

Recombinant Virus Like Particles as Drug Delivery System

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Abstract: The drug delivery system described here is based on a virus like particle consisting of the recombinant expressed major capsid protein of Polyomavirus, VP1. Polyoma, a murine virus belonging to the *Papovaviridae*, forms a non-enveloped icosahedral capsid. These capsids are organized as a double shell composed of three different proteins: VP1, VP2 and VP3. The outer shell of the virus is composed of 360 VP1 molecules arranged as 72 pentamers. These capsids have a diameter of about 50 nm. The VP1 protein acts as a major ligand for certain membrane receptors during virus infection. Furthermore, the N-terminus of the VP1 protein contains a DNA-binding domain and a nuclear localization sequence. The recombinant production of the VP1 protein offers a save way to obtain a highly purified, non-pathogenic pharmaceutical excipient. Combining these aspects, VP1 proteins provide a targeting as well as a drug binding site when used as a save drug carrier for gene therapy. Current applications are also including oligonucleotides as well as small molecules as well as vaccines.

Key Words: Artificial virus, virus like particle, VP1 capsids, drug delivery system.

1. INTRODUCTION

Drug delivery is one of the main challenges for pharmaceutical biotechnology. Recombinant products could be used as excipients more frequently in the future such as viral proteins and polymers. In this review we will focus on a viral protein derived from *Papovaviridae*.

The family name *Papovaviridae* is made up of the initials of the most important representatives: rabbit papilloma virus (**pa**), mouse polyoma virus (**po**) and vacuolating virus (=simian virus 40) (**va**) [1]. Representatives of this family are characterised by capsids of small, non enveloped icosahedral viruses [2]. The genome of these viruses is composed of a covalently closed circular double-stranded DNA molecule [3]. The *Papovaviridae*-family can be subdivided into two independent subfamilies: *Papillomaviridae* and *Polyomaviridae*. They differ in their genomic organisation and in their composition of the capsoids. *Papillomaviridae* are defined as virions, which are 55 nm in diameter and the molecular weight of the DNA amounts to $5 \cdot 10^6$ Dalton. Whereas *Polyomaviridae* are defined as virions, which are smaller in their diameter (45 nm) and the molecular weight of the DNA adds up to $3 \cdot 10^6$ Dalton. These viruses are oncogenic in hosts [4]. An abridgement of the most important *Papovaviridae* is shown in Table 1, as well as the genomic quantity and the molecular weight of the capsid proteins. Table 2 displays the molecular weight and the percentage arrangement of viral proteins of the two primary representatives of *Papillomaviridae*.

Two exponents of the *Polyomaviridae* utilise humans as their host: JC-virus and BK-virus. JC virus is the etiological

agent of progressive multifocal leukoencephalopathy (PML), which is the only known viral demyelinating disease of humans [5]. PML is the result of JC virus lytic infection of oligodendrocytes [6], whereas BK virus was first isolated from the urine of an immunosuppressed renal transplant recipient [7]. It infects human in childhood and persists in the kidney, apparently without causing disease [8]. These two viruses are thought to have similar structures to simian virus 40 (SV 40) and murine polyomavirus based on their high sequence homology as well as similarity of their gene organization [7].

For the first time SV 40 – very similar to murine polyomavirus – was identified as a contaminant in polio vaccines produced from rhesus monkey kidney cell cultures [8]. This monkey virus is understood to be oncogenic for newborn hamsters. Large epidemiological studies have not appeared an increased cancer risk in humans who received SV 40-contaminated vaccines, but fragments of SV 40 DNA have retrieved in specific human tumours [9].

The most important papilloma viruses are named after their host: rabbit, human, bovine, canine and hamster. They differ in their composition of the capsoids from *polyomaviridae*.

2. STRUCTURE

Polyomaviruses and papilloma viruses belong to the papovavirus family. In turn this virus family belongs to the non enveloped dsDNA viruses. Both subfamilies are alike in their apparition, but their capsids vary in their capsomer morphology and the intercapsomere associations, which cause capsids assembly [10]. Because murine polyomavirus, simian virus 40 (SV 40) and human polyomaviruses are characterized best due to their small genome size [8], we will turn our main attention to these viruses. Most properties are transferable to the other representatives as well.

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Table 1. Survey of the Subfamily Polyomaviridae

Virus	Host	Genomic quantity [bp]	Virus capsid protein [kDa]		
			VP1	VP2	VP3
Polyomavirus	Mice	5392	42	35	22
Simian Virus 40 (SV 40)	Rhesus monkey	5243	40	39	26
JC-Virus	Man	5130	39	38	25
BK-Virus	Man	5133	40	39	26
Lymphotropic Papovavirus	Vervet monkey	5270	40	39	26
Bovine Polyomavirus	Cow	4967	40	39	26
Hamster Polyomavirus	Hamster	5366	41	38	24
Kirstenvirus	Mice	4754	41	35	24
Rabbit Polyomavirus	Rabbit	n.d.	n.d.	n.d.	n.d.
Rat Polyomavirus	Rat	n.d.	n.d.	n.d.	n.d.
Simian Agent 12	Baboon	n.d.	n.d.	n.d.	n.d.
Budgerigar fledgling disease virus	Budgerigar	4980	38	38	26

n.d.= not determined

Modified assumed of [1]

Table 2. Survey of the Subfamily Papillomaviridae

Virus	Polypeptide classification	Molecular Weight [kDa]	Overall protein [%]
Rabbit papilloma virus	VP1	85	11
	VP2	70	21
	VP3	60	48
	VP4	19	9
	VP5	15	7
	other		4
Human papilloma virus	VP1	115	8
	VP2	100	1
	VP3	85	9
	VP4	63	60
	VP5	51	17
	VP6	14	1
	other		2

Modified assumed of [4]

The capsids of polyomaviruses are non enveloped and icosahedral in shape [2, 11]. The diameter of these capsids amounts to approximately 45 nm [12]. They are made up of three viral-encoded proteins, VP1, VP2 and VP3, which embed the viral chromatin composed of virus DNA and the cellular histones H2A, H2B and H4 [13, 14].

The three structural proteins VP1, VP2 and VP3 of the capsids are found in different ratios. VP1 displays with 75 % the major structural protein [15]. The outer shell of the capsid is composed of 72 pentameric VP1 proteins also referred to as capsomeres [3] (Fig. 2). These pentamers are set up in 60 hexavalent pentamers and 12 pentavalent

pentamers. Each pentavalent pentamer is encircled by further five pentamers, whereas each hexavalent pentamer by further six pentamers [16] (Fig. 1). The carboxy-terminal arms of VP1 stabilize the pentamers among each other [13], whereupon the amino-terminal arms are of importance in the case of binding polyomavirus DNA [17]. Every VP1 pentamer is associated with one of the two related minor coat proteins VP2 or VP3 [17]. The C-terminus of VP2 or VP3 is linked into the axial loop of a VP1 pentamer *via* hydrophobic interactions [18]. These minor structural proteins are not essential for capsids formation [19]. They are thought to connect the viral chromatin to the VP1 capsomers [13].

The polyoma genome is made up of covalently closed circular double-stranded DNA molecules [20] about 5000 base-pairs in size (Table 1). The replication cycle is classified into early and late stages [7] of infection. The early and late regions of the virus genome are responsible for this. During virus infection in permissive cells first of all protein biosynthesis of the early proteins takes place, then the virus DNA replication starts and finally the late proteins are synthesized. The transcription processes bi-directional from the initial point. The early and late mRNAs are read off from the contraire DNA strands.

The early region comprises the sequence for proteins referred to as tumour antigens (T antigens). SV 40 and related viruses codify for the small T and the large T antigen, whereas murine polyomavirus and related codify additional for the middle T antigen [8]. The various antigens have different features. The large T antigen is mainly located in the nucleus and regulates the early transcription *via* binding to viral DNA in the region of the early promoter [8]. The small T antigen is placed between the nucleus and the cytoplasm and concerns in the accumulation of viral DNA [8], whereas the middle T antigen of murine polyomavirus is

mainly detectable in the plasma membrane. It is fundamental for cell transformation [8].

During late stage of infection the three structural proteins VP1, VP2 and VP3 are synthesized. The accordant genes overlap on the genome, but are translated from three different mRNAs [21]. In the case of SV 40 and akin viruses there exists a fourth late protein referred to as agnoprotein. So far its role is unknown [8].

The genetic information of the N-terminal of the major protein VP1 overlaps with the C-terminal of the minor capsids proteins VP2 and VP3 encoding region. The sequence of VP3 is an integral part of the gene of VP2. They share the same stop codon [17].

Six distinct species of VP1 were found in the total polyomavirus, whereas the capsids were composed of only four subspecies [22]. The amino acid sequences of all six species are identical. The differences between the subspecies arise in the degree of modification like phosphorylation, acetylation, sulfation and hydroxylation [23]. They can be separated due to their different pI's [22]. Because the structural proteins are synthesized at the ribosomes of the rough endoplasmic reticulum in the cytoplasm, the single components of the virus have to be transported into the cell nucleus to accumulate. A nuclear localization signal could be identified for all of the three capsid proteins. In the case of VP1 this sequence was demonstrated to be located within the first eleven amino acids of the N-terminus [24], whereas for VP2 and VP3 twelve amino acids of the C-terminus are responsible for transportation into the nucleus [25, 26]. Compared to VP2 and VP3, VP1 is able to migrate into the cell nucleus on its own. It could be shown that VP1 interacts with VP2 and VP3 in the cytoplasm to efficiently transport the minor proteins into the nucleus [21, 27].

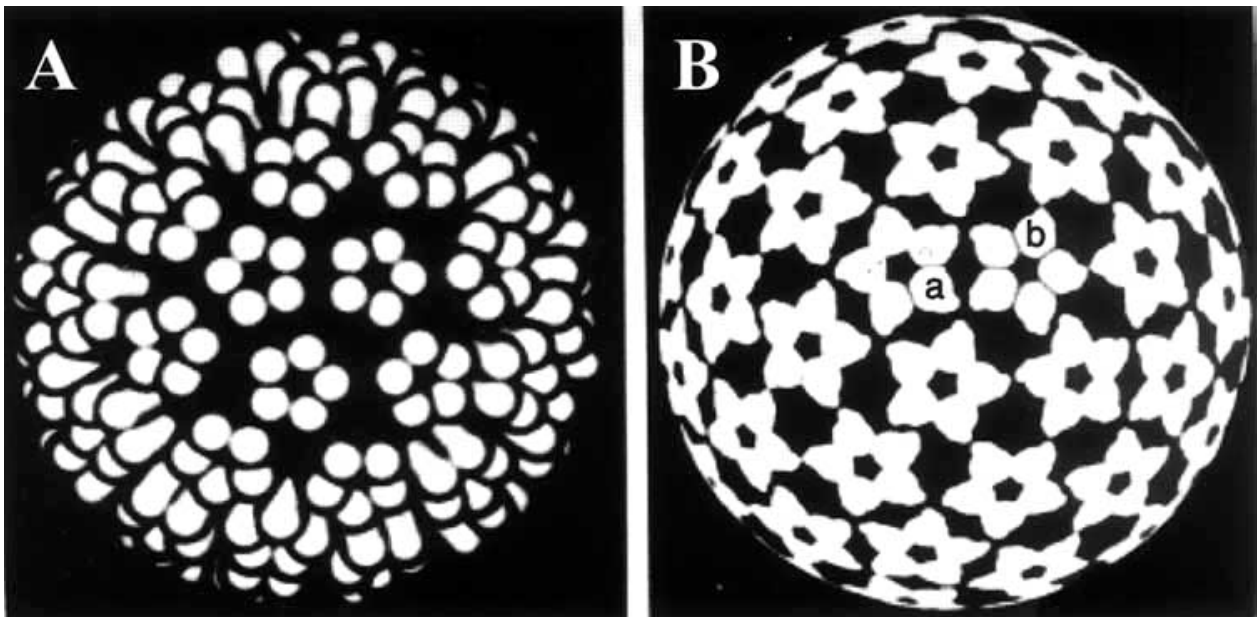


Fig. (1). Computer graphical illustration of VP1 pentamers of the polyomavirus capsid.

Figure A displays the surface morphology of the capsid. The diameter of the capsid amounts to approximately 50 nm and whereas the diameter of the pentamers adds up to 8.5 nm. Figure B shows the pentavalent pentamers (a) and the hexavalent pentamers (b). The figures are modified assumed of [19].

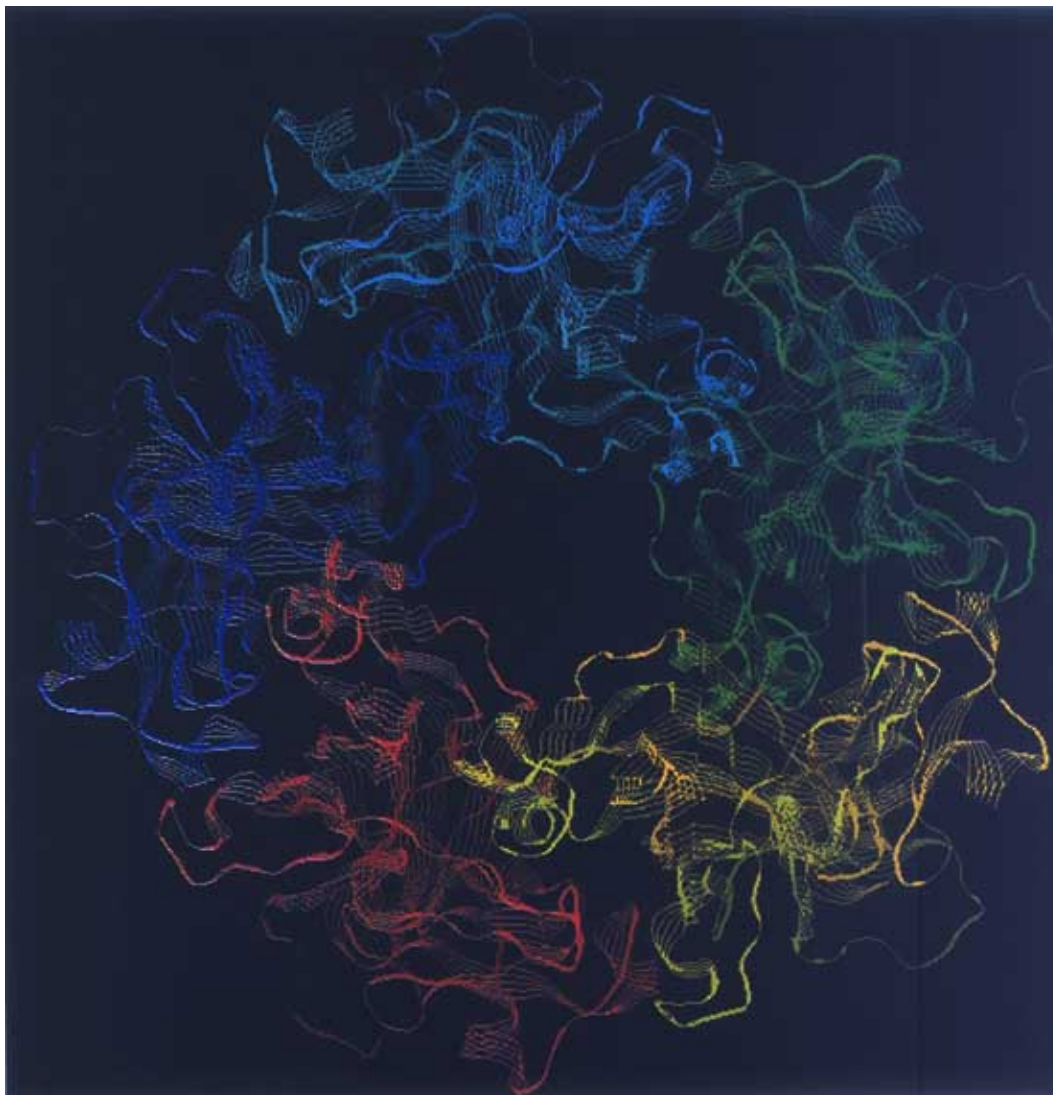


Fig. (2). Computer graphical illustration of the structure of one VP1 pentamer.

The figure shows the outside of the VP1 pentamer complex in a computer graphical adaptation with RasMol based on the coordinates of Stehle and Harrison [53]. The VP1 monomers are demonstrated in separate colours.

3. CELLULAR UPTAKE

Mackay and Consigli observed two forms of penetration in primary fetal mouse kidney cells (MKC), which were permissive for the polyomavirus [28]. On the one hand virus particles were taken up by monopinocytotic vesicles through the cell membrane. The virus was enclosed by the membrane and was carried through the cytoplasm to the nucleus. Normally, the native virus is then uncoated and the replication process can begin (productive infection).

On the other hand empty capsids were absorbed as phagocytotic vesicles into the cells and were degraded in lysosomes (non-productive infection). In this case a specific transport to the outer cell membrane could not be observed.

Further investigation by Griffith [29] approved that the cellular uptake of the viruses depended on the binding on specific receptors (receptor mediated endocytosis). The receptor binding is an important attribute for the pathology

of the different types of polyomaviruses. For the receptor recognition it seems to be important that N-acetyl neuraminic acid (sialic acid) is presented on the cell surface [30-35].

The transport of certain proteins to the nucleus has been shown to depend on nuclear localization signal (NLS) sequences residing in the proteins. NLS are highly basic amino acid stretches which were also found in VP1 (Ala₁-Cys₁₁) and VP2/3 (Arg₃₀₁-Pro₃₁₉). These sequences target the capsid to the nucleus [23].

4. PRODUCTION

Expression Systems

There are different cell lines used for recombinant expression of the structural proteins VP1, VP2 and VP3 of polyomaviruses. Insect cells, *Escherichia coli* and yeast are the mainly used cell types.

The structural proteins of murine polyomavirus [36], JC virus [37], SV 40 [13], avian polyomavirus [38] and monkey B-lymphatic papovavirus (LPV) [39] have been shown to be expressible in insect cells by a baculovirus vector [6]. Expression of the major structural protein VP1 of JC virus in the insect cell line Sf158 was achieved with the help of recombinant baculoviruses [5]. Another eucaryotic baculovirus-insect expression system was mentioned in [40] and [36]. In place they cloned the genes including the sequences for the VP1, VP2 and VP3 proteins of murine polyomavirus into a p2Bac dual multiple cloning site vector. This vector and the linear DNA of Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) are cotransfected into *Spodoptera frugiperda* (Sf9) cells. These cells are now able to express the adequate proteins.

Another frequently used expression system for VP1 from murine polyomavirus [19, 41, 42], avian polyomavirus, budgerigar fledgling disease virus [43] and L1 capsid protein of papilloma virus [44] displays *E. coli*.

The third procedure to express VP1 utilizes *Saccharomyces cerevisiae* (INVSC1) yeast cells. The VP1 gene is cloned into a yeast-expressing plasmid. Afterwards this plasmid is transfected into yeast cells [45, 46].

The integration of a complete mouse polyoma genome into the c-myc gene locus of tumour sublines arises the expression of c-myc significantly because of interactions between the polyoma proteins and c-myc [47].

Purification

After lyses of the producing cells and separation of cell constituents different purification methods can be used to achieve pure recombinant proteins.

This cellular lysate can be purified *via* cesium chloride (CsCl) density gradient centrifugation [24, 36, 48], whereas [12] purified the protein *via* chitin affinity chromatography. Another method of purification takes advantage of expression of the protein as a glutathione S-transferase (GST) fusion protein [18]. These fusion proteins can be deterged using affinity chromatography on immobilized glutathione [49]. The cleavage of the fusion proteins from the carrier results from digestion with site-specific proteases. Thrombin or blood coagulation factor Xa can act as such proteases [17, 49-51]. As well purification can be achieved *via* dialysis and proceeding to a phosphocellulose (P-11) column [41]. Over and over precipitation of the protein by addition of ammonium sulphate is also possible to purify the cell lysate [41]. To verify the amount of the purification the structural proteins could be separated by SDS-PAGE [24, 36, 37, 52].

Assembly

Expressed and purified VP1 monomers immediately accumulate to pentamers [19]. These capsomeres assemble into capsids and polymorphic aggregates by increasing the ionic strength of the solution [19, 50]. To stabilize these virus like particles even at low ionic strength, calcium has to be added [16, 50]. In addition disulfide bounds stabilize the capsids. By the use of a VP1 mutant with only two of the originally six cysteines (position 19 and 114) it could be

shown that only intrapentamer but no interpentamer disulfid bounds were necessary for stabilization and complete virus assembly [12]. By the deletion of 57 amino acids at the C-terminus of the major structural protein VP1 it was shown that capsomeres are formed, but these pentamers are unable to associate into capsid structures like the wild type [52]. This flexible C-terminus establishes a connection between proximate VP1 capsomeres [53].

Loading

Barr *et al.* (1979) described for the first time the production of polyoma like particles (PLP) [54]. These particles were composed of purified, empty capsids and polyoma DNA. The DNA uptake was achieved by an osmotic shock. First of all a complex between the empty capsids and the DNA was performed. Afterwards an incubation in sterile distilled water at 37°C took place to load the capsids [20, 50]. These particles were stable in high salt concentrations. The integrated double-stranded DNA was protected against pancreatic DNase degradation [55] and the molecular weight amounted to approximately $1,1 \times 10^6$ Dalton [54]. Polyoma virus pseudocapsids or as well referred to as virus like particles (VLP) just composed of VP1 are able to incorporate exogenous DNA till 3kbp [56]. Not only linear, circular or supercoiled polyoma DNA but also single-stranded DNA (antisense oligonucleotides), rRNA and synthetic homopolymers are shown to be encapsulated into VLPs to enhance cellular uptake into cell [51, 57-59]. To increase the transfer of larger DNA fragments (greater than 3kbp), a possible application could be DNA transfection for gene therapy, the DNA was complexed with the polycation polylysine. Afterwards these complexes were encapsulated in VLPs to protect the DNA against degradation [56]. Pseudocapsids are shown to interact *in vitro* with double-stranded DNA in two different ways. On the one hand VP1-VLPs form very strong complexes depending on the free ends of the DNA, on the other hand they interact feebles with internal fragments of the DNA [60].

The major structural protein VP1 is able to bind DNA in contrast to both minor structural proteins VP2 and VP3 [61]. The DNA binding domain was detected in the N-terminal first five amino acids of VP1 (Ala-1-Pro-Lys-Arg-Lys-5). This domain overlaps with the nuclear localization signal area and displays to be non-sequence specific referring to the DNA [62]. It is shown that VP1 binds to the DNA in a very high affine way, but this affinity is stronger for single-stranded DNA than for double-stranded DNA [63].

It could be demonstrated that virus like particles composed of VP1 (VP1-VLP) are more efficient in transporting heterologous DNA into different tissues than DNA on its own [64]. These particles are an useful tool to transfer biologically functional genetic informations into targeted cells [65, 66]. Using VLPs loaded with DNA significant higher long-term levels of expression could be achieved [67].

Murine polyomavirus VP1 virus like particles reveal a great immune response to mice. Both serum and mucosal antibodies are accumulated and cells start to proliferate. That is inappropriate for gene therapy but may be useful for vaccination [68].

VP1-VLPs are shown to be an useful tool to incorporate not only DNA but also small biological molecules and to improve the specific delivery of these molecules to their target cells. That was proved using the fluorescence dye propidium iodide (PI), which is easily detectable by flow cytometry [69].

Targeting

VLPs have demonstrated their potential in transfecting mammalian cells [50, 51, 58, 59, 65]. In the case of cell transfection, the VLPs are bound to sialic acid residues which are present on almost all cells of higher eukaryotes. To achieve a cell type specific targeting of this potential useful vector system, new functional domains have to be established on the surface of VP1. One attempt is the insertion of single chain Fv fragments, which are the smallest antigen binding antibody fragments. However, this strategy is limited by the modification of the virus coat protein and the development of single chain Fv fragments. Another strategy is the insertion of an immunoglobulin binding domain from *Staphylococcus aureus*, the protein Z [70-72]. This allows working with a once modified and characterized viral coat protein in several different applications.

5. OUTLOOK

Virus like particles display an excellent system for drug delivery *in vitro*. *In vivo* application turns out to be more difficult. Murine VP1-VLPs causes an antibody response in normal and immunodeficient mice [64, 73]. T-cell-independent antiviral IgM and IgG response was tested as well on T-cell-deficient mice. These animals were infected with the soluble structural protein VP1, virus like particles and live polyomavirus. IgM response arose from each of the viral antigens, whereas IgG production was only detectable *via* immunization with the live virus [74]. These properties can be exploited to clone highly immunogenic virus like particles in order to develop new vaccines [75]. The current status of using VLPs as subunit vaccines is described by Noad (2003). Thereabouts it is reported that virus like particles mimic the structure of real virus particles without being infectious because of the lack of the pathogenous genome. Thereby safe, they are very capable due to their authentic conformation. There seems to be no limits in the use of different virus types or families [76].

In addition Beyer *et al.* [77] described the great potential of VP1 capsids as heterologous drug carriers, especially for the delivery of foreign protein antigens. VLPs were well suited for evoking a protective immune response on several routes of vaccine administration. Both, humoral and cell-mediated immunity was observed. The prominent advantage of these drug delivery devices was presented by the capacity to target antigenic proteins or DNA vaccines to immature dendritic cells along their maturation pathway.

In contrast to the polyomaviruses, virus like particles made up of the major capsid protein L1 of the genital human papilloma viruses (HPVs) are meantime in phase III clinical trials for vaccination. They are to be applied against low-grade benign genital infections [78, 79].

Another idea to improve virus like particles for the use as drug delivery systems displays the employment of the structural protein VP2 as molecular anchor [18]. This is intended to arise the loading rate of the particles, because the active agents are covalently bound. These agents are associated with VP2 *via* cross-linker. In turn each pentamer of the VP1 capsid is ligated to one of the VP2 molecules. According to this the maximum capacity of loading comes up to 72 VP2 proteins. The linkage is even more stable and specific [18].

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