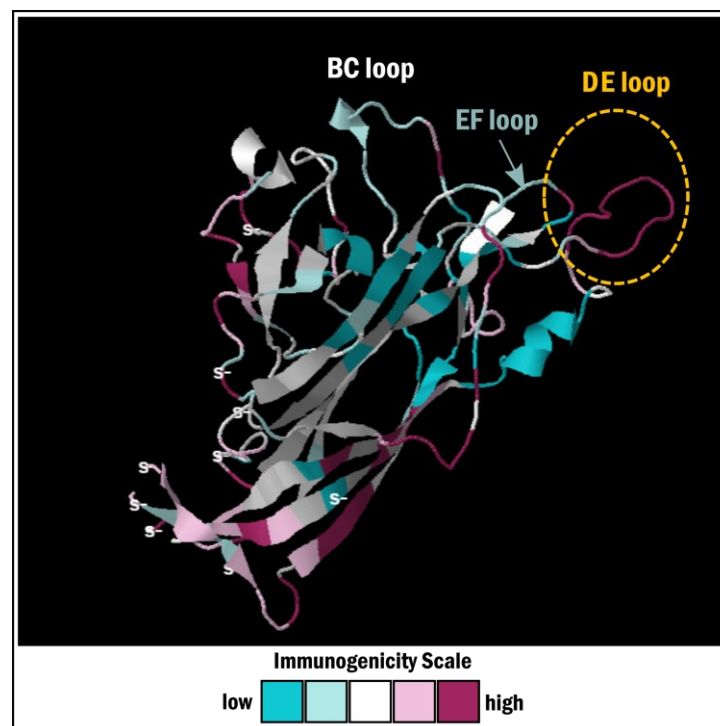


Figure 6: Immunogenicity of BKPyV VP1. The immunogenicity of BKPyV surface loops was predicted by Epitopia Server by Rubinstein *et al.* (2009) and depicted on BKPyV VP1 monomer (based on published structure by Neu *et al.*, 2013; PDB: 4MJ1).

For the investigation of BKPyV prevalence and the use of multiple subtypes of BKPyV for antibody detection, we produced a number of polyomaviral VPs that served as antigens for ELISA testing. The created ELISA tests were used for a seroprevalence study where we discovered the importance of using multiple BKPyV subtypes (see Chapter 4.1.1.1). Nonetheless, simple ELISA

with coated VPs is not sufficient for distinguishing the reaction with subtype-specific and cross-reacting antibodies. Therefore, one of our conclusions was that the VPs derived from different subtypes could serve as a competing antigen in ACA. Therefore, in the follow-up project (see *Figure 7*), we intended to optimize this approach and further utilize these VPs in ACA (described in Chapter 2.2.3.3). Additionally, we intended to study subtype-specific antibody recognition and related epitopes further. We hypothesize that BKPyV-derived VPs carrying mutations in two different surface loops (DE and EF loop) will react differently with patient sera, which points us to the role of these loops in reactivity with antibodies. We propose to investigate not only their reactivity but also their potential for further applications (e.g., in ELISA or ACA).

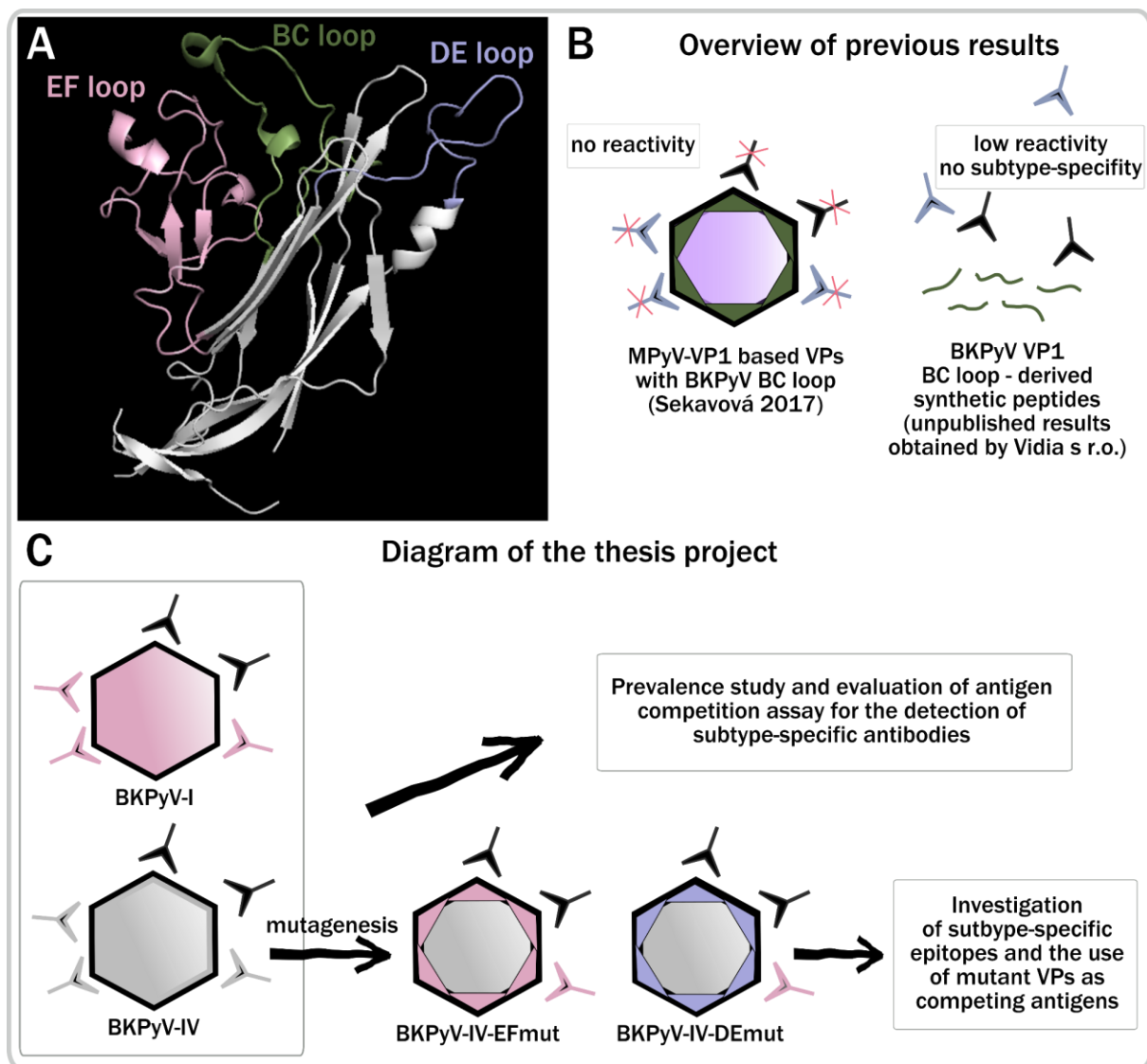


Figure 7: Overview of Part I project. A) Schematic representation of the polyomaviral VP1 protein with color-coded surface loops (BC loop - green, DE loop - blue, EF loop -pink). Created in The PyMOL Molecular Graphics System, Version 1.8, based on published structure PDB: 4MJ1 (Neu *et al.*, 2013); B) Previous work on related projects in our lab or by colleagues from Vidia spol. s r.o. Included exposition of BKPyV BC loop on MPyV-derived capsid in the form of VLPs (left) and preparation of synthetic BC loop-

derived peptides (right) and their subsequent testing for reactivity with BKPyV-positive sera. C) A diagram of the project is presented in this thesis.

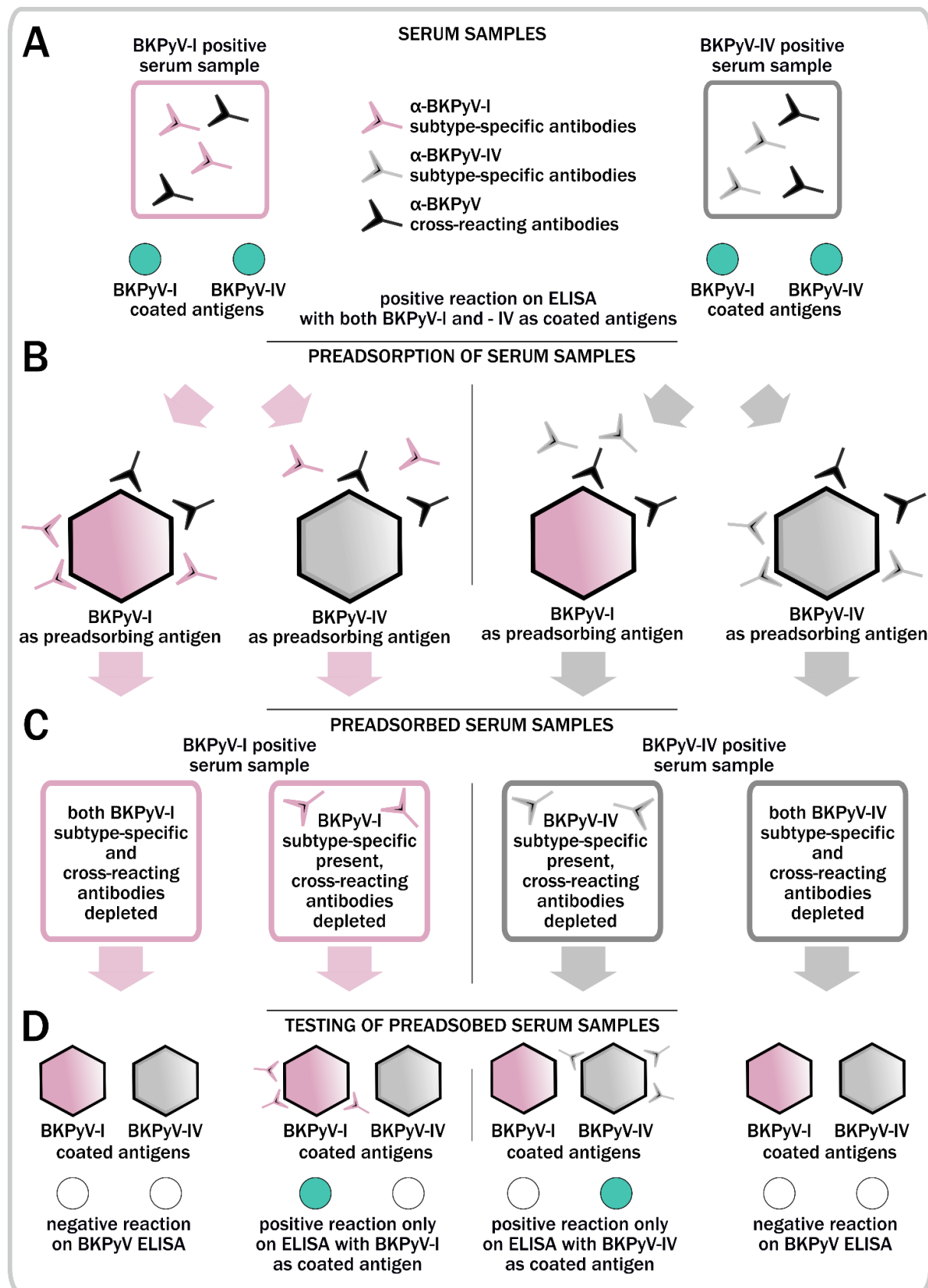


Figure 8: **The principle of ACA:** A) Schematic depiction of antibody content in BKPyV-positive serum sample with a mixture* of subtype-specific (depicted as pink and grey) antibodies and subtype-common or

cross-reacting antibodies (black). Both serum samples could test positive on both BKPyV-I and BKPyV-IV ELISA initially (ELISA read-out schematically shown as circles with green (positive) or white (negative) reaction);

B) The sample is first incubated with “pre-adsorbing antigen.” Here, either BKPyV-I VLPs (pink) are added in order to adsorb all BKPyV-I specific (pink) and subtype-common (black) antibodies, or BKPyV-IV (grey) are added in order to adsorb all BKPyV-IV specific (grey) and subtype-common (black) antibodies;

C) The resulting antibody content in preadsorbed serum samples: the added pre-adsorbing antigen should deplete the sample of subtype-common and homologous subtype-specific antibodies;

D) The resulting reactivity of the pre-adsorbed serum sample with the coated antigen: The serum samples incubated with heterologous pre-adsorbing antigen should now react in a subtype-specific manner with the coated antigen

*The ratio of subtype-specific and cross-reacting antibodies differs between samples: the subtype-specific reaction is possible, as well as a high content of cross-reacting antibodies in samples with titers of antibodies demanding a careful optimization of the pre-adsorption protocol.

4.1.1 Part I - Results

The VPs derived from BKPyV-I and BKPyV-IV were used in a seroprevalence study in collaboration with Vidia s.r.o, where we also evaluated the use of ACA (see Chapter 2.2.3.3) as a method for the detection of subtype-specific antibodies (*Figure 8*).

4.1.1.1 Prevalence of antibodies against BKPyV subtype I and IV in kidney transplant recipients and in the general Czech population

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Journal of Medical Virology, 2019, IF₂₀₁₉ = 2,021

see Attachment 3

We prepared three ELISA tests coated with BKPyV VLPs (BKPyV-I only, BKPyV-IV only, or with a mixture of the two) and tested 50 plasma samples from KTR and 111 samples from healthy blood donors. We demonstrated the importance of using antigens derived from multiple subtypes of BKPyV in serological testing and the need to analyze the cross-reactivity of antibodies against two subtypes, BKPyV-I and BKPyV-IV. The screening showed that ELISA coated with BKPyV-I only (which is usually used in seroepidemiology studies) does not detect all the truly positive samples, as some contain antibodies reacting only with BKPyV-IV, as 9% of healthy donors and 20% of KTR showed reactivity specific to BKPyV-IV, which is usually not used in diagnostic testing. By using our ELISA tests, we have found no significant correlation between the levels of antibodies against BKPyV-I and BKPyV-IV (Spearman's $r_s = 0.176$, $p > 0.2$). Additionally, the correlation of antibodies against BKPyV and JCPyV (closely related human polyomavirus) was tested but also not significant (Spearman's $r_s = -0.071$, $p > 0.6$). The cross-reactivity between BKPyV-I and BKPyV-IV was tested by ACA: plasma samples were preincubated with each of the antigens to deplete them of subtype-common antibodies, and then

the sample was then tested for remaining subtype-specific antibodies. ACA confirmed that subtype-common cross-reacting antibodies might be responsible for the reactivity with both tested BKPyV subtypes in some of the samples. Thus, employing ACA could contribute to better risk assessment in kidney transplant patients.

Contribution

I prepared the VLPs, did preliminary screenings of sera, and optimized the ACA. I participated in the statistical analysis of the data, writing and editing of the manuscript, and prepared the figures (with the exception of Figure 2).

Our next objective was the investigation of surface epitopes responsible for the binding of the subtype-specific (neutralizing) antibodies. As discussed at the beginning of this chapter, our previous results suggest that another surface loop (in addition to the BC loop) is needed for the binding of subtype-specific antibodies. Sequence alignment of BKPyV I-IV VP1 shows that the sequences differ mostly in the surface loops (*Figure 9*), and some of these differences are responsible for the differences in tropism, pathogenesis, and antibody recognition. We argue that the EF loop is the most probable candidate, but the role of the DE loop in the recognition by antibodies (either subtype-specific or cross-reacting) is worth exploring.

In the DE loop, BKPyV VP1 subtypes differ in AA 139 (*Figure 9*). Even though this AA is in close proximity to the residues of the BC loop that are probably part of the neutralization epitope (Pastrana *et al.*, 2013), this residue was not yet studied in the context of neutralization and BKPyV subtypes. Another potential epitope of interest is the EF loop, where BKPyV-I and BKPyV-IV differ in amino acids located on the side of the capsid (*Figure 10A, B*). Although the EF loop probably does not participate in the receptor attachment, it contains two amino acid differences between BKPyV-I and -IV and could be important for the recognition by the neutralizing antibody, as suggested in the work of Lindner *et al.*, who mapped a neutralizing antibody in this very loop, along with BC loop and C-end terminus of VP1 (Lindner *et al.*, 2019).

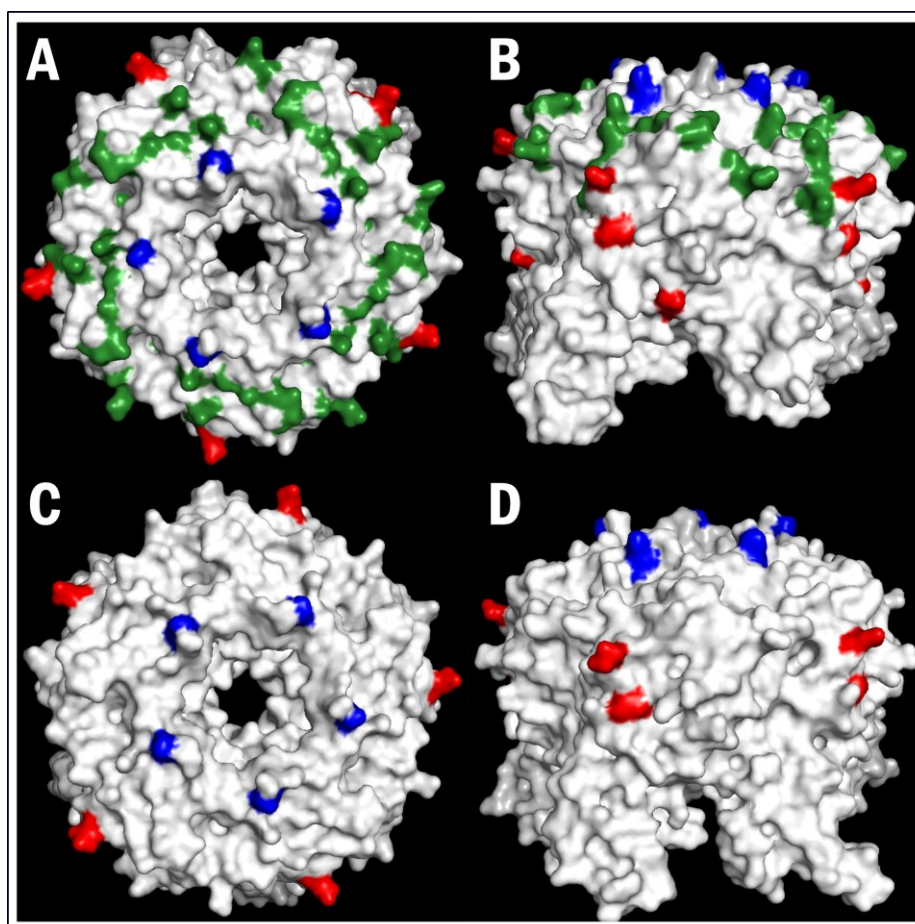


Figure 10: **BKPyV pentamer with highlighted differences between BKPyV-I and -IV.** VP1 pentamer (A - top view) and (B - side view) with marked AA residues that differ between the I and IV BKPyV subtypes. Green – subtype-specific AA residues in hypervariable BC loop, blue - AAs in DE loop, red - AAs in EF loop. VP1 pentamer (C - top view) and (D - side view) with marked AA residues we planned to mutate. Blue marks N139H in the DE loop, and red marks E175D and V178I in the EF loop. AAs on the side of the pentamer are not accessible to the antibodies in the whole capsid, which consists of 72 VP1 pentamers Created in PyMol, based on the published structure PDB: 4MJ1 (Neu *et al.*, 2013).

4.1.1.2 Preparation of BKPyV mutant VPs and characterization of their reactivity with antibodies

Therefore, we designed two mutant VPs: BKPyV-DEmut and BKPyV-EFmut. Using site-directed mutagenesis, AAs typical for BKPyV-I were introduced into the corresponding sequence of BKPyV-IV. Thus, BKPyV-I loops (DE loop in BKPyV-DEmut, EF loop in BKPyV-EFmut) will be displayed on the BKPyV-IV carrier VP. We expected decreased reactivity of these mutants with BKPyV-IV-positive serum samples, which would suggest that the mutation targeted an important AA for antibody recognition.

Both mutant variants of BKPyV-IV VPs (BKPyV-DEmut with N139H substitution and BKPyV-EFmut with (E175D and V178I substitutions) were prepared by Tereza Tomanová as a part of her diploma thesis (Tomanová, 2019). She optimized protocol for their purification and characterized them.

The prepared mutants performed interestingly in ELISA tests and were then used in the following project. I was the principal investigator in the project Serological properties of BK polyomaviruses: mutation analysis, which was funded by the Grant Agency of Charles University (GA UK). My co-investigator Helena Caisová further analyzed the mutants as a part of her diploma thesis. Specifically, she investigated their potential as competing antigens in ACA in order to explore the subtype-specific reactivity of anti-BKPyV VP1 antibodies with the changed surface loops (Caisová, 2023).

As the principal investigator and consultant to these theses, I participated in the overall project concept and design of the mutant VPs and assisted in the preparation of the VPs and their visualization by TEM. I assisted in the planning of the experiments, oversaw their execution, and consulted the results. The results are part of the published study:

4.1.1.3 The role of the DE and EF loop of BKPyV VP1 in the serological cross-reactivity between subtypes

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Virus Research, 2023, IF₂₀₂₁ = 6.286
see Attachment 4

We characterized the reactivity of fourteen serum samples with known BKPyV viremia and viruria, and Cytotect CP (commercially available immunoglobulin preparations) by ELISA, using BKPyV-I and BKPyV-IV antigens. We then proceeded with ACA using not only BKPyV-I and -IV, but also with the prepared mutants BKPyV-IV-DEmut and BKPyV-IV-EFmut.

In agreement with our initial hypothesis, changing the EF loop of BKPyV-IV VP1 into the EF loop of BKPyV-I leads to the loss of reactivity with anti-BKPyV-IV antibodies. Moreover, it also leads to loss of reactivity with anti-BKPyV-I antibodies, indicating the importance of EF loop not only in the subtype-specific antibody recognition, but BKPyV VP1 antibody recognition in general.

Interestingly, the single AA change introduced into the DE loop results in the stronger reaction of the BKPyV-IV mutant variant with anti-VP1 BKPyV-I antibodies in some samples. This confirms that the DE loop indeed attracts antibodies that do not depend on the subtype-specific context of the capsid.

Although previous research (including our results, see Chapter 4.1.1.1) suggested the ACA as a plausible way to distinguish between BKPyV serotypes, here we show that the interpretation of this assay might prove difficult, especially as even subtle changes in the variable epitopes can rapidly change the reactivity with the respective presumably subtype-specific antibodies.

Contribution

As the principal investigator of the project funded by GAUK, I participated in the overall project concept and design of the mutant VPs. I did preliminary screens of sera and optimized the ACA, assisted in the planning of the experiments, and oversaw the preparation of the VPs and the experimental work by Tereza Tomanová and Helena Caisová (as a consultant to their master theses). I prepared Figure 1, wrote the original manuscript, and participated in its editing and in the statistical analysis of the data.

4.2 Part II - Viral particles as containers for intracellular delivery

In the second part of the project, we investigated the barriers of intracellular delivery by VPs, namely the formation of protein corona (see Chapter 2.1.3.1) and endolysosomal sequestration (see Chapter 2.1.3.2). One of our goals was to assess the protein corona formation on MPyV-derived VPs and its effect on the successful targeting of these particles. Next, a major part of the research for this thesis is my contribution to a project investigating CPPs and their use in the enhancement of intracellular delivery mediated by MPyV-derived VPs. One of the main goals of this project was to examine how CPPs enhance polyomaviral infection and nuclear delivery by polyomavirus-derived VPs. In the hope that the CPPs will facilitate the endosomal escape of the VPs, we set out to modify polyomaviral VPs in two different ways: either by simple coinubation (creating noncovalently bound complexes) with CPPs or by genetically linking the CPP to the minor capsid protein VP3, which in the wild type virus has the viroporin properties (see Chapter 2.2.1). Our goal then was to investigate whether CPPs promote gene delivery and cytosolic release of cargo delivered by polyomavirus-derived VPs and compare these two modes of CPP association.

4.2.1 Part II A - Characterization of the interaction of polyomaviral VPs with the extracellular environment

4.2.1 Part II A - Results

One of the long-term objectives of our laboratory is the utilization of polyomaviral (with an emphasis on MPyV) VPs for theranostics. Their targeting of cancer cells was the overall aim of my colleague Dr. Jiřina Žáčková Suchanová. In her doctoral research, she used transferrin and an inhibitor of glutamate carboxypeptidase II to retarget MPyV VPs (Žáčková Suchanová, 2018).

Since the Tf-TfR targeting system might serve as a clinically relevant model to investigate the targeting properties of potential drug carriers, she also utilized these MPyV VPs targeted on transferrin receptor (VLPs*Tf, see *Figure 5*) (Zackova Suchanova et al., 2017) to explore the effect of protein corona on the targeting of VPs of polyomavirus origin and consequently their ability to potentially deliver cargo *in vivo*.

4.2.1.1 The Protein Corona Does Not Influence Receptor-Mediated Targeting of Virus-like Particles

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Bioconjugate Chemistry, 2020, IF₂₀₂₀ = 4,774
see Attachment 5

We investigated how serum proteins adsorb on MPyV VLPs and VLPs*Tf by their coincubation with serum and subsequent ultracentrifugation through a 20% sucrose cushion. The presence of protein corona after the centrifugation step was examined by dynamic light scattering analysis on the SDS page and TEM). We found that only a small amount of an additional protein (probably human serum albumin) was associated with the VLPs and that VLPs*Tf interacted with serum proteins more readily than unmodified VLPs.

We assessed the ability of the targeted VLPs*Tf to interact with TfR in the presence of sera. We tested sera from different species (human, mouse, bovine, and chicken) to show that although the interaction is inhibited in human sera, it is due to the presence of free transferrin competing with the particles. The interaction of VLPs*Tf with TfR in the presence of bovine sera was also tested on cells expressing TfR. VLPs*Tf were found indeed to bind these cells and enter cells in a clathrin-dependent manner (as typical for Tf). This contrasts with previously published findings that a serum-rich environment destroys the targeting specificity of Tf-functionalized silica NPs and supports our notion that MPyV VLPs can possess valuable characteristics for *in vivo* use.

Contribution

I optimized and carried out experiments for the detection of serum proteins adsorbed on VLPs surface, the competitive ELISA tests assessing the interaction with TfR and helped with the experiments on cell lines. I participated in editing the manuscript and preparing the graphic abstract.

4.2.2 Part II B - Functionalization of polyomaviral VPs with cell-penetrating peptides and characterization of their interaction with cells

4.2.2.1 Part II B - Materials and methods for unpublished data

In Chapter 4.2.2.2, I present unpublished results, and the respective methods and materials are described here. The materials and methods used for published results are described in the respective publications included in the Attachments.

Equipment

- Centrifuge GS-15R (Beckman)
- Centrifuge Microfuge (Beckman)
- Cultivation shaker Orbital Shaker (Forma Scientific)
- CO₂ incubator (Forma Scientific)
- Epoch microplate spectrophotometer (BioTek Instruments)
- Fluorescence microscope BX-60 Olympus
- Laminar Flow Box (Forma Scientific)
- Microscope Leica DMi1 (Leica)
- pHmeter S20 Seven Easy (MettlerToledo)
- Qubit® fluorometer (Invitrogen)
- Sonicator Soniprep 150 (Schoeller Pharmacia)
- Termobox LBT 165 (Zanussi)
- Thermoblock Bio TDB-100, Dry block thermostat (Biosan)
- Transmission electron microscope JEOL JEM-1011
- UV transilluminator Electronic EAC-20 DualLight Transilluminator (UltraLum)
- Vortex-Genie 2 (Scientific Industries)
- Water bath (GrantInstruments)
- Weighing scale 440-33 (Kern)

Software and online tools

- NIS-Elements AR 2.30 (Nikon) for fluorescence microscope BX-60 Olympus
- The itTEM 5.1 (Olympus soft imaging solutions GmbH) software
- Gen5 1.10 (Bio Tek) for spectrophotometric measurements on Epoch™ Microplate Spectrophotometer

Solutions

- Phosphate Buffered Saline (PBS)
- EDTA-NaOH (pH 8.0): 0.5 M EDTA, pH adjusted to 8.0 by NaOH
- Serum-free medium for culture tissues - Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich D5796)
- Medium for culture tissues with serum - DMEM supplied with 10% Fetal bovine serum (Sigma-Aldrich)

Peptides

The four used peptides (CPPs, see *Table 1*) were synthesized by Synpeptide Co., Ltd. (Shanghai, China). Peptides were obtained and modified by N-terminal acetylation and C-terminal amidation, prepared with 95% purity, dissolved in DMSO at the 25 mM concentration, and stored at – 20 °C. For each experiment, peptides were dissolved in serum-free media or PBS shortly before use to achieve the working concentrations as specified in each experiment, usually 20 uM.

CPP	amino acid sequence
LAH4	KKALLALALHHLAHLALHLALALKKAGC
KH27K	KHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHK
R8	CGRRRRRRRR
FUSO	CGLFEALLELLESLWELLLEA

Table 1: Used cell-penetrating peptides

Virus and virus-based particles

- MPyV: wild-type virus, strain BG (GenBank accession number AF442959.1, prof. Griffin, Royal Postgraduate School, London) was prepared by Jakub Soukup (Soukup, 2015)
- VLPs *- Fluorescently labeled VLPs were prepared by Jana Váňová
 - MPyV VP1-based (BG strain) VLPs were produced in a baculovirus expression system and modified by Alexa Fluor 488 NHS Ester (Váňová 2016)
- AdV5 - adenovirus 5, prepared at and gifted by Cardiff University (Cloning genes into the AdZ vectors and making virus: available at <https://www.cardiff.ac.uk/>), propagated and isolated by Jana Váňová

Primary antibodies

- Mouse- α -MPyV-VP1: monoclonal antibody against VP1 of MPyV, clone D4 prepared by Alena Morávková (Morávková, 2001), diluted 1:20
- Rabbit- α -MPyV-VP1: polyclonal antibody against MPyV VP1 (Exbio), diluted 1:500
- Rabbit- α -AF488: monoclonal antibody against AlexaFluor-488 (ThermoFisher Scientific), diluted 1:1000
- Mouse- α -Hsp90: monoclonal antibody against Hsp90 (ThermoFisher Scientific), diluted 1:100
- Mouse- α -Galectin-3, monoclonal antibody against galectin-3, A3A12 (Invitrogen), diluted 1:100

Secondary antibodies

- Goat- α -Mouse-HRP: Goat antibody against mouse immunoglobulins conjugated with horseradish peroxidase (BioRad), diluted 1:1000 (BioRad)
- Donkey- α -Mouse-AF488, Donkey antibody against mouse immunoglobulins conjugated with AlexaFluor-488, A21202, diluted 1:1000 (ThermoFisher Scientific)

Cells

- NIH 3T6: mouse fibroblast cell line (prof. Griffin, Royal Postgraduate School, London)
- HF-CAR: human-derived fibroblasts expressing coxsackievirus and adenovirus receptor (CAR) (prof. Wilkinson, University of Wales College of Medicine, Cardiff)

Measurement of protein concentration

Protein concentration was measured by Qubit® Protein Assay Kit (ThermoFisher Scientific) according to the manufacturer's protocol.

ELISA

MPyV was diluted in serum-free media for the immunodetection of CPP/VP complexes. The samples were coated onto the 96-well half-area microplate (20 ng / well) in a final volume of 50 µl / well and left to incubate overnight at 4°C. Next, MPyV samples to be coincubated with CPPs were aspirated, and the diluted CPP (20µM) was added (50 µL / well) and left to incubate for 30 minutes. Each well was then washed 3x with 1x PBS/0,1% Tween-20 (Sigma-Aldrich) (v/v) and blocked by 5% milk (100uL / well) for 1 hour at room temperature. The wells were then again washed 3x with 1x PBS/0,1% Tween-20 (v/v), primary antibody (mouse-αMPyV-VP1) diluted 1:20 in 5% milk was added (50 µl / well) and left for 1 hour at room temperature. The wells were then again washed 3x with 1x PBS/0,1% Tween-20 (v/v), secondary antibody (Goat-αMouse-HRP) diluted 1000x in 5% milk was added (50 µl / well) and left for 30 minutes at room temperature. The wells were then again washed 4x with 1xPBS/0,1% Tween-20 (v/v), ABTS substrate (Sigma-Aldrich) was added, and (50 µl / well), and absorbance at 415 was measured (Epoch BioTek). Wells with serum-free media instead of samples were processed in the same manner as other samples and used as a negative control.

Transmission electron microscopy

Standard preparation of samples:

Viral particles were mixed with LAH4 (final concentration 20 µM in PBS) or PBS (control) and incubated for 30 min at room temperature. A carbon-coated formvar copper grid (Electron Microscopy Sciences, Hatfield, PA, USA) was then laid on a seven µL drop of each sample and left to adsorb for 5 min. Grids were transferred on a drop of redistilled H₂O to wash (2 x 30 s) and then onto a drop of 2% (w/v) solution of phosphotungstic acid, pH 7.3 (2x 1 min).

An improved protocol for visualization of CPP-VP aggregates:

Viral particles were mixed with LAH4 (final concentration 20 µM in PBS) or PBS (control) and incubated for 30 min at room temperature. The samples were dropped (7 µL) directly on a carbon-coated formvar copper grid and left to dry for over an hour. A drop (7 µL) of fixating agent (1.5% glutaraldehyde) was then applied and left for 1 min. The grid was then transferred onto a 45

μL drop of 1.5% glutaraldehyde and incubated for 1 min. Then, the grid was washed on a 45 μL drop of ddH₂O for 1 minute. The final steps were a 2-minute incubation on a μL drop of the contrasting agent (uranyl acetate solution) and a 1-minute wash on a drop of ddH₂O.

The samples were observed with a JEOL JEM (Akishima, Tokyo, Japan) using the itTEM 5.1 (Olympus soft imaging solutions GmbH) software.

Cell cultivation

The 3T6 and HF CAR cells were grown on Petri dish (6 cm, Jet Biofil) in 37 °C, 5% CO₂ and usually passaged twice a week in 1:6 (3T6) or 1:6 - 1:12 (HF CAR) ratio.

Testing the autofluorescence of LAH4 after immunostaining

The cells were seeded on coverslips in a 24-well plate: 6×10^4 in 1 ml of media supplemented with FBS and incubated overnight (37°C, 5% CO₂). Media was aspirated, and 20 μM LAH4 in serum-free media was added. Cells were incubated for 6 hours at 37°C and fixed by 3.7% paraformaldehyde, then washed 3x with PBS. Samples on coverslips were then treated for 5 minutes with 0.5% Triton-X-100, washed 3x with PBS, and mounted into 50% glycerol with DAPI (Sigma) to stain cell nuclei.

Preparation of samples for proximity ligation assay

The cells were seeded on coverslips on a Permaxox® 8-chamber slide (Nunc® Lab-Tek® Chamber Slide™ system, Sigma-Aldrich), 2×10^4 cells per chamber, in media supplemented with FBS and incubated overnight (37°C, 5% CO₂). The next day, samples were diluted in serum-free media or serum-free media containing five μM CPPs, mixed well by vortexing, and left to incubate for 30 minutes. Media from cells was aspirated, and the diluted samples were added (0.250 μg of VLPs* or MPyV per chamber). Serum-free media or serum-free media containing five μM of used CPPs was also used as a negative control.

After 1 hour (37°C, 5% CO₂), cells were washed with serum-free media, and complete media was added. The media was aspirated after another 2 hours of incubation (37°C, 5% CO₂) and fixed by 3.7% paraformaldehyde for 15 minutes at room temperature, then washed twice by 1x PBS and permeabilized by 0.5% Triton-X-100 for 5 minutes.

Proximity ligation assay

Samples were prepared, fixed, and permeabilized as described above. Then, Duolink® blocking solution was dropped on the samples. Samples were incubated for 1 hour in a 37°C humidity chamber. Then, the primary antibodies were added in pairs, diluted in the Duolink® antibody diluent:

- RbαVP1 + MαVP1 (VP1-VP1 detection for control for entering virus: two different anti-VP1 antibodies for self-colocalization of the viral particles)
- RbαAF488 + MαHsp90 or RbαVP1 + MαHsp90 for the detection of VLPs*

Samples were incubated for 1 hour at room temperature in a humidity chamber. The slides were washed twice for 5 minutes in the supplied Wash Buffer A. Then, the Duolink probes were added (α Mouse and α Rabbit), diluted 1:5 in the Duolink® antibody diluent. Samples were incubated for 1 hour at 37°C in a humidity chamber. The slides were washed twice for 5 minutes in Wash Buffer A. The supplied ligase was diluted 1:40 in a 1:5 diluted Ligase buffer, added to samples, and incubated for 30 minutes at 37°C in a humidity chamber. Then, samples were washed twice for 2 minutes with Wash Buffer A. The supplied polymerase was diluted 1:80 in a 1:5 diluted amplification buffer, added to samples, and incubated for 100 minutes at 37°C in a humidity chamber.

For the fluorescent detection with Duolink® In Situ Red (Sigma-Aldrich), samples were washed twice for 10 minutes with the supplied Wash Buffer B and for 1 minute in a 0.01x Wash Buffer B. Duolink In Situ Mounting Medium with DAPI was dropped on the samples and covered with a coverslip, left for 15 minutes and inspected by fluorescence microscope.

For the brightfield detection with Duolink® In Situ Brightfield (Sigma-Aldrich), the slides were washed twice for 2 minutes with Wash Buffer A. Next, Detection Brightfield Stock diluted 1:5 was added, and samples were incubated for 60 minutes in a humidity chamber at room temperature. Slides were washed twice for 2 in Wash Buffer A, then the substrate reagents were diluted (A - 1:70, B - 1:100, C - 1:100, D - 1:50) and added to samples and left to incubate. Then, slides were washed twice for 2 in Wash Buffer A, and a Nuclear stain was added and left to incubate for 2 minutes. Then, samples were washed for 10 minutes under running deionized water and dehydrated by incubation in 96% EtOH for 2 minutes, in 99.7% EtOH for 2 minutes, in xylene for 10 minutes, and observed under a brightfield microscope.

Galectin assay with immunofluorescence staining

The HF CAR cells were seeded on coverslips in a 24-well plate: 8×10^4 cells / well in 1 ml of complete DMEM media. After an overnight incubation (37°C, 5% CO₂), the cells were washed with serum-free media, and AdV5 diluted in serum-free media (3×10^4 virus particles/cell) or cell-penetrating peptides (20 μ M) were added to the cells. Untreated cells in serum-free media were used as a negative control.

The cells were incubated for 1 hour (37°C, 5% CO₂), then washed with 1x PBS and fixed with 3.7% paraformaldehyde for 15 minutes at room temperature. The cells were then again

washed twice with 1x PBS, treated with 0.5% Triton-X-100 for 5 minutes, washed three times in 1x PBS, and then incubated overnight at 4°C in blocking solution (0.25% BSA, 0.25% gelatine in PBS). The blocking solution was aspirated, and the primary antibody (Mouse- α -galectin-3) diluted 100x in the blocking solution was added and incubated for 1 hour. The samples were then washed 3x with PBS, and a secondary antibody (donkey- α -mouse-488) diluted 1000x in the blocking solution was added for 30 minutes. The samples were then washed 3x with PBS, and the coverslips were mounted into drops of 50% glycerol with DAPI (Sigma) on glass slides.

4.2.2.2 Part II B – Results

Since the sequestration of nanoparticles in the endolysosomal system is thought to be the major limitation of successful intracellular delivery (Chapter 2.1.3.2), the main idea in this part was to help polyomaviral VPs overcome vesicular barriers after cellular entry through the action of CPPs. While CPPs have been demonstrated to enhance the cellular uptake of various cargos, their efficiency in facilitating endosomal escape remains unclear. A significant unresolved question is whether permanent association, such as covalent bonding, between the cargo and CPP is preferable to non-covalent or reversible conjugation. Multiple reports suggest that non-covalent association may be the more viable approach. During our project, we investigated both strategies (*Figure 11 A.1*).

For the VPs' modification, we used several types of VPs (*Figure 11 A.2*) and peptides of different properties: As candidate peptides, we selected two histidine-rich peptides (LAH4 a KH27K), positively charged octaarginine (R8) and a fusogenic peptide derived from hemagglutinin (FUSO) (*Table 1*, Chapter 4.2.2.1, *Figure 11 B*). These CPPs were reported to substantially enhance intracellular delivery of drug delivery systems based on various nanoparticles, including large polyplexes (Vaysse *et al.* 2000; Khalil *et al.* 2011; Ferrer-Miralles *et al.* 2011; Fenard *et al.* 2013, Koshkaryev *et al.* 2013, Liu *et al.* 2014, Deshpande *et al.* 2018).

We took advantage of the versatility of polyomaviral VPs, and for the external modifications, we utilized infectious virus, fluorescently labeled VLPs, as well as pseudovirions containing luciferase reporter gene. For the genetic modification by CPPs (that ensures covalent association of CPPs with VPs), we exclusively used PsVs containing luciferase reporter. Our investigation effort can be categorized into three parts (*Figure 11 A.3*):

First, by using VPs with externally and non-covalently associated CPPs (VirPorters-EX), we set out to characterize the influence of CPPs on the interaction between VPs and cells and on

compare the impact of CPPs that were associated with VPs non-covalently as VirPorters-EX or covalently (by genetic attachment with minor structural viral proteins) as VirPorters-IN. We hypothesized that in VirPorters-IN (with CPPs encapsulated inside VPs), CPPs would act only upon partial disassembly in endosomes (and not during cellular entry), leading to an improved release of particles into the cytosol (*Figure 11 A.3: right panel*).

4.2.2.2.1 - Influence of cell-penetrating peptides on the activity and stability of virus-based nanoparticles

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International Journal of Pharmaceutics, 2020, IF₂₀₂₀ = 5.875
see Attachment 6

We first tested the effect of CPPs on the infectious virus and indeed saw increased infectivity in the virus coincubated with R8, LAH4, or KH27K peptides. Our next goal was to determine the mechanism that contributed to the enhancement of viral infection.

We characterized the stability of viral capsids after coincubation with the peptides by TEM and DLS.

CPPs on the capsid surface or in the media might enhance the polyomavirus infection in a different way than we intended, e.g., by enhancing the VPs uptake by the cells via increased binding to the cell surface. Therefore, we investigated the cell surface binding properties of these CPP-VP* complexes (using fluorescently labeled VPs coincubated with CPPs; see diagram in (*Figure 11*)). The resulting images from confocal microscopy further confirmed the aggregation of particles after LAH4 or KH27K treatment (*Figure 3a in Attachment 6*). These aggregates readily interacted with cellular membranes but also stuck to the surface in between the cells. No visible changes and/or aggregates were observed in samples with R8 or FUSO. These results were also confirmed by flow cytometry of similarly treated cells in suspension (*Figure 3b in Attachment 6*). When CPP-VP* complexes were allowed to enter cells, we also quantified the cellular uptake. Interestingly, none of the CPPs significantly increased the uptake into cells during 2-hour incubation (*Figure 3c in Attachment 6*).

We also addressed the possibility that CPP could reroute the VPs to a different entry pathway. By using inhibitors of clathrin-mediated endocytosis (hypertonic sucrose, 0.45 M) and macropinocytosis (5-(N-ethyl-N-isopropyl)amiloride; i.e., EIPA), we determined clathrin-mediated endocytosis to be important internalization route for all CPP-VP* complexes as well as VPs* alone. Our results also indicate a role of macropinocytosis in the internalization of VLP*/KH27K, which was significantly inhibited in EIPA-treated cells (*Figure 4 in Attachment*

6). However, more specific inhibitors to investigate these pathways further could not be used, as the cells did not withstand both adding the quite cytotoxic inhibitors and CPP-VP* complexes at the same time.

Lastly, we focused on the effect that CPPs have on the *intranuclear* delivery of pseudovirions containing the luciferase reporter gene. Here, we took advantage of pseudovirions of various origins that we had available, and, besides MPyV-derived VPs, we also used SV40 and mouse papillomavirus (MusPV) based pseudovirions. Indeed, the transduction was substantially enhanced after coincubation with LAH4 and KH27K. R8 enhanced the transduction by SV40 and MusPV pseudovirions, and FUSO only slightly enhanced the transduction by MusPV pseudovirions (Figure 5 in Attachment 6). Additionally, we showed that these effects were not dependent on cellular heparan sulfates. In doing so, we found that heparin did decrease the transduction efficiency of MPyV- and SV40-based CPP-VPs, but we attributed this effect to the destabilization of VP capsid rather than the interaction of the CPP-VPs with heparan sulfate receptors, as heparinase treatment of cells did not result in any significant transduction decrease (Figure 6 in Attachment 6).

Overall, we show that the cationic R8 and histidine-rich peptides LAH4 and KH27K enhance gene delivery and probably do so partly due to destabilization of the capsid and (in the case of LAH4 and KH27K) increased association with the cellular membrane, even though the uptake of particles is actually lower than that of unmodified VPs. The high histidine content, as a common characteristic of two of the promising CPPs, points to a possible site of enhancement in the endocytic compartment.

Contribution

I optimized and performed the cell-binding assay including the visualisation by confocal microscopy. I carried out the statistical analysis, participated in the editing of the manuscript and prepared the figures (with the exception of figure 4).

4.2.2.2.2 The detection and quantification of endosomal escape of CPP-modified VPs

As we saw that CPPs increase the MPyV infectivity by different means than increased uptake into the cells, we further investigated the mechanisms behind the enhancement and focused on the initially proposed idea that even LAH4 (or other CPPs) bound on the capsid exterior may function after internalization. Our focus, therefore, moved to the endosomal escape as we aspired to establish an assay for reliable quantification of the efficiency of the VPs release into the cytosol. The detection of endosomal escape is possible by several methods (see Chapter 2.1.3.2.2), yet they all have certain limitations (Chapter 5.2.2.2).

To properly interpret the potential quantitative differences of VPs present in the cytosol among various modified VPs, it is necessary to take into account the possible changes in uptake (either amount of VPs or difference in timing or the employed pathway) compared to the unmodified VPs that served as a control. In other words, if we detect, e.g., more VirPorters-EX than control VPs in the cytosol, we need to normalize these data to the total amount of internalized VirPorters-EX and control VPs. Only by using such normalized data can we draw accurate conclusions about the „endosomal escape properties“ of VirPorters-EX. Thus, precise quantitative assays are crucial to determine whether control VPs are sequestered in endolysosomal compartments while VirPorters-EX has successfully evaded this fate.

In addition, we also wanted to employ some rapid assay that would allow us to predict the endosomolytic properties of candidate CPPs. The hemolytic assay is often used for this purpose. Since histidine-rich peptides might act in a pH-dependent manner and facilitate the escape of VPs from late endosomes, I performed the assay in various settings, i.e., varying pH mimicking the conditions throughout the endolysosomal system (see Supporting information in Attachment 7). The only CPP that showed detectable penetrating ability at neutral pH was LAH4 and, to a lesser extent, FUSO. This was further enhanced with decreasing pH, where we also saw hemolysis by the FUSO and KH27K peptide. R8 did not lyse the cells in any tested pH. These results then contributed to a published study (see Attachment 7, *Váňová et al., 2022*).

After assessing the endosome-penetrating potential of the peptides, we employed several methods to determine whether the enhancing effect of CPPs in VirPorters results in destabilization of vesicles in cells or increased appearance of VPs in cytosol. I focused mainly on two methods: proximity ligation assay and galectin assay.

We attempted to test a previously unreported approach for quantifying endosomal escape and utilize proximity ligation assay as a method to quantify viral particles (VPs) in the cytosol by detecting them in close proximity to cytosolic markers. Proximity ligation assay indicates whether two proteins are in close proximity (30-40 nm) through immunolabeling. Supplied probes labeled with oligonucleotides bind to the primary antibody, and in case the two proteins are indeed in close proximity, the two oligonucleotides ligate during the ligation step. This enables subsequent rolling circle amplification with labeled nucleotides, resulting in a detectable spot-like signal. The assay is used to detect protein-protein interactions (Alam, 2018), and we intended to use it to determine whether the modified VPs are present in the cytosol in larger quantities than unmodified VPs.

Galectin assay enables the visualization of endosomal damage by the immunolabeling of galectin-3, which is usually evenly distributed in the cytosol but binds to the galactose residues that are exposed during the rupture. Its concentration around the damaged spots enables its

immunofluorescent labeling (reviewed in Martinez *et al.*, 2013). This assay was first used to detect vacuole lysis during bacterial infection (Dupont *et al.*, 2009; Paz *et al.*, 2010) and later also served to visualize endosomal escape of adenovirus (Maier *et al.*, 2012; Martinez *et al.*, 2013).

Unfortunately, we encountered several methodological difficulties, and not all experiments yielded consistent results, as shown below. Most of the presented data come from experiments still in the optimization stage, when most samples are used in duplicates in order to save enough material (e.g., viral preparation) for the final tests intended for publication. Since we encountered technical issues in virtually every run, we constantly slightly changed the conditions of the experiments to overcome these challenges. Therefore, these results remain unpublished, and the main objective of this chapter is to provide insight into the methodological issues of the approaches we used. Results that we saw consistently and were part of the published manuscripts will be clearly indicated throughout this segment of the chapter. The presented methods (Chapter 4.2.2.1) and some of their limitations will be discussed throughout this chapter, but further discussion in a broader context will follow (Chapter 5.2).

Endosome escape detection by the proximity ligation assay

Immunolabeling is an essential step in the proximity ligation assay. Therefore, we needed to determine whether the coincubation of VPs with CPPs does not compromise the subsequent immunodetection (e.g., by blocking the epitopes on VP1 or by partial disassembly of the capsid that could lead to an increased interaction with the antibodies).

We performed an ELISA (Chapter 4.2.2.1) in various settings to test this. While we show that coincubation with 20 μ M of selected CPPs (LAH4 or R8, two CPPs we used the most in immunoassays) for 30 minutes does not interfere with the virus-antibody interaction (*Figure 12A*), we also saw a rapid decrease of reactivity in samples of virus incubated with LAH4 overnight, but not with R8 (*Figure 12B*).

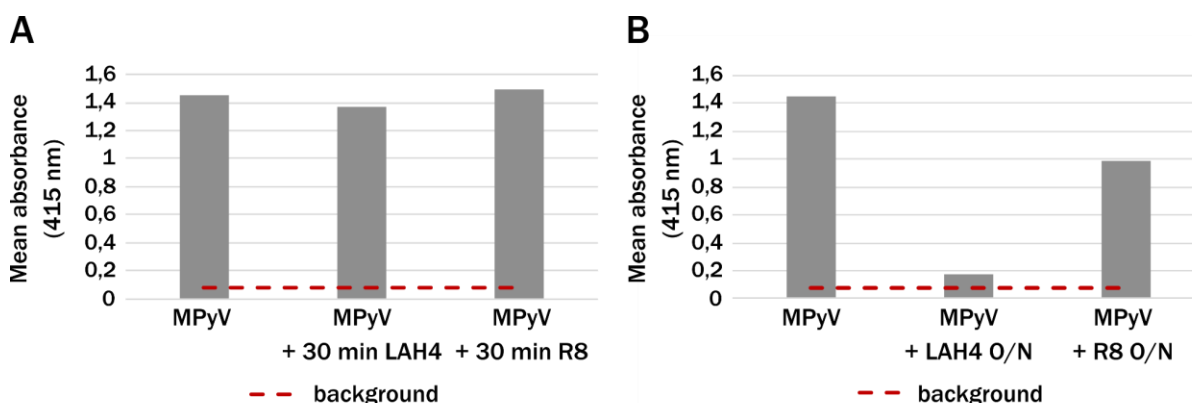


Figure 12: Immunodetection of virus-CPP complexes on ELISA. A - Detection of MPyV after 30 min coincubation with LAH4 or R8; B - Detection of MPyV (virus) after an overnight (O/N) coincubation with

LAH4 or R8; the dashed line represents the background, which is defined as the average absorbance of primary antibody in wells without the added sample (serum-free media only)

We further hypothesized that the decrease in reactivity of antibodies could be caused by the aggregates that tend to form during the coincubation of LAH4 with VPs, as was implied by DLS measurements and as we saw in the cell-binding assay (*Váňová et al., 2020*). In this particular case, these aggregates might be washed away or prevent the antibodies from effectively accessing the viral epitopes.

I visualized these aggregates by transmission electron microscopy. We noticed some loss of material on electron microscopy grids when using our standard preparation of samples for electron microscopy (i.e., incubation of electron microscopy grid, twice wash with water and negative contrasting with 2% phosphotungstic acid; see Chapter 4.2.2.1), suggesting that the large aggregates are washed away during the procedure. Therefore, I tried a different approach where the sample was left to dry on the grid, then fixed with 2.5% glutaraldehyde and contrasted with uranyl acetate solution (Chapter 4.2.2.1). With this sample preparation method, we were able to successfully visualize the aggregates (*Figure 13*).

Then, we moved to the detection of VPs in the cytosol by PLA. However, finding an optimal strategy for comparing modified and unmodified VPs posed new challenges. Since the CPPs added to the cell surface might change the timing of intracellular events, our initial thought was to map the passage of individual VPs by colocalizing them with various cellular markers at different time points. We also needed to use the "self-colocalization" of VP1-VP1 by double immunolabeling to control for the amount of the entering virus and the overall amount of VPs present in the cell.

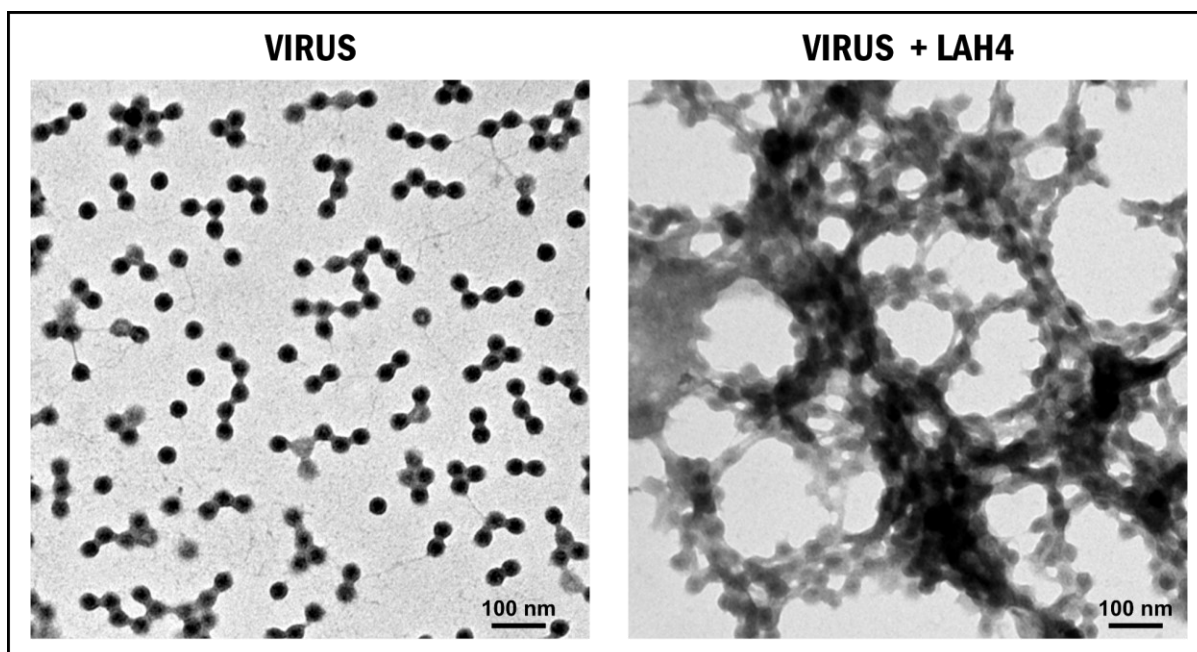


Figure 13: The aggregates of MPyV after coincubation with LAH4. Electron micrograph of virus (left) and virus coincubated with LAH4 (right) showing the aggregates that form during the 30-minute coincubation.

We then compared several strategies for detecting/quantifying viral particles by proximity ligation assay - several combinations of anti-VP1 antibodies and, eventually, anti-AF488, which can be used for immunolabeling of VLPs-AF488. During the optimization of the proximity ligation assay, we gradually overcame several obstacles. One of our unfortunate findings was that the polyclonal rabbit anti-VP1 antibody we intended to use for the VP1-VP1 colocalization (since it is necessary to use primary antibodies from two different animals) generates a high background that greatly varies between iterations of the experiments. Additionally, the VP1-VP1 detection resulted in an uncountable amount of dots (*Figure 14A*), further complicated by the high fluorescent background of LAH4 (*Figure 14B*). To eliminate this issue, we also tried proximity ligation for brightfield microscopy visualization (with horseradish peroxidase-generated colocalization signal). However, we were not able to quantify the colocalization spots in the brightfield microscope either (*Figure 14C*).

We also encountered a technical issue: the proximity ligation for brightfield microscopy visualization includes an incubation step with xylene. As xylene dissolves the plastic, this prevented the use of 8-chambered Permanox slides, which we previously utilized to process more samples at a time. Unfortunately, glass slides were not ideal either, as the cells seeded on them did not withstand the incubation and washing steps during the assay, and the use of coverslips for seeding the cells complicated the processing of multiple samples at once.

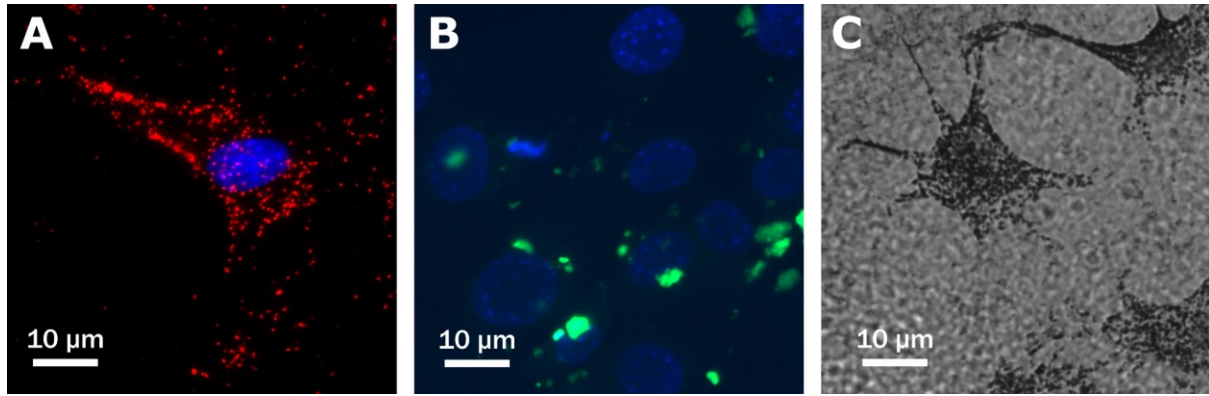


Figure 14: The challenges of PLA. **A - The uncountable amount of dots in VP1-VP1 detection for control for entering virus:** 3T6 cells were infected with MPyV as in standard PLA experiments (Chapter 4.2.2.1) and fixed 3 hours post-infection. The PLA procedure was carried out using two different anti-VP1 antibodies for self-colocalization of the viral particles; **B - The autofluorescence of cells treated with LAH4:** 3T6 cells were incubated with 20 μ M LAH4 (green signal) for 6 hours, fixed by paraformaldehyde and mounted into 70% glycerol with DAPI to stain cell nuclei (blue); **C - The VP1-VP1 detection in PLA with nonfluorescent HRP labeling** did not produce a clearer picture for the quantification of the entering virus.

In the end, we decided to perform a fluorescent variant of the PLA assay and use R8 (which does not generate the fluorescent background) for optimization steps. For the detection of VP1 presence in the cytosol, we colocalized VP1 with Hsp90. Hsp90 was selected as a suitable cytosolic marker based on a series of PLA tests. It is a highly abundant protein that is almost exclusively cytosolic, with only a small fraction found in the nucleus. Additionally, well-performing commercially available antibodies of various types are accessible, all of which have been tested in the lab for different labeling methods. This allowed us to use various antibody pairs as well as double labeling of Hsp90 as a control (not shown) with highly satisfactory results. However, we faced another unexpected obstacle: the negative control using only R8 treatment on cells generated an inexplicably high background (*Figure 15 B, D*), and we saw distinct dots with the Hsp90-VP1 detection even in negative controls (samples without VPs). To avoid issues with the detection of VP1-VP1, we attempted to use VLP-AF488 in the assay. The reasoning behind the use of VLPs-AF488 was that the fluorescent label would serve as a means to control for the number of VPs entering the cell, while anti-AF488 rabbit antibodies are commercially available for PLA labeling.

In order to follow the VPs during their path through the cell, we originally wanted to colocalize VP1 with selected cellular markers (e.g., BiP, DNAJB14) at different time points. We started with the colocalization of AF-488 with cytosolic Hsp90, after 3 hours after the addition of VLPs-AF488 to the cells, since at this time point the cytosolic release of VPs (and its expected enhancement) should be detectable.

At first, the quantification of the dots suggested that CPPs indeed change the passage through the cell and/or its timing (*Figure 15A*). To our surprise, we observed that VLPs-AF488 do

rapidly colocalize with Hsp90 (i.e., cytosolic marker), while VLPs-AF488 coincubated with CPPs do not. We planned to continue with the investigation using various cellular markers at different times post-infection to further characterize the CPP-VPs way through the cell. Before that, however, we again attempted to replicate these promising results with anti-VP1 antibodies: we again used the VLPs-AF488 as in the previous successful experiment but used anti-VP1 labeling for colocalization with Hsp90 at the same time point (3h). This should yield basically identical results, and the ultimate goal was to use the same approach for VirPorters-IN (undetectable with anti-AF488 antibodies). Unfortunately, we were unable to reproduce the initial results reliably (compare results shown for the action of FUSO peptide in *Figure 15 A, B*).

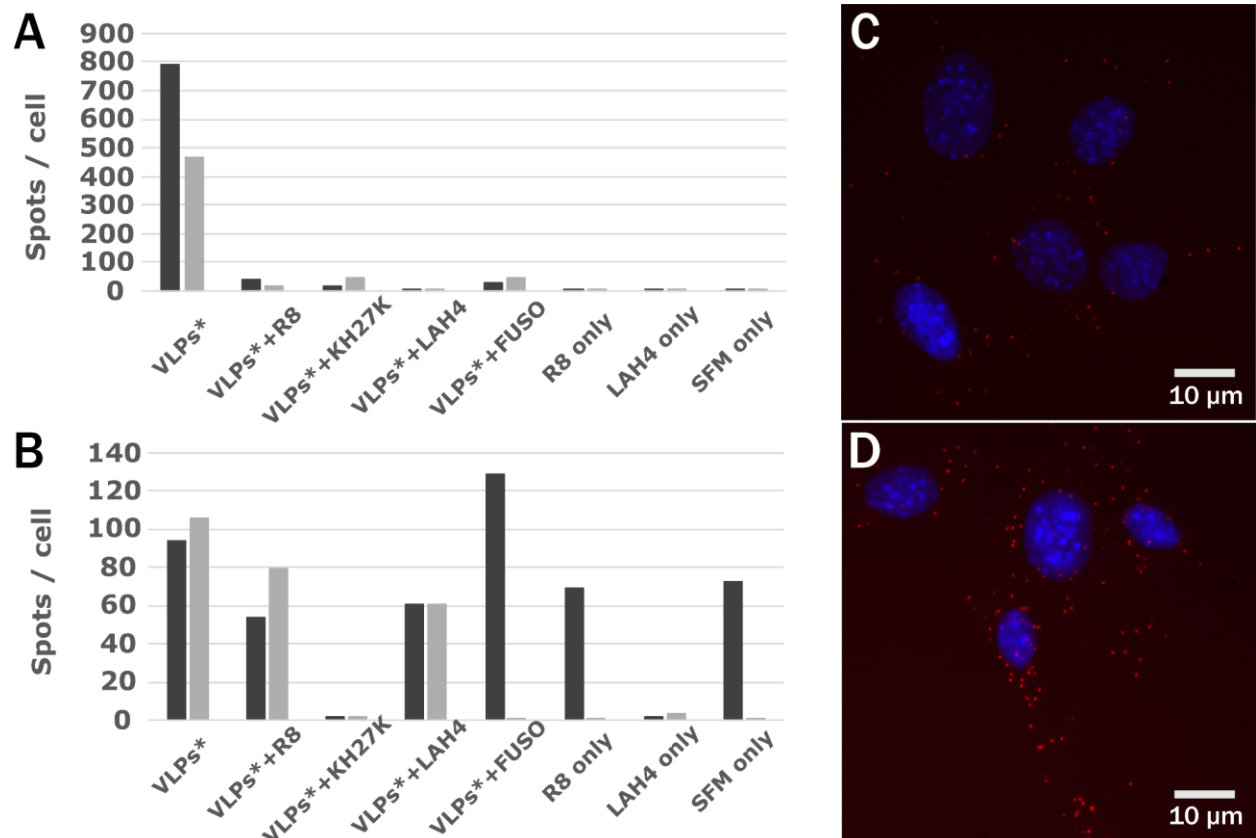


Figure 15: Examples of varying results of PLA for detection of VPs in the cytosol. A) VLPs-AF488 (VLPs*) were incubated with or without CPPs and added to 3T6 cells. Three hours later, cells were fixed and processed for PLA by using antibodies RbαAF488 + MaHsp90 (A) or RbαVP1 + MaHsp90 (B). The graph shows the results of the analysis of widefield microscopic images of approximately 50 cells per sample; the number of colocalization spots per cell that were manually counted is on the y-axis. The experiment was carried out in duplicates, and the results are presented separately to illustrate inconsistencies. With αVP1 + αHsp90 antibodies, we also observed a high positive signal (red spots) in negative controls using untreated cells in serum-free media (SFM) only (C) and cells treated with R8 without VLPs* (D). Slides were mounted into DAPI containing Duolink® mounting media to stain cell nuclei (blue).

To summarize, in each repetition, we observed inconsistencies and high background in negative controls (both untreated cells, *Figure 15C* and cells treated with CPP, specifically R8,

Figure 15D), especially when using anti-VP1 antibody for the detection of VPs. Our goal was to develop a PLA assay for quantifying all types of VPs (virus, VLPs, pseudovirions) without using AF488 labeling and anti-AF488 antibodies, and since we failed to optimize the method despite testing various antibody combinations (for VP1 and a cytosolic marker), we concluded that PLA is not suitable for reliable quantification of endosomal escape (see Chapter 5.2.2.2). We decided to adopt an alternative strategy for the detection of VPs in the cytosol, and I collaborated on establishing the subcellular fractionation techniques to monitor the intracellular fate of VPs. This method was eventually used for quantification of endosomal escape of VPs in a published study (see Attachment 7, *Váňová et al., 2022*).

Endosome escape detection by the galectin assay

We attempted to optimize the galectin assay based on galectin-3 (Chapter 4.2.21) for quantification of endosomal escape. We chose the galectin-3 assay to quantify endosomal escape, assuming that the frequency of particle escape correlates with the formation of galectin-3 spots/puncta since galectin-3 marks sites of endo-lysosomal damage by recruiting to these locations. As adenovirus 5 (AdV5) is a virus with well-documented endosomal escape abilities and galectin-3 assay was used for the detection of AdV5 egress from endosomes, we aspired to establish AdV5 as a model in endosomal escape detection (e.g., positive control for the endosomal escape of VPs).

The assay was optimized with AdV5 and CPPs using the AdV5-permissive HF CAR cell line (human-derived fibroblasts expressing coxsackie and adenovirus receptor; CAR). Even though we saw puncta caused by the CPPs, mainly LAH4 peptide added to cells (*Figure 16*), we encountered difficulties with the AdV5 positive control, as the puncta we saw were much less convincing. The poor condition of the infected cells (even after just 1-hour post-infection) led to a significant loss of cells that were washed away during the procedure. Therefore, we did not attempt to quantify the dots.

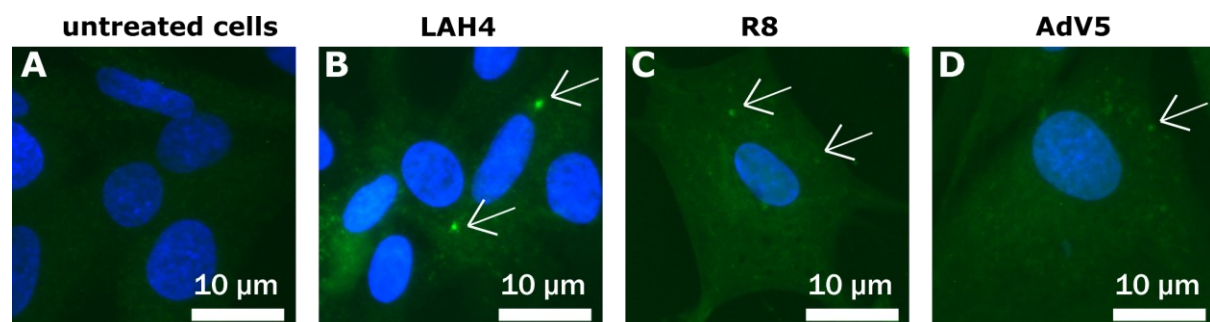


Figure 16: Galectin assay with the HF CAR cells. We saw galectin puncta after the treatment with CPPs (B- LAH4, C - R8), subsequent 1-hour incubation, and immunolabeling for galectin-3, but the puncta we detected after AdV5 infection (D) were much less visible.

Overall, our many attempts to use AdV5 as a control in these methods were unsuccessful. Due to a limited amount of available AdV5, we decided to proceed without this control and decided to use different positive control in future experiments and opted for L-Leucyl-L-Leucine methyl ester (LLOME), which is known to cause endosomal damage (Radulovic *et al.*, 2018). While AdV5 offered the advantage of immunolabeling the positive control, LLOME, on the other hand, was more likely to work reliably in the MPyV-permissive 3T6 cell line. However, the anti-galectin antibody of mouse origin we had available and initially used with HF CAR did not yield any results on 3T6 cells. Afterward, my colleague Jana Váňová tried to optimize the assay with a new antibody (monoclonal antibody rabbit-anti-galectin, Abcam), but when the issues continued (including observations of distinct spots in untreated cells), she established a HeLa-derived cell line expressing galectin-3-mCherry. This allowed us to observe the overall dynamics of the galectin puncta formation without the need for the fixation of cells. The fixation process is known to distort the results of endosomal escape detection (see Chapter 2.1.3.2.2) and limits the observation to one time-point only, which is hard to pinpoint and omits the potential changes in the timing of modified VPs' passage through the cell. With this cell line, she successfully performed the assay with free CPPs and VirPorters-IN (see Attachment 7).

4.2.2.2.3 VirPorters: Insights into the action of cationic and histidine-rich cell-penetrating peptides

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International Journal of Pharmaceutics, 2022, IF₂₀₂₁ = 6.510
see Attachment 7

In our final study on this topic, we investigated the effect of encapsidated CPPs, which we proposed would act upon partial disassembly in endosomes, resulting in enhanced release of particles into the cytosol. We first assessed the hemolytic abilities and their pH dependency (as an approximation of their endosomolytic properties) of the CPPs used for the construction of the VirPorters. While the R8 peptide did not show any hemolytic activity, the polyhistidine peptide KH27K showed some (15%) in the lowest tested pH (5.5). LAH4 showed the most prominent and pH-dependent hemolytic properties, ranging from 25% in neutral pH to 80% on pH 5.5 (see Figure S1 in Supporting information in Attachment 7).

With these peptides, we constructed two types of modified MPyV pseudovirions, VirPorters, that carry the luciferase reporter gene. VirPorters-IN have the CPP genetically attached to the capsid interior - either at the C-terminus of the minor capsid protein VP3 or at the N-terminus of the truncated VP3 (CPP-tVP3). As VirPorters-EX, we used pseudovirions with noncovalently

associated CPPs, similar to those we used previously (*Váňová et al., 2020*). However, in this setting, they lacked the VP2 minor capsid protein to provide an adequate comparison for the VirPorters-IN.

In the transduction assay, the most effective for gene delivery (6-fold change over control) were VP1/R8-tVP3 VirPorters-IN. Interestingly, their variant with R8 linked to the C-terminus of VP3 (VP1/ VP3-R8) did not perform as well (1.5 fold change). Both KH27K-containing VirPorters-IN exhibited enhanced gene delivery properties (Figure 2 in Attachment 7). This enhancement, however, does not necessarily reflect only the improved endosomal escape. Additionally, even VirPorters-IN without enhanced transduction rate might have successfully escaped the endosomal entrapment and might be useful in cytosolic delivery.

To quantify VP escape into the cytosol; we were compelled to use subcellular fractionation and densitometric quantification of immunolabeled VP1. Interestingly, our results suggested that VP escape from the endo-lysosomal system is not associated with membrane damage detectable by the galectin-3 sensor. Specifically, we investigated the subcellular fractions at 4 hours after adding VPs to the cell culture. We aimed to characterize the passage of modified VPs through the cell and possibly detect the accumulation of VPs in the cytosol, indicating the endosomal escape. We found the cytosolic presence of some VirPorters-IN is enhanced, confirming the extra endosomal escape-enhancing potential of KH27K. While noncovalently associated LAH4 is the most effective transduction enhancer even in this setting (without the VP2 in VirPorters-EX), it actually decreased the transduction when encapsidated in VirPorters-IN (Figure 3 in Attachment 7). Interestingly, LAH4 prevented the efficient interaction with cells when covalently attached, presumably because of potential interference with the necessary conformational changes upon cellular entry. Even though some VirPorters-IN (those containing KH27K or VP1/R8-tVP3) were found abundant in the cytosol, the galectin assay showed no detectable endomembrane damage caused by the VirPorters-IN (Figure 4 in Attachment 7).

We designed a novel system of MPyV-based VPs containing CPPs fused to the minor capsid protein. Our approach shows potential for enhanced gene delivery by VPs via genetic modification with CPPs. Our work also provides insight into some drawbacks in the research of endosomal releasing properties of gene carriers.

Contribution

I optimized and performed the hemolytic assay. I also participated in the optimization of the fractionation and galectin assays. I participated in the statistical analysis and the editing of the manuscript.

5. DISCUSSION

Polyomaviruses and their particles are a fascinating and active area of research in the field of biomedicine. Polyomaviruses are small DNA viruses that can cause various diseases and have the ability to manipulate host cell signaling pathways. On the other hand, polyomavirus-based particles are highly versatile and can be engineered for various biomedical applications such as gene therapy, vaccines, imaging, and drug delivery due to their unique properties.

The current goals of polyomavirus-based particle research are to improve their efficiency for gene delivery, optimize their design for vaccine development, enhance their drug delivery capabilities, and advance our understanding of the biology of polyomaviruses. Despite considerable progress in this field, there are still several challenges that need to be addressed.

In this thesis, we focused on two areas: the use of polyomavirus-based VPs as antigens in serological assays and their use in intracellular delivery, specifically their ability to overcome its limitations and means of enhancement.

5.1 Part I - Viral particles as antigens in serodiagnostics

In the first part of the thesis, we focused on the use of VPs in the detection of BKPyV subtype-specific antibodies and the role of VP1 loops on the surface of VP in their recognition. The importance of the topic stems from the post-transplant complications in kidney transplant recipients caused by this usually innocuous and ubiquitous polyomavirus. *De novo* infection with a subtype not neutralized by a patient's preexisting antibodies is considered one of the main risk factors for adverse outcomes. Simple and fast serological assessment of patient-donor pairs with the distinction of neutralizing antibodies could, therefore, help identify patients at risk in order to adjust their immunosuppression regime.

5.1.1 The use of VPs in BKPyV serodiagnostics

An excellent feature of VPs is that the epitopes of VP1 adopt the same configuration as in live virus, with the advantage of omitting the minor capsid proteins (which would decrease specificity) and bypassing the need to work with an infectious virus. This renders VPs an exceptional antigen for various serological assays, not only as an antigen for coating in ELISA.

We first showed that for reliable detection of BKPyV seropositivity, particles derived from more subtypes than the usually used BKPyV-I are needed. In the seroprevalence study, we used ELISA tests coated with BKPyV VLPs derived from the two most prevalent subtypes (BKPyV-I and BKPyV-IV) as well as the mixture of the two. The use of only one subtype underestimates the seropositivity, as some samples evidently react in a subtype-specific manner and show no cross-

reactivity. Among our samples, 9% of healthy donors and 20% of KTRs were seroreactive only with BKPyV-IV; thus, it would be viewed as seronegative in many studies using BKPyV-I VPs based ELISAs.

Until now, most seroprevalence studies usually used antigens derived from BKPyV-I, e.g., a study also performed in the healthy Czech population detected seropositivity ranging between 55.7-79.9% depending on the age group (Šrolller *et al.*, 2014), which is comparable with the 79% of samples reactive with BKPyV-I in our study. However, we detected overall higher seropositivity (88% in healthy subjects and 84% in KTR) due to the use of the BKPyV-IV antigen.

Among KTR in our study, 64% of samples react with BKPyV-I. This is also comparable with the 61.9% of positive KTR in a recent Czech study (Saláková *et al.*, 2022) which uses ELISA based on BKPyV-I antigen. However, we detected additional 20% KTR serum samples reactive exclusively with BPvV-IV.

Surprisingly, in this study by Saláková *et al.*, two (out of 210 patients included in the post-transplant examination) initially seronegative recipients developed BKPyV-DNAemia after transplantation of a kidney from a seronegative donor. The authors do not disclose whether they characterized the BKPyV genotype found in these recipients and do not address the possible undetected BKPyV-IV seropositivity in either recipient or donor. Another recipient in the study remained seronegative on BKPyV-I ELISA 12 months after the transplantation even though they developed BKPyV-DNAemia (Saláková *et al.* 2022). This illustrates the need for the use of antigens of multiple BKPyV subtypes, as with the use of BKPyV-I ELISA, the antibodies specific to other subtypes could remain undetected.

Interestingly, in a longitudinal study of seroprevalence in a group of organ transplant recipients from 5 European countries (The Netherlands, United Kingdom, Germany, France, and Italy) by Antonsson *et al.* (2013), the detected seroprevalence reached even 100% depending on the age group (in the youngest age category - up to 29 years of age). Although the authors do not mention the used subtype, we can presume it was BKPyV-I, which is the most used subtype in these types of studies at that time. Most notably, they used Luminex technology with the GST-VP1 antigen. This agrees with the high seroprevalence (up to 99.2%) found in the Dutch population (of healthy blood donors) in a study that also uses Luminex and GST-VP1 (Kamminga *et al.*, 2018a). More specifically, this assay uses BKPyV of genotype I, which was previously found to be species-specific for BKPyV (Kamminga *et al.*, 2018b).

A lower seroprevalence was detected in another study - up to 78.9% depending on the age group (Viscidi *et al.*, 2011). In this case, the researchers used VLP-ELISA (also of undisclosed subtype) while controlling for cross-reactivity (with Merkel Cell Polyomavirus and JCPyV) with

ACA. This is more comparable to the above-mentioned results (seroprevalence up to 79.9%) from another VLP-ELISA-based study using BKPyV-I VP1 VLPs (Šroller *et al.*, 2014) and our own results using BKPyV-I VLPs. Comparison of these results might imply a higher degree of subtype cross-reactivity for GST-VP1, thus possibly compensating for the missing antigens of other subtype(s). Indeed, in our publication, we argue that native VP1 in VLPs might offer greater subtype-specificity than GST-VP1 constructs (or disassembled VP1 protein in general). In fact, overwhelming evidence shows that both the subtype specificity and sensitivity of ELISA are greatly influenced by the type of used antigen.

Curiously, when the reactivity of GST-VP1 and VLPs of the same (but undisclosed) subtype was directly compared in VLP-based and GST-VP1-based ELISA by Bodaghi *et al.* (2009), GST-VP1 was less sensitive, similar to denatured VLPs. According to their results, the use of GST-VP1 is less sensitive as 39 serum samples (from healthy donors and dialysis patients) were positive on VLP ELISA but negative when the GST-VP1 was used. The overall reactivity with GST-VP1 was also much lower. Notably, different amounts of each antigen were used, specifically, 25 ng of GST-VP1 BKPyV and 50 ng of BKPyV VLPs per well. Given that GST (26kDa) makes up a third of the GST-VP1 (62kDa), the content of the coated actual VP1 is more than twice lower than in the case of coated VLPs. Therefore, from the available information, it cannot be excluded that a comparable sensitivity could be achieved while using GST-VP1 as an antigen if coated in a proper amount. Moreover, the GST-VP1 antigen could actually capture antibodies directed against other subtypes and thus result in higher assay sensitivity but lower subtype-specificity, as suggested by other studies (Kamminga *et al.*, 2018a; Wunderink *et al.*, 2019b). In these studies (with GST-VP1 antigen in Luminex), the overall detected seroprevalence against subtype I was 91-100% depending on the source population (healthy donors had slightly lower seroprevalence than kidney transplant recipients) and specific genotype (Ib1, Ib2, and Ic). This is in agreement with the above-discussed results of other studies using the subtype I GST-VP1. The authors, however, are quite confident in their good comparability with VLPs and support this by their previous results where a strong correlation was observed between data generated with the Luminex BKPyV GST-VP1 fusion protein assay and the BKPyV VP1 VLP ELISA (Wunderink *et al.*, 2017b). This also further supports the notion that a sufficient amount of GST-VP1 can serve as a satisfyingly sensitive antigen despite the results of Bodaghi *et al.* (2009).

However, serodiagnostics must be tailored to specific clinical needs. To assess the prevalence of BKPyV infection in the population, it is essential to use antigens that detect antibodies against all serotypes, as described above. Also, the use of methods capable of detecting antibodies that react with multiple subtypes is advantageous, as they allow for the identification of

so-called cross-reactive, or subtype-common, antibodies. Notably, the detection of (cross)reactive antibodies might be affected not only by using GST-VP1 antigen but also by using disassembled VP1 or assembled capsids, and their composition might play a role. For example, in the study of Pastrana *et al.* (2012), authors observed a much stronger correlation between individual subjects' anti-BKPyV-I and anti-BKPyV-IV titers on ELISA than we show in our publication. The authors admit that this might be the result of the cross-reactivity of BKPyV-I and -IV on ELISA, as their neutralization assay showed no significant correlation between BKPyV-I and -IV titers. In our work, the correlation between BKPyV-I and -IV titers in tested individuals was not significant. We explain this difference by using VP1 only VLPs in our study, while Pastrana *et al.* used PsVs containing minor capsid proteins. Moreover, they produced PsVs of all subtypes with minor capsid protein derived from the same subtype (BKPyV-IV). Even though the VP2 and VP3 proteins are buried within the capsid, they can be accessible to antibodies when PsVs are not perfectly intact and are known to participate in cross-reactivity between some polyomaviral species (Moens *et al.*, 2013).

Although cross-reactivity of type-common antibodies against denatured VP1 was observed, it did not seem to confound VLP-based ELISA tests (Moens *et al.*, 2013). For BKPyV and JCPyV (polyomavirus with the highest, 78% VP1 sequence similarity), this was confirmed by us and other teams (Viscidi and Clayman, 2006; Šroller *et al.*, 2014; Kamminga *et al.*, 2018b; *Hejtmánková et al.*, 2019). There is evidence of cross-reactivity with SV40 (Viscidi *et al.*, 2003), which was thought to be seroprevalent due to known contamination of poliovirus vaccines between 1955 and 1963. However, there is currently no evidence of it being prevalent in the human population (Carter *et al.*, 2003). Several seroprevalence studies of polyomaviruses control for potential species cross-reactivity with the use of the ACA (Carter *et al.*, 2003; Viscidi *et al.*, 2003; Warnke *et al.*, 2013; Kardas *et al.*, 2015).

ACA, in general, appears to be a potential solution for cases where it is necessary to evaluate not seroprevalence but the presence of type- or subtype-specific antibodies. Typically, in the context of post-transplant management, precise serological differentiation of the infecting subtype in both the donor and recipient is required. In our ELISA study, a number of samples were reactive in both BKPyV-I and BKPyV-IV VLPs antigens. Therefore, we performed an ACA to improve the distinction of subtype-specific antibodies in these serum samples. We confirmed that cross-reacting antibodies are present in some of these samples, contribute to this double reactivity, and can be distinguished by the use of ACA. A study published simultaneously with ours also used ACA to differentiate between the subtypes: previously, the Luminex assay with VP1-GST antigen was used for the investigation of polyomavirus seroprevalence (Kamminga *et al.*, 2018a;

Kamminga *et al.*, 2018b) and now Wunderink *et al.* (2019b) used it for the serotyping of BK polyomavirus and six serum samples were further investigated with an ACA. Their data show a “little chance of cross-reactivity” between the seroreactivity against BKPyV I and IV. In addition to that, they used antigens derived from all subtypes: Ib1, Ib2, Ic, II, III, and IV (Ia VP1 was not included because its amino acid sequence is identical to Ib1 VP1) and showed that the assay could distinguish even between the three genotypes of subtype I. Interestingly, the cross-reactivity of subtypes II and III with other subtypes was quite high, and the reactivity was almost completely inhibited by antigens of other subtypes in the ACA.

The study also compares the results of Luminex BKPyV GST–VP1 assay, Luminex BKPyV GST–VP1-based ACA, and neutralization assay. Three out of six samples were conclusively seroreactive against the same subtype (Ib1). Another was highly reactive with both Ib1 and BKPyV-IV in the non-competing Luminex assay but was determined as BKPyV-IV positive in both ACA and neutralization assay. Interestingly, for two samples, there were major discrepancies between the results of ACA and neutralization assay.

To conclude this part, the seroprevalence detected using the BKPyV-I subtype only corresponds with previously published results obtained with the same antigen in ELISA. However, increased sensitivity can be achieved by adding additional BKPyV subtypes into the assay as some samples contain only subtype-specific antibodies against, e.g., BKPyV-IV. While using the Luminex multiplex assay indeed offers a great advantage of distinguishing the subtypes on a one-well basis using different color coding for each subtype, an obvious disadvantage of this approach over ELISA is the need for special equipment. Competitive ELISA could replace neutralization assay to detect subtype-specific antibodies against BKPyV in samples containing low levels of cross-reacting antibodies. VLPs-based ELISA might offer additional advantages as major capsid protein VP1 is in its native conformation (opposed to, e.g., GST-VP1 antigen), and the absence of minor capsid proteins could further decrease the cross-reactivity.

5.1.2 Modification of BKPyV VP surface loops and characterization of their role in the serological cross-reactivity

Following our previous findings, we then aimed to prepare novel antigens to investigate the epitopes responsible for (subtype-specific) antibody recognition. We prepared two mutant variants (BKPyV-IV-DEmut and BKPyV-IV-EFmut), which are derived from BKPyV-IV VP1 but contain mutations to have their DE or EF loop AA sequence identical to the loops in BKPyV-I VP1.

We chose to mutate the variable surface loops, which are a favorable target of changes and usually do not restrict the stability of the mutant polyomaviral particle. While the BC loop is quite well mapped by previous studies (Pastrana *et al.*, 2013) and contains many inter-subtype differences, we chose to alter the DE and EF loops, which differ only in a small number of amino acids between the most common subtypes, BKPyV-I and BKPyV-IV. We hypothesized that the introduced changes would change the antibody-binding capacity of the resulting antigen and their behavior as the competing antigen in an ACA. This would provide important information about the epitopes reacting with the subtype-specific and cross-reacting antibodies against BKPyV.

As we aimed, the changes introduced in the EF loop of BKPyV-IV VP1 (D175E and I178V) led to the loss of reactivity with anti-BKPyV-IV antibodies. Interestingly, even anti-BKPyV-I antibodies reacted less with the antigen containing the residues typical for the BKPyV-I subtype. This suggests that the EF loop is a part of an important antibody-binding site not only for subtype-specific antibodies. This is in agreement with the findings of Lindner *et al.* (2019) that the EF loop is part of an epitope that interacts with a broadly cross-neutralizing antibody. Actually, one of the charged residues in BKPyV-IV-EFmut (AA 175) was part of the investigated antibody binding site in multiple studies (Lindner *et al.*, 2019; Abend *et al.*, 2024). In other polyomaviruses, the EF loop was previously identified as a site of neutralization escape mutations (Murata *et al.*, 2008; Fleury *et al.*, 2015).

Replacing the DE loop of BKPyV-IV with its BKPyV-I counterpart resulted in a stronger reaction with anti-BKPyV VP1 antibodies in some sera. This is in agreement with the prediction of the DE loop as a highly immunodominant epitope (*Figure 6*). The site is in close proximity to the BC loop residues that contribute to the neutralizing epitope (Pastrana *et al.*, 2013). This epitope could, therefore, be the target of both neutralizing and non-neutralizing antibodies depending on the other epitope contributing to the antibody binding.

5.1.3 Modified BKPyV VPs as a tool in functional assays

The prepared mutants could also serve as a tool in a functional study to characterize the site of the effect of neutralizing antibodies.

Such functional analyses of mutant BKPyV variants were previously mostly focused on the BC loop, which contains a hypervariable region with frequent differences among the subtypes. With the use of chimeric pseudovirions, some of these variable residues of subtypes Ib1 and Ib2 (positions 73 and 82) were shown to define the serotypes in the neutralization-based assay (Pastrana *et al.*, 2013). The hypervariable loop is also part of the sialic-acid binding epitope and target of frequent mutations under the selection pressure during experimental cultivation (positions

62 and 82) of BKPyV (Lindner *et al.*, 2019). This was confirmed by tracing the intra-patient evolution by Krautkrämer *et al.* (2009), who detected frequent mutations at positions 73, 77, and 82 (and also 139 in the DE loop) and by Peretti *et al.* (2018) at positions 62, 73, and 77. By comparing the median viral load of the isolates, Krautkrämer *et al.* determined that the observed mutations did not change the efficiency of viral replication *in vivo*. This agrees with the results of previous experiments *in vitro* (Dugan *et al.*, 2007). Dugan *et al.* also found that some residues within the BC loop (61, 64, 65, 79, and 82) are critical for capsid stability and interaction with the receptor. Even though Krautkrämer *et al.* did not observe the specific mutations of said residues *in vivo* and note that these would inhibit viral growth and thus the selective pressure works against them (Krautkrämer *et al.*, 2009), Peretti *et al.* later showed that these residues participate in the receptor binding and their mutations lead to neutralization escape (Peretti *et al.*, 2018). Indeed, a higher diversity of this region in the BC loop (positions 61-83) was detected in patients with BKPyVAN (Luo *et al.*, 2012, reviewed in McIlroy *et al.*, 2019). Interestingly, Peretti *et al.* show that the found mutants still depend on sialic acid for cellular entry (Peretti *et al.*, 2018). Later, it was confirmed that over time, VP1 mutations in the BC loop (residues 60, 62, 69, 72, 73, 75, 77, and 82) accumulate in patients with prolonged viremia (McIlroy *et al.*, 2020). These mutations lead to neutralization escape but, again, did not change the sialic acid dependence of the resulting virus. Surprisingly, a recent study showed that certain mutations at positions 69 and 82 (K69N and E82Q) lead to changes in the sialic acid binding pocket, sterically preventing the interaction. Moreover, these mutants remained infectious. The follow-up experiments also led to evidence of a sialic acid-independent entry receptor for BKPyV (Sorin *et al.*, 2023). Interestingly, mutations at position 139 (DE-loop) were also observed among BKPyV isolates from kidney transplant recipients (Krautkrämer *et al.*, 2009). Specifically, a reverse variant (BKPyV-I with H139N substitution) of the mutation we introduced (N139H in BKPyV-IV) was found in an isolate of BKPyV genotype Ib-2 and another (N139K) in an isolate of BKPyV genotype IV. In a later study, the H139N mutation was observed in a patient with persistent viremia and, together with other found mutations in the BC loops, was determined to form a “ring around the sialic acid binding pocket” (McIlroy *et al.*, 2020). Mutant BKPyV variants with changes in other surface loops are yet to be thoroughly explored.

Consequently, our initial plan was to perform such functional assays with our mutants and assess both transduction efficiency and potential escape from neutralization on well-characterized sera from KTx patients. We aimed to produce mutant-based pseudovirions containing also minor capsid proteins and reporter gene. Unfortunately, we were unable to achieve successful pseudovirion production within the timeframe of this project. The underlying reason appears to be

insufficient expression of the minor capsid proteins (Caisová, 2023), which compromised the efficiency of pseudoinfection, rendering it inadequate for subsequent neutralization assays.

It is also worth noting that the production and isolation of BKPyV-based VPs in the form of pseudovirions were accompanied by unexpected challenges, which ultimately affected the assessment of antibody reactivity. The introduction of a new pseudovirion isolation protocol, inspired by the adenovirus preparation procedure and incorporating tetrachloroethylene extraction (Baker, 2019), demonstrated that despite the successful isolation of intact particles (confirmed by TEM, SDS-PAGE, and hemagglutination), antibody reactivity on ELISA was significantly reduced compared to the conventional VLP preparation method (Tomanová, 2019).

Later, the experiments were repeated with the pseudovirions prepared by Helena Caisová (Caisová, 2023), who used the common isolation method in an Optiprep gradient (Buck *et al.*, 2004). This protocol emphasizes the importance of an overnight incubation step for capsid maturation (Buck *et al.*, 2004), and it was found that different preparation methods may yield incomplete capsids (Sorin *et al.*, 2023), which can be, in turn, relevant even for the interaction of antigens with antibodies. Interestingly, we also observed a difference in the properties of BKPyV-IV-EFmut PsVs preparations purified by two different methods. To conclude, we believe that establishing inter-laboratory standard antigens would facilitate more reliable result comparisons in serological assessment of BKPyV infections and in future research.

5.1.4 On solving the serological cross-reactivity of BKPyV subtypes

The current consensus on the management of BKPyV infection in kidney transplant recipients is the monitoring of viremia and eventual decrease of immunosuppression (Saleh *et al.*, 2020). The majority of scientific literature on the topic agrees that a pretransplant serological assessment of both donors and recipients is beneficial in predicting the outcomes and identifying the patients at risk (Bohl *et al.*, 2008; Sood *et al.*, 2013; Abend *et al.*, 2017; Wunderink *et al.*, 2017b).

Although the BKPyV genotypes were categorized into the serotype groups by the neutralization assay, implying that the neutralizing antibodies are, by definition, subtype-specific, the existence of cross-neutralizing antibodies was demonstrated. Randhawa *et al.* characterized monoclonal neutralizing antibodies that neutralized the infection of BKPyV but not JCPyV or SV40, suggesting that neutralizing epitopes are usually species-specific. However, some of the tested neutralizing antibodies did cross-react across different species in ELISA (Randhawa *et al.*, 2009). The work of Lindner *et al.* also described several antibodies that neutralized the infection of more than one BKPyV subtype and some also JCPyV (Lindner *et al.*, 2019). Similarly, it was

possible to achieve cross-neutralizing antibody response in mice even by BKPyV monovalent vaccines when booster vaccines were used (Pastrana *et al.*, 2012). According to the authors, this could be attributed to the restimulation of memory B cells, which then secrete cross-neutralizing antibodies, as was previously seen for West Nile Virus (Purtha *et al.*, 2011). It is entirely possible that such cross-neutralizing antibodies are present in patients with multiple exposures to BKPyV infection and could complicate the subtype-specific diagnostic tests, which, on the other hand, ultimately would not be necessary in cases when a broadly neutralizing immunity restricts the BKPyV reactivation.

In recently published commentaries, some researchers question whether recipient pre-transplant anti-VP1 BKPyV levels can be used as a predictive tool to manage clinical BKPyV infection (Blazquez-Navarro *et al.*, 2022; Brochot, 2023). Blazquez-Navarro *et al.* argue that high levels of antibodies do not protect against virus reactivation after KTx but rather serve as a marker for virus levels in peripheral blood and probably also in tissue (early reactivation was significantly associated with higher antibody levels). However, they do not take into account the neutralizing antibodies. Interestingly, Blazquez-Navarro *et al.* use only the AS strain (genotype III) in their ELISA test to determine the recipient's seropositivity and/or antibody level. As Brochot (2023) notes in their response, the used strain is fairly uncommon (a prevalence of over 10% is detected only in African populations) but is highly cross-reactive with the other subtypes (Wunderink *et al.*, 2019b). Even though 99.5% of the tested sample was seropositive, the antibody levels might have been underestimated because of this. The study sample was also influenced by an uncharacteristically high percentage of viremic patients before the transplantation. Both commentaries agree that there is a pressing need for robust data, specifically studies measuring neutralizing antibodies and cellular immunity.

Some recent publications address this question and conclude that the recipient's serostatus before transplantation had no effect on posttransplant BKPyV replication, while the donor serostatus was a reliable predictor of adverse outcomes (Saláková *et al.*, 2022). Their results contrast with the previous findings of Dakroub *et al.* (2021), who collected data that support the importance of recipients' seropositivity in this regard. As discussed above (Chapter 5.1.1), the differences in methodology (specifically the subtypes of used antigens) might significantly affect antibody detection. Indeed, these two studies differ in the used antigens: while Saláková *et al.* used BKPyV-I VLPs, Dakroub *et al.* used both BKPyV-I and -IV VLPs as antigens in ELISA.

Overall, the need to develop a simple and fast serological assessment of patient-donor pairs with the distinction of neutralizing antibodies remains. Additionally, including antigens of

multiple BKPyV subtypes in the testing would allow a thorough investigation of the serostatus and the associated risk factors for renal transplants.

5.1.5 The imminent future of BKPyV research

As subtype cross-reactivity is still considered a major challenge in the subtype differentiation for BKPyV serodiagnostics, it would be interesting to address its source and specify the role of each BKPyV epitope. VP1 sequences of known BKPyV isolates show over 90% homology, and their differences cluster in the BC loop. Even though it is quite a frequent subject of published studies, there is still potential for further research. A detailed mutation analysis of the region could help decipher the exact positions needed for the subtype-specific antibody recognition.

As was hinted above (Chapter 5.1.3), the investigation of the *in vivo* BKPyV genetic diversity and the role of each genotype and specific mutations in the pathogenesis is currently a hot topic in the field (Peretti *et al.*, 2018; McIlroy *et al.*, 2019; McIlroy *et al.*, 2020; Signorini *et al.*, 2020; Gras *et al.*, 2023, also reviewed in Blackard *et al.*, 2020; Furmaga *et al.*, 2021).

Since the discovery of BKPyV in the 70s, the topic has been vastly explored, but there is still much unknown about the mechanisms of persistence, pathogenesis, and management of patients at risk, and new discoveries constantly come to light. Recently, the need for routine BKPyV screening was shown to be advisable in patients undergoing lung transplantation (Dube *et al.*, 2023). BKPyV is also investigated as a risk factor for alloreactivity after transplant as the detection of donor-specific antibodies is associated with BK viremia (Hod-Dvorai *et al.*, 2023), and the infection also increases the risk of acute graft rejection (Alonso *et al.*, 2022). To date, there is no approved specific anti-viral treatment, and post-transplant BK viremia is usually managed by the reduction of immunosuppression with continuous monitoring of the viremia (reviewed in Saleh *et al.*, 2020; Kant *et al.*, 2022; Qeska *et al.*, 2022).

A novel human monoclonal antibody (MAU868) against the BKPyV-VP1 was recently characterized and assessed for its use *in vivo*. It was found to neutralize the infection by all four major BKPyV subtypes (Abend *et al.*, 2024). In the first-in-human study, the MAU868 was safe and well-tolerated and showed potential to be an antiviral agent for the treatment of BKPyV-associated disease (Abend *et al.*, 2024). The authors also identified the key binding residues (Y169, R170, and K172) that are part of the interaction surface that we mutated in the BKPyV-IV-EF mutant (substitutions E175D and V178I). Indeed, the mutations we introduced in this region diminished the reactivity with the antibodies. There are indeed other positive reports, such as a potential multivalent vaccine against polyomaviruses that was already successfully used in

macaques (Peretti *et al.*, 2023) and also the very recent advances by Duty *et al.* (2024). Their not-yet peer-reviewed study shows that experimental vaccination of VelocImmune® mice² can yield broadly neutralizing monoclonal antibodies. The mice were immunized with expression plasmids encoding BKPyV VP1 from some of the four subtypes together with BKPyV-IV VP2 and VP3 in various combinations. Then, a final boost of the respective PsVs was administered. The largest percentage of cross-reactive antibodies was generated by first immunizing against BKPyV-II and -III VP1, followed by BKPyV-I and -IV VP1 (Duty *et al.*, 2024). Taken together, the future of BKPyV research will focus on potential vaccination and antibody treatment, for which the production of VPs or even their modified versions to enhance the cross-reactions of elicited antibody response will be essential.

5.2 Part II - Viral particles as containers for intracellular delivery

Polyomaviral particles are a promising tool for intracellular delivery. Their use has been supported by a significant amount of literature (Chapter 2.3.1). However, there are still some areas that require further investigation and improvement. While the viral infection is evolutionarily optimized for a “best infection rate,” which is not necessarily the “highest infection rate” (which would lead to rapid immune response), administration of lower doses might be desirable and call for higher efficiency in the case of particles for intracellular delivery of drugs and gene therapy.

We have focused on overcoming two known major barriers: the protein corona (Chapter 4.2.1) and endosomal entrapment (Chapter 4.2.2). This may help to further enhance the efficacy of polyomaviral particles for intracellular delivery and ultimately improve their potential for a wide range of applications in the field of medicine and biotechnology.

5.2.1 The effect of protein corona on MPyV-VPs

The unwanted interaction of nanoparticles (including polyomaviral VPs) with the extracellular environment might be the first potential barrier after entering the bloodstream. We first delved into the hypothesis that animal viruses should be molded by evolution to evade obstacles presented by the extracellular environment, specifically the formation of the protein corona. We assessed the protein corona formation on MPyV VPs and its effect on the successful targeting of these particles.

In the initial phase of our research (Žáčková Suchanová, 2018), we focused on comparing VPs and NPs regarding their ability to interact with serum. We demonstrated that, unlike model polystyrene nanoparticles, MPyV VPs do not interact with serum. While polystyrene NPs

² Mice expressing human variable light and heavy chains in their antibodies (Murphy *et al.*, 2014)

underwent aggregation and extensive protein adsorption (as demonstrated by an increased hydrodynamic radius, TEM analysis, and analysis on protein electrophoresis), MPyV VPs remained non-aggregated after serum incubation, with only minimal protein adsorption. However, we encountered several technical challenges that are currently a subject of vigorous discussion (Ashkarran *et al.*, 2022). Standard procedures (Ashkarran *et al.*, 2024) for isolating the protein corona on NPs, such as simple centrifugation (14,000 x g) to remove non-adsorbed proteins, could not be applied to VPs due to their inability to pellet at such centrifugal forces. Initially, we chose dialysis used in previous studies (Kristensen and Gram, 1982; Lin *et al.*, 2005; Zeitlinger *et al.*, 2011), and we found (despite optimizing conditions like pore size, dialysis volumes, and durations) that HS does not pass through the used dialysis tubing, which makes this step unnecessary and leads to an avoidable loss of material. Therefore, we resorted to ultracentrifugation through a sucrose cushion, as previously used for the assessment of hard corona on VLPs by Pitek *et al.* (2018). However, by using HS alone as a control, we showed that this allows co-sedimentation of some serum proteins, especially following longer incubation steps.

The analysis of protein corona was also performed on modified VLPs*Tf to further investigate if the serum environment can affect the targeting of those particles to Tf receptors. We found that VLPs*Tf interact with serum proteins more readily than unmodified VLPs. However, these additional proteins present in the purified fraction of VLPs*Tf generally did not interfere with the interaction with TfR on ELISA. This contrasts strongly with the findings of Salvati *et al.* (2013), who demonstrated that transferrin-functionalized nanoparticles (50 nm fluorescent silica nanoparticles) lose their targeting capabilities due to the adsorption of a biomolecular corona on their surface.

The presence of human sera, however, partially inhibits the interaction of VLPs*Tf with TfR in the ELISA test (whereas the presence of various animal sera does not). In her thesis, Dr. Žáčková Suchanová attributed this to the presence of free transferrin and showed that the preincubation of 10% human sera with an adequate amount of anti-Tf antibody prevents free Tf from inhibiting the VLPs*Tf binding to cells. We then confirmed these results in our ELISA experiment with additional human serum samples (from various blood types) and an additional type of animal sera (chicken serum). We show that this inhibition by human sera is dose-dependent and varies depending on the inhibitory concentration of Tf in the serum sample, thus can truly be attributed to the presence of free Tf binding human TfR rather than the formation of the protein corona. This was further supported by the analysis of cellular uptake of VPs. In contrast to unmodified MPyV VPs, which utilize clathrin-independent endocytic pathways for entry

(Richterová *et al.*, 2001), MPyV*Tf VPs employed the clathrin-mediated entry pathway, as previously shown for free transferrin (Dautry-Varsat, 1986), independent of serum presence.

While the targeting abilities of nanoparticles are generally thought to be hindered by the formation of the protein corona (reviewed in Xiao *et al.*, 2022; Liu *et al.*, 2023), we showed that it does not have to be the case for virus-derived particles.

Interestingly, in some cases, the protein corona can actually help the nanocarriers, e.g., crossing the blood-brain barrier (Xiao *et al.*, 2021). Therefore, understanding protein corona formation around nanoparticles is crucial not only to avoid its unwanted effects but also to potentially use it to our advantage. For example, the purposeful coating of NPs with a corona of specific composition can improve their targeting and prevent aggregation (Safi *et al.*, 2011; Dominguez-Medina *et al.*, 2013). Proteins adsorbed on nanoparticles are known to change their various characteristics and, ultimately, the cellular response to these nanoparticles (reviewed in Dawson and Yan, 2021), and it is essential to embrace the effects of protein corona and take them into account when designing nanocarriers (reviewed in Khan *et al.*, 2022).

Interestingly, some virus-derived nanoparticles can attract a protein corona and benefit from protein corona replacement (Huang *et al.*, 2023). In contrast, others, as demonstrated in our work with MPyV VPs, exhibit only limited interaction with serum proteins. This underscores that the investigation of the protein corona is a fruitful area of research, even within virology. Many viral vectors could improve their clinical applications through potential "protein corona engineering," offering a unique approach to modify particles, enhance their strengths, balance potential weaknesses, and enable them to fulfill their intended purpose effectively.

5.2.2 Functionalization of polyomaviral VPs with cell-penetrating peptides and characterization of their interaction with cells

Endosomal entrapment is considered to be another major obstacle in intracellular delivery. To bypass this, we used two types (covalent and non-covalent) of VP modifications by CPPs (LAH4, R8 KH27K, and FUSO) and investigated their effect. Furthermore, we aimed to establish a reliable method for quantifying the endosomal escape of VPs in order to determine whether the desired enhancement by CPPs truly stems from the induction of VPs' release into the cytosol.

5.2.2.1 Enhancing intracellular delivery by MPyV VPs with CPPs

My colleagues successfully prepared several variants of VPs modified by CPPs, either externally by non-covalent attachment (VirPorters-EX) or internally by genetically engineering

the particles with the CPP covalently attached to the interior capsid proteins (VirPorters-IN). We tested their interaction with the cellular membrane as well as the influence of CPPs on the particles and overall gene-delivery properties.

In the two published studies, we found that CPPs change the physical properties of the capsids, increase MPyV infectivity and transduction by MPyV VPs (with the exception of FUSO), change the mode of interaction with the cellular membrane, and influence the passage of VPs through the cell (see *Table 2* and *Table 3*). The changes in physical properties may play a role in the overall effect of CPPs on VPs. We employed several methods to find out where the action of CPPs actually takes place.

The detection of CPP-VP complexes by fluorescent microscope (necessary for some of these methods) can be quite tricky. Fluorescent labels attached to CPPs change their properties and can interfere with the experiments (reviewed in *Váňová et al., 2019*) and would make the comparison with unmodified VPs impossible. Therefore, either immunodetection of VPs or the use of pre-labeled VPs is needed. Indeed, for some previous experiments, we used VLPs labeled with Alexa Fluor 488 (VLP-AF488). However, one has to keep in mind that the label itself can also interact with the CPPs and change the noncovalent association formed during coincubation compared to unmodified VPs.

Even when using the usual VP1 immunolabeling, the CPPs could change the interaction of antibodies with the capsid surface. Therefore, we first inspected the potential changes of antibody interaction due to the surface-bound CPPs and verified that the VP1 protein is still detectable by our commonly used antibodies even after coincubation with CPP using an ELISA (Chapter 4.2.2.2.2). Given our results (some samples showed a decrease in reactivity with antibodies after longer incubation time), we hypothesized that some peptides (LAH4) cause the aggregation of VPs. This was in agreement with the measurements from DLS and observations in the cell binding assay. To visualize the aggregates, we optimized a new contrasting protocol for TEM using uranyl acetate.

Unequivocally, the immunolabeling of VirPorters-EX requires careful interpretation of data as the presence of aggregates or partially disassembled capsids can inhibit or amplify the signal, respectively. Therefore, we used immunofluorescence assays with VirPorters-EX mainly to optimize the methodology for future assessment of VirPorters-IN action.

	VirPorter-EX											
CPP	physical state			infectivity of MPyV	cell binding assay		uptake measured	effect of endocytic inhibitors on VLPs*/CPP entry		transduction		
										MPyV	SV40	MusPV
	hydrodynamic diameter (DLS)	Zeta potential	stability (TEM)		microscopy	FACS	FACS	sucrose	EIPA	luciferase assay		
CPP concentration	20 µM			20 µM	20 µM		20 µM	20 µM	20 µM	various concentration		
no CPP	~116 nm	negative surface potential						decrease	no change			
LAH4	PDI too high (unsuitable for measurement)	positive surface potential	enlargement; frequent 4.6% disassembly; aggregation	substantial increase	clusters	increased attachment	decrease	decrease	increase	increase	increase	increase
R8	~89 nm	negative surface potential	0.8% disassembly	substantial increase	no detectable changes	no measurable changes	substantial decrease	decrease	none	no changes	slight increase	increase
KH27K	heterogeneous	high PDI positive surface potential	0% disassembly; aggregation	increase	clusters	no measurable change	no measurable change	decrease	decrease	substantial increase	substantial increase	increase
FUSO	~123 nm	more negative surface potential than control	0% disassembly	no change	no detectable change	decreased attachment	decrease	N/A	N/A	no changes	no changes	no changes

Table 2 : **The summary of the results** of our investigation of CPP influence on MPyV VPs (*Váňová et al., 2020*).

CPP	composition	transduction	intracellular location of VP1 at 4 hpi			hemolytic activity (at 20uM)		
			cytosol (% of total)	membrane fraction (% of total)	nucleus (% of total)	pH		
						7.4	6.5	5.5
no CPP	VP1/VP3		~34	~66	no signal			
LAH4	VP1/VP3-LAH4	decrease (1.6x)	48	52	no signal	31.5%	47.8%	86.5%
	VirPorter-EX (VP1/VP3 + LAH4)	increase (40x)	26	38	36			
R8	VP1/R8-tVP3	increase (6x)	23	63	14	6.6%	4.7%	8.2%
	VP1/VP3-R8	increase (1.5x)	13	84	3			
	VirPorter-EX (VP1/VP3 + R8)	increase (1.6x) (ns)	16	84	no signal			
KH27K	VP1/KH27K-tVP3	increase (2.7x)	60	34	6	2.9%	7.1%	17.0%
	VP1/VP3-KH27K	increase (3.5x)	41	48	11			
	VirPorter-EX (VP1/VP3 + KH27K)	decrease (4.1x)	40	58	2			

Table 3: **The summary of the results** of our investigation of CPP influence on MPyV VPs (*Váňová et al., 2022*).

Since CPPs are known to bind to the cellular membrane (Richard *et al.*, 2003), we assessed how this applies to VP-CPP complexes. In a cell binding assay, VPs coincubated with LAH4 formed large clusters that indeed adhered to cells. In a sample of VPs coincubated with R8, we saw no apparent changes. This result was seen consistently in various settings and was also confirmed by measurement on FACS. The increased adhesion to cells, however, did not lead to increased cellular uptake (*Váňová et al.*, 2020).

5.2.2.2 The detection and quantification of endosomal escape

The presumed mode of action of CPPs was the facilitation of endosome escape. The hemolytic activity of CPPs was tested as an approximation of their endosomolytic properties. Given the correlation between pH-dependent disruption of erythrocytes and gene delivery by synthetic transfer systems (Plank *et al.*, 1994), several studies used hemolytic assay as a pilot test of “endosome escaping properties” (Murthy *et al.*, 1999; Wang *et al.*, 2007; Lee *et al.*, 2010; Evans *et al.*, 2013; Algayer *et al.*, 2019) and membrane destabilization by CPPs (Saar *et al.*, 2005). In our setup, the erythrocyte represents the endolysosomal membrane, and the various pH conditions we used correspond to the respective pH at different points of the endocytic pathway (i.e., cellular membrane in pH 7.4, early endosomal membrane in pH 6.4 and late endosomal membrane in pH 5.5).

Both used histidine-rich peptides (KH27K and LAH4) showed pH-dependent hemolytic properties, with LAH4 having the most pronounced hemolytic activity. Moreover, LAH4 lyses red blood cells even at neutral pH, which is in agreement with previously published findings (Vogt and Bechinger, 1999). This implies that the histidine-rich peptides could work in the intended way and penetrate the endosome upon acidification. R8 did not exhibit any hemolytic properties at any tested pH. In the works of Medina *et al.* (2016), the R8 peptide exhibited some hemolytic activity on human red blood cells, but the measured EC₅₀ (concentration for 50% of the maximal effect) at neutral pH was quite high (370.8 μ M), which is much higher than the concentration we used for most experiments (20 μ M) and even the highest concentration tested by us in the hemolysis assay (40 μ M).

According to Evans *et al.* (2013), hemolysis assay can be used as rapid, high-throughput screen for the cytocompatibility and endosomolytic activity of intracellular drug delivery systems, but should not be the sole used tool for assessing cytosolic delivery agents, as the endosomal membrane content vary between cell types and the red blood cell membrane does not represent these membranes accurately.

While hemolysis assays provide valuable insights into the potential endosomolytic properties of CPPs, they are limited in their ability to accurately represent the complex environment of cellular endosomes. Therefore, to further elucidate how CPPs enhance VirPorter functionality and to directly observe their role in facilitating endosomal escape within cells, we transitioned from indirect hemolysis assays to more direct visualization techniques using fluorescent microscopy.

We intended to visualize the escape event using fluorescent microscopy assays, such as calcein assay, leakage assay, and galectin assay (Chapter 2.1.3.2.2). With fluorescent calcein and dextran, the release of VNPs should be visible under a microscope as the fluorescent pattern shifts from punctuate to dispersed. However, we were not able to reliably determine the difference in the signal after incubation of the cells with LAH4 and other histidine-rich peptides (unpublished results). We fully agree with Teo *et al.* (2021) that “this approach does not provide a direct measurement of cargo escape. Determining what constitutes endosomal escape is therefore highly subjective.” As VirPorters-IN contains fewer CPPs than VirPorters-EX, the differences between them would be even more subtle. Therefore, we abandoned this approach.

We then explored proximity ligation assay (PLA) as a method to quantify the endosomal escape of VPs into the cytosol, with a focus on how CPPs might enhance this process. The goal was to detect VPs in close proximity to cytoplasmic markers, indicating successful release from endosomes. The PLA method was previously employed as a tool for protein quantification and detection of protein-protein interactions. It is regarded as highly sensitive and specific. PLA was seen as a promising alternative to biochemical methods for the detection of stable protein-protein interactions or traditional protein quantification methods, largely because of its ability to detect proteins directly within cells and tissues in situ. These features make PLA an innovative tool for both research and diagnostics (reviewed in Greenwood *et al.*, 2015; Wang *et al.*, 2021). To the best of our knowledge, PLA has never before been used to detect endosomal escape and its quantification.

Unfortunately, we observed inconsistencies in repeated experiments, and PLA did not prove to be a suitable method.

Key issues include:

- Finding an optimal strategy to compare modified and unmodified VPs using different anti-VP1 and anti-AF488 antibody combinations proved difficult.
- Attempts at "self-colocalization" using double immunolabeling for VP1-VP1 to control for virus entry were hard to quantify.

- The fluorescent variant of PLA encountered problems, such as a high background in control samples and autofluorescence of LAH4.
- The brightfield microscopy version of PLA required incubation with xylene, precluding the use of 8-chamber Permanox slides, and glass slides were unsuitable because the cells were damaged during the assay.

Due to the technical issues, we first shifted to using VLPs labeled with AF488 (VLPs-AF488), and we observed that VLPs-AF488 do rapidly colocalize with Hsp90 (i.e., cytosolic marker), while VLPs-AF488 coincubated with CPPs do not. This finding was consistently observed across all repetitions of the experiment and was surprising, as it suggested that maximum efficiency of cytosolic release was achieved without the presence of CPP. This was not evident from previous observations of MPyV and MPyV-derived VLP trafficking that indicated that VPs mainly remain in the vesicular system (Richterová *et al.* 2001; Mannová and Forstová 2003; Liebl *et al.* 2006). Instead, it raised the question of whether the cytosolic marker Hsp90 was appropriately chosen. Hsp90 is commonly used in biochemical analyses to detect the cytosolic fraction, but it can also be present in the nucleus in small amounts (Vermeulen *et al.* 2019). However, our PLA results from control double-labeling tests using two types of anti-Hsp90 antibodies did not indicate nuclear localization (data not shown). Interestingly, in tumor cells, Hsp90 has been reported to be exported extracellularly through unconventional protein secretion (Liao *et al.* 2000). Given that VP1 has been shown to directly interact with Hsp90 (Horníková *et al.* 2017), it is possible that a strong interaction between VPs and Hsp90 occurred in the absence of CPP on the cell surface. The association of CPPs with VPs may have partially inhibited this interaction.

Originally, we planned to test additional cytosolic markers, but due to the overall inconsistency of the results, we ultimately abandoned this approach. From a technical perspective, it remains intriguing why we were unable to establish PLA for our purposes despite previous successful reports of its use in various applications. That said, some reports have also described unsatisfactory PLA performance, which might explain our difficulties.

For example, an article preprint published in 2018 highlights significant limitations, particularly concerning quantification and the potential for false positives regarding protein-protein interactions (Alsemarz *et al.*, 2018). While the authors thoroughly discuss the impossibility of discriminating between random proximity and actual interactions between proteins, this was not something we aimed for. However, other issues brought up in the article are highly relevant to our problems with PLA optimization. According to the authors, the PLA reaction is stochastic by nature, and the signal is influenced by multiple factors, including the antibody distribution in the

sample. The binding of antibodies is also subject to variability based on antibody affinity, fixation sensitivity, antigen availability, and epitope accessibility. This means the PLA signal is not a direct measure of the molecular quantity or position, creating an obstacle for quantitative comparisons. The signal is dependent on both the distance and the relative orientation of the epitopes and their antibodies. If the epitopes are masked, the binding of two antibodies simultaneously is less likely. This results in inconsistent signal generation. The assay is also, according to the authors, difficult to standardize, and the comparison of results between different laboratories is therefore complicated. The authors also bring to attention that commonly used controls (usually omitting one primary antibody) only verify the specificity of secondary antibodies but fail to ensure that the PLA reaction itself is specific. The authors highlight the need to interpret the results with caution and awareness of the limitations (preprint by Alsemarz *et al.*, 2018). Some of the issues align with the difficulties we encountered during the attempt to use PLA to quantify the endosomal escape of VPs, namely the inconsistency of results and high background.

When we troubleshoot the protocol, we first consulted the Duolink® PLA Troubleshooting Guide, e.g., we followed the suggestion to try Brightfield Duolink® PLA when samples with LAH4 exhibited significant autofluorescence, tried an alternative primary antibody to the same target (or switched from VP1 detection to AF488 detection, to be exact) to address the unspecific binding of primary antibodies (Merck: Duolink® PLA Troubleshooting Guide). When we later searched for answers elsewhere, we encountered a discussion about issues with the kit on ResearchGate.net. Here, several users express their frustration with the protocol and problems (such as uneven signal), which, according to some, started around the same time when we first attempted the assay and are blamed by some on the alleged change of manufacturer of the kits (unpublished communication³ on ResearchGate.net 2025).

The problem specific to our approach is the somewhat unreliable binding of antibodies against VP1 in samples of VirPorters-EX (as seen on ELISA) - the antibody recognition can be influenced by disassembly (higher signal, especially when using polyclonal antibodies) and aggregation (lower signal, due to unavailability of epitopes). We encountered troubles with both autofluorescent background and background generated by nonspecific antibody reactions, rendering the results difficult to reliably replicate. However, we were not able to optimize the protocol for brightfield microscopy due to technical limitations (see Chapter 4.2.2.2.2).

We encountered technical issues in virtually every run of the optimization stage, and the assay results were quite inconsistent. Therefore, we considered that a) each combination of CPP and

³ https://www.researchgate.net/post/Problems_with_proximity_ligation_assay_DouLink

cellular marker might potentially need separate optimization (as each CPP could influence the unspecific binding of antibodies against each cellular marker differently), and b) we would need to assess each combination at multiple timepoints using several different samples. That, along with the lack of material, eventually prevented us from pursuing the optimizations further and using the PLA assay for VirPorters-IN. Therefore, we concluded that PLA is not a suitable method for our goals. Currently, the SLEEQ method, which utilizes split NanoLuc luciferase complementation (Teo *et al.*, 2021), is being prepared for quantitatively measuring endosomal escape in the follow-up project. My main conclusion is that although proximity ligation assay could be in theory used for the detection of endosomal escape, the optimization would be extremely time-consuming and costly and with uncertain results and seems unrealistic to do so in our settings (given the amount of samples and controls we would need to properly interpret the assay results). An alternative approach, subcellular fractionation, was adopted.

Galectin-3 assay also required extensive optimization that led to establishing galectin-3-mCherry expressing cell line (prepared by Jana Váňová). Accumulation of various galectins (including galectin-1, -3, -4, -8, and -9) was seen in cells infected by variety of pathogens (e.g. *Shigella*, *Salmonella*, adenovirus and picornaviruses) as well as cells treated by cell-penetrating peptides or otherwise damaged cells (reviewed in Hong *et al.* 2021). Although recent studies lean more towards the use of galectin-8 and -9 as markers of endosomal damage (Kilchrist *et al.* 2019, (Kilchrist *et al.* 2019; Munson *et al.* 2021), in the early studies galectin-3 was used as an indicator of endosomal rupture during adenoviral infection (Maier *et al.* 2012) or vacuolar lysis in *Shigella* infected cells (Dupont *et al.* 2009; Paz *et al.* 2010). Therefore, when we chose to visualize galectin-3 spots, we used adenovirus-infected cells as a control. Nevertheless, the optimization of the assay eventually led us to the use of LLOME which induces galectin-3 (Kimura *et al.* 2017) as well as galectin-8 and -9 accumulation (Jia *et al.* 2018). We performed the galectin assay with free CPPs, VirPorters-EX and VirPorters-IN at 4 hours after pseudoinfection - at this time the enhancement of cytosolic release is most likely to occur. To our surprise, the galectin assay showed no detectable endomembrane damage caused by the VirPorters-IN or VirPorters-EX (Figure 4, S5 and S6 in Attachment 7). This includes VirPorters containing LAH4, which on its own induced detectable damage and exhibited strong hemolytic ability.

This contrasts with our finding that KH27K- and LAH4-VirPorter-EX are found in the cytosolic fraction 4 hours after being added to the cells (Figure 3 in Attachment 7). We know that the increased cytosolic presence seen in fractionation did not stem from enhanced uptake. However, the material found in the cytosolic fractions might have accumulated there over the course of incubation or gotten there at an earlier time.

We also argue that the CPPs might be facilitating the release of VPs into the cytosol through a mechanism other than endosomal disruption - the VirPorters could enter cytosol predominantly from different compartments, such as ER, or the crossing of endosomal membrane does not cause membrane damage sufficient to trigger galectin response. Moreover, other mechanisms of endosomal escape have been suggested, including “back-fusion” of intraluminal vesicles from the multivesicular body (LeCher *et al.* 2017; Griffiths *et al.* 2022).

CPPs could also directly translocate small cargo across the membrane which was observed for the delivery of nucleic acids (reviewed in Gori *et al.*, 2023), but that is highly unlikely for a 50 nm particle, such as a VPs. This would also result in higher uptake and increased amounts of VPs present in the cytosol. However, we saw that CPPs on VirPorters-EX do not increase the overall uptake, and their action is inhibited by sucrose. Thus, endocytosis plays a role in the entry (Váňová *et al.*, 2020). For VirPorters-IN, the effect must take place upon (at least partial) disassembly of the capsid as the CPP is buried within. Therefore, it is unlikely that the effect we observed in the transduction assay was caused by changes in effectiveness and/or mode of entry.

VirPorters-IN action could be better understood by assessing their properties/action in various pH, i.e., deciphering whether the membrane-penetrable abilities manifest upon the acidification of endosomal vesicles. Indeed, we tried the hemolysis assay not only with CPPs but also with VirPorters-IN. It was, however, difficult to achieve a sufficient concentration of CPPs using VirPorters-IN, which only contained 72 molecules of CPP per particle. Also, the viral particles agglutinate the erythrocytes (and this is also pH-dependent), so the interpretation of results is dubious (unpublished results). Another approach would be to compare transduction by VirPorters-IN in various extracellular pH. If VirPorters-IN acts in lower pH as intended, the transduction by VirPorters-IN in lower pH should resemble the transduction by VirPorters-EX in normal pH (though again, the number of CPPs will be much lower in VirPorters-IN than in VirPorters-EX).

In the luciferase assays, we saw that histidine-rich peptides LAH4 and KH27K increase the transduction when associated non-covalently with the VPs exterior, and arginine-rich peptide R8 and KH27K increase transduction when buried inside of the capsid (Figure 2 in Attachment 7). The rationale behind our design was to free the VPs stuck in the non-productive pathway and thus increase the overall gene delivery. Unfortunately, we were unable to confirm the presumed site of action. We did, however, see that LAH4 on the capsid exterior facilitates its way to the nucleus. The biggest increase in cytosolic presence was seen for VP1/KH27K-tVP3, which were indeed the most successful transducers. The action of R8 in VirPorters-IN remains a mystery, as we saw it mostly associated with membranes, suggesting that it acts at a different time or in an unknown manner.

6. CONCLUSION

Polyomaviruses are actively researched in biomedicine and can be engineered for various biomedical applications such as gene therapy, vaccines, imaging, and drug delivery. This dissertation emphasizes two primary areas of polyomavirus-based VP research: their application as antigens in serological assays and their utilization in intracellular delivery.

Part 1: Viral particles as antigens in serodiagnostics

In serological applications, the preparation and modification potential of VPs allows for the detection of antibodies against specific strains.

- We tested the seroprevalence of the Czech population on BKPyV VLPs ELISA and found that both BKPyV-I and BKPyV-IV antigens are needed in order to avoid false negativity.
- We found that the ACA allows subtype-specific antibody testing with BKPyV VLPs.
- We successfully prepared two mutant BKPyV variants and characterized their reactivity with patient sera, confirming the role of DE and EF loops in antibody recognition, which can guide the development of novel antigens for BKPyV serodiagnostics

Part 2: Viral particles as containers for intracellular delivery

In the realm of intracellular delivery, VPs can be modified to overcome barriers such as protein corona formation and endolysosomal sequestration, as well as enhanced delivery into cells by cell-penetrating peptides.

Part 2.A: Characterization of the interaction of polyomaviral VPs with the extracellular environment

- We assessed the formation of protein corona on MPyV-based VLPs and found that some serum proteins (most likely human serum albumin) adhere to the particles more readily in the case of modified VLPsTf.
- We investigated the effect of these adhered proteins on the interaction of VLPs*Tf with their target receptor, TfR, and concluded that the proteins do not interfere with the particle-receptor interaction, but the human serum environment inhibits the interaction due to the presence of competing free transferrin.
- We examined the ability of VLPs*Tf to enter their target cells in the serum environment and confirmed that even in the presence of serum proteins, the particles do enter the cells in the intended clathrin-dependent manner.

Part 2.B: Functionalization of polyomaviral VPs with cell-penetrating peptides and characterization of their interaction with cells

- We investigated the enhancement of VPs by several CPPs, namely LAH4, R8 KH27K, and FUSO, in various settings. We found that:
 - Some CPPs (LAH4, R8 KH27K) do indeed increase the infectivity of MPyV and influence the physical state of the capsid (causing destabilization and/or partial disassembly);
 - histidine-rich peptides LAH4 and KH27K increase aggregation of VPs and their interaction with the cellular membrane but do not increase the uptake into cells;
 - the observed enhancement of transduction can be inhibited by sucrose, which primarily blocks clathrin-mediated endocytosis. Additionally, macropinocytosis might play some role in the internalization of KH27K-modified VPs;
 - although we did not detect endosomal damage by galectin assay, we found that some of the VPs were abundant in the cytosol (especially VirPorters containing KH27K and VP1/VP3-LAH4) and LAH4-VirPorters-EX could even be found in the nucleus just 4 hours after pseudo infection.
- We emphasize the complexity of endosomal escape and the need for rigorous methodologies to study it effectively.
- We established a novel approach for CPP-modified VPs and showed their potential in gene delivery and cargo delivery into the cytosol.

The dissertation makes several contributions to the fields of diagnostics and intracellular delivery using polyomavirus-based virus-like particles (VLPs) and pseudovirions (PsVs). **A major finding is that using a mixture of antigens from different BKPyV subtypes in ELISA tests improves antibody detection.** We also identified **specific amino acids in the DE and EF loops of BKPyV VP1 as crucial for antibody recognition** and that mutations in these loops alter antibody reactivity, suggesting their role in cross-reactivity. Furthermore, we found that **protein corona does not influence receptor-mediated targeting of polyomaviral particles**, which is important for *in vivo* applications. Additionally, we developed the "VirPorter" system for studying CPP-mediated gene delivery and showed that **CPPs, particularly when associated non-covalently with the exterior of VPs, can enhance the cytosolic release of viral particles.**

7. REFERENCES

- Abbing, A. *et al.* (2004) “Efficient intracellular delivery of a protein and a low molecular weight substance via recombinant polyomavirus-like particles,” *The Journal of biological chemistry*, 279(26), pp. 27410–27421. <https://doi.org/10.1074/jbc.M313612200>
- Abend, J.R. *et al.* (2017) “Correlation of BK Virus Neutralizing Serostatus With the Incidence of BK Viremia in Kidney Transplant Recipients,” *Transplantation*, 101(6), pp. 1495–1505. <https://doi.org/10.1097/TP.0000000000001261>
- Abend, J.R. *et al.* (2024) “Nonclinical and clinical characterization of MAU868, a novel human-derived monoclonal neutralizing antibody targeting BK polyomavirus VP1,” *American journal of transplantation*, 24(11), pp. 1994–2006. <https://doi.org/10.1016/j.ajt.2024.07.002>
- Alam, M.S. (2018) “Proximity Ligation Assay (PLA),” *Current protocols in immunology*, 123(1), p. e58. <https://doi.org/10.1002/cpim.58>
- Algayer, B. *et al.* (2019) “Novel pH Selective, Highly Lytic Peptides Based on a Chimeric Influenza Hemagglutinin Peptide/Cell Penetrating Peptide Motif,” *Molecules*, 24(11). <https://doi.org/10.3390/molecules24112079> .
- Aljabali, A.A. *et al.* (2021) “The viral capsid as novel nanomaterials for drug delivery,” *Future science OA*, 7(9), p. FSO744. <https://doi.org/10.2144/fsoa-2021-0031>
- Allen, R.J. and Byrnes, A.P. (2019) “Interaction of adenovirus with antibodies, complement, and coagulation factors,” *FEBS letters*, 593(24), pp. 3449–3460. <https://doi.org/10.1002/1873-3468.13649>
- Alonso, M. *et al.* (2022) “Impact of BK Polyomavirus Plasma Viral Load in Kidney Transplant Outcomes,” *Transplantation proceedings*, 54(9), pp. 2457–2461. <https://doi.org/10.1016/j.transproceed.2022.10.012>
- Alsemarz, A. *et al.* (2018) “Limited significance of the in situ proximity ligation assay,” *bioRxiv*. bioRxiv. <https://doi.org/10.1101/411355>.
- An, K. *et al.* (1999) “Use of the baculovirus system to assemble polyomavirus capsid-like particles with different polyomavirus structural proteins: analysis of the recombinant assembled capsid-like particles,” *The Journal of general virology*, 80 (Pt 4), pp. 1009–1016. <https://doi.org/10.1099/0022-1317-80-4-1009>
- Andrian, T. *et al.* (2021) “Nanoscopy for endosomal escape quantification,” *Nanoscale advances*, 3(1), pp. 10–23. <https://doi.org/10.1039/d0na00454e>
- Anggraeni, M.R. *et al.* (2013) “Sensitivity of immune response quality to influenza helix 190 antigen structure displayed on a modular virus-like particle,” *Vaccine*, 31(40), pp. 4428–4435. <https://doi.org/10.1016/j.vaccine.2013.06.087>
- Antonsson, A. *et al.* (2013) “Longitudinal study of seroprevalence and serostability of the human polyomaviruses JCV and BKV in organ transplant recipients,” *Journal of medical virology*, 85(2), pp. 327–335. <https://doi.org/10.1002/jmv.23472>
- Aposhian, H.V. *et al.* (1975) “Formation of nucleoprotein complexes between polyoma empty capsids and DNA,” *Journal of virology*, 15(3), pp. 645–653. <https://doi.org/10.1128/JVI.15.3.645-653.1975>
- Ashkarran, A.A. *et al.* (2022) “Measurements of heterogeneity in proteomics analysis of the nanoparticle protein corona across core facilities,” *Nature communications*, 13(1), p. 6610.

<https://doi.org/10.1038/s41467-022-34438-8>

Ashkarran, A.A. *et al.* (2024) “Standardizing protein corona characterization in nanomedicine: A multicenter study to enhance reproducibility and data homogeneity,” *Nano letters*, 24(32), pp. 9874–9881. <https://doi.org/10.1021/acs.nanolett.4c02076>

Ashley, C.E. *et al.* (2011) “Cell-specific delivery of diverse cargos by bacteriophage MS2 virus-like particles,” *ACS nano*, 5(7), pp. 5729–5745. <https://doi.org/10.1021/nn201397z>

Azzi, A. *et al.* (2006) “BK virus regulatory region sequence deletions in a case of human polyomavirus associated nephropathy (PVAN) after kidney transplantation,” *Journal of clinical virology*, 35(1), pp. 106–108. <https://doi.org/10.1016/j.jcv.2005.08.003>

Baker, A.T. (2019) *Structural and functional insights into species D adenovirus receptor usage: Implications for oncolytic virotherapy*. Cardiff University. <https://doi.org/10.1126/sciadv.aax3567>

Baksh, F.K. *et al.* (2001) “Molecular genotyping of BK and JC viruses in human polyomavirus-associated interstitial nephritis after renal transplantation,” *American journal of kidney diseases*, 38(2), pp. 354–365. <https://doi.org/10.1053/ajkd.2001.26101>

Banerjee, D. *et al.* (2010) “Multivalent display and receptor-mediated endocytosis of transferrin on virus-like particles,” *Chembiochem: a European journal of chemical biology*, 11(9), pp. 1273–1279. <https://doi.org/10.1002/cbic.201000125>

Bar, H. *et al.* (2008) “Killing cancer cells by targeted drug-carrying phage nanomedicines,” *BMC biotechnology*, 8, p. 37. <https://doi.org/10.1186/1472-6750-8-37>

Barouch, D.H. and Harrison, S.C. (1994) “Interactions among the major and minor coat proteins of polyomavirus,” *Journal of virology*, 68(6), pp. 3982–3989. <https://doi.org/10.1128/JVI.68.6.3982-3989.1994>

Bauer, P.H. *et al.* (1995) “Genetic and structural analysis of a virulence determinant in polyomavirus VP1,” *Journal of virology*, 69(12), pp. 7925–7931. <https://doi.org/10.1128/JVI.69.12.7925-7931.1995>

Bedi, A. *et al.* (1995) “Association of BK virus with failure of prophylaxis against hemorrhagic cystitis following bone marrow transplantation,” *Journal of clinical oncology*, 13(5), pp. 1103–1109. <https://doi.org/10.1200/JCO.1995.13.5.1103>

Berardi, A. *et al.* (2018) “Stability of plant virus-based nanocarriers in gastrointestinal fluids,” *Nanoscale*, 10(4), pp. 1667–1679. <https://doi.org/10.1039/c8nr09067j>

Berardi, A. *et al.* (2019) “Viral nanoparticles can elude protein barriers: exploiting rather than imitating nature,” *Nanoscale*, 11(5), pp. 2306–2316. <https://doi.org/10.1039/c7nr07182e>

Binet, I. *et al.* (1999) “Polyomavirus disease under new immunosuppressive drugs: a cause of renal graft dysfunction and graft loss,” *Transplantation*, 67(6), pp. 918–922. <https://doi.org/10.1097/00007890-199903270-00022>

Blackard, J.T. *et al.* (2020) “BK polyomavirus diversity-Why viral variation matters,” *Reviews in medical virology*, 30(4), p. e2102. <https://doi.org/10.1002/rmv.2102>

Blazquez-Navarro, A. *et al.* (2022) “Lack of predictive capacity of pre-transplant anti-BK virus antibodies for post-transplant reactivation,” *Journal of nephrology* [Preprint]. <https://doi.org/10.1007/s40620-022-01487-7>.

Bodaghi, S. *et al.* (2009) “Antibody responses to recombinant polyomavirus BK large T and VP1

- proteins in young kidney transplant patients,” *Journal of clinical microbiology*, 47(8), pp. 2577–2585. <https://doi.org/10.1128/JCM.00030-09>
- Bohl, D.L. *et al.* (2008) “BK virus antibody titers and intensity of infections after renal transplantation,” *Journal of clinical virology*, 43(2), pp. 184–189. <https://doi.org/10.1016/j.jcv.2008.06.009>
- Boura, E. *et al.* (2005) “Polyomavirus EGFP-pseudocapsids: analysis of model particles for introduction of proteins and peptides into mammalian cells,” *FEBS letters*, 579(29), pp. 6549–6558. <https://doi.org/10.1016/j.febslet.2005.10.062>
- Brandenburg, B. *et al.* (2005) “A novel system for efficient gene transfer into primary human hepatocytes via cell-permeable hepatitis B virus-like particle,” *Hepatology (Baltimore, Md.)*, 42(6), pp. 1300–1309. <https://doi.org/10.1002/hep.20950>
- Brasch, M. *et al.* (2011) “Encapsulation of phthalocyanine supramolecular stacks into virus-like particles,” *Journal of the American Chemical Society*, 133(18), pp. 6878–6881. <https://doi.org/10.1021/ja110752u>
- Brochot, E. (2023) “Commentary on: Lack of predictive capacity of pre-transplant anti-BK virus antibodies for post-transplant reactivation,” *Journal of nephrology* [Preprint]. <https://doi.org/10.1007/s40620-023-01579-y>.
- Buck, C.B. *et al.* (2004) “Efficient intracellular assembly of papillomaviral vectors,” *Journal of virology*, 78(2), pp. 751–757. <https://doi.org/10.1128/jvi.78.2.751-757.2004>
- Caisová, H. (2023) *Analysis of serological cross-reactivity between antibodies against BK polyomavirus variants*. Edited by L. Horníková. Charles University.
- Cantin, G.T. *et al.* (2011) “Comparison of Methods for Chemical Conjugation of an Influenza Peptide to Wild-Type and Cysteine-Mutant Virus-Like Particles Expressed in *Pseudomonas fluorescens*,” *International journal of peptide research and therapeutics*, 17(3), pp. 217–224. <https://doi.org/10.1007/s10989-011-9259-7>
- Capehart, S.L. *et al.* (2013) “Controlled integration of gold nanoparticles and organic fluorophores using synthetically modified MS2 viral capsids,” *Journal of the American Chemical Society*, 135(8), pp. 3011–3016. <https://doi.org/10.1021/ja3078472>
- Carter, J.J. *et al.* (2003) “Lack of serologic evidence for prevalent simian virus 40 infection in humans,” *Journal of the National Cancer Institute*, 95(20), pp. 1522–1530. <https://doi.org/10.1093/jnci/djg074>
- Caruso, M. *et al.* (2003a) “Alpha4beta1 integrin acts as a cell receptor for murine polyomavirus at the postattachment level,” *Journal of virology*, 77(7), pp. 3913–3921. <https://doi.org/10.1128/jvi.77.7.3913-3921.2003>
- Caruso, M. *et al.* (2003b) “Role of sialic acid-containing molecules and the alpha4beta1 integrin receptor in the early steps of polyomavirus infection,” *The Journal of general virology*, 84(Pt 11), pp. 2927–2936. <https://doi.org/10.1099/vir.0.19369-0>
- Chang, K.-S. *et al.* (2007) “Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture,” *Journal of virology*, 81(24), pp. 13783–13793. <https://doi.org/10.1128/JVI.01091-07>
- Chao, C.-N. *et al.* (2015) “Inhibition of human diffuse large B-cell lymphoma growth by JC polyomavirus-like particles delivering a suicide gene,” *Journal of translational medicine*, 13(1), pp. 1–9. <https://doi.org/10.1186/s12967-015-0389-0>

- Chao, C.-N. *et al.* (2016) “Gene Therapy for Human Lung Adenocarcinoma Using a Suicide Gene Driven by a Lung-Specific Promoter Delivered by JC Virus-Like Particles,” *PloS one*, 11(6), p. e0157865. <https://doi.org/10.1371/journal.pone.0157865>
- Chao, C.-N. *et al.* (2018) “Gene therapy for human glioblastoma using neurotropic JC virus-like particles as a gene delivery vector,” *Scientific reports*, 8(1), p. 2213. <https://doi.org/10.1038/s41598-018-19825-w>
- Chatterji, A. *et al.* (2004) “New addresses on an addressable virus nanoblock; uniquely reactive Lys residues on cowpea mosaic virus,” *Chemistry & biology*, 11(6), pp. 855–863. <https://doi.org/10.1016/j.chembiol.2004.04.011>
- Chen, C.H. *et al.* (2001) “A regulatory region rearranged BK virus is associated with tubulointerstitial nephritis in a rejected renal allograft,” *Journal of medical virology*, 64(1), pp. 82–88. <https://doi.org/10.1002/jmv.102>
- Chen, H.-Z. *et al.* (2011) “Membrane penetrating peptides greatly enhance baculovirus transduction efficiency into mammalian cells,” *Biochemical and biophysical research communications*, 405(2), pp. 297–302. <https://doi.org/10.1016/j.bbrc.2011.01.032>
- Chen, L.-S. *et al.* (2010) “Efficient gene transfer using the human JC virus-like particle that inhibits human colon adenocarcinoma growth in a nude mouse model,” *Gene therapy*, 17(8), pp. 1033–1041. <https://doi.org/10.1038/gt.2010.50>
- Chen, Z. *et al.* (2022) “Harnessing protein corona for biomimetic nanomedicine design,” *Biomimetics (Basel, Switzerland)*, 7(3), p. 126. <https://doi.org/10.3390/biomimetics7030126>
- Chesters, P.M. *et al.* (1983) “Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues,” *The Journal of infectious diseases*, 147(4), pp. 676–684. <https://doi.org/10.1093/infdis/147.4.676>
- Chou, M.-I. *et al.* (2010) “In vitro and in vivo targeted delivery of IL-10 interfering RNA by JC virus-like particles,” *Journal of biomedical science*, 17(1), p. 51. <https://doi.org/https://doi.org/10.1186/1423-0127-17-51>
- Chuan, Y.P. *et al.* (2013) “Effects of pre-existing anti-carrier immunity and antigenic element multiplicity on efficacy of a modular virus-like particle vaccine,” *Biotechnology and bioengineering*, 110(9), pp. 2343–2351. <https://doi.org/10.1002/bit.24907>
- Clark, B. *et al.* (2001) “Immunity against both polyomavirus VP1 and a transgene product induced following intranasal delivery of VP1 pseudocapsid–DNA complexes,” *Journal of General Virology*, pp. 2791–2797. <https://doi.org/10.1099/0022-1317-82-11-2791>
- Dakroub, F. *et al.* (2021) “The impact of pre-graft serology on the risk of BKPyV infection post-renal transplantation,” *Nephrology, dialysis, transplantation*. <https://doi.org/10.1093/ndt/gfab279>
- Dalianis, T. and Hirsch, H.H. (2013) “Human polyomaviruses in disease and cancer,” *Virology*, 437(2), pp. 63–72. <https://doi.org/10.1016/j.virol.2012.12.015>
- Dashti *et al.* (2018) “Programmable In Vitro Coencapsidation of Guest Proteins for Intracellular Delivery by Virus-like Particles,” *ACS nano*, 12(5), pp. 4615–4623. <https://doi.org/10.1021/acsnano.8b01059>
- Dashti, N.H. and Sainsbury, F. (2020) “Virus-Derived Nanoparticles,” *Methods in molecular biology*, 2073, pp. 149–162. https://doi.org/10.1007/978-1-4939-9869-2_9

- Dautry-Varsat, A. (1986) "Receptor-mediated endocytosis: the intracellular journey of transferrin and its receptor," *Biochimie*, 68(3), pp. 375–381. [https://doi.org/10.1016/s0300-9084\(86\)80004-9](https://doi.org/10.1016/s0300-9084(86)80004-9)
- Dawson, K.A. and Yan, Y. (2021) "Current understanding of biological identity at the nanoscale and future prospects," *Nature nanotechnology*, 16(3), pp. 229–242. <https://doi.org/10.1038/s41565-021-00860-0>
- Degors, I.M.S. *et al.* (2019) "Carriers Break Barriers in Drug Delivery: Endocytosis and Endosomal Escape of Gene Delivery Vectors," *Accounts of chemical research*, 52(7), pp. 1750–1760. <https://doi.org/10.1021/acs.accounts.9b00177>
- Deprey, K. *et al.* (2019) "Trapped! A Critical Evaluation of Methods for Measuring Total Cellular Uptake versus Cytosolic Localization," *Bioconjugate chemistry*, 30(4), pp. 1006–1027. <https://doi.org/10.1021/acs.bioconjchem.9b00112>
- Deshpande, P. *et al.*, 2018. Transferrin and octaarginine modified dual-functional liposomes with improved cancer cell targeting and enhanced intracellular delivery for the treatment of ovarian cancer. *Drug delivery*, 25(1), pp.517–532. <https://doi.org/10.1080/10717544.2018.1435747>
- Destito, G. *et al.* (2007) "Folic acid-mediated targeting of cowpea mosaic virus particles to tumor cells," *Chemistry & biology*, 14(10), pp. 1152–1162. <https://doi.org/10.1016/j.chembiol.2007.08.015>
- Dominguez-Medina, S. *et al.* (2013) "Adsorption of a protein monolayer via hydrophobic interactions prevents nanoparticle aggregation under harsh environmental conditions," *ACS sustainable chemistry & engineering*, 1(7), pp. 833–842. <https://doi.org/10.1021/sc400042h>
- Doronin, K. *et al.* (2012) "Coagulation factor X activates innate immunity to human species C adenovirus," *Science*, 338(6108), pp. 795–798. <https://doi.org/10.1126/science.1226625>
- Dube, G.K. *et al.* (2023) "BK DNAemia and native kidney polyomavirus nephropathy following lung transplantation," *American journal of transplantation*, 23(2), pp. 284–290. <https://doi.org/10.1016/j.ajt.2022.11.007>
- Dugan, A.S. *et al.* (2007) "Identification of amino acid residues in BK virus VP1 that are critical for viability and growth," *Journal of virology*, 81(21), pp. 11798–11808. <https://doi.org/10.1128/JVI.01316-07>
- Dupont, N. *et al.* (2009) "Shigella phagocytic vacuolar membrane remnants participate in the cellular response to pathogen invasion and are regulated by autophagy," *Cell host & microbe*, 6(2), pp. 137–149. <https://doi.org/10.1016/j.chom.2009.07.005>
- Durán-Meza, A.L. *et al.* (2020) "Encapsulation of Different Plasmonic Gold Nanoparticles by the CCMV CP," *Molecules*, 25(11). <https://doi.org/10.3390/molecules25112628>.
- Durymanov, M. and Reineke, J. (2018) "Non-viral Delivery of Nucleic Acids: Insight Into Mechanisms of Overcoming Intracellular Barriers," *Frontiers in pharmacology*, 9, p. 971. <https://doi.org/10.3389/fphar.2018.00971>
- Duty, J.A. *et al.* (2024) "Heterologous immunization strategy developed broadly reactive human monoclonal antibodies against the BK virus," *Microbiology*. bioRxiv. <https://www.biorxiv.org/content/10.1101/2024.12.18.629307v1>.
- Ebi.ac.uk. (2023). Clustal Omega. [online] <https://www.ebi.ac.uk/jdispatcher/msa/clustalo> [Accessed 2025].

- Egli, A. *et al.* (2009) “Inhibition of polyomavirus BK-specific T-Cell responses by immunosuppressive drugs,” *Transplantation*, 88(10), pp. 1161–1168. <https://doi.org/10.1097/TP.0b013e3181bca422>
- Eiden, M. *et al.* (2021) “Vaccination with Prion Peptide-Displaying Polyomavirus-Like Particles Prolongs Incubation Time in Scrapie-Infected Mice,” *Viruses*, 13(5). <https://doi.org/10.3390/v13050811>.
- Enomoto, T. *et al.* (2013) “Viral protein-coating of magnetic nanoparticles using simian virus 40 VP1,” *Journal of biotechnology*, 167(1), pp. 8–15. <https://doi.org/10.1016/j.jbiotec.2013.06.005>
- Epitopia Server by Rubinstein, N.D., Mayrose, I., Martz, E. and Pupko, T. The Epitopia Server. <http://epitopia.tau.ac.il> [Accessed 2018] No longer available.
- Eriksson, M. *et al.* (2011) “Murine polyomavirus virus-like particles carrying full-length human PSA protect BALB/c mice from outgrowth of a PSA expressing tumor,” *PloS one*, 6(8), p. e23828. <https://doi.org/10.1371/journal.pone.0023828>
- Eto, Y. *et al.* (2009) “Transduction of adenovirus vectors modified with cell-penetrating peptides,” *Peptides*, 30(8), pp. 1548–1552. <https://doi.org/10.1016/j.peptides.2009.05.017>
- Evans, B.C. *et al.* (2013) “Ex vivo red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs,” *Journal of visualized experiments: JoVE*, (73), p. e50166. <https://doi.org/10.3791/50166>
- Ezzat, K. *et al.* (2019) “The viral protein corona directs viral pathogenesis and amyloid aggregation,” *Nature communications*, 10(1), p. 2331. <https://doi.org/10.1038/s41467-019-10192-2>
- Fang, C.-Y. *et al.* (2012) “Analysis of the size of DNA packaged by the human JC virus-like particle,” *Journal of virological methods*, 182(1–2), pp. 87–92. <https://doi.org/10.1016/j.jviromet.2012.03.017>
- Fang, C.-Y. *et al.* (2015) “Inhibition of human bladder cancer growth by a suicide gene delivered by JC polyomavirus virus-like particles in a mouse model,” *The Journal of urology*, 193(6), pp. 2100–2106. <https://doi.org/10.1016/j.juro.2015.01.084>
- Fenard, D. *et al.* (2013) “Infectivity enhancement of different HIV-1-based lentiviral pseudotypes in presence of the cationic amphipathic peptide LAH4-L1,” *Journal of Virological Methods*, pp. 375–378. <https://doi.org/10.1016/j.jviromet.2013.02.005>.
- Ferrer-Miralles, N. *et al.*, 2011. Biological activities of histidine-rich peptides; merging biotechnology and nanomedicine. *Microbial cell factories*, 10(1), p.101. <https://doi.org/10.1186/1475-2859-10-101>
- Fields, B.N. *et al.* (2013) *Fields Virology*. Lippincott Williams & Wilkins, Philadelphia. ISBN: 9781451105636
- Finch, J.T. (1974) “The surface structure of polyoma virus,” *The Journal of general virology*, 24(2), pp. 359–364. <https://doi.org/10.1099/0022-1317-24-2-359>
- FitzGerald, L.I. *et al.* (2020) “A molecular sensor to quantify the localization of proteins, DNA and nanoparticles in cells,” *Nature communications*, 11(1), p. 4482. <https://doi.org/10.1038/s41467-020-18082-8>
- Fleury, M.J.J. *et al.* (2015) “Identification of the neutralizing epitopes of Merkel cell polyomavirus major capsid protein within the BC and EF surface loops,” *PloS one*, 10(3), p.

e0121751. <https://doi.org/10.1371/journal.pone.0121751>

Forstová, J. *et al.* (1995) “Polyoma virus pseudocapsids as efficient carriers of heterologous DNA into mammalian cells,” *Human gene therapy*, 6(3), pp. 297–306. <https://doi.org/10.1089/hum.1995.6.3-297>

Fraiberk, M. *et al.* (2017) “Exploitation of stable nanostructures based on the mouse polyomavirus for development of a recombinant vaccine against porcine circovirus 2,” *PloS one*, 12(9), p. e0184870. <https://doi.org/10.1371/journal.pone.0184870>

Freund, R. *et al.* (1991) “A single-amino-acid substitution in polyomavirus VP1 correlates with plaque size and hemagglutination behavior,” *Journal of virology*, 65(1), pp. 350–355. <https://doi.org/10.1128/JVI.65.1.350-355.1991>

Furmaga, J. *et al.* (2021) “BK Polyomavirus-Biology, Genomic Variation and Diagnosis,” *Viruses*, 13(8). <https://doi.org/10.3390/v13081502>.

Gan, B.K. *et al.* (2018) “Targeted Delivery of Cell Penetrating Peptide Virus-like Nanoparticles to Skin Cancer Cells,” *Scientific reports*, 8(1), p. 8499. <https://doi.org/10.1038/s41598-018-26749-y>

Gardner, S. *et al.* (1971) “NEW HUMAN PAPOVAVIRUS (B.K.) ISOLATED FROM URINE AFTER RENAL TRANSPLANTATION,” *The Lancet*, 297(7712), pp. 1253–1257. [https://doi.org/10.1016/S0140-6736\(71\)91776-4](https://doi.org/10.1016/S0140-6736(71)91776-4)

Gedvilaite, A. *et al.* (2000) “Formation of immunogenic virus-like particles by inserting epitopes into surface-exposed regions of hamster polyomavirus major capsid protein,” *Virology*, 273(1), pp. 21–35. <https://doi.org/10.1006/viro.2000.0392>

Gedvilaite, A. *et al.* (2015) “Evaluation of Trichodysplasia Spinulosa-Associated Polyomavirus Capsid Protein as a New Carrier for Construction of Chimeric Virus-Like Particles Harboring Foreign Epitopes,” *Viruses*, 7(8), pp. 4204–4229. <https://doi.org/10.3390/v7082818>

Gee, G.V. *et al.* (2004) “Modeling a sialic acid binding pocket in the external loops of JC virus VP1,” *The Journal of biological chemistry*, 279(47), pp. 49172–49176. <https://doi.org/10.1074/jbc.M409326200>

Geiger, R. *et al.* (2011) “BAP31 and BiP are essential for dislocation of SV40 from the endoplasmic reticulum to the cytosol,” *Nature cell biology*, 13(11), pp. 1305–1314. <https://doi.org/10.1038/ncb2339>

Geiger, R. *et al.* (2013) “Investigating Endocytic Pathways to the Endoplasmic Reticulum and to the Cytosol Using SNAP-Trap,” *Traffic*, pp. 36–46. <https://doi.org/10.1111/tra.12018>.

Gilbert, J. *et al.* (2005) “Ganglioside GD1a restores infectibility to mouse cells lacking functional receptors for polyomavirus,” *Journal of virology*, 79(1), pp. 615–618. <https://doi.org/10.1128/JVI.79.1.615-618.2005>

Gillitzer, E. *et al.* (2002) “Chemical modification of a viral cage for multivalent presentation,” *Chemical communications*, (20), pp. 2390–2391. <https://doi.org/10.1039/b207853h>

Gillock, E.T. *et al.* (1997) “Polyomavirus major capsid protein VP1 is capable of packaging cellular DNA when expressed in the baculovirus system,” *Journal of virology*, 71(4), pp. 2857–2865. <https://doi.org/10.1128/JVI.71.4.2857-2865.1997>

Gleiter, S. and Lilie, H. (2001) “Coupling of antibodies via protein Z on modified polyoma virus-like particles,” *Protein science*, 10(2), pp. 434–444. <https://doi.org/10.1110/ps.31101>

- Gleiter, S. *et al.* (1999) “Changing the surface of a virus shell fusion of an enzyme to polyoma VP1,” *Protein science*, 8(12), pp. 2562–2569. <https://doi.org/10.1110/ps.8.12.2562>
- Goldmann, C. *et al.* (2000) “Packaging of small molecules into VP1-virus-like particles of the human polyomavirus JC virus,” *Journal of virological methods*, 90(1), pp. 85–90. [https://doi.org/10.1016/s0166-0934\(00\)00226-3](https://doi.org/10.1016/s0166-0934(00)00226-3)
- Gori, A. *et al.* (2023) “Cell penetrating peptides: Classification, mechanisms, methods of study, and applications,” *ChemMedChem*, 18(17), p. e202300236. <https://doi.org/10.1002/cmdc.202300236>
- Gosert, R. *et al.* (2008) “Polyomavirus BK with rearranged noncoding control region emerge in vivo in renal transplant patients and increase viral replication and cytopathology,” *The Journal of experimental medicine*, 205(4), pp. 841–852. <https://doi.org/10.1084/jem.20072097>
- Gras, J. *et al.* (2023) “BK virus genotypes and humoral response in kidney transplant recipients with BKV associated nephropathy,” *Transplant infectious disease*, p. e14012. <https://doi.org/10.1111/tid.14012>
- Greenwood, C. *et al.* (2015) “Proximity assays for sensitive quantification of proteins,” *Biomolecular detection and quantification*, 4, pp. 10–16. <https://doi.org/10.1016/j.bdq.2015.04.002>
- Griffiths, G. *et al.* (2022) “Nanoparticle entry into cells; the cell biology weak link,” *Advanced drug delivery reviews*, 188(114403), p. 114403. <https://doi.org/10.1016/j.addr.2022.114403>
- Grove, J. and Marsh, M. (2011) “The cell biology of receptor-mediated virus entry,” *The Journal of cell biology*, 195(7), pp. 1071–1082. <https://doi.org/10.1083/jcb.201108131>
- Merck. Duolink® PLA Troubleshooting Guide. *Merck*, [online] 1(1). <https://www.sigmaaldrich.com/CZ/cs/technical-documents/technical-article/protein-biology/protein-and-nucleic-acid-interactions/duolink-troubleshooting-guide> (Accessed: February 15, 2025).
- Hassani-Mehraban, A. *et al.* (2015) “Feasibility of Cowpea chlorotic mottle virus-like particles as scaffold for epitope presentations,” *BMC biotechnology*, 15, p. 80. <https://doi.org/10.1186/s12896-015-0180-6>
- Hausig-Punke, F. *et al.* (2022) “Tracking the Endosomal Escape: A Closer Look at Calcein and Related Reporters,” *Macromolecular bioscience*, 22(10), p. e2200167. <https://doi.org/10.1002/mabi.202200167>
- Heidari, S. *et al.* (2000) “Persistence and tissue distribution of DNA in normal and immunodeficient mice inoculated with polyomavirus VP1 pseudocapsid complexes or polyomavirus,” *Journal of virology*, 74(24), pp. 11963–11965. <https://doi.org/10.1128/jvi.74.24.11963-11965.2000>
- Hejtmánková, A. *et al.* (2019) “Prevalence of antibodies against BKPyV subtype I and IV in kidney transplant recipients and in the general Czech population,” *Journal of medical virology*, 91(5), pp. 856–864. <https://doi.org/10.1002/jmv.25388>
- Hejtmánková, A. *et al.* (2021) “Cell-penetrating peptides in the intracellular delivery of viral nanoparticles,” *Vitamins and hormones*, 117, pp. 47–76. <https://doi.org/10.1016/bs.vh.2021.06.010>
- Henriksen, S. *et al.* (2015) “Human BK Polyomavirus Plasmid pBKV (34-2) (Dunlop) contains mutations not found in the originally published sequences,” *Genome announcements*, 3(2).

<https://doi.org/10.1128/genomeA.00046-15>. <https://doi.org/10.1128/genomeA.00046-15>

Heritage, J. *et al.* (1981) “The persistence of papovavirus BK DNA sequences in normal human renal tissue,” *Journal of medical virology*, 8(2), pp. 143–150.
<https://doi.org/10.1002/jmv.1890080208>

Hirsch, H.H. (2002) “Polyomavirus BK nephropathy: a (re-)emerging complication in renal transplantation,” *American journal of transplantation*, 2(1), pp. 25–30.
<https://doi.org/10.1034/j.1600-6143.2002.020106.x>

Hirsch, H.H. *et al.* (2005) “Polyomavirus-associated nephropathy in renal transplantation: interdisciplinary analyses and recommendations,” *Transplantation*, 79(10), pp. 1277–1286.
<https://doi.org/https://doi.org/10.1097/01.tp.0000156165.83160.09>

Hirsch, H.H. *et al.* (2013) “Polyomavirus BK replication in de novo kidney transplant patients receiving tacrolimus or cyclosporine: a prospective, randomized, multicenter study,” *American journal of transplantation*, 13(1), pp. 136–145. <https://doi.org/10.1111/j.1600-6143.2012.04320.x>

Hirsch, H.H. *et al.* (2013) “BK polyomavirus in solid organ transplantation,” *American journal of transplantation*, 13 Suppl 4, pp. 179–188. <https://doi.org/10.1111/ajt.12110>

Hisdome, Y. *et al.* (2020) “Association of Pretransplant BK Polyomavirus Antibody Status with BK Polyomavirus Infection After Kidney Transplantation: A Prospective Cohort Pilot Study of 47 Transplant Recipients,” *Transplantation proceedings*, 52(6), pp. 1762–1768.
<https://doi.org/10.1016/j.transproceed.2020.01.164>

Hod-Dvorai, R. *et al.* (2023) “Development of de novo donor-specific antibodies in renal transplant recipients with BK viremia managed with immunosuppression reduction,” *Transplant infectious disease*, 25(1), p. e13993. <https://doi.org/10.1111/tid.13993>

Hoffmann, D.B. *et al.* (2016) “In Vivo siRNA Delivery Using JC Virus-like Particles Decreases the Expression of RANKL in Rats,” *Molecular therapy. Nucleic acids*, 5(3), p. e298.
<https://doi.org/10.1038/mtna.2016.15>

Hong, M.-H. *et al.* (2021) “Intracellular galectins sense cytosolically exposed glycans as danger and mediate cellular responses,” *Journal of biomedical science*, 28(1), p. 16.
<https://doi.org/10.1186/s12929-021-00713-x>

Horníková, L. *et al.* (2015) “Mouse Polyomavirus: Propagation, Purification, Quantification, and Storage,” *Current protocols in microbiology*, 38, p. 14F.1.1-26.
<https://doi.org/10.1002/9780471729259.mc14f01s38>

Horníková, L. *et al.* (2017) “VP1, the major capsid protein of the mouse polyomavirus, binds microtubules, promotes their acetylation and blocks the host cell cycle,” *The FEBS journal*, 284(2), pp. 301–323. <https://doi.org/10.1111/febs.13977>

Hovlid, M.L. *et al.* (2014) “Encapsidated atom-transfer radical polymerization in Q β virus-like nanoparticles,” *ACS nano*, 8(8), pp. 8003–8014. <https://doi.org/10.1021/nn502043d>

Hrusková, V. *et al.* (2009) “Bcr-Abl fusion sequences do not induce immune responses in mice when administered in mouse polyomavirus based virus-like particles,” *International journal of oncology*, 35(6), pp. 1247–1256. <https://doi.org/10.3892/ijo.00000441>

Huang, H. *et al.* (2023) “Virus-protein corona replacement strategy to improve the antitumor efficacy of intravenously injected oncolytic Adenovirus,” *ACS nano*, 17(15), pp. 14461–14474.
<https://doi.org/10.1021/acsnano.3c00847>

- Huang, X. *et al.* (2007) “Self-assembled virus-like particles with magnetic cores,” *Nano letters*, 7(8), pp. 2407–2416. <https://doi.org/10.1021/nl071083l>
- Huérffano, S. *et al.* (2017) “Hydrophobic domains of mouse polyomavirus minor capsid proteins promote membrane association and virus exit from the ER,” *The FEBS journal*, 284(6), pp. 883–902. <https://doi.org/10.1111/febs.14033>
- Hurdiss, D.L. *et al.* (2018) “The Structure of an Infectious Human Polyomavirus and Its Interactions with Cellular Receptors,” *Structure*, 26(6), pp. 839–847.e3. <https://doi.org/10.1016/j.str.2018.03.019>
- Hustedová, A. (2020) *Targeting of Viral Nanoparticles to CD44 via Hyaluronic Acid*. Edited by H. Španielová. Charles University.
- Inoue, T. *et al.* (2008) “Engineering of SV40-based nano-capsules for delivery of heterologous proteins as fusions with the minor capsid proteins VP2/3,” *Journal of biotechnology*, 134(1–2), pp. 181–192. <https://doi.org/10.1016/j.jbiotec.2007.12.006>
- Iwamoto, S. *et al.* (2002) “BK virus-associated fatal renal failure following late-onset hemorrhagic cystitis in an unrelated bone marrow transplantation,” *Pediatric hematology and oncology*, 19(4), pp. 255–261. <http://dx.doi.org/10.1080/08880010252899424>.
- Jia, J. *et al.* (2018) “Galectins control mTOR in response to endomembrane damage,” *Molecular cell*, 70(1), pp. 120–135.e8. <http://dx.doi.org/10.1016/j.molcel.2018.03.009>.
- Jin, L., *et al.* (1993a) “Genomic typing of BK virus in clinical specimens by direct sequencing of polymerase chain reaction products,” *Journal of medical virology*, 41(1), pp. 11–17. <http://dx.doi.org/10.1002/jmv.1890410104>.
- Jin, L., *et al.* (1993b) “BK virus antigenic variants: sequence analysis within the capsid VP1 epitope,” *Journal of medical virology*, 39(1), pp. 50–56. <http://dx.doi.org/10.1002/jmv.1890390110>.
- Johnston, A.P.R. (2017) “Life Under the Microscope: Quantifying Live Cell Interactions to Improve Nanoscale Drug Delivery,” *ACS sensors*, 2(1), pp. 4–9. <http://dx.doi.org/10.1021/acssensors.6b00725>.
- Kamminga, S. *et al.* (2018a) “Seroprevalence of fourteen human polyomaviruses determined in blood donors,” *PloS one*, 13(10), p. e0206273. <http://dx.doi.org/10.1371/journal.pone.0206273>.
- Kamminga, S. *et al.* (2018b) “Development and Evaluation of a Broad Bead-Based Multiplex Immunoassay To Measure IgG Seroreactivity against Human Polyomaviruses,” *Journal of clinical microbiology*, 56(4). <https://doi.org/10.1128/JCM.01566-17>. <http://dx.doi.org/10.1128/JCM.01566-17>.
- Kamminga, S. *et al.* (2019) “Prevalence of DNA of fourteen human polyomaviruses determined in blood donors,” *Transfusion*, 59(12), pp. 3689–3697. <http://dx.doi.org/10.1111/trf.15557>.
- Kant, S. *et al.* (2022) “BK Virus Nephropathy in Kidney Transplantation: A State-of-the-Art Review,” *Viruses*, 14(8). <https://doi.org/10.3390/v14081616>. <http://dx.doi.org/10.3390/v14081616>.
- Kardas, P. *et al.* (2015) “Optimizing JC and BK polyomavirus IgG testing for seroepidemiology and patient counseling,” *Journal of clinical virology*, 71, pp. 28–33. <http://dx.doi.org/10.1016/j.jcv.2015.07.305>.
- Kasiske, B.L. *et al.* (2010) “KDIGO clinical practice guideline for the care of kidney transplant

recipients: a summary,” *Kidney international*, 77(4), pp. 299–311.
<http://dx.doi.org/10.1038/ki.2009.377>.

Kawano, M. *et al.* (2013) “SV40 virus-like particles as an effective delivery system and its application to a vaccine carrier,” *Expert review of vaccines*, 12(2), pp. 199–210.
<http://dx.doi.org/10.1586/erv.12.149>.

Kawano, M. *et al.* (2014) “Chimeric SV40 virus-like particles induce specific cytotoxicity and protective immunity against influenza A virus without the need of adjuvants,” *Virology*, 448, pp. 159–167. <http://dx.doi.org/10.1016/j.virol.2013.10.010>.

Keppeler, A. *et al.* (2003) “A general method for the covalent labeling of fusion proteins with small molecules in vivo,” *Nature biotechnology*, 21(1), pp. 86–89.
<https://doi.org/10.1038/nbt765>

Khalil, I.A. *et al.*, 2011. Octaarginine- and pH sensitive fusogenic peptide-modified nanoparticles for liver gene delivery. *Journal of controlled release*, 156(3), pp.374–380.
<https://doi.org/10.1016/j.jconrel.2011.08.012>.

Khan, S. *et al.* (2022) “Artificial engineering of the protein corona at bio-nano interfaces for improved cancer-targeted nanotherapy,” *Journal of controlled release*, 348, pp. 127–147.
<http://dx.doi.org/10.1016/j.jconrel.2022.05.055>

Kida, S. *et al.* (2010) “Evaluation of synthetic cell-penetrating peptides, Pro-rich peptide and octaarginine derivatives, as adenovirus vector carrier,” *Protein and peptide letters*, 17(2), pp. 164–167. <http://dx.doi.org/10.2174/092986610790225978>.

Kilchrist, K.V. *et al.* (2019) “Gal8 visualization of endosome disruption predicts carrier-mediated biologic drug intracellular bioavailability,” *ACS nano*, 13(2), pp. 1136–1152.
<https://doi.org/10.1021/acsnano.8b05482>

Kim, J.-S. *et al.* (2015) “Quantitative assessment of cellular uptake and cytosolic access of antibody in living cells by an enhanced split GFP complementation assay,” *Biochemical and biophysical research communications*, 467(4), pp. 771–777.
<http://dx.doi.org/10.1016/j.bbrc.2015.10.066>.

Kim, P.-H. *et al.* (2010) “The effect of surface modification of adenovirus with an arginine-grafted bioreducible polymer on transduction efficiency and immunogenicity in cancer gene therapy,” *Biomaterials*, 31(7), pp. 1865–1874.
<http://dx.doi.org/10.1016/j.biomaterials.2009.11.043>.

Kimchi-Sarfaty, C. *et al.* (2002) “In Vitro-Packaged SV40 Pseudovirions as Highly Efficient Vectors for Gene Transfer,” *Human Gene Therapy*, pp. 299–310.
<https://doi.org/10.1089/10430340252769815>.

Kimura, T. *et al.* (2017) “Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy,” *The EMBO journal*, 36(1), pp. 42–60.
<http://dx.doi.org/10.15252/embj.201695081>.

Kitai, Y. *et al.* (2011) “Cell selective targeting of a simian virus 40 virus-like particle conjugated to epidermal growth factor,” *Journal of biotechnology*, 155(2), pp. 251–256.
<http://dx.doi.org/10.1016/j.jbiotec.2011.06.030>.

Klug, A. and Finch, J.T. (1965) “STRUCTURE OF VIRUSES OF THE PAPILLOMA-POLYOMA TYPE. I. HUMAN WART VIRUS,” *Journal of molecular biology*, 11, pp. 403–423. [http://dx.doi.org/10.1016/s0022-2836\(65\)80066-3](http://dx.doi.org/10.1016/s0022-2836(65)80066-3).

- Knowles, W.A. *et al.* (1989) “Serological typing scheme for BK-like isolates of human polyomavirus,” *Journal of medical virology*, 28(2), pp. 118–123.
<https://doi.org/10.1002/jmv.1890280212>
- Koshkaryev, A. *et al.* 2013. Bleomycin in octaarginine-modified fusogenic liposomes results in improved tumor growth inhibition. *Cancer letters*, 334(2), pp.293–301.
<https://doi.org/10.1016/j.canlet.2012.06.008>.
- Krautkrämer, E. *et al.* (2009) “Mutations in the BC-loop of the BKV VP1 region do not influence viral load in renal transplant patients,” *Journal of medical virology*, 81(1), pp. 75–81.
<http://dx.doi.org/10.1002/jmv.21359>.
- Krauzewicz, N. *et al.* (2000) “Sustained ex vivo and in vivo transfer of a reporter gene using polyoma virus pseudocapsids,” *Gene therapy*, 7(13), pp. 1094–1102.
<http://dx.doi.org/10.1038/sj.gt.3301219>.
- Kristensen, C.B. and Gram, L.F. (1982) “Equilibrium dialysis for determination of protein binding of imipramine – evaluation of a method,” *Acta pharmacologica et toxicologica*, 50(2), pp. 130–136. <https://doi.org/10.1111/j.1600-0773.1982.tb00954.x>.
- Kuppachi, S. *et al.* (2016) “BK polyoma virus infection and renal disease in non-renal solid organ transplantation,” *Clinical kidney journal*, 9(2), pp. 310–318.
<http://dx.doi.org/10.1093/ckj/sfv143>.
- Kwak, E.J. *et al.* (2002) “Pathogenesis and management of polyomavirus infection in transplant recipients,” *Clinical infectious disease*, 35(9), pp. 1081–1087. <http://dx.doi.org/10.1086/344060>.
- Lai, W.-H. *et al.* (2021) “Peptide-guided JC polyomavirus-like particles specifically target bladder cancer cells for gene therapy,” *Scientific reports*, 11(1), p. 11889.
<http://dx.doi.org/10.1038/s41598-021-91328-7>.
- Lasickienè, R. *et al.* (2012) “The use of recombinant pseudotype virus-like particles harbouring inserted target antigen to generate antibodies against cellular marker p16INK4A,” *TheScientificWorldJournal*, 2012, p. 263737. <http://dx.doi.org/10.1100/2012/263737>.
- LeCher, J.C. *et al.* (2017) “Breaking in and busting out: cell-penetrating peptides and the endosomal escape problem,” *Biomolecular concepts*, 8(3–4), pp. 131–141.
<https://pmc.ncbi.nlm.nih.gov/articles/PMC5640260/>.
- Lee, P. *et al.* (2013) “A second-generation ELISA (STRATIFY JCV™ DxSelect™) for detection of JC virus antibodies in human serum and plasma to support progressive multifocal leukoencephalopathy risk stratification,” *Journal of clinical virology*, 57(2), pp. 141–146.
<http://dx.doi.org/10.1016/j.jcv.2013.02.002>.
- Lee, Y.-J. *et al.* (2010) “Modeling of the endosomolytic activity of HA2-TAT peptides with red blood cells and ghosts,” *Biochemistry*, 49(36), pp. 7854–7866.
<http://dx.doi.org/10.1021/bi1008408>.
- Lehmusvaara, S. *et al.* (2006) “Utility of cell-permeable peptides for enhancement of virus-mediated gene trans to human tumor cells,” *BioTechniques*, 40(5), pp. 573–4, 576.
<http://dx.doi.org/10.2144/000112152>.
- Leung, A.Y. *et al.* (2001) “Quantification of polyoma BK viruria in hemorrhagic cystitis complicating bone marrow transplantation,” *Blood*, 98(6), pp. 1971–1978.
<http://dx.doi.org/10.1182/blood.v98.6.1971>.
- Lewis, J.D. *et al.* (2006) “Viral nanoparticles as tools for intravital vascular imaging,” *Nature*

medicine, 12(3), pp. 354–360. <http://dx.doi.org/10.1038/nm1368>.

Li, F. *et al.* (2009) “Imaging viral behavior in Mammalian cells with self-assembled capsid-quantum-dot hybrid particles,” *Small*, 5(6), pp. 718–726. <http://dx.doi.org/10.1002/sml.200801303>.

Liao, D.F. *et al.* (2000) “Purification and identification of secreted oxidative stress-induced factors from vascular smooth muscle cells,” *The journal of biological chemistry*, 275(1), pp. 189–196. [http://jbc.org/article/S0021-9258\(19\)52851-4/fulltext](http://jbc.org/article/S0021-9258(19)52851-4/fulltext).

Liebl, D. *et al.* (2006) “Mouse polyomavirus enters early endosomes, requires their acidic pH for productive infection, and meets transferrin cargo in Rab11-positive endosomes,” *Journal of virology*, 80(9), pp. 4610–4622. <http://dx.doi.org/10.1128/JVI.80.9.4610-4622.2006>.

Liepold, L. *et al.* (2007) “Viral capsids as MRI contrast agents,” *Magnetic resonance in medicine*, 58(5), pp. 871–879. <http://dx.doi.org/10.1002/mrm.21307>.

Lin, M.-C. *et al.* (2019a) “Gene therapy for castration-resistant prostate cancer cells using JC polyomavirus-like particles packaged with a PSA promoter driven-suicide gene,” *Cancer gene therapy*, 26(7–8), pp. 208–215. <http://dx.doi.org/10.1038/s41417-019-0083-0>.

Lin, M.-C. *et al.* (2019b) “Inhibition of human lung adenocarcinoma growth and metastasis by JC polyomavirus-like particles packaged with an SP-B promoter-driven CD59-specific shRNA,” *Clinical science*, 133(21), pp. 2159–2169. <http://dx.doi.org/10.1042/CS20190395>.

Lin, Z.J. *et al.* (2005) “In vitro plasma protein binding determination of flunarizine using equilibrium dialysis and liquid chromatography-tandem mass spectrometry,” *Journal of pharmaceutical and biomedical analysis*, 37(4), pp. 757–762. <https://doi.org/10.1016/j.jpba.2004.10.050>.

Lindner, J.M. *et al.* (2019) “Human Memory B Cells Harbor Diverse Cross-Neutralizing Antibodies against BK and JC Polyomaviruses,” *Immunity*, 50(3), pp. 668–676.e5. <http://dx.doi.org/10.1016/j.immuni.2019.02.003>.

Liu, Q. *et al.* (2023) “Current research trends of nanomedicines,” *Acta pharmaceutica Sinica. B*, 13(11), pp. 4391–4416. <https://doi.org/10.1016/j.apsb.2023.05.018>

Liu, Y. *et al.* (2014) “Enhancing gene delivery of adeno-associated viruses by cell-permeable peptides,” *Molecular therapy. Methods & clinical development*, 1, p. 12. <http://dx.doi.org/10.1038/mtm.2013.12>.

Loo, L. *et al.* (2006) “Controlled encapsidation of gold nanoparticles by a viral protein shell,” *Journal of the American Chemical Society*, 128(14), pp. 4502–4503. <http://dx.doi.org/10.1021/ja057332u>.

Loo, L. *et al.* (2007) “Encapsidation of nanoparticles by red clover necrotic mosaic virus,” *Journal of the American Chemical Society*, 129(36), pp. 11111–11117. <http://dx.doi.org/10.1021/ja071896b>.

Loo, L. *et al.* (2008) “Infusion of dye molecules into Red clover necrotic mosaic virus,” *Chemical communications*, (1), pp. 88–90. <http://dx.doi.org/10.1039/b714748a>.

Lopez-Gordo, E. *et al.* (2014) “The importance of coagulation factors binding to adenovirus: historical perspectives and implications for gene delivery,” *Expert opinion on drug delivery*, 11(11), pp. 1795–1813. <http://dx.doi.org/10.1517/17425247.2014.938637>.

Lorentzen, E.M. *et al.* (2020) “Early fulminant BK polyomavirus-associated nephropathy in two

- kidney transplant patients with low neutralizing antibody titers receiving allografts from the same donor,” *Virology journal*, 17(1), p. 5. <http://dx.doi.org/10.1186/s12985-019-1275-9>.
- Lucchino, M. *et al.* (2021) “Absolute quantification of drug vector delivery to the cytosol,” *Angewandte Chemie (International ed. in English)*, 60(27), pp. 14824–14830. <https://doi.org/10.1002/anie.202102332>
- Luo, C. *et al.* (2012) “VP-1 quasispecies in human infection with polyomavirus BK,” *Journal of medical virology*, 84(1), pp. 152–161. <http://dx.doi.org/10.1002/jmv.22147>.
- Machida, K. and Imataka, H. (2015) “Production methods for viral particles,” *Biotechnology letters*, 37(4), pp. 753–760. <http://dx.doi.org/10.1007/s10529-014-1741-9>.
- Maier, O. *et al.* (2012) “Spatiotemporal dynamics of adenovirus membrane rupture and endosomal escape,” *Journal of virology*, 86(19), pp. 10821–10828. <http://dx.doi.org/10.1128/JVI.01428-12>.
- Majdoul, S. *et al.* (2016) “Molecular Determinants of Vectofusin-1 and Its Derivatives for the Enhancement of Lentivirally Mediated Gene Transfer into Hematopoietic Stem/Progenitor Cells,” *The Journal of biological chemistry*, 291(5), pp. 2161–2169. <http://dx.doi.org/10.1074/jbc.M115.675033>.
- Mannová, P. *et al.* (2002) “Analysis of mouse polyomavirus mutants with lesions in the minor capsid proteins,” *Journal of General Virology*, pp. 2309–2319. <https://doi.org/10.1099/0022-1317-83-9-2309>.
- Mannová, P. and Forstová, J. (2003) “Mouse polyomavirus utilizes recycling endosomes for a traffic pathway independent of COPI vesicle transport,” *Journal of virology*, 77(3), pp. 1672–1681. <http://dx.doi.org/10.1128/jvi.77.3.1672-1681.2003>.
- Manzenrieder, F. *et al.* (2011) “Stabilization of virus-like particles with poly(2-oxazoline)s,” *Angewandte Chemie*, 50(11), pp. 2601–2605. <http://dx.doi.org/10.1002/anie.201006134>.
- Martens, T.F. *et al.* (2014) “Intracellular delivery of nanomaterials: How to catch endosomal escape in the act,” *Nano today*, 9(3), pp. 344–364. <https://doi.org/10.1016/j.nantod.2014.04.01>
- Martinez, R. *et al.* (2013) “High temporal resolution imaging reveals endosomal membrane penetration and escape of adenoviruses in real time,” *Methods in molecular biology*, 1064, pp. 211–226. http://dx.doi.org/10.1007/978-1-62703-601-6_15.
- Martino, M.L. *et al.* (2021) “Single-Point Mutations in Q β Virus-like Particles Change Binding to Cells,” *Biomacromolecules*, 22(8), pp. 3332–3341. <http://dx.doi.org/10.1021/acs.biomac.1c00443>.
- Mayberry, C.L. *et al.* (2021) “Sending mixed signals: polyomavirus entry and trafficking,” *Current opinion in virology*, 47, pp. 95–105. <http://dx.doi.org/10.1016/j.coviro.2021.02.004>.
- Mazeike, E. *et al.* (2012) “Induction of insert-specific immune response in mice by hamster polyomavirus VP1 derived virus-like particles carrying LCMV GP33 CTL epitope,” *Virus research*, 163(1), pp. 2–10. <http://dx.doi.org/10.1016/j.virusres.2011.08.003>.
- McAleer, W.J. *et al.* (1984) “Human hepatitis B vaccine from recombinant yeast,” *Nature*, 307(5947), pp. 178–180. Available at: <http://dx.doi.org/10.1038/307178a0>.
- McIlroy, D. *et al.* (2020) “Persistent BK Polyomavirus Viruria is Associated with Accumulation of VP1 Mutations and Neutralization Escape,” *Viruses*, 12(8). <https://doi.org/10.3390/v12080824>.

- McIlroy, D. *et al.* (2019) “Intra-patient viral evolution in polyomavirus-related diseases,” *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 374(1773), p. 20180301. <http://dx.doi.org/10.1098/rstb.2018.0301>.
- Medina, S.H. *et al.* (2016) “An intrinsically disordered peptide facilitates non-endosomal cell entry,” *Angewandte Chemie (International ed. in English)*, 55(10), pp. 3369–3372. <https://doi.org/10.1002/anie.201510518>
- Mehrvar, A. *et al.* (2021) “Successful Treatment with Intravenous and Intravascular Cidofovir for BK Virus-Associated Hemorrhagic Cystitis after Allogeneic Hematopoietic Stem Cell Transplantation: A Case Report,” *Case reports in oncology*, 14(2), pp. 892–895. <http://dx.doi.org/10.1159/000516269>.
- Mellid-Carballal, R. *et al.* (2023a) “Viral protein nanoparticles (Part 1): Pharmaceutical characteristics,” *European journal of pharmaceutical sciences*, 187(106460), p. 106460. <http://dx.doi.org/10.1016/j.ejps.2023.106558>
- Mellid-Carballal, R. *et al.* (2023b) “Viral protein-based nanoparticles (part 2): Pharmaceutical applications,” *European journal of pharmaceutical sciences*, 189(106558), p. 106558. <https://doi.org/10.1016/j.ejps.2023.106460>
- Mezes, B. and Amati, P. (1994) “Mutations of polyomavirus VP1 allow in vitro growth in undifferentiated cells and modify in vivo tissue replication specificity,” *Journal of virology*, 68(2), pp. 1196–1199. <http://dx.doi.org/10.1128/JVI.68.2.1196-1199.1994>.
- Middelberg, A.P.J. *et al.* (2011) “A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines,” *Vaccine*, 29(41), pp. 7154–7162. <http://dx.doi.org/10.1016/j.vaccine.2011.05.075>.
- Milech, N. *et al.* (2015) “GFP-complementation assay to detect functional CPP and protein delivery into living cells,” *Scientific reports*, 5, p. 18329. <http://dx.doi.org/10.1038/srep18329>.
- Minten, I.J. *et al.* (2009) “Controlled encapsulation of multiple proteins in virus capsids,” *Journal of the American Chemical Society*, 131(49), pp. 17771–17773. <http://dx.doi.org/10.1021/ja907843s>.
- Moens, U. *et al.* (2013) “Serological cross-reactivity between human polyomaviruses,” *Reviews in medical virology*, 23(4), pp. 250–264. <http://dx.doi.org/10.1002/rmv.1747>.
- Moens, U. *et al.* (2017) “ICTV Virus Taxonomy Profile: Polyomaviridae,” *The Journal of general virology*, 98(6), pp. 1159–1160. <http://dx.doi.org/10.1099/jgv.0.000839>.
- Mohsen, M.O. *et al.* (2017) “Major findings and recent advances in virus-like particle (VLP)-based vaccines,” *Seminars in immunology*, 34, pp. 123–132. <http://dx.doi.org/10.1016/j.smim.2017.08.014>.
- Montross, L. *et al.* (1991) “Nuclear assembly of polyomavirus capsids in insect cells expressing the major capsid protein VP1,” *Journal of virology*, 65(9), pp. 4991–4998. <http://dx.doi.org/10.1128/JVI.65.9.4991-4998.1991>.
- Morávková, A. (2001) *Příprava protilátek proti hlavnímu strukturnímu proteinu myšího polyomaviru*. Charles University, Faculty of Science.
- Moreland, R.B. *et al.* (1991) “Characterization of the DNA-binding properties of the polyomavirus capsid protein VP1,” *Journal of virology*, 65(3), pp. 1168–1176. <http://dx.doi.org/10.1128/JVI.65.3.1168-1176.1991>.

- Munson, M.J. *et al.* (2021) “A high-throughput Galectin-9 imaging assay for quantifying nanoparticle uptake, endosomal escape and functional RNA delivery,” *Communications biology*, 4(1), p. 211. <https://doi.org/10.1038/s42003-021-01728-8>
- Murata, H. *et al.* (2008) “Identification of a neutralization epitope in the VP1 capsid protein of SV40,” *Virology*, 381(1), pp. 116–122. <http://dx.doi.org/10.1016/j.virol.2008.07.032>.
- Murphy, A.J. *et al.* (2014) “Mice with megabase humanization of their immunoglobulin genes generate antibodies as efficiently as normal mice,” *Proceedings of the National Academy of Sciences of the United States of America*, 111(14), pp. 5153–5158. <http://dx.doi.org/10.1073/pnas.1324022111>.
- Murthy, N. *et al.* (1999) “The design and synthesis of polymers for eukaryotic membrane disruption,” *Journal of controlled release*, 61(1–2), pp. 137–143. [https://doi.org/10.1016/s0168-3659\(99\)00114-5](https://doi.org/10.1016/s0168-3659(99)00114-5)
- Najafabadi, M. M. *et al.* (2020) “Treatment protocols for BK virus associated hemorrhagic cystitis after hematopoietic stem cell transplantation,” *American journal of blood research*, 10(5), pp. 217–230. ISSN: 2160-1992/AJBR0117096
- Neburkova, J. *et al.* (2018) “Inhibitor-GCPII Interaction: Selective and Robust System for Targeting Cancer Cells with Structurally Diverse Nanoparticles,” *Molecular pharmaceuticals*, 15(8), pp. 2932–2945. <http://dx.doi.org/10.1021/acs.molpharmaceut.7b00889>.
- Neu, U. *et al.* (2013) “A structure-guided mutation in the major capsid protein retargets BK polyomavirus,” *PLoS pathogens*, 9(10), p. e1003688. <http://dx.doi.org/10.1371/journal.ppat.1003688>.
- Nicol, J.T.J. *et al.* (2012) “Seroprevalence and cross-reactivity of human polyomavirus 9,” *Emerging infectious diseases*, 18(8), pp. 1329–1332. <https://doi.org/10.3201/eid1808.111625>.
- Nicol, J.T.J. *et al.* (2013) “Age-specific seroprevalences of merkel cell polyomavirus, human polyomaviruses 6, 7, and 9, and trichodysplasia spinulosa-associated polyomavirus,” *Clinical and vaccine immunology: CVI*, 20(3), pp. 363–368. <http://dx.doi.org/10.1128/CI.00438-12>.
- Nigatu, A.S. *et al.* (2013) “Evaluation of cell-penetrating peptide/adenovirus particles for transduction of CAR-negative cells,” *Journal of pharmaceutical sciences*, 102(6), pp. 1981–1993. <http://dx.doi.org/10.1002/jps.23556>.
- Niikura, K. *et al.* (2013) “Virus-like particles with removable cyclodextrins enable glutathione-triggered drug release in cells,” *Molecular bioSystems*, 9(3), pp. 501–507. <http://dx.doi.org/10.1039/c2mb25420d>.
- Nukuzuma, S. *et al.* (2006) “Subtype I BK polyomavirus strains grow more efficiently in human renal epithelial cells than subtype IV strains,” *The Journal of general virology*, 87(Pt 7), pp. 1893–1901. <http://dx.doi.org/10.1099/vir.0.81698-0>.
- O'Donnell, P.H. *et al.* (2009) “BK virus infection is associated with hematuria and renal impairment in recipients of allogeneic hematopoietic stem cell transplants,” *Biology of blood and marrow transplantation*, 15(9), pp. 1038–1048.e1. <http://dx.doi.org/10.1016/j.bbmt.2009.04.016>.
- Oh, M.H. *et al.* (2015) “Genetically Programmed Clusters of Gold Nanoparticles for Cancer Cell-Targeted Photothermal Therapy,” *ACS applied materials & interfaces*, 7(40), pp. 22578–22586. <http://dx.doi.org/10.1021/acsami.5b07029>.
- Olsen, G.-H. *et al.* (2006) “Genetic variability in BK Virus regulatory regions in urine and kidney biopsies from renal-transplant patients,” *Journal of medical virology*, 78(3), pp. 384–393.

<http://dx.doi.org/10.1002/jmv.20551>.

Osterman, J.V. *et al.* (1970) "DNA and gene therapy: uncoating of polyoma pseudovirus in mouse embryo cells," *Proceedings of the National Academy of Sciences of the United States of America*, 67(1), pp. 37–40. <http://dx.doi.org/10.1073/pnas.67.1.37>.

Ou, W.C. *et al.* (1999) "The major capsid protein, VP1, of human JC virus expressed in *Escherichia coli* is able to self-assemble into a capsid-like particle and deliver exogenous DNA into human kidney cells," *The Journal of general virology*, 80 (Pt 1), pp. 39–46. <http://dx.doi.org/10.1099/0022-1317-80-1-39>.

Panda, S.K. *et al.* (2015) "Recombinant Hepatitis E virus like particles can function as RNA nanocarriers," *Journal of nanobiotechnology*, 13, p. 44. <http://dx.doi.org/10.1186/s12951-015-0101-9>.

Panthi, S. *et al.* (2021) "Paramyxovirus-Like Particles as Protein Delivery Vehicles," *Journal of virology*, 95(20), p. e0103021. <http://dx.doi.org/10.1128/JVI.01030-21>.

Pareek, V. *et al.* (2018) "Formation and Characterization of Protein Corona Around Nanoparticles: A Review," *Journal of nanoscience and nanotechnology*, 18(10), pp. 6653–6670. <http://dx.doi.org/10.1166/jnn.2018.15766>.

Park, S.-H. *et al.* (2010) "Branched oligomerization of cell-permeable peptides markedly enhances the transduction efficiency of adenovirus into mesenchymal stem cells," *Gene therapy*, 17(8), pp. 1052–1061. <http://dx.doi.org/10.1038/gt.2010.58>.

Pastrana, D.V. *et al.* (2004) "Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18," *Virology*, 321(2), pp. 205–216. <http://dx.doi.org/10.1016/j.virol.2003.12.027>

Pastrana, D.V. *et al.* (2009) "Quantitation of human seroresponsiveness to Merkel cell polyomavirus," *PLoS pathogens*, 5(9), p. e1000578. <https://doi.org/10.1371/journal.ppat.1000578>

Pastrana, D.V. *et al.* (2012) "Neutralization serotyping of BK polyomavirus infection in kidney transplant recipients," *PLoS pathogens*, 8(4), p. e1002650. <http://dx.doi.org/10.1371/journal.ppat.1002650>.

Pastrana, D.V. *et al.* (2013) "BK polyomavirus genotypes represent distinct serotypes with distinct entry tropism," *Journal of virology*, 87(18), pp. 10105–10113. <http://dx.doi.org/10.1128/JVI.01189-13>.

Pattinson, D.J. *et al.* (2019) "Chimeric Murine Polyomavirus Virus-Like Particles Induce Plasmodium Antigen-Specific CD8+ T Cell and Antibody Responses," *Frontiers in cellular and infection microbiology*, 9, p. 215. <http://dx.doi.org/10.3389/fcimb.2019.00215>.

Paz, I. *et al.* (2010) "Glectin-3, a marker for vacuole lysis by invasive pathogens," *Cellular microbiology*, 12(4), pp. 530–544. <http://dx.doi.org/10.1111/j.1462-5822.2009.01415.x>.

Peralta, A. *et al.* (2009) "Chimeric recombinant rotavirus-like particles as a vehicle for the display of heterologous epitopes," *Virology journal*, 6, p. 192. <http://dx.doi.org/10.1186/1743-422X-6-192>.

Peretti, A. *et al.* (2018) "Characterization of BK Polyomaviruses from Kidney Transplant Recipients Suggests a Role for APOBEC3 in Driving In-Host Virus Evolution," *Cell host & microbe*, 23(5), pp. 628–635.e7. <http://dx.doi.org/10.1016/j.chom.2018.04.005>.

- Peretti, A. *et al.* (2023) “A multivalent polyomavirus vaccine elicits durable neutralizing antibody responses in macaques,” *Vaccine* [Preprint].
<https://doi.org/10.1016/j.vaccine.2023.02.002>.
- Pitek, A.S. *et al.* (2016) “The Protein Corona of Plant Virus Nanoparticles Influences their Dispersion Properties, Cellular Interactions, and In Vivo Fates,” *Small*, 12(13), pp. 1758–1769.
<https://pubmed.ncbi.nlm.nih.gov/29869268/>.
- Pitek, A.S. *et al.* (2018) “Interactions between plant viral nanoparticles (VNPs) and blood plasma proteins, and their impact on the VNP in vivo fates,” *Methods in molecular biology*, 1776, pp. 591–608. <http://dx.doi.org/10.1002/sml.201502458>.
- Plank, C. *et al.* (1994) “The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems,” *The Journal of biological chemistry*, 269(17), pp. 12918–12924. <https://www.ncbi.nlm.nih.gov/pubmed/8175709>.
- Pleckaityte, M. *et al.* (2015) “Construction of polyomavirus-derived pseudotype virus-like particles displaying a functionally active neutralizing antibody against hepatitis B virus surface antigen,” *BMC biotechnology*, 15, p. 85. <http://dx.doi.org/10.1186/s12896-015-0203-3>.
- Plummer, E.M. and Manchester, M. (2011) “Viral nanoparticles and virus-like particles: platforms for contemporary vaccine design,” *Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology*, 3(2), pp. 174–196. <http://dx.doi.org/10.1002/wnan.119>.
- Pokorski, J.K. *et al.* (2011) “Cell targeting with hybrid Q β virus-like particles displaying epidermal growth factor,” *Chembiochem: a European journal of chemical biology*, 12(16), pp. 2441–2447. <http://dx.doi.org/10.1002/cbic.201100469>.
- Polidarová, M. (2016) *Utilization of mouse polyomavirus derived virus-like particles for cargo delivery into cells*. Edited by H. Španielová. Charles University.
- Porta, C. *et al.* (1994) “Development of cowpea mosaic virus as a high-yielding system for the presentation of foreign peptides,” *Virology*, 202(2), pp. 949–955.
<http://dx.doi.org/10.1006/viro.1994.1417>.
- Purighalla, R. *et al.* (1995) “BK virus infection in a kidney allograft diagnosed by needle biopsy,” *American journal of kidney diseases*, 26(4), pp. 671–673.
[http://dx.doi.org/10.1016/0272-6386\(95\)90608-8](http://dx.doi.org/10.1016/0272-6386(95)90608-8).
- Purtha, W.E. *et al.* (2011) “Memory B cells, but not long-lived plasma cells, possess antigen specificities for viral escape mutants,” *The Journal of experimental medicine*, 208(13), pp. 2599–2606. <http://dx.doi.org/10.1084/jem.20110740>.
- Qasba, P.K. and Aposhian, H.V. (1971) “DNA and gene therapy: transfer of mouse DNA to human and mouse embryonic cells by polyoma pseudovirions,” *Proceedings of the National Academy of Sciences of the United States of America*, 68(10), pp. 2345–2349.
<http://dx.doi.org/10.1073/pnas.68.10.2345>.
- Qeska, D. *et al.* (2022) “Incidence, risk factors, outcomes, and clinical management of BK viremia in the modern era of kidney transplantation,” *Transplant infectious disease*, 24(6), p. e13915. <http://dx.doi.org/10.1111/tid.13915>.
- Radulovic, M. *et al.* (2018) “ESCRT-mediated lysosome repair precedes lysophagy and promotes cell survival,” *The EMBO journal*, 37(21), p. e99753.
<https://doi.org/10.15252/embj.201899753>
- Rainey-Barger, E.K. *et al.* (2007) “A chaperone-activated nonenveloped virus perforates the

- physiologically relevant endoplasmic reticulum membrane,” *Journal of virology*, 81(23), pp. 12996–13004. <http://dx.doi.org/10.1128/JVI.01037-07>.
- Randhawa, P.S. *et al.* (1999) “HUMAN POLYOMA VIRUS-ASSOCIATED INTERSTITIAL NEPHRITIS IN THE ALLOGRAFT KIDNEY,” *Transplantation*, 67(1), p. 103. <https://journals.lww.com/co-criticalcare/00007890-199901150-00018.fulltext>
- Randhawa, P.S. *et al.* (2002) “DNA sequencing of viral capsid protein VP-1 region in patients with BK virus interstitial nephritis,” *Transplantation*, 73(7), pp. 1090–1094. <http://dx.doi.org/10.1097/00007890-200204150-00013>.
- Randhawa, P.S. *et al.* (2009) “Identification of species-specific and cross-reactive epitopes in human polyomavirus capsids using monoclonal antibodies,” *The Journal of general virology*, 90(Pt 3), pp. 634–639. <http://dx.doi.org/10.1099/vir.0.008391-0>.
- Randhawa, P.S. *et al.* (2011) “Impact of genomic sequence variability on quantitative PCR assays for diagnosis of polyomavirus BK infection,” *Journal of clinical microbiology*, 49(12), pp. 4072–4076. <http://dx.doi.org/10.1128/JCM.01230-11>.
- Randhawa, P.S. and Demetris, A.J. (2000) “Nephropathy due to polyomavirus type BK,” *The New England journal of medicine*, pp. 1361–1363. <http://dx.doi.org/10.1056/NEJM200005043421809>.
- Rashidijahanabad, Z. *et al.* (2022) “Virus-like Particle Display of *Vibrio cholerae* O-Specific Polysaccharide as a Potential Vaccine against Cholera,” *ACS infectious diseases*, 8(3), pp. 574–583. <http://dx.doi.org/10.1021/acsinfecdis.1c00585>.
- Rayment, I. *et al.* (1982) “Polyoma virus capsid structure at 22.5 Å resolution,” *Nature*, 295(5845), pp. 110–115. <https://doi.org/10.1038/295110a0>
- Regules, J.A. *et al.* (2011) “The RTS,S vaccine candidate for malaria,” *Expert review of vaccines*, 10(5), pp. 589–599. <http://dx.doi.org/10.1586/erv.11.57>.
- Ren, Y. *et al.* (2007) “Folic acid-conjugated protein cages of a plant virus: a novel delivery platform for doxorubicin,” *Bioconjugate chemistry*, 18(3), pp. 836–843. <http://dx.doi.org/10.1021/bc060361p>.
- ResearchGate.net (2025) *Researchgate.net, ResearchGate*. https://www.researchgate.net/post/Problems_with_proximity_ligation_assay_DouLink/ (Accessed: February 15, 2025).
- Richard, J.P. *et al.* (2003) “Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake,” *The Journal of biological chemistry*, 278(1), pp. 585–590. <http://dx.doi.org/10.1074/jbc.M209548200>.
- Richterová, Z. *et al.* (2001) “Caveolae are involved in the trafficking of mouse polyomavirus virions and artificial VP1 pseudocapsids toward cell nuclei,” *Journal of virology*, 75(22), pp. 10880–10891. <http://dx.doi.org/10.1128/JVI.75.22.10880-10891.2001>.
- Rivera-Hernandez, T. *et al.* (2013) “Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS),” *Vaccine*, 31(15), pp. 1950–1955. <http://dx.doi.org/10.1016/j.vaccine.2013.02.013>.
- Roberson, J.A. *et al.* (2007) “Differentiation of West Nile and St. Louis encephalitis virus infections by use of noninfectious virus-like particles with reduced cross-reactivity,” *Journal of clinical microbiology*, 45(10), pp. 3167–3174. <http://dx.doi.org/10.1128/JCM.01143-07>.

- Rockstroh, A. *et al.* (2017) “Specific detection of dengue and Zika virus antibodies using envelope proteins with mutations in the conserved fusion loop,” *Emerging microbes & infections*, 6(11), p. e99. <http://dx.doi.org/10.1038/emi.2017.87>.
- Roldão, A. *et al.* (2010) “Virus-like particles in vaccine development,” *Expert review of vaccines*, 9(10), pp. 1149–1176. <http://dx.doi.org/10.1586/erv.10.115>.
- Rose, R.C. *et al.* (1994) “Human papillomavirus (HPV) type 11 recombinant virus-like particles induce the formation of neutralizing antibodies and detect HPV-specific antibodies in human sera,” *The Journal of general virology*, 75 (Pt 8), pp. 2075–2079. <http://dx.doi.org/10.1099/0022-1317-75-8-2075>.
- Rubinstein, N.D. *et al.* (2009) “Epitopia: a web-server for predicting B-cell epitopes,” *BMC bioinformatics*, 10(1), p. 287. <https://doi.org/10.1186/1471-2105-10-287>
- Saar, K. *et al.* (2005) “Cell-penetrating peptides: a comparative membrane toxicity study,” *Analytical biochemistry*, 345(1), pp. 55–65. <https://doi.org/10.1016/j.ab.2005.07.033>
- Safi, M. *et al.* (2011) “The effects of aggregation and protein corona on the cellular internalization of iron oxide nanoparticles,” *Biomaterials*, 32(35), pp. 9353–9363. <https://doi.org/10.1016/j.biomaterials.2011.08.048>
- Saláková, M. *et al.* (2022) “Pretransplantation seroreactivity in kidney donors and recipients as a predictive factor for posttransplant BKPyV-DNAemia,” *Frontiers in immunology*, 13, p. 929946. <http://dx.doi.org/10.3389/fimmu.2022.929946>.
- Saleh, A. *et al.* (2020) “Update on the Management of BK Virus Infection,” *Experimental and clinical transplantation*, 18(6), pp. 659–670. <http://dx.doi.org/10.6002/ect.2019.0254>.
- Salunke, D.M. *et al.* (1986) “Self-assembly of purified polyomavirus capsid protein VP1,” *Cell*, 46(6), pp. 895–904. [http://dx.doi.org/10.1016/0092-8674\(86\)90071-1](http://dx.doi.org/10.1016/0092-8674(86)90071-1).
- Salvati, A. *et al.* (2013) “Transferrin-functionalized nanoparticles lose their targeting capabilities when a biomolecule corona adsorbs on the surface,” *Nature nanotechnology*, 8(2), pp. 137–143. <https://doi.org/10.1038/nnano.2012.237>
- Saundh, B.K. *et al.* (2013) “Early BK polyomavirus (BKV) reactivation in donor kidney is a risk factor for development of BKV-associated nephropathy,” *The Journal of infectious diseases*, 207(1), pp. 137–141. <http://dx.doi.org/10.1093/infdis/jis642>.
- Schiller, J.T. and Lowy, D.R. (1996) “Papillomavirus-like particles and HPV vaccine development,” *Seminars in cancer biology*, 7(6), pp. 373–382. <http://dx.doi.org/10.1006/scbi.1996.0046>.
- Schmidt, S. *et al.* (2015) “Detecting Cytosolic Peptide Delivery with the GFP Complementation Assay in the Low Micromolar Range,” *Angewandte Chemie*, 54(50), pp. 15105–15108. <http://dx.doi.org/10.1002/anie.201505913>.
- Schmidt, U. *et al.* (2001a) “Protein and peptide delivery via engineered polyomavirus-like particles,” *FASEB journal*, 15(9), pp. 1646–1648. <http://dx.doi.org/10.1096/fj.00-0645fje>.
- Schmidt, U. *et al.* (2001b) “Binding of external ligands onto an engineered virus capsid,” *Protein engineering, design & selection: PEDS*, 14(10). <https://doi.org/10.1093/protein/14.10.769>.
- Schmitt, C. *et al.* (2014) “Donor origin of BKV replication after kidney transplantation,” *Journal of clinical virology*, 59(2), pp. 120–125. <http://dx.doi.org/10.1016/j.jcv.2013.11.009>.
- Schwarz, A. *et al.* (2016) “Viral Origin, Clinical Course, and Renal Outcomes in Patients With

- BK Virus Infection After Living-Donor Renal Transplantation,” *Transplantation*, 100(4), pp. 844–853. <http://dx.doi.org/10.1097/TP.0000000000001066>.
- Sekavova, A. (2017) *Preparation of polyomaviral nanostructures for diagnostics of BK virus infections*. Edited by H. Spanielova. Mgr. Charles University.
- Seth, A. *et al.* (2016) “Modular virus-like particles for sublingual vaccination against group A streptococcus,” *Vaccine*, 34(51), pp. 6472–6480. <http://dx.doi.org/10.1016/j.vaccine.2016.11.008>.
- Shen, C.-H. *et al.* (2021) “Suppression of bone metastatic castration-resistant prostate cancer cell growth by a suicide gene delivered by JC polyomavirus-like particles,” *Gene therapy* [Preprint]. <https://doi.org/10.1038/s41434-021-00280-8>.
- Shete, H.K. *et al.* (2014) “Endosomal escape: a bottleneck in intracellular delivery,” *Journal of nanoscience and nanotechnology*, 14(1), pp. 460–474. <http://dx.doi.org/10.1166/jnn.2014.9082>.
- Shin, Y.C. and Folk, W.R. (2003) “Formation of polyomavirus-like particles with different VP1 molecules that bind the urokinase plasminogen activator receptor,” *Journal of virology*, 77(21), pp. 11491–11498. <http://dx.doi.org/10.1128/jvi.77.21.11491-11498.2003>.
- Shirley, J.L. *et al.* (2020) “Immune Responses to Viral Gene Therapy Vectors,” *Molecular therapy*, 28(3), pp. 709–722. <http://dx.doi.org/10.1016/j.ymthe.2020.01.001>.
- Signorini, L. *et al.* (2020) “Viral Genomic Characterization and Replication Pattern of Human Polyomaviruses in Kidney Transplant Recipients,” *Viruses*, 12(11). <https://doi.org/10.3390/v12111280>.
- Simpson, J.D. *et al.* (2019) “Engineered Polymeric Materials for Biological Applications: Overcoming Challenges of the Bio–Nano Interface,” *Polymers*, 11(9), p. 1441. <https://doi.org/10.3390/polym11091441>
- Singh, P. *et al.* (2006) “Canine parvovirus-like particles, a novel nanomaterial for tumor targeting,” *Journal of nanobiotechnology*, 4, p. 2. <http://dx.doi.org/10.1186/1477-3155-4-2>.
- Skrastina, D. *et al.* (2008) “High immunogenicity of a hydrophilic component of the hepatitis B virus preS1 sequence exposed on the surface of three virus-like particle carriers,” *Vaccine*, 26(16), pp. 1972–1981. <http://dx.doi.org/10.1016/j.vaccine.2008.02.030>.
- Smith, S.A. *et al.* (2019) “The Endosomal Escape of Nanoparticles: Toward More Efficient Cellular Delivery,” *Bioconjugate chemistry*, 30(2), pp. 263–272. <http://dx.doi.org/10.1021/acs.bioconjchem.8b00732>.
- Soeda, E. *et al.* (1998) “Enhancement by polylysine of transient, but not stable, expression of genes carried into cells by polyoma VP1 pseudocapsids,” *Gene therapy*, 5(10), pp. 1410–1419. <http://dx.doi.org/10.1038/sj.gt.3300748>.
- Solis, M. *et al.* (2018) “Neutralizing Antibody-Mediated Response and Risk of BK Virus-Associated Nephropathy,” *Journal of the American Society of Nephrology: JASN*, 29(1), pp. 326–334. <http://dx.doi.org/10.1681/ASN.2017050532>.
- Sood, P. *et al.* (2013) “Donor and recipient BKV-specific IgG antibody and posttransplantation BKV infection: a prospective single-center study,” *Transplantation*, 95(6), pp. 896–902. <http://dx.doi.org/10.1097/TP.0b013e318282ba83>.
- Sorin, M.N. *et al.* (2023) “Structural and functional analysis of natural capsid variants suggests sialic acid-independent entry of BK polyomavirus,” *Cell reports*, 42(2), p. 112114.

<http://dx.doi.org/10.1016/j.celrep.2023.112114>.

Sosnick, T.R. *et al.* (1992) “Distances between the antigen-binding sites of three murine antibody subclasses measured using neutron and X-ray scattering,” *Biochemistry*, 31(6), pp. 1779–1786. <https://doi.org/10.1021/bi00121a028>

Soukup, J., 2015. Optimization of methods for analysis of early steps of mouse polyomavirus life cycle. Charles University, Faculty of Science.

Spanielová, H. *et al.* (2014) “The encapsidation of polyomavirus is not defined by a sequence-specific encapsidation signal,” *Virology*, 450–451, pp. 122–131. <http://dx.doi.org/10.1016/j.virol.2013.12.010>.

Šroller, V. *et al.* (2014) “Seroprevalence rates of BKV, JCV, and MCPyV polyomaviruses in the general Czech Republic population,” *Journal of medical*. <https://doi.org/10.1002/jmv.23841>

Staring, J. *et al.* (2018) “Viral escape from endosomes and host detection at a glance,” *Journal of cell science*, 131(15). <https://doi.org/10.1242/jcs.216259>.

Stehle, T. *et al.* (1994) “Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment,” *Nature*, 369(6476), pp. 160–163. <http://dx.doi.org/10.1038/369160a0>.

Stehle, T. *et al.*, 1996. The structure of simian virus 40 refined at 3.1 Å resolution. *Structure* (London, England: 1993), 4(2), pp.165–182. [https://doi.org/10.1016/s0969-2126\(96\)00020-2](https://doi.org/10.1016/s0969-2126(96)00020-2)

Steinmetz, N.F. (2010) “Viral nanoparticles as platforms for next-generation therapeutics and imaging devices,” *Nanomedicine: nanotechnology, biology, and medicine*, 6(5), pp. 634–641. <http://dx.doi.org/10.1016/j.nano.2010.04.005>.

Steinmetz, N.F. (2013) “Viral nanoparticles in drug delivery and imaging,” *Molecular pharmaceutics*, 10(1), pp. 1–2. <http://dx.doi.org/10.1021/mp300658j>.

Stetefeld, J. *et al.* (2016) “Dynamic light scattering: a practical guide and applications in biomedical sciences,” *Biophysical reviews*, 8(4), pp. 409–427. <https://doi.org/10.1007/s12551-016-0218-6>

Stokrová, J. *et al.* (1999) “Interactions of heterologous DNA with polyomavirus major structural protein, VP1,” *FEBS letters*, 445(1), pp. 119–125. [http://dx.doi.org/10.1016/s0014-5793\(99\)00003-4](http://dx.doi.org/10.1016/s0014-5793(99)00003-4).

Stolt, A. *et al.* (2003) “Seroepidemiology of the human polyomaviruses,” *The Journal of general virology*, 84(Pt 6), pp. 1499–1504. <http://dx.doi.org/10.1099/vir.0.18842-0>.

Stracke, S. *et al.* (2003) “Polyoma virus-associated interstitial nephritis in a patient with acute myeloid leukaemia and peripheral blood stem cell transplantation,” *Nephrology, dialysis, transplantation*, 18(11), pp. 2431–2433. <http://dx.doi.org/10.1093/ndt/gfg361>.

Takahashi, R.-U. *et al.* (2008) “Presentation of functional foreign peptides on the surface of SV40 virus-like particles,” *Journal of biotechnology*, 135(4), pp. 385–392. <http://dx.doi.org/10.1016/j.jbiotec.2008.05.012>.

Takasaka, T. *et al.* (2004) “Subtypes of BK virus prevalent in Japan and variation in their transcriptional control region,” *The Journal of general virology*, 85(Pt 10), pp. 2821–2827. <http://dx.doi.org/10.1099/vir.0.80363-0>.

Tan, Y.H. *et al.* (2008) “A nanoengineering approach for investigation and regulation of protein immobilization,” *ACS nano*, 2(11), pp. 2374–2384. <https://pmc.ncbi.nlm.nih.gov/articles/PMC4512660/>

- Tegerstedt, K. *et al.* (2003) “Murine pneumotropic virus VP1 virus-like particles (VLPs) bind to several cell types independent of sialic acid residues and do not serologically cross react with murine polyomavirus VP1 VLPs,” *The Journal of general virology*, 84(Pt 12), pp. 3443–3452. <http://dx.doi.org/10.1099/vir.0.19443-0>.
- Tegerstedt, K. *et al.* (2005a) “A Single Vaccination with Polyomavirus VP1/VP2Her2 Virus-Like Particles Prevents Outgrowth of HER-2/neu–Expressing Tumors,” *Cancer research*, 65(13), pp. 5953–5957. <https://doi.org/10.1158/0008-5472.CAN-05-0335>
- Tegerstedt *et al.* (2005b) “Murine polyomavirus virus-like particles (VLPs) as vectors for gene and immune therapy and vaccines against viral infections and cancer,” *Anticancer research*, 25(4), pp. 2601–2608. <https://www.ncbi.nlm.nih.gov/pubmed/16080500>.
- Tekewe, A. *et al.* (2017) “Integrated molecular and bioprocess engineering for bacterially produced immunogenic modular virus-like particle vaccine displaying 18 kDa rotavirus antigen,” *Biotechnology and bioengineering*, 114(2), pp. 397–406. <http://dx.doi.org/10.1002/bit.26068>.
- Teo, S.L.Y. *et al.* (2021) “Unravelling cytosolic delivery of cell penetrating peptides with a quantitative endosomal escape assay,” *Nature communications*, 12(1), p. 3721. <http://dx.doi.org/10.1038/s41467-021-23997-x>.
- Tomanová, T. (2019) *Analýza protilátkové odpovědi u BK virové infekce*. Edited by H. Španielová. Přírodovědecká fakulta, Univerzita Karlova.
- Tooker, G.M. *et al.* (2020) “Intravesicular Cidofovir in the Treatment of BK Virus-Associated Hemorrhagic Cystitis Following Hematopoietic Stem Cell Transplantation,” *The Annals of pharmacotherapy*, 54(6), pp. 547–553. <http://dx.doi.org/10.1177/1060028019897896>.
- Touzé, A. *et al.* (2001) “Gene transfer using human polyomavirus BK virus-like particles expressed in insect cells,” *The Journal of general virology*, 82(Pt 12), pp. 3005–3009. <http://dx.doi.org/10.1099/0022-1317-82-12-3005>.
- Touzé, A. *et al.* (2010) “Generation of Merkel cell polyomavirus (MCV)-like particles and their application to detection of MCV antibodies,” *Journal of clinical microbiology*, 48(5), pp. 1767–1770. <http://dx.doi.org/10.1128/JCM.01691-09>
- Tremolada, S. *et al.* (2010b) “Mutations in the external loops of BK virus VP1 and urine viral load in renal transplant recipients,” *Journal of cellular physiology*, 222(1), pp. 195–199. <http://dx.doi.org/10.1002/jcp.21937>.
- Tremolada, S. *et al.* (2010c) “Polymorphisms of the BK virus subtypes and their influence on viral in vitro growth efficiency,” *Virus research*, 149(2), pp. 190–196. <http://dx.doi.org/10.1016/j.virusres.2010.01.017>.
- Tremolada, S. *et al.* (2010a) “Rare subtypes of BK virus are viable and frequently detected in renal transplant recipients with BK virus-associated nephropathy,” *Virology*, 404(2), pp. 312–318. <http://dx.doi.org/10.1016/j.virol.2010.05.012>.
- Tsai, B. *et al.* (2003) “Gangliosides are receptors for murine polyoma virus and SV40,” *The EMBO journal*, 22(17), pp. 4346–4355. <http://dx.doi.org/10.1093/emboj/cdg439>
- Tsai, W.-Y. *et al.* (2020) “Enzyme-linked immunosorbent assays using virus-like particles containing mutations of conserved residues on envelope protein can distinguish three flavivirus infections,” *Emerging microbes & infections*, 9(1), pp. 1722–1732. <http://dx.doi.org/10.1080/22221751.2020.1797540>.
- Váňová, J. (2016) *Usměrněná evoluce myšího polyomaviru*. Charles University, Faculty of

Science.

Váňová, J. *et al.* (2019) “The Utilization of Cell-Penetrating Peptides in the Intracellular Delivery of Viral Nanoparticles,” *Materials*, 12(17). <https://doi.org/10.3390/ma12172671>.

Váňová, J. *et al.* (2020) “Influence of cell-penetrating peptides on the activity and stability of virus-based nanoparticles,” *International journal of pharmaceutics*, 576, p. 119008. <http://dx.doi.org/10.1016/j.ijpharm.2019.119008>.

Váňová, J. *et al.* (2022) “VirPorters: Insights into the action of cationic and histidine-rich cell-penetrating peptides,” *International journal of pharmaceutics*, 611, p. 121308. <http://dx.doi.org/10.1016/j.ijpharm.2021.121308>.

Vaysse, L. *et al.*, 2000. Improved transfection using epithelial cell line-selected ligands and fusogenic peptides. *Biochimica et biophysica acta*, 1475(3), pp.369–376. [https://doi.org/10.1016/S0304-4165\(00\)00093-3](https://doi.org/10.1016/S0304-4165(00)00093-3).

Vera-Sempere, F.J. *et al.* (2006) “Renal Donor Implication in the Origin of BK Infection: Analysis of Genomic Viral Subtypes,” *Transplantation Proceedings*, pp. 2378–2381. <https://doi.org/10.1016/j.transproceed.2006.08.049>.

Verghese, P.S. *et al.* (2015) “The impact of donor viral replication at transplant on recipient infections posttransplant: a prospective study,” *Transplantation*, 99(3), pp. 602–608. <http://dx.doi.org/10.1097/TP.0000000000000354>.

Vermeulen, K. *et al.* (2019) “Evaluation of [11C]NMS-E973 as a PET tracer for in vivo visualisation of HSP90,” *Theranostics*, 9(2), pp. 554–572. <https://doi.org/10.7150/thno.27213>

Vervoort, D.F.M. *et al.* (2021) “Dual Site-Selective Presentation of Functional Handles on Protein-Engineered Cowpea Chlorotic Mottle Virus-Like Particles,” *Bioconjugate chemistry*, 32(5), pp. 958–963. <http://dx.doi.org/10.1021/acs.bioconjchem.1c00108>.

Villagrana-Escareño, M.V. *et al.* (2019) “VLPs Derived from the CCMV Plant Virus Can Directly Transfect and Deliver Heterologous Genes for Translation into Mammalian Cells,” *BioMed research international*, 2019, p. 4630891. <http://dx.doi.org/10.1155/2019/4630891>.

Viscidi, R.P. *et al.* (2003) “Serological cross-reactivities between antibodies to simian virus 40, BK virus, and JC virus assessed by virus-like-particle-based enzyme immunoassays,” *Clinical and diagnostic laboratory immunology*, 10(2), pp. 278–285. <http://dx.doi.org/10.1128/cdli.10.2.278-285.2003>.

Viscidi, R.P. *et al.* (2011) “Age-specific seroprevalence of Merkel cell polyomavirus, BK virus, and JC virus,” *Clinical and vaccine immunology: CVI*, 18(10), pp. 1737–1743. <http://dx.doi.org/10.1128/CVI.05175-11>.

Viscidi, R.P. and Clayman, B. (2006) “Serological cross reactivity between polyomavirus capsids,” *Advances in experimental medicine and biology*, 577, pp. 73–84. http://dx.doi.org/10.1007/0-387-32957-9_5.

Vogt, T.C. and Bechinger, B. (1999) “The interactions of histidine-containing amphipathic helical peptide antibiotics with lipid bilayers. The effects of charges and pH,” *The journal of biological chemistry*, 274(41), pp. 29115–29121. <https://doi.org/10.1074/jbc.274.41.29115>

Voronkova, T. *et al.* (2007) “Hamster polyomavirus-derived virus-like particles are able to transfer in vitro encapsidated plasmid DNA to mammalian cells,” *Virus genes*, 34(3), pp. 303–314. <http://dx.doi.org/10.1007/s11262-006-0028-1>.

- Waddington, S.N. *et al.* (2008) “Adenovirus serotype 5 hexon mediates liver gene transfer,” *Cell*, 132(3), pp. 397–409. <http://dx.doi.org/10.1016/j.cell.2008.01.016>
- Wang, M. *et al.* (2004) “Inhibition of simian virus 40 large tumor antigen expression in human fetal glial cells by an antisense oligodeoxynucleotide delivered by the JC virus-like particle,” *Human gene therapy*, 15(11), pp. 1077–1090. <http://dx.doi.org/10.1089/hum.2004.15.1077>.
- Wang, P. *et al.* (2021) “Proximity ligation assay: an ultrasensitive method for protein quantification and its applications in pathogen detection,” *Applied microbiology and biotechnology*, 105(3), pp. 923–935. <https://doi.org/10.1007/s00253-020-11049-1>
- Wang, Q. *et al.* (2002) “Natural supramolecular building blocks. Cysteine-added mutants of cowpea mosaic virus,” *Chemistry & biology*, 9(7), pp. 813–819. [http://dx.doi.org/10.1016/s1074-5521\(02\)00166-7](http://dx.doi.org/10.1016/s1074-5521(02)00166-7).
- Wang, R.Y.L. *et al.* (2016) “The association between polyomavirus BK strains and BKV viruria in liver transplant recipients,” *Scientific reports*, 6, p. 28491. <http://dx.doi.org/10.1038/srep28491>.
- Wang, X.-L. *et al.* (2007) “Novel polymerizable surfactants with pH-sensitive amphiphilicity and cell membrane disruption for efficient siRNA delivery,” *Bioconjugate chemistry*, 18(6), pp. 2169–2177. <https://doi.org/10.1021/bc700285q>
- Warnke, C. *et al.* (2013) “An assay to quantify species-specific anti-JC virus antibody levels in MS patients,” *Multiple sclerosis*, 19(9), pp. 1137–1144. <http://dx.doi.org/10.1177/1352458513475489>.
- Waterboer, T. *et al.* (2005) “Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins,” *Clinical chemistry*, 51(10), pp. 1845–1853. <https://doi.org/10.1373/clinchem.2005.052381>
- Wei, B. *et al.* (2009) “Development of an antisense RNA delivery system using conjugates of the MS2 bacteriophage capsids and HIV-1 TAT cell-penetrating peptide,” *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, 63(4), pp. 313–318. <http://dx.doi.org/10.1016/j.biopha.2008.07.086>.
- Wibowo, N. *et al.* (2014) “Protective efficacy of a bacterially produced modular capsomere presenting M2e from influenza: extending the potential of broadly cross-protecting epitopes,” *Vaccine*, 32(29), pp. 3651–3655. <http://dx.doi.org/10.1016/j.vaccine.2014.04.062>.
- Wollebo, H.S. *et al.* (2015) “Persistence and pathogenesis of the neurotropic polyomavirus JC,” *Annals of neurology*, 77(4), pp. 560–570. <http://dx.doi.org/10.1002/ana.24371>.
- Wong, A.S.Y. *et al.* (2007) “Relationship of pretransplantation polyoma BK virus serologic findings and BK viral reactivation after hematopoietic stem cell transplantation,” *Clinical infectious diseases*, 44(6), pp. 830–837. <https://doi.org/10.1086/511863>
- Wu, Y. *et al.* (2019) “Patchy Amphiphilic Dendrimers Bind Adenovirus and Control Its Host Interactions and in Vivo Distribution,” *ACS nano*, 13(8), pp. 8749–8759. <http://dx.doi.org/10.1021/acsnano.9b01484>.
- Wunderink, H.F. *et al.* (2017a) “Stability of BK polyomavirus IgG seroreactivity and its correlation with preceding viremia,” *Journal of clinical virology*, 90, pp. 46–51. <https://doi.org/10.1016/j.jcv.2017.03.015>
- Wunderink, H.F. *et al.* (2017b) “Pretransplantation donor-recipient pair seroreactivity against BK Polyomavirus predicts viremia and nephropathy after kidney transplantation,” *American*

journal of transplantation, 17(1), pp. 161–172. <https://doi.org/10.1111/ajt.13880>

Wunderink, H.F. *et al.* (2019a) “Source and Relevance of the BK Polyomavirus Genotype for Infection After Kidney Transplantation,” *Open forum infectious diseases*, 6(3), p. ofz078. <http://dx.doi.org/10.1093/ofid/ofz078>.

Wunderink, H.F. *et al.* (2019b) “Development and evaluation of a BK polyomavirus serotyping assay using Luminex technology,” *Journal of clinical virology*, 110, pp. 22–28. <http://dx.doi.org/10.1016/j.jcv.2018.11.009>.

Xiao, Q. *et al.* (2022) “The effects of protein corona on in vivo fate of nanocarriers,” *Advanced drug delivery reviews*, 186(114356), p. 114356. <https://doi.org/10.1016/j.addr.2022.114356>

Xiao, W. *et al.* (2021) “The protein corona hampers the transcytosis of transferrin-modified nanoparticles through blood-brain barrier and attenuates their targeting ability to brain tumor,” *Biomaterials*, 274(120888), p. 120888. <https://doi.org/10.1016/j.biomaterials.2021.120888>

Xu, Y.-F. *et al.* (2006) “Papillomavirus virus-like particles as vehicles for the delivery of epitopes or genes,” *Archives of virology*, 151(11), pp. 2133–2148. <http://dx.doi.org/10.1007/s00705-006-0798-8>.

Yildiz, I. *et al.* (2011) “Applications of viral nanoparticles in medicine,” *Current opinion in biotechnology*, 22(6), pp. 901–908. <http://dx.doi.org/10.1016/j.copbio.2011.04.020>.

Youn, J.-I. *et al.* (2008) “Enhanced delivery efficiency of recombinant adenovirus into tumor and mesenchymal stem cells by a novel PTD,” *Cancer gene therapy*, 15(11), pp. 703–712. <http://dx.doi.org/10.1038/cgt.2008.45>.

Yu, D. *et al.* (2011) “Adenovirus with hexon Tat-protein transduction domain modification exhibits increased therapeutic effect in experimental neuroblastoma and neuroendocrine tumors,” *Journal of virology*, 85(24), pp. 13114–13123. <http://dx.doi.org/10.1128/JVI.05759-11>.

Zackova Suchanova, J. *et al.* (2017) “Retargeting Polyomavirus-Like Particles to Cancer Cells by Chemical Modification of Capsid Surface,” *Bioconjugate chemistry*, 28(2), pp. 307–313. <http://dx.doi.org/10.1021/acs.bioconjchem.6b00622>.

Žáčková Suchanová, J. (2018) *Targeting of viral nanoparticles to cancer specific receptors*. Edited by H. Španielová. PhD. Charles University.

Zeitlinger, M.A. *et al.* (2011) “Protein binding: do we ever learn?,” *Antimicrobial agents and chemotherapy*, 55(7), pp. 3067–3074. <https://doi.org/10.1128/AAC.01433-10>.

Zeng, Q. *et al.* (2013) “Cucumber mosaic virus as drug delivery vehicle for doxorubicin,” *Biomaterials*, 34(19), pp. 4632–4642. <http://dx.doi.org/10.1016/j.biomaterials.2013.03.017>

Zhang, L. *et al.* (2023) “Virus-like Particles as Antiviral Vaccine: Mechanism, Design, and Application,” *Biotechnology and bioprocess engineering: BBE*, pp. 1–16. <http://dx.doi.org/10.1007/s12257-022-0107-8>.

Zhao, Q. *et al.* (2011) “Self-assembled virus-like particles from rotavirus structural protein VP6 for targeted drug delivery,” *Bioconjugate chemistry*, 22(3), pp. 346–352. <http://dx.doi.org/10.1021/bc1002532>.

Zheng, H.-Y. *et al.* (2007) “Relationships between BK virus lineages and human populations,” *Microbes and infection / Institut Pasteur*, 9(2), pp. 204–213. <http://dx.doi.org/10.1016/j.micinf.2006.11.008>.

Zhong, S. *et al.* (2009) “Distribution patterns of BK polyomavirus (BKV) subtypes and

- subgroups in American, European and Asian populations suggest co-migration of BKV and the human race,” *Journal of General Virology*, pp. 144–152. <https://doi.org/10.1099/vir.0.83611-0>.
- Zielonka, A. *et al.* (2006) “Generation of virus-like particles consisting of the major capsid protein VP1 of goose hemorrhagic polyomavirus and their application in serological tests,” *Virus research*, 120(1–2), pp. 128–137. <http://dx.doi.org/10.1016/j.virusres.2006.02.010>.
- Zielonka, A. *et al.* (2011) “Detection of chimpanzee polyomavirus-specific antibodies in captive and wild-caught chimpanzees using yeast-expressed virus-like particles,” *Virus research*, 155(2), pp. 514–519. <http://dx.doi.org/10.1016/j.virusres.2010.12.009>.
- Zielonka, A. *et al.* (2012) “Serological cross-reactions between four polyomaviruses of birds using virus-like particles expressed in yeast,” *The Journal of general virology*, 93(Pt 12), pp. 2658–2667. <http://dx.doi.org/10.1099/vir.0.044917-0>.
- Zila, V. *et al.* (2014) “Involvement of microtubular network and its motors in productive endocytic trafficking of mouse polyomavirus,” *PloS one*, 9(5), p. e96922. <http://dx.doi.org/10.1371/journal.pone.0096922>.
- Zvirbliene, A. *et al.* (2006) “Generation of monoclonal antibodies of desired specificity using chimeric polyomavirus-derived virus-like particles,” *Journal of immunological methods*, 311(1–2), pp. 57–70. <http://dx.doi.org/10.1016/j.jim.2006.01.007>.
- Zvirbliene, A. *et al.* (2014) “The use of chimeric virus-like particles harbouring a segment of hantavirus Gc glycoprotein to generate a broadly-reactive hantavirus-specific monoclonal antibody,” *Viruses*, 6(2), pp. 640–660. <http://dx.doi.org/10.3390/v6020640>.