Viruses as a tool in nanotechnology and target for conjugated polymers

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Chapter 1 Virus-like particles with natural and unnatural cargoes

1.1 Viruses – general introduction

A virus is a supramolecular assembly of a nucleic acid and a dense, protective layer of proteins. Certain viruses are assembled in a more complex way than others. They can carry along own enzymes or they can be enveloped with a lipid membrane of the host origin, such as the Human Immunodeficiency Virus (HIV). Some also exhibit morphologically distinct domains, for instance the bacteriophage T4, which has a head, where genetic material is stored, and a tail responsible for cell puncture and gene transfer. Viruses have evolved into extremely efficient gene delivery vehicles that can infect cells from every taxonomic kingdom[1,2]. They are able to replicate in the host cell using hijacked cellular machinery, however, they do not belong to living matter. The genome of viruses consists of DNA or RNA and is relatively easy to manipulate. In their "life cycle" they need to withstand harsh extracellular conditions, which is only possible because of the outstanding protective performance of the viral capsid.

The first virus was discovered in 1898 by Dutch biologist Martinus Beijerinck. He extracted an agent infecting tobacco leaves, which was later named as the *tobacco mosaic virus* (TMV). TMV was also the first virion to be purified from infected plant extracts and crystallized in 1935. Therefore, it has served as a model to study and understand

properties of viruses already for more than 100 years. The thorough understanding of TMV makes it the perfect candidate for versatile applications in biomaterial science[3]. Today TMV can be purified in gram scale, subjected to multiple re-assembly cycles and equipped with functionalities which can be controlled on a genomic level.

1.1.1 Diversity in viral morphology

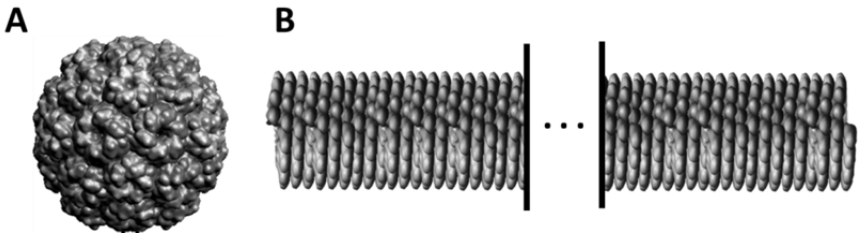

Figure 1.1. Two main morphologies of viruses represented by: (A) icosahedral CCMV, and (B) filamentous TMV. ***Structures obtained from RCSB protein data bank (TMV, www.pdb.org) and http://viperdb.scripps.edu (CCMV.)***

Viruses come in wide variety of size and shape[4]. The most abundant form is a spherical/icosahedral viral capsid represented by Cowpea Chlorotic Mottle Virus (CCMV, Figure 1.1A), bacteriophage MS2 or adenoviruses. Rod-like geometries are represented by TMV (Figure 1.1B), Potato Virus X (PVX) and bacteriophage M13. Their size ranges from 20 to 500 nm of diameter for icosahedral particles and can reach 2 μm for tubular ones. The cages can be hollow or massive, and exhibit different degree or porosity[4,5]. Furthermore the naturally occurring morphologies can be altered by changing the assembly conditions, which was extensively explored with plant viruses. For example, TMV subunits have been shown to form wedges, discs or stacks upon changes of pH and ionic strength of the environment[5]. CCMV on the other hand can yield particles with sizes smaller or bigger than the wild type (WT) virus or even tubes when its cargo is appropriately shaped[6,7].

1.1.2 Virus-like particles

Viruses and their empty capsids quickly filled the niche where nanosized, precisely defined objects with functionalizable groups were necessary. The term virus-like particle (VLP) was introduced to describe a biomimetic self-assembly that resembles a mature virion. The VLPs are however mostly non-infectious because their genetic material was

removed and/or replaced by a functional cargo. Infectious VLPs are often considered as a separate group and will be shortly discussed in section 1.2.6. It has to be noted that cages formed by non-viral proteins are also classified as VLPs, with ferritin as a prominent example[6]. However, they will not be discussed here.

In general, the morphology of VLP corresponds to that of the WT virus. Hence rod-like viruses were used to organize defined arrays of (poly)peptides, organic molecules and inorganic nanoparticles (NPs)[5]. Spherical capsids were in turn employed as nano-sized containers for bioimaging applications, reactors, drug delivery vehicles but also as templates to synthesise narrowly dispersed materials[4]. However, usually native viruses don't exhibit properties required for sophisticated applications in microelectronics, vaccine development or drug delivery, or at least, their assets are not sufficient. Thus they have to be subjected either to chemical modifications or genetic engineering to obtain the required functionality[8].

Currently the majority of all virus material is produced by plants, bacteria and yeasts or in cellular extracts[9]. Mammalian cells are used only in special cases to ensure specific properties[10,11].

1.1.3 Coat proteins

The unique properties of virus coat proteins (CPs) determine the possibilities of VLP construction. Viral CPs evolved to assemble into a highly symmetric protein shell around the genetic material. Their common feature is the presence of a positively charged domain, which is responsible for electrostatic interactions with the nucleic acids[12]. For example, the CP of CCMV has nine basic residues at the N-terminal region, while the entire CP of Ross River-type alphavirus (RRV) has an isoelectric point above 9.5[13,14]. Some viruses that lack positively charged domains are known to co-encapsulate a substantial number of polyamines[12]. Although complexation with the genome is important for self-assembly, it often is not crucial. It was shown that when the basic N'-terminus of CCMV and BMV CPs is removed, viruses still assemble in vivo in smaller, non-infective empty particles.[12] Moreover, side chains of CPs are often charged or polar and therefore respond to changes in pH and ionic strength of the environment, which might cause virus re-assembly. The main driving force of capsid formation is the hydrophobic interaction between apolar patches of the capsid proteins, which is

strong enough to overcome electrostatic repulsion of charged side chains[15-17]. Hydrogen bonds, salt bridges and Caspar carboxylate pairs play a secondary role in capsid stabilization although they regulate capsid stability upon changes in the environment. Overall the attractive interactions between CPs are rather weak to avoid formation of kinetic traps that would stop the virus from amplification[18,19]. Finally, the very conserved character of many viral CPs resulted in development of an artificial coat protein, which was subsequently assembled into the first synthetic virus[20].

1.1.4 Nucleation

Although virus capsid formation is often described as a spontaneous event, it is in fact a complicated thermodynamic process for a large class of viruses[16]. There are two factors that influence nucleation of a new capsid. The first parameter is the statistical probability of having a sufficient number of CPs together at one place for assembly. The other one is the recognition of a nucleation promoting factor, e.g. a RNA sequence or protein, which initiate conformational changes of the first CP to an associable conformation[21]. However, this is highly dependent on the virus. For example Brome Mosaic Virus (BMV) and Red Clover Necrotic Mosaic Virus (RCNMV) require a tRNA-like structure to initiate assembly, which is exhibited at the 3' end of the genomic RNAs[12,22]. On the other hand, TMV's CP forms a cylindrical disc composed of two layers of coat protein, a structure that requires the initial recognition of a specific RNA hairpin sequence[3]. In contrast, CCMV capsid assembly does not seem to be triggered by any specific sequence nor nucleating event. Regardless of the need of recognition, once the nucleation is triggered, conformational changes within CPs promote step-wise assembly of the virus. Theferofe it was suggested that an RNA molecule must be saturated with loosely bound CP, present in large excess[23,24].

1.1.5 The self-assembly

The self-assembly of viral proteins into a rigid shell protecting the viral genome is a fundamental step of its replication. Thus, understanding of the viral self-assembly is highly beneficial for the development of new antiviral therapies. It also plays a crucial role in the design of VLP drug delivery systems. Capsid formation is a sequence of reversible reactions involving gain and loss of single CP molecules[16]. In nature this process is self-regulated and any flaws in assembled virions can lead to

reassembly of the nucleoprotein complexes[16]. Still, viral CPs exhibit relatively high degree of cooperativity[25]. Under the right salinity, pH, protein concentration and temperature, many viruses, including Hepatitis B Virus (HBV), Human Papillomavirus (HPV), and already mentioned CCMV, BMV and TMV, form capsids that exhibit a morphology that is identical to the WT virus. Interestingly, non-native structures emerge too. It is known that a virus capsid does not exhibit a fixed radius of curvature. In such a case there would be only one possible capsid size per virion and a limited range of RNA sizes would fit in. However, the capsid diameters of natural viruses do not increase proportionally to increase of RNA length. Therefore, there must be a constrain and certain predetermined capsid size which the coat protein favours. The conformational switching of CPs is still poorly understood although it was extensively studied with CCMV, BMW, and TMV[16,25,26].

1.2. Virus-like particles – core modification

The size- and shape-defined interior of the viral capsid was promptly recognized as a container that could revolutionize the field of nanotechnology. First proof-of-principle experiments were conducted nearly 50 years ago[27-29]. The WT viruses were disassembled in vitro and, after removal of their genetic material, assembled again into VLPs (Figure 1.2A). Noteworthy is that native as well as altered morphologies were already mentioned in these first reports[30]. The early findings led to development of several strategies for VLP formation, in which particles could be equipped with diverse cargo. The first possibility is to employ passive loading, which utilizes pores that are present in the protein shell. This does not require capsid disassembly and can be performed with native viruses. Many viruses tend to swell upon pH changes, which is a natural phenomenon involved in the cargo release in the intracellular environment. Here, small molecules can enter the swollen capsid and remain trapped in the protein cage after the pores close (Figure 1.2B). In a similar fashion the protein skeleton can be saturated with inorganic anions or cations that mineralize in the interior of the capsid (Figure 1.2C). The third possibility is the random statistical entrapment of cargo in a protein shell upon re-assembly of the virus (Figure 1.2D). This strategy is based on reversible assembly of RNA-free VLPs at low pH. As such virtually any payload can be entrapped in the virus capsids when added to neutral CP solutions, which are subsequently dialyzed against a low pH buffer. However, the low pH is

not required for VLP assembly in the presence of polyanionic species as these mimic interactions of the CP with genetic material (Figure 1.2E).

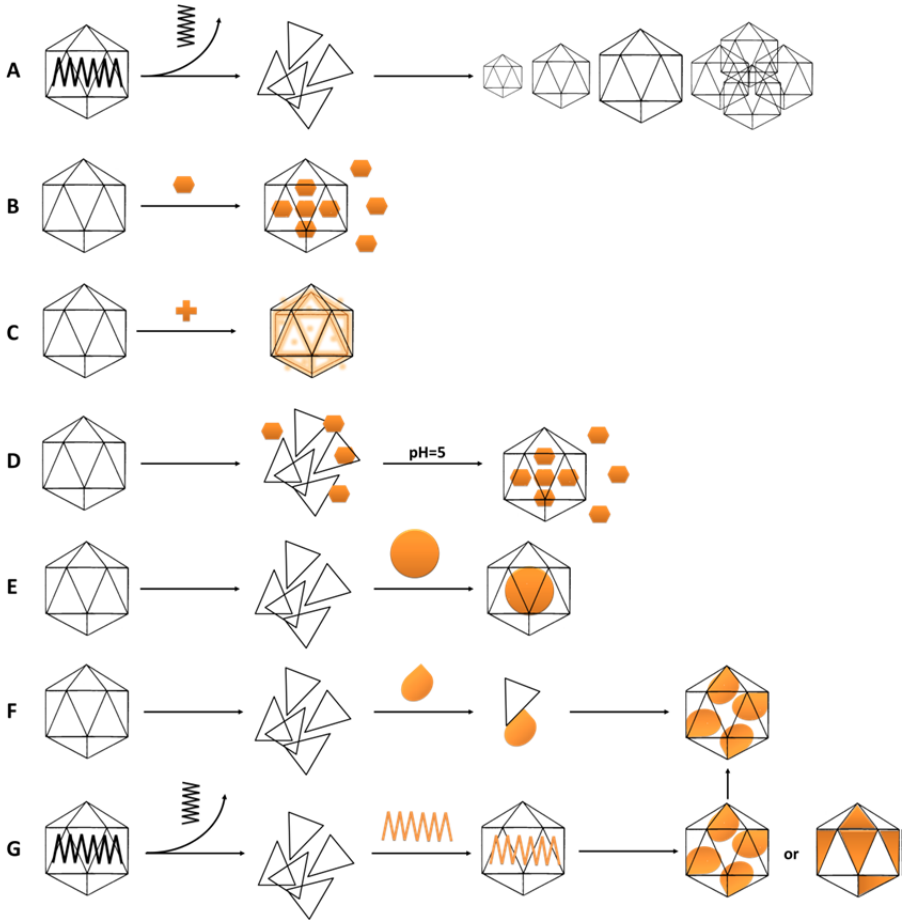

Figure 1.2. Strategies towards assembly of functional virus-like particles. (A) Reassembly of capsids in vitro can lead to an altered morphology; (B) Virus capsid in a swollen state can be saturated with small molecules in a passive way; (C) Saturation of interior of the virus cage with anionic or cationic species involves electrostatic interactions with amino acids and can be utilized in (bio)mineralization of the NPs; (D) Statistical entrapment of any cargo is accomplished by utilizing low pH, which induces VLP self-assembly; (E) CPs can assemble into VPLs on anionic templates yielding wild type or altered morphology. Precisely positioned modifications in the interior of the capsid are achieved by chemical modification of the CP (F) or via genetic manipulations (G).

This method represents a more elegant strategy for VLP formation because it resembles the natural process. However, it is not easily accomplished for small molecules. Last, the interior of the capsid can be subjected to chemical (Figure 1.2F) or genetic (Figure 1.2G) manipulations in order to introduce functional moieties inside of the VLP. There are also less general approaches, which are often virus specific. For example, P22 bacteriophage utilizes scaffolding proteins, which can be also modified with a molecule of choice and co-packed[31].

The intriguing capsid self-assembly processes resulted in incorporation of a wide range of molecular structures. This section highlights the diversity of cargos that were encapsulated in protein shells of viral origin. Proof-of-concept experiments were conducted with genetic material extracted from other species and with synthetic polymers. Subsequently spherical inorganic nanoparticles (INPs) for various applications were incorporated in the virus hybrids, followed by small molecules, including metal cations, dyes and drugs. Moreover, amphiphiles were exploited for loading of hydrophobic payloads while loading of proteins led to the development of enzymatic reactors.

1.2.1 Polymers

Negatively charged polymers were quickly recognized and evaluated as alternative cargo to template VLPs[30]. In fact they were the second evaluated scaffold that induced VLP formation, after foreign RNA. Polystyrene sulfonic acid (PSS) is the most widely explored synthetic polyanion that facilitated formation of CCMV (Figure 1.3), as well as Hibiscus Chlorotic Ringspot Virus (HCRSV) and BMV VLPs. Furthermore, polyacrylic acid and dextran sulfate were also successfully employed for capsid formation, which shows that the phosphate backbone is not a prerequisite for CP assembly[30]. Also molecular dynamic simulations studies were performed to further understand the process[32].

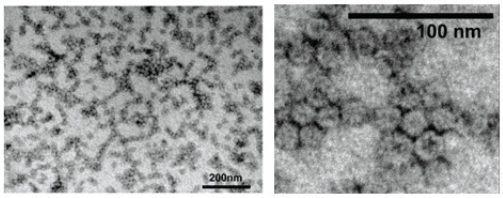

Figure 1.3. VLPs formed by CCMV coat protein on 70 kDa PSS template, decorated with 3 kDa PEG units on the exterior. ***Figures reproduced from reference [33].***

Polyanions up to 3.4 MDa were successfully evaluated to act as cargo in several experiments. However, it was found that application of high molecular weight PSS led to formation of closely connected spherical capsids with different geometries. Therefore, it was suggested that long polymers are encapsulated in multiple virus particles[34]. Interestingly, systems comprised of two polymers were also introduced. For example, CCMV CP chemically modified with polyethylene glycol (PEG) chains was successfully assembled on a PSS-template as shown in Figure 1.3[33]. Additionally, PSS proved to facilitate loading of doxorubicin (DOX) as functional cargo, which was suggested as a general co-loading strategy for small molecules[35].

1.2.2 Inorganic nanoparticles and metallic species

Gold NPs

Gold nanoparticles (AuNPs) exhibit unique optical and physical properties which makes them widely applicable in sensing systems, drug delivery and in the development of photothermally active agents[36]. These inorganic NPs are available in different sizes and they are relatively easy to work with due to straightforward surface functionalization with thiols. Therefore they were extensively utilized in VLP nucleation experiments. The AuNPs-containing VLPs were successfully formed by BMV (Figure 1.4A), Red Clover Necrotic Mosaic Virus (RCNMV), and several mammalian viruses: Simian Virus 40 (SV40), Ross River alphavirus (RRV) and even HIV capsid proteins[6,37]. In all cases, AuNPs needed to be surface-functionalized with negatively charged species, for example carboxylated PEG oligomers or short DNA sequences. The optimal core size was found to be in the range of 6 to 12 nm. Aside from such systematic studies on cores sizes or ligands facilitating assembly, encapsulated gold nanoclusters can be utilized to develop spectroscopic detection techniques and therapeutic or diagnostic agent delivery vehicles[38].

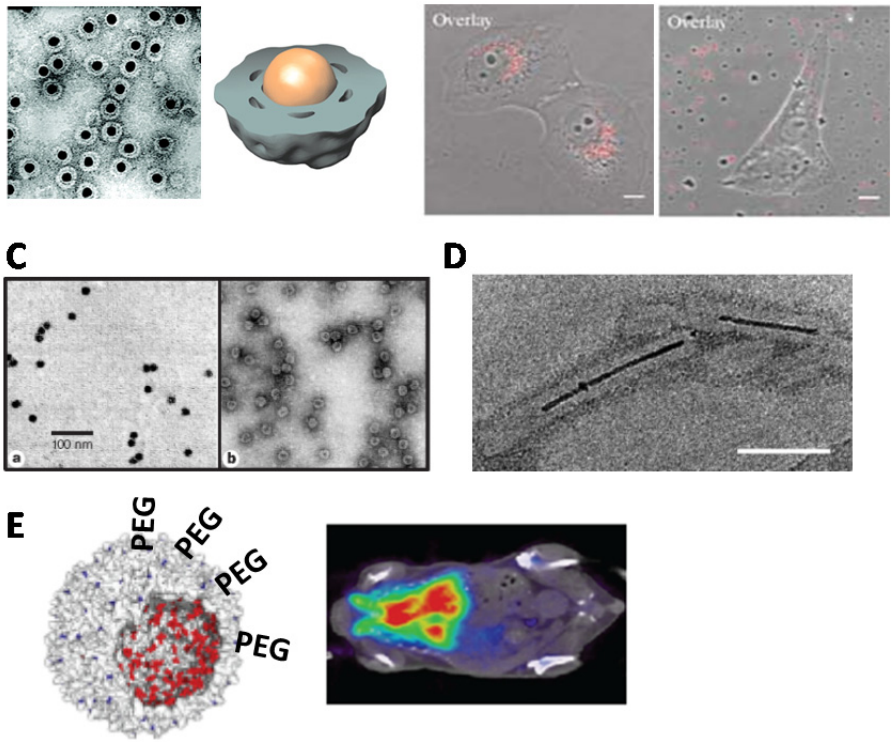

Figure 1.4. VLP core modifications with INPs and metallic cargoes: (A) Negatively stained electron microscopy image of BMV VLP assembled on 12 nm Au core and its 3-D reconstruction; (B) Overlay of phase contrast and fluorescence images of Vero cells treated with QDs (red). QDs encapsulated in SV40 VLP (left) are internalized by the cells whereas uncoated QDs (right) show random distribution; (C) Unstained (left) and negatively stained (right) electron microscopy picture of paratungstate mineralized in CCMV capsid; (D) Electron microscopy micrograph of nickel wire in the inner cavity of TMV; (E) Schematic representation of 64Cu loaded MS2 particle designed for in vivo imaging and a rat PET scan showing accumulation of the VLPs in the heart region which indicates presence in blood circulation even 24 h after administration.
Figure A (left) reproduced from ref [39], Figure A (right) reproduced from ref [38], Figure B reproduced from ref [40], Figure C reproduced from ref [41], Figure D reproduced from ref [42], Figure E reproduced from ref [43].

Quantum dots

Quantum dots (QDs) are spherical semiconductor nanoparticles with outstanding luminescence properties. They emerged as promising imaging tools due to their broad absorption spectrum and narrow emission bandwidth. Furthermore, they outperform conventional fluorescent dyes regarding their brightness and photostability. Additionally, QDs are large enough to facilitate attachment of multiple

targeting units which make them an ideal candidate for development of diagnostic nanoprobes[44]. The QDs in a bare form are cytotoxic due to their elemental composition (cadmium) and insoluble in water. However, their surface can be functionalized in a similar fashion as AuNPs, which allows encapsulation in viruses. BMV, RCNMV and SV40 were successfully investigated employing strategies similar to those previously established for AuNPs[45]. It was found that BMV readily assembled on QDs functionalized with carboxyl-modified PEGs. In contrast, for RCNMV the presence of the original assembly sequence was needed on the QD's surface. The QD-VLPs were already evaluated in mammalian cells for cellular markers detection purposes. SV40-QD hybrids were internalized by Vero cells and concentrated in the subnuclear region while uncoated QDs were randomly distributed as shown in Figure 1.4B.[40]

Magnetic NPs

The third class of inorganic nanoparticles that gained significant attention are nanoparticles exhibiting magnetic properties. Such NPs can be employed in magnetic resonance imaging (MRI), which is a non-invasive, high resolution, in vivo imaging technique commonly utilized in medicine. MRI is based on the application of agents which enhance the contrast between different tissues. So far, the field was dominated by gadolinium chelates, which are small and not tissue specific. Undoubtedly, there is a high demand to obtain contrast enhancing vehicles targeted for specific locations in the body[46]. As a result the field of protein-based MRI agents is rapidly emerging.

A lot of effort was put in development of techniques to form a protein shell around magnetic cores. The pioneering work was accomplished with CCMV capsids, which templated biomineralization of paratungstate ($H_2W_{12}0_{10}^{42-}$) and decavanadate ($V_{10}O_6^{28-}$)[41]. First, CCMV capsids were saturated with anionic precursors and subsequently subjected to a pH-induced formation of NPs inside the protein cages, as depicted in Figure 1.4C[41]. Shortly after, CCMV capsids were genetically engineered to display negatively charged amino acids in their interior, which would attract cationic NPs precursors. Indeed, it was shown that capsids could be saturated with iron cations, which spontaneously mineralized to yield monodisperse ferrite oxide particles[47]. This experiment also proved that such level of genetic manipulations did not hinder capsid assembly. Additionally, pre-synthesized iron oxide and cobalt ferrite

nanoparticles were incorporated into a VLP using a templating strategy[22,48].

Regarding rod-like viruses, the inner cavity of the TMV was subjected to similar experiments. The direct binding of metal ions was utilized to template synthesis of 3 nm thick cobalt and nickel nanowires by chemical reaction or catalyst assisted mineralization (Figure 1.4D)[42,49]. These experiments were extremely challenging when compared to metallization of the external surface.

Metal cations and metal wires

The interior of many viruses exhibits natural metal-binding sites. However metal chelating domains can be also genetically introduced. It was demonstrated that divalent metal ions bound to such domains are able to stabilize formed CCMV capsids[50]. This direct loading was utilized to saturate CCMV VLPs with Gd^{3+} ions[51]. Loading of the same species was also accomplished with viruses that were chemically modified with chelating agents[52,53]. Another study was performed with MS2 phage, which was infused with ^{64}Cu radionuclide and evaluated for Positron Emission Tomography (PET). The VLPs were additionally equipped with external PEGs in order to avoid rapid systemic clearance. It was found that the radioactive VLPs were significantly longer detected in the circulation system in vivo when compared to free ^{64}Cu, which rapidly accumulated in the liver and bladder. Moreover, loaded capsids were found in the tumour although no targeting ligand was present. Authors attributed this behaviour to the enhanced permeation and retention effect. The presented system demonstrated the possibility of VLPs altering the distribution distribution patterns of small ions (Figure 1.4D)[43].

1.2.3 Small molecules

Loading of small molecules with defined absorption or fluorescence properties was established using several approaches. The simplest way is soaking of the assembled virus in the chromophore solution as demonstrated with Cowpea Mottle Virus (CPMV)[54]. It was shown that this process is particularly successful with DNA intercalating dyes, as they were retained in the virus through interactions with its genome. These VLPs were specifically internalized by cancer cells due to CPMV's intrinsic properties (Figure 1.5A)[54]. Aside from passive loading, the interior of the virus shell can also be addressed via chemical

modifications utilizing functional groups of naturally occurring amino acids or via a genetically introduced label[55,56]. These strategies ensure that the loaded molecules will not rapidly diffuse out of the VLP. Here, a variety of viruses was employed including human JC polyomavirus (JCV), CCMV, MS2 and CPMV. In such systems, a wide range of chromophores was employed to serve as a model for therapeutic cargo. However, these fluorescent VLPs can also greatly facilitate imaging of the virus biodistribution patterns upon exposure to cells or living organisms. Therefore, such particles can promote understanding the mechanisms of viral infections and serve as a tool to develop new techniques to study these processes[57]. Finally, it was shown that DNA modified with chromophores, which stiffened the structure by π-π stacking, templated CCMV reassembly into tubular structures[58].

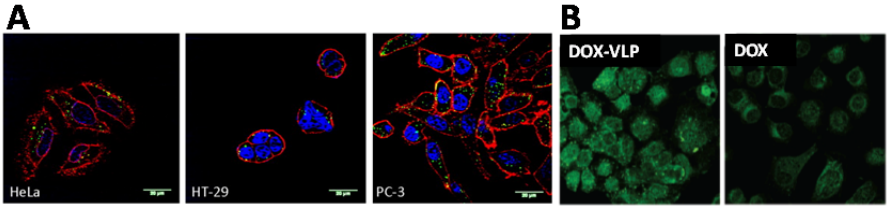

Figure 1.5. VLP-based drug delivery vehicles are readily internalized by mammalian cells; (A) DAPI-loaded CPMV delivered the cargo to cell nucleus, which in the result got stained (blue). No additional nuclei stain was employed; (B) Doxorubicin (green) loaded in folic acid functionalized VLPs was more efficient in cargo delivery to the cancer cells than the free drug supplemented with same amount of folic acid. ***Figure A reproduced from ref. [54], Figure B reproduced from ref. [35]***

Loading of drugs in protein cages is one of the ultimate goals of VLP development. Such drug delivery systems ensure that the active pharmaceutical ingredient is not exposed to the environment and therefore its potential toxicity, instability or rapid clearance characteristics can be avoided. Above all, a targeting unit can be attached to the vehicle to render the therapy more efficient. Doxorubicin is by far the most studied example. The free drug was already efficiently co-loaded in HCRSV when utilizing PSS as scaffolding moiety. These VLPs were additionally surface-modified with folic acid to achieve cancer cell targeting as shown in Figure 1.5B[35]. Furthermore, it was shown that this loading strategy was more efficient than surface modification of CPMV[59]. Similarly to HRSCV, chemically modified doxorubicin was bound to Rotavirus CP, which was subsequently assembled in VLPs intended for targeted drug delivery[60]. Such particles

were shown to prevent cargo from leaking out before being internalized by hepatoma cells in vitro. Another interesting class of compounds are photosensitizers utilized for photodynamic therapy (PDT). Here sulfonated zinc phthalocyanine (ZnPc) was successfully encapsulated into CCMV capsids and promising in vitro activity was demonstrated. The incorporation of this compound, however, was quickly improved by employing micelle-assisted loading, which will be discussed in the next section[61,62].

1.2.4 Amphiphilic systems

To extend the scope of VLPs as versatile drug carriers, the loading of synthetic amphiphilic systems was investigated. Such a hierarchical two-step self-assembly approach allows for indirect charging interior of a VLP with hydrophobic drugs, loaded in the core of micelles. As already mentioned, this system was suggested to improve performance of VLPs used for PDT. Indeed, it was shown that the micelle mediated approach greatly reduced aggregating behaviour of ZnPc and these VLPs were successfully internalized by macrophages[62]. Similarly, micelles formed by lipid-modified gadolinium chelates were also successfully incorporated in VLPs[62].

DNA-based amphiphiles were successfully implemented in VLP technology as well[63]. This class of compounds exhibits a hydrophobic core and hydrophilic corona built of short single stranded DNA oligonucleotides. The resulting micelles allowed for multimodal cargo loading i.e. in the core or via complementary DNA strands. Additionally both strategies can be employed at the same time (Figure 1.6A). It was proven that the DNA micelles template the virus assembly in neutral pH due to the negatively charged corona.

Interestingly, virus capsid formation was also achieved using a nanoemulsion template. Therefore, polydimethoxylsiloxane oil (PDMS) nanodroplets were stabilized in water with the anionic surfactant, sodium dodecylsulfate (SDS). Formation of several morphologies of the virus capsids around the emulsion particles was observed upon dialysis against different pH and ionic strength buffers. In such systems, CCMV CP could form virus-like droplets with a diameter reaching twice the size of WT CCMV (Figure 1.6B)[64].

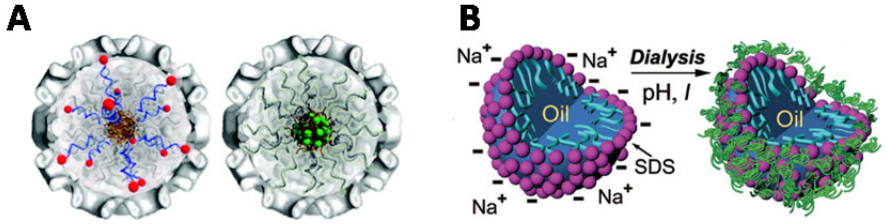

Figure 1.6. Amphiphilic systems encapsulated in VLPs: (A) DNA-based amphiphiles template CCMV capsid formation. Cargo can be loaded in the micelle core (green) or via functionalization of the complementary DNA strand (red); (B) Schematic representation of SDS stabilized PDMS droplets in CCMV CP shells. ***Figure A reproduced from ref. [63], Figure B reproduced from ref.[64]***

1.2.5 Capsid Reactors: enzyme loaded micro-compartments

The interior of many VLPs is spacious enough to accommodate multiple proteins. Hence, VLPs were applied as nanoreactors to study performance of enzymes in a confined compartment, which resembles its natural, intracellular environment. Here, viral capsids exhibit superior properties over previously applied liposomes, polymeric vesicles or micelles, which are not as defined regarding morphology and have limited substrate accessibility[65]. A study performed with horse radish peroxidase (HRP) encapsulated in CCMV revealed that the capsid pores ensure permeability of the VLP for low molecular weight substrates and products (Figure 1.7). It has to be noted that various proteins can be simultaneously present in one capsid. It was demonstrated with CCMV and P22, both encapsulating an enzyme and green fluorescent protein (GFP)[31,66]. This kind of entrapment was accomplished either in a statistical way or by means of noncovalent conjugation with CPs. The latter approach can be performed using coiled coil interactions (CCMV), coupling to the RNA hairpin that initiates packing of the genome (Qβ) or by fusion with phage-own scaffolding protein (P22)[45]. Moreover, general strategies emerged which rely on decorating of the VLP's interior with a universal tag, like His_6[55]. Recently a sortase A (SrtA) working principle was implemented in VLP technology. SrtA is an enzyme anchoring proteins onto the cell wall of Gram positive bacteria. It recognizes two amino acid tags and subsequently fuses proteins exhibiting such labels. Thus, SrtA tags were genetically introduced in CCMV interior and on the model protein (GFP). Upon addition of SrtA, CP fusion proteins were formed which

afterwards could be assembled into VLPs. The authors suggest that any protein can be anchored within CCMV capsid following this strategy[67].

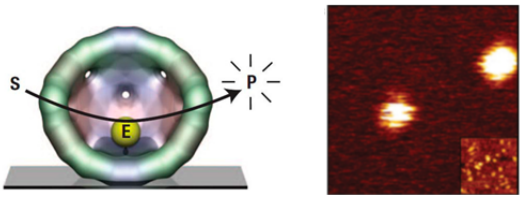

Figure 1.7. Single VLP-based enzymatic reactors imaged under confocal microscope. Fluorescent product of the HRP enzyme initially accumulates in the cavity of the VLP and afterwards diffuses out. ***Figures reproduced from ref.*** **[68]**

In general, confined enzymes exhibit changes in at least one kinetic parameter[69]. For example lipase PalB showed better performance when compared to free protein solution, while the catalytic efficiency of aspartate dipeptidase E (PepE) encapsidated in bacteriophage Qβ was reduced three fold[69,70]. Therefore, an attempt was made to evaluate the technological advantage of such systems. After confirming that encapsulation of thermostable glycosidase (CelB) in a P22 cage did not affect enzyme efficiency, such catalytic VLPs were immobilized in a gel matrix, lyophilized and grinded. Unfortunately, authors did not observe any benefit of VLP cage during immobilization process regarding the enzyme activity. However, the morphology of protein capsid after immobilization procedure was not assessed[66].

1.2.6 Nucleic acids

Foreign nucleic acids

Foreign RNA was the first payload evaluated as template for VPL formation. Studies, which were performed with CPs of spherical viruses and the RNA of filamentous TMV or with mixtures of different viral CPs revealed the flexibility of the capsid assembly process[27,28]. In succeeding experiments on CCMV, BMV and Broad Bean Mottle Virus (BBMV) synthetic homopolynucleotides (nt=100) successfully yielded spherical VLPs, whereas calf thymus double stranded DNA induced formation of elongated viral morphologies[30] (Figure 1.8A). The tubular morphology was also confirmed in a more recent study where CCMV CP efficiently assembled into tubes on a 500 bp ds DNA scaffold[71].

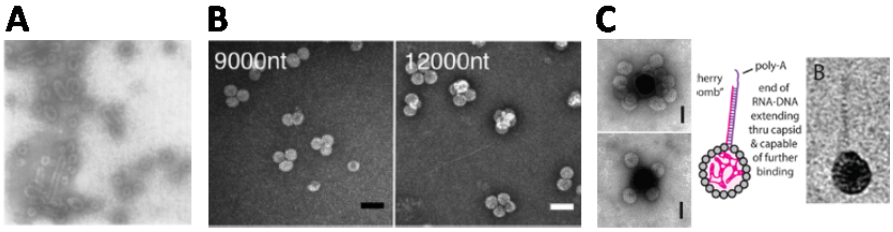

Figure 1.8. VLP assembly on nucleic acids scaffolds: (A) Calf thymus DNA induces formation of elongated morphologies of BMV; (B) Long RNA strands are encapsulated by multiple spherical CCMV VLPs; (C) CCMV assembly in a "cherry bomb" morphology with DNA-RNA strand available for further manipulations e.g. attachment to the 30-nm AuNP. ***Figure A reproduced from ref.[[30]]; Figure B reproduced from ref. [[24]]; Figure C reproduced from ref.[[72]].***

After first experiments on viral reassembly, questions about the influence of RNA size on the encapsidation process were addressed. The CCMV assembly was investigated using genomic RNA from different species (0.14-11.7 knt), including genetic material of other viruses such as BMV, TMV and the Sinbdis virus. The results showed that mulitplets of capids were formed when RNA length exceeded 4500 nt[24] (Figure 1.8B), while shorter nucleotides were packed in one spherical VLP. This systematic approach was complemented with studies employing various molecular weights of PSS. However, it was pointed out that the synthetic polymer does not fold into secondary structures like tRNA and therefore could underestimate the maximal length of polyanionic scaffolds that can template VLPs[24].

Recently CCMV "cherry bombs" were created, underlining the possibility of formation of stable but not completely closed capsids. These structures were designed to have a DNA-RNA hybrid strand poking out of the capsid enabling e.g. attachment to a functional surface (Figure 1.8C). Finally, one needs to keep in mind that nucleic acids can template assembly of VLP even when they are chemically modified or involved in formation of a superstructures, as mentioned before[58,63].

Viruse like particles with functional genetic material

Discussion on VLPs is often being restricted to non-infectious species. However, there is a large group of infective viral particles developed for therapeutic purposes. In this context, maintaining infectivity is of crucial importance as it distinguishes viruses from other therapeutic vehicles. In the same time, these viruses are largely reconstructed to meet requirements of medical procedures. In fact, these VLPs truly show the

applicability and possibilities of viral engineering. Oncolytic virotherapy and viral gene therapy represent two important accomplishments, which already advanced from experimental protocols to clinical trials and approved therapies[8,73].

The idea of oncolytic virotherapy emerged from clinical case reporting cancer regression that coincided with a viral infection[73]. Today virotherapy treatment options include a number of viruses from different families, including Adenoviridae, Picornaviridae, Herpesviridae, Paramyxoviridae or Rhabdoviridae. However, the majority of these viruses is heavily engineered to boost tumour specificity and cargo efficacy. For example genetic or chemical modifications can be employed to obtain greater specificity of tumour targeting. Here DARPins, repeat-motif proteins smaller than a single antibody chain, were successful applied to redirect measles virus to target two different tumour types[74].

Safety of the oncolytic virotherapy was confirmed in many clinical trials, where neither severe side effects nor transmission to other patients was evidenced[73]. Unfortunately, most viral therapies faced serious challenges. Administration of virus-based therapeutic agents exposes them to pre-existing antibodies of the human immune system. To prevent neutralization, several measures can be undertaken. One consist of serotype exchange. It is achieved by generating VLPs with original core but with surface proteins that belong to a different virus. Additionally, viruses can be equipped with a few "artificial serotypes". In these cases a library of virus particles is shuffled through the therapy so patients don't develop an immune response. Still, the manipulated serotype should not hinder desired virus tropism. The serotype-exchange approach is more challenging for non-enveloped particles because capsid modifications can drastically influence virus stability[75]. Another method is chemical shielding, which is accomplished with polymers e.g. PEGs or poly-(*N*-2-hydroxypropyl) methacrylamide. However, applicability of those polymers greatly depends on the ability of the viruses to enter the cell, which should not be compromised by the polymer modification[76].

Finally, infecting and killing of all tumour cells is a challenging task even for viral systems. Thus, several strategies were developed that lead to a drastic change of the cell environment upon entry. For example, oncolytic viruses were equipped with genes encoding prodrug

convertases that activate non-toxic drug precursors and thereby generate highly toxic metabolites only in the tumour microenvironment[73]. Such enzymes include cytosine deaminase and purine nucleoside phosphorylase, which partially degrade nucleotides leading to dysfunctional genes[77]. In another approach, viruses can stimulate overexpression of particular ion channels that later promote uptake of e.g. radioactive nuclides[73]. Lastly, viruses can be applied to provoke immune system response to a tumour. They are able to sensitise a patient to weakly immunogenic targets overexpressed by cancer cells. This was demonstrated with CPMV chemically modified with tumour assisted cancer antigen[78]. In these approaches, systemic toxicity of therapy is minimalized[59].

Viral gene therapy is based on VLPs equipped with therapeutic nucleic acid material which is supposed to replace or neutralize a malfunctioning gene[8,79,80]. It relies on the ability of the virus to cross the cellular membranes and deliver their genome to the host cell. Such therapies were developed with several viral vectors including adenoviruses, retroviruses and adeno-associated viruses[8]. The virus of interest has to address specific cell types, for which they carry a recognition systems and it needs to match the target cell character. For example retro- and lentiviruses integrate their genome within the genome of the host and therefore they are applied to achieve stable expression of therapeutic genes in intensively proliferating cells, e.g. bone marrow. On the other hand, non-dividing cells are often treated with adenoviral vectors which do not integrate their DNA in the host genome[76]. Besides that, gene delivery vehicles have to evade the immune system, just as the viruses used in oncolytic therapy. Therefore, they were equipped with a chemically or genetically modified surface[8]. Although the infectivity of viral vectors can be compromised upon structural changes of the capsid, they still feature some advantages over liposomes and lipoplexes.

Unfortunately, up to date most clinical trials did not confirm efficacy as well as seen in preclinical models. However, the VLP tool box is continuously expanding. The current generation of viruses already went through serious improvements in tumour targeting, delivery of therapeutic payload and evading neutralisation by the cells of the immune system.

1.2.8 Summary

The diversity of VLP cargoes discussed in this section is summarized in Table 1.1. They are grouped according to the nature of the cargo and the type of virus. Furthermore, the assembly mode and corresponding reference is given.

Table 1.1. Overview of the cargos used in VLPs formation experiments

Cargo	Virus	Mode of assembly	Reference
Polymers			
Polystyrene sulfonic acid	CCMV	Templated	33,34,81
	HCRSV	Templated	82
Polyacrylic acid	HCRSV	Templated	82
Polyvinyl sulfate	CCMV	Templated	30
	BMV	Templated	30
Dextran sulfate	CCMV	Templated	30
	BMV	Templated	30
Inorganic nanoparticles and metallic species			
AuNP	BMV	Templated	38,39
	RCNMV	Templated	22
	RRV	Templated	13
	SV40	Templated	83
	HIV-Gag	Templated	37
QDs (CdSe/ZnS)	BMV	Templated	12
	SV40	Templated	84,85
	RCNMV	Templated	22
Fe_xO_y	BMV	Templated	48
	CCMV	Mineralization	47
	P22	Templated	86
TiO_2	CCMV	Mineralization	87
$H_2W_{12}O_{10}^{42-}$	CCMV	Mineralization	41
$V_{10}O_6^{28-}$	CCMV	Mineralization	41
$CoFe_2O_4$	RCNMV	Templated	22
Gd^{3+}	CCMV	Passive	51,52
	P22	Chemical modification of CP	53
Ni	TMV	Mineralization	49,88
Tb^{3+}	CCMV	Passive	51
Co	TMV	Mineralization	49

CoPt	TMV	Mineralization	42
	Tomato Mosaic Virus	Mineralization	89
$FePt_3$	TMV	Mineralization	42
^{64}Cu	MS2	Passive	43
Dyes			
Propidium iodide	CPMV	Passive	54
	JCV	Statistical	90
Sulforhodamine nitrilotriacetic acid derivative	JCV	Chemical modification of CP	55
naphthalene, stilbene, oligo(p-phenylenevinylene)	CCMV	Templated	58
Fluorescein	MS2	Chemical modification of CP	91
Acridine orange	CPMV	Passive	54
DAPI	CPMV	Passive	54
Alexa Fluor 680	MS2	Passive	92
Drugs			
Doxorubicin	RCNMV	Passive	93
	HCRSV	Templated	35,59
	Rotavirus	Chemical modification of CP	60
Ricin A	MS2	Templated	94
5-fluorouridine	MS2	Templated	94
phthalocyanine	CCMV	Templated	61,62
porphirine	MS2	Chemical modification of CP	95
Amphiphilic systems			
DNA micelle	CCMV	Templated	63
Lipid modified Gd3+ chelator	CCMV	Templated	62
PDMS nanoemulsion	CCMV	Templated	64
Proteins			
HRP	CCMV	Statistical	68
GFP/EGFP	CCMV	Statistical	96
		Modification of CP	50,67,70,97
	P22	Fusion with scaffolding protein	31

	MS2	Templated	[98]
	SV40	Fusion protein with CP	[99]
Alkaline phosphatase (PhoA)	MS2	Templated	[98]
Asptartate peptidase (PepE)	Qβ	Fusion protein with CP	[69]
Lucifersae	Qβ	Statistical	[69]
Lipase (Pal1)	CCMV	Statistical	[70]
Galactosidase (CelB)	P22	Fusion with scaffolding protein	[66]
Alcohol dehydrogenase (AdhD)	P22	Fusion with scaffolding protein	[100]
Nucleic acids[1]			
ds DNA	CCMV	Templated	[30,71]
	BMV	Templated	[30]
RNA 140 to 12k nt	CCMV	Templated	[23,27]
Homopolynucleotides 100 nt poly(A), poly(C), and poly(U)[2]	CCMV	Templated	[30]

[1] only studies where nucleic acids were used for systematic investigation are included
[2] poly(G) was investigated to lesser extent due to formation of secondary structures

1.3. Outer shell modification

The outer surface of the viral capsid is responsible for cell recognition, virus entry and virion spreading from cell to cell[12]. Thus, any modifications can have significant consequences for the functionality of the virus. Nonetheless, the easily accessible exterior of the capsid can be subjected to multiple conjugation procedures designed to label, re-target or strengthen wild type virions.

From a technological point of view, manipulation of the capsid exterior leads to the development of a nanocarrier which displays newly introduced entities on its surface. In this case, the virus capsid does not shield the probe, which remains exposed to the outer environment. This means that toxicity of the applied probe or its degradation profile may be problematic despite successful targeting. Hence, only the general principles of outer shell modification will be discussed here as they can be beneficial for application of VLPs with loaded interior[8,10,45,59,101-103].

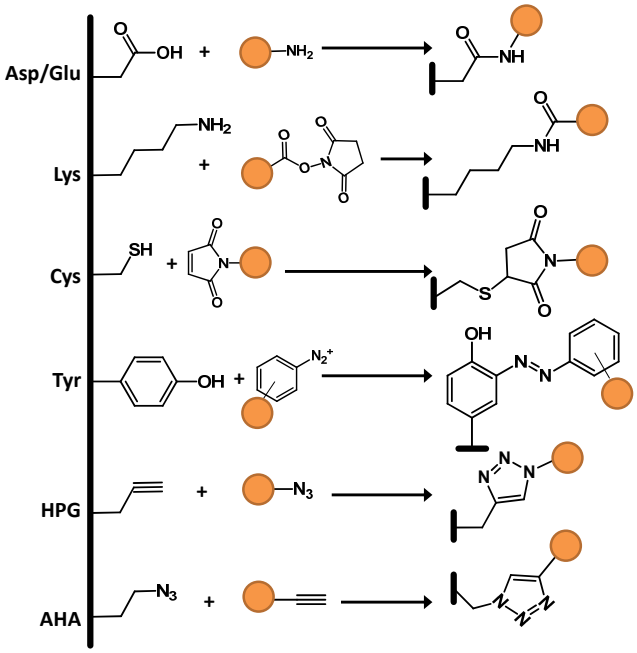

Figure 1.9. Bioconjugation strategies towards modification of virus capsids (non-comprehensive list).

The modifications of the viral outer shell to a large extent follow the same strategies that are applicable to other proteins. The functional groups of amino acids positioned on the exterior of a virus capsid, like amines, thiols and carboxylic acids, can be addressed to implement a broad range of additional functional moieties (Figure 1.9). Noteworthy, capsids offer precise control of modification site because their composition and shape is strictly defined on the genetic level. For example, per single CCMV capsid one can address over 500 amine and carboxylic acid groups and over 100 thiols evenly distributed over all CPs[6]. These functionalities can be subjected to N-hydroxysuccinimidyl chemistry, Michael additions, and carbodiimide activation for attachment of e.g. small molecules, polymers or nucleic acids (Figure 1.9)[102]. Additionally, tyrosine side chains can be addressed by performing diazonium coupling, which requires aniline derivatives of the molecules to be conjugated and their subsequent diazonation[91,104]. However, these derivatives are not commercially available which makes this approach more cumbersome resulting in less frequent application. Nevertheless, MS2 and TMV viruses were used as showcases to develop

this strategy. To broaden the scope of available conjugation chemistries, VLPs can also be decorated with functional groups that allow for bioorthogonal coupling strategies, i.e. azides and alkynes. CPMV, HBV and bacteriophage Qβ were selectively modified employing copper-catalyzed azide–alkyne cycloaddition (CuAAC, "click chemistry") to efficiently conjugate a variety of payloads including sugars, small molecules, fullerenes, proteins, and RNA[105,106]. These additional functionalities can be introduced via chemical modifications of natural side chains or via incorporation of unnatural amino acids. For example homopropargyl glycine (HPG) or azidohomoalanine (AHA) were introduced in CPs produced in methionine-starved E. coli culture with >95% efficiency and were readily accessible for CuAAC reactions[102].

As already mentioned, genetic manipulations of viral CPs is feasable. Hence the incorporation of functional domains into the CP scaffolds quickly emerged as a powerful technique to equip VLPs with versatile functionalities[102]. It is noteworthy that single amino acids as well as long polypeptides can be introduced on the VLP surface using genetic engeneering techniques.

Thus by following either chemical modification strategies or genetic manipulations of the virus exterior, a great variety of spherical and rod-shaped VLPs was created[107-109]. Metal NPs, including Pt, Pd, Ag and Ni, was deposited on the surface of CPMV and TMV[45]. Moreover, CPMV, TMV, M13 and P22 viruses were decorated with gold nanoparticles and sometimes used to arrange AuNPs in 3-D superstructures of various sizes[110,111]. Similarly, QDs were coupled to P22, M13 and SV40 capsids. The TMV and PVX capsids were coated with silica and M13 was employed to decorate surface of single walled carbon nanotubes[5]. Additionally, TMV was exploited as a scaffold to polymerize aniline and pyrolle, which resulted in conducting polymeric wires[45]. Apart from that, a variety of targeting units was introduced onto the VLPs. In this way the tropism of these viruses was modulated due to the presence of i.a. folic acid, transferrin, epidermal vascular growth factor, RGD peptides, cell penetrating peptides, glycans or fibrin targeting units[59,92,112]. A lot of interest was put in development of universal non-covalent coupling strategies that allow for conjugation of various moieties to the exterior, such as His_6-tags or streptavidin binding motifs introduced on the surface of CPMV[110,113]. Additionally, a range of small molecules was chemically coupled to the VLP exterior. Dyes were employed to mimic functional materials, for imaging purposes or to

serve as light harvesting system[114]. Furthermore, VLPs intended for biomedical use were decorated with drugs or contrast agents, including DOX, hygromycin, nicotin and gadolinium ions[8]. Above all, the coupling of PEG chains has to be mentioned here, as it altered immunogenicity of the viral protein cage and greatly improved its circulation time[4]. It was demonstrated for i.a. TMV, PVX, CCMV, CPMV and MS2[33,46].

1.3.1 Vaccines

The medical application of VLPs with altered capsid surface was already introduced in section 1.2.6. However, there the capsid needed to be shielded from immune system to ensure success of the therapy. This already points out that proteins exposed on the viral surface provoke a very strong immune reaction. Indeed, the highly ordered and repetitive CPs are perfectly suited for immunestimulation[11]. Immune response is triggered with entire capsids, but also by capsid proteins or isolated epitopes. VLP-based vaccines devoid infectious material already revolutionized prevention of infectious diseases. Two very prominent examples are vaccines developed against HBV and HPV, obtained from yeast (Gardasil®) and insect cells (Cervarix®), respectively. For example crude HPV VLPs produced in yeast do not exhibit a uniform morphology. However after purification and in vitro re-assembly, monodisperse VLPs are formed. Additionally, they exhibit improved thermal stability.[115] The HBV particle is very versatile. It can serve as a scaffold presenting novel immunogenic antigens on its surface (Figure 1.10D)[11,116]. Such systems were used to study epitope presentation of viruses that were not yet reconstituted in vitro, e.g. HIV, and therefore they could greatly increase the chance of developing a vaccine against such viruses[11].

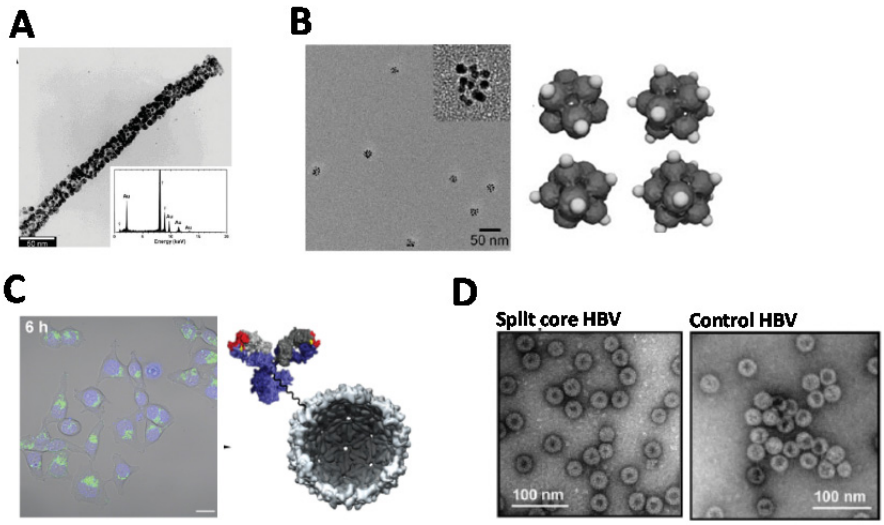

Figure 1.10. (A) TMV used as a scaffold for assembly of AuNPs; (B) 3-D AuNPs superstrustures assembled on SV40 VLP; (C) MS2 bacteriphage VLP (green) equipped with antibody targeting tumour markers is internalized by cancer cell line (blue nucleus stain, overlay with phase contast picture); (D) Engineerered HBV "Split core" capsids allow genetically controlled presentation of polypeptides up to 500 amino acids without affecting VLP assembly. ***Figure A reproduced from ref. [117]; Figure B reproduced from ref. [111]; Figure C reproduced from ref. [118]; Figure D reproduced from ref. [116].***

1.4 Scope, limitations, and future perspectives

Viral components, especially the coat proteins are an exciting class of materials to fabricate highly defined nanoparticles or rod-like structures. Regarding structural perfection, VLPs are superior compared to inorganic nanoparticles in the same size range. Foreign functional materials can be controllably loaded in the inner cavity, on the exterior of VLPs, or in both, leading to multifunctional systems. Such tailoring of viruses can be achieved by following three general approaches: i) classical bioconjugation chemistries, ii) unique reassembly routes, and finally, iii) genetic manipulations. In general all three are compatible with each other and can be used simultaneously. Remarkably, virus capsids exhibit unusual plasticity and tolerate a high degree of modification. As such, spherical viruses can be turned into tubes and tubular ones into spheres[58,119]. Their production on larger scales can be easily achieved when employing infected host cells[9]. So far VLPs

derived from plant viruses and bacteriophages didn't show systemic toxicity, however, very little is known about their possible interactions with the human immune system[46,59].

Nevertheless, there are a few shortcomings of these systems, which will be shortly addressed here. The efficient entrapment of small molecules in VLPs can be challenging due to porosity of the capsid. Thus the release profile might not be controllable in a biological setup upon changes of ionic strength and the pH of the local environment. Moreover, there are obvious limitations regarding protein or peptide expression on the VLP's surface. Hybrids made by genetic manipulations can have a limited stability and altered reassembly behaviour. In turn, the stability and biodegradable character of the protein shell can be considered as advantage or disadvantage depending on the application. From a material science point of view, it is desired that the protein shell is as stable as possible. Therefore hyperstable viral particles from mutants naturally occurring in extreme environments, or chemically engineered VLPs were investigated[8,120-122]. On the other hand, for delivery of medical cargos in vivo degradation is desired.

Nanoscale engineering of viruses has provided important tools for science and research of today. Above all, it revolutionized the way how we prevent diseases by vaccination and might have a great impact on the way how we detect and treat them. Natural properties of the capsids were combined with physical properties of diverse cargoes often scarcely resembling the regular one. As such over dozens of virus-like particles can be employed as vaccines, gene vectors, targeted drug delivery vehicles, contrast agents and as numerous research tools, thereby constantly pushing the frontiers of biomedicine, nanotechnology and biotechnology.

1.5 Motivation and thesis overview

Viruses are unique and very diverse biological entities that continuously remain in the focus of medicine and science. On one hand, treating viral infections still poses a serious challenge and new medications are in high demand. On the other hand, viruses and their isolated capsids gained significant attention as functionalizable bionanoparticles due to their robust, defined architectures and intrinsic monodispersity. Although virus engineering progressed rapidly in the last years, there are still many more options to explore. In view of that,

in the first two chapters of this thesis, viral proteins will be employed as a tool to create novel hybrid materials. However, we also urged to propose novel materials that offer the possibility to manipulate the virus itself. Therefore, in the last two chapters viruses are taken as a biological target to explore new possible viricide strategies.

In Chapter 2 and Chapter 3, viruses are explored as a building block for nanotechnology. In Chapter 2 we propose single walled carbon nanotubes (SWCNTs) as a new type of template for virus-like particle formation. SWCNTs attract a lot of attention in multiple research disciplines due to their outstanding mechanical and electronic properties. Combination of this synthetic material with highly ordered proteins will possibly allow for implementation of such hybrid in biosensing devices. It may also lead to protein composite material with novel, mechanically strengthened properties. Up to now, the SWCNTs have been challenging to implement in experiments involving biological molecules due to their unspecific interactions with proteins and nucleic acids. We recognized viral capsid proteins as promising protein source for such studies. To meet the requirements of templated assembly of viral coat protein, DNA is employed as dispersing agent. Afterwards, we probe three plant viruses: Tobacco Mosaic Virus (TMV), Potato Virus X (PVX) and Cowpea Chlorotic Mottle Virus (CCMV) as capsid protein donors. In these experiments, we employ transmission electron microscopy to study interactions of virus capsids with SWCNTs and to explore their new assembly possibilities. We reveal a rationally designed and nature inspired stepwise process that results in encapsulation of this carbonaceous synthetic material within the viral capsid.

In Chapter 3, we investigate electrical properties of SWCNTs in a protein shell. To do so the methodology presented in the previous chapter is adapted to obtain virus-SWCNT hybrids that exhibit semiconducting properties. We propose a method that yields water soluble, electronically active carbon material, which is additionally encapsulated in a protein shell of viral origin. First, the electrical properties of the formed hybrids are evaluated on a single-object level, utilizing an atomic force microscope working in conducting mode. Subsequently, bulk measurements are performed to characterize the electrical properties of VLP-coated SWCNT network.

In the second part of this work, we concentrate on investigating the interactions of a conjugated polymer with viruses. In Chapter 4, we reveal the potential of charged polyfluorenes for versatile applications in virology. We introduce negatively and positively charged polymer candidates and evaluate their activity on the human immunodeficiency virus (HIV). The chosen retroviral system allows us to fully exploit the biological potential of the synthesized materials. Additionally, dimeric and monomeric forms of both polymers are included in the study to obtain systematic information about possible structure-activity relationships.

In Chapter 5, we elucidate a probable mode of action of the charged conjugated polymers presented before. We hypothesise that the observed polyfluorene activity is based on interactions with the phospholipid membranes. Therefore, we design and study a model liposomal system and its interactions with the conjugated polymers. A set of biophysical experiments is conducted and electron microscopy is performed to investigate interactions of oppositely charged polyfluorene backbones with the virion- and cell-mimicking membranes. The membrane stability is assessed by fluorescence based experiments, using dynamic light scattering and cryo electron microscopy. These studies give an insight into the possible mechanisms of action of agents targeting enveloped viruses and they are good reference points for observations made from biological experiments employing membrane active polyelectrolytes.

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