Virus-based nanocarriers for drug delivery ☆

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ABSTRACT

New nanocarrier platforms based on natural biological building blocks offer great promises in revolutionalizing medicine. The usage of specific protein cage structures: virus-like particles (VLPs) for drug packaging and targetted delivery is summarized here. Versatile chemical and genetic modifications on the outer surfaces and inner cavities of VLPs facilitate the preparation of new materials that could meet the biocompatibility, solubility and high uptake efficiency requirements for drug delivery. A full evaluation on the toxicity, biodistribution and immunology of these materials are envisaged to boost their application potentials.

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Contents

1. Introduction

1.1. Development of nanocarrier platforms for drug delivery and targeting

With the recent rapid development of nanotechnology, advances in protein engineering and materials science are offering great promises to revolutionize medicine [1]. New nanocarrier platforms exhibiting great potentials in improving drug packaging, delivery, and targeting efficiencies are currently emerging. These systems could offer several advantages, e.g. (*i*) prevention of premature drug degradation or interaction with its biological environment, (*ii*) enhancement of drug adsorption to the desired tissues (e.g. tumors,) and (*iii*) control over the drug tissue distribution profile [1]. Examples of nanocarrier platforms that have been extensively studied include dendrimers [2], liposomes [3], polymersomes [4], micelles [5], and virus-like particles [6]. Each of these systems has its advantages and disadvantages regarding the versatile requirements for therapeutic applications. From a material point of view, the range of building blocks that can be applied for various nanocarrier platforms has been extended enormously over the past few decades. Following explorations on the design and synthesis of small compounds and polymers as well as the self-assembly of these synthetic systems, scientists are now also focusing on the direct use of natural, i.e. biological building blocks for the fabrication of nanocarriers. At the same time the investigation of self-assembly at all length scales has also proceeded to the stage of mimicking biological entities by studying hierarchical assembly behavior. All of these new developments are aimed at improving the performance of drug nanocarriers, in making them more biocompatible, water-soluble, or colloidal, displaying reduced toxicity and high differential uptake efficiencies [1].

1.2. Biological nanocontainers composed of protein cages

Protein cages are perfect examples of nanocarrier platforms built from natural materials by a process of hierarchical (supramolecular) assembly. One of the characteristic features of protein cage structures is their size uniformity, i.e. each cage exhibits an extremely homogeneous size distribution. Nature has developed a variety of protein cages with diverse sizes as nanocarriers for metal ions and minerals, which has inspired researchers to design new systems for drug packaging and delivery based on these materials. Protein cages can be chemically or biologically engineered in many ways, making them uniquely attractive platforms for drug delivery applications. One of the most famous examples is the iron storage protein ferritin. Ferritins are produced in almost all living organisms, including bacteria, plants, and animals. They consist of a central core of hydrated iron (III) oxide encapsulated within a 24-subunit protein shell. The use of ferritin's cage structure was first explored in the area of biomineralization [7]. A later approach involved the biomedical application of ferritin as a delivery vehicle for the MRI contrast agent Gadolinium (Gd) [8].

Another extensively studied biological cage structure is the small heat shock protein (sHsp). sHsp originates from the species *Methanococcus jannaschii* and consists of 24 subunits that self-assemble into a cage with a 12 nm outer diameter and a 6.5 nm inner diameter [9]. sHsp differs from ferritin in that it has large 3 nm pores that allow the free exchange of substances between the interior and exterior environments [10]. sHsp has been studied for the encapsulation and release of the antitumor agent doxorubicin (Dox) [11]. Recently, the use of sHsp as a MRI contrast agent with extremely efficient relaxivity [12] and as a vehicle for the targeted delivery of imaging agents for the diagnosis of atherosclerosis [13] has been reported, highlighting their great potential in biomedical applications.

Other examples of biological cage structures include *Lumazine synthase* (LS) [14], a hollow icosahedral bacterial enzyme and vaults [15], which are self-assembled ribonucleoprotein nanocapsules found in nearly all eukaryotic cells. However, the application potential of these compounds is yet to be evaluated.

This review will mainly focus on drug delivery applications based on a specific and important type of protein cages, i.e. viruses. Viruses, especially bacterial viruses or bacteriophages, are the most abundant

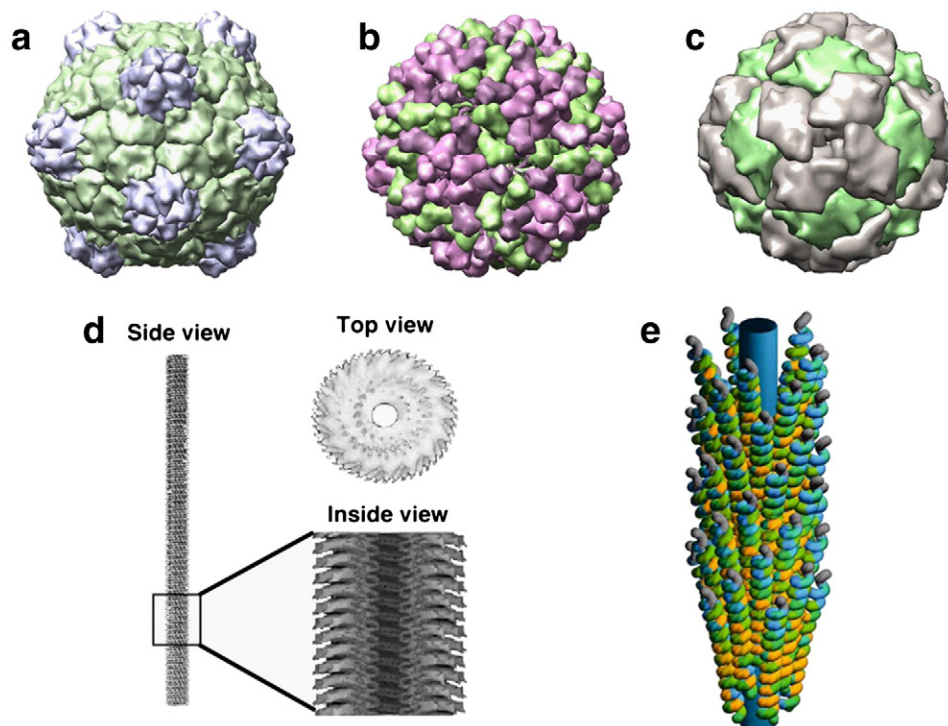**Fig. 1.** Structures of some of the viruses discussed in this review: a) Cowpea mosaic virus (CPMV), 31 nm in diameter; b) Cowpea chlorotic mottle virus (CCMV), 28 nm in diameter; c) bacteriophage MS2, 27 nm in diameter; d) tobacco mosaic virus (TMV), 18 nm in diameter and 300 nm in length [19]; e) bacteriophage M13, 6.5 nm in diameter and 900 nm in length [20]. a)–c) were obtained from the RCSB protein data bank (www.pdb.org).

biological entities on earth, playing a key role in biological systems [16]. Here, the structure and chemistry of different types of viruses will first be discussed, followed by a summary of recent advances in drug delivery related packaging and targeting strategies involving viruses. In the last part of this review, initial results on in vivo studies will be discussed in order to evaluate the toxicity and immunology aspect of virus assemblies in drug delivery applications. It should be mentioned that the broad topic of viruses with its native DNA/RNA as viral vectors for gene therapy will not be the main focus of the present review. The development of virus-like particles for diagnostic imaging before 2006 has been covered in a review by Manchester et al. [17], hence only the most recent discoveries in this area will be discussed here.

2. Virus-based nanocarriers

Virus particles typically consist of several hundreds to thousands of protein molecules, which self-assemble to form a hollow scaffold packaging the viral nucleic acid. It has been shown in recent years that viruses are tailorable at the genetic level, for applications as reagents, catalysts, and scaffolds for chemical reactions [18]. As an emerging and important nanocarrier platform, virus-like particles offer the great advantages of morphological uniformity, biocompatibility, and easy functionalization. Moreover, they range in sizes from ~10 nm to over a micron and can be found in a variety of distinctive shapes (most abundantly icosahedrons, spheres, also in tubes, see examples shown in Fig. 1). In this paragraph various virus-based materials that have been studied in the field of nanotechnology for drug delivery will be summarized.

2.1. CCMV

The cowpea chlorotic mottle virus (CCMV) is a member of the bromovirus group of *Bromoviridae*, a family of plant viruses [21]. CCMV can be obtained in high yields (ca. 1–2 mg/g) from infected plants, but yeast-based expression systems can also be used to produce CCMV coat proteins, which self-assemble into empty virus-like particles lacking the genetic material [22]. CCMV consists of 180 identical coat protein subunits surrounding the central RNA. The protein subunits are arranged as 20 hexamers and 12 pentamers, forming an icosahedral shell with a diameter of 28 nm (T = 3 symmetry). Each of the CCMV coat proteins contains 190 amino acids with the N terminus located on the inside of the viral capsid. The residues on the N terminus (amino acids 1–25) are predominantly basic and responsible for the interaction with the negatively charged RNA. When the N terminus is truncated, the capsid proteins are also known to assemble into T = 1 particles (18 nm in diameter) or pseudo T = 2 particles (22 nm in diameter), consisting of 60 and 120 capsid proteins, respectively [23]. The pseudo T = 2 particles are not icosahedral-shaped but are dodecahedrons.

One of the most attractive characteristics of CCMV lies in the fact that it has a dynamic structure. CCMV undergoes a reversible pH-dependent swelling, resulting in the formation of 60 separate openings with diameters of 2 nm at the quasi-threefold axes [21].

Depending on the pH and ionic strength, the 180 coat protein subunits are able to self-assemble and disassemble in vitro (Fig. 2). By making use of this feature, the viral RNA could be removed from the virus and be replaced with functional species. The inner cavity of CCMV is 18 nm in diameter, around twice the diameter of ferritin. Since the interior surface carries a high positive charge density due to presence of the arginine and lysine residues, CCMV is especially useful for the encapsulation of negatively charged species.

2.2. CPMV

The cowpea mosaic virus (CPMV) is another icosahedral plant virus belonging to the *Comoviridae* family. CPMV can be produced in 0.8–1.0 mg/g yields from infected black eyed pea plant leaves [24]. It is measured 30 nm in diameter, containing 60 copies of an asymmetric unit that is made of two different proteins: a small (S) and a large (L) subunit. CPMV is stable at a rather wide range of temperatures (up to 60 °C), in the pH range of 3–9, and in the presence of certain organic solvents. The outstanding stability of CPMV made it a very-well studied virus for nanotechnology and drug delivery [25].

2.3. RCNMV

The red clover necrotic mosaic virus (RCNMV) is a plant virus belonging to the *Dianthovirus* genus and the family of *Tombusviridae*. RCNMV is a soil-transmitted virus that consists of 180 identical protein subunits (37 kDa) arranged in the form of a T = 3 icosahedral virion with an outer diameter of 36 nm and an inner diameter of 17 nm [26]. It has been shown that in response to divalent cations, RCNMV can undergo a structural transition resulting in the formation of surface pores extending through the capsid. This structural transition in response to cytoplasmic levels of divalent cations is associated with the release mechanism of the bipartite RNA genome in vivo. This opening of surface pores by varying the Ca^{2+} and Mg^{2+} concentrations has been demonstrated to be useful in the packaging of molecules inside the RCNMV capsid (vide infra).

2.4. MS2

MS2 is a RNA-containing bacteriophage of which the phage head displays an icosahedral symmetry with an average diameter of 27 nm [27]. The spherical viral capsid comprises 180 identical protein monomers, which can be expressed independently in bacteria by recombinant methods and afterwards be assembled to form virus-like particles (VLPs) [28]. A yield of 30 mg/L has been reported for pure VLPs from an *E. coli* culture [29]. Moreover, the genome RNA could be easily removed during the VLP assembly in *E. coli*, resulting in empty capsids [30]. The empty capsid contains 32 pores, each 1.8 nm in diameter [28], which allows modifications of the capsid interior. The capsid is rather stable under variations in pH (3–10), temperature, and the surrounding environment [31].
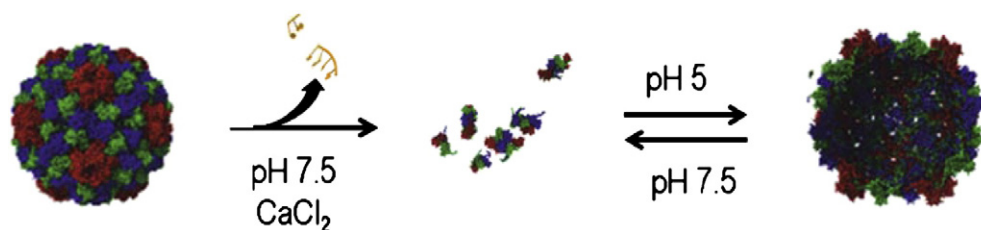

Fig. 2. Schematic representation of the disassembly of CCMV into viral RNA and protein dimers at pH 7.5 in the presence of CaCl₂. Following the isolation of the viral RNA, the protein dimers can be re-assembled again into empty capsids by lowering the pH to 5.

2.5. Qβ

The bacteriophage Qβ is a member of the *Leviviridae* family, forming icosahedral capsids from 180 coat protein subunits around a central RNA genome [32]. Qβ is approximately 28 nm in diameter and closely related to the bacteriophage MS2 [33]. In particular, Qβ VLPs are remarkably stable since they contain multiple inter-monomer disulfide bonds arising from cysteine residues at positions 74 and 80, which are present near the 5- and 3-fold axes of symmetry. In this way, each protein dimer is connected to the rest of the capsid by 4 disulfide bonds [34]. The high stability of Qβ renders it possible to make genetic alternations and chemical attachments to the side-chain residues of its 180 identical subunits [35–37].

2.6. M13

M13 bacteriophage is a rod-like virus (880 nm long and 6.6 nm wide for the wild-type variant) that specifically infects bacteria [38]. It is composed of a circular, single-stranded DNA that is encapsulated by approximately 2700 copies of the highly ordered, genetically modifiable major coat protein P8 and capped with 5 copies of four different minor coat proteins (P9, P7, P6, and P3) at its ends. The two ends of the rod like virus (see Fig. 1e) can be engineered to display a peptide that can recognize a specific target (e.g. cells, tissues, or organs) through a technique called phage display [39]. The versatility of phage display has made M13 a very popular element for the fabrication of functional hybrids in nanotechnology [38,40,41]. Phage display and bioconjugation on M13 has also facilitated its biomedical applications in targeted drug delivery and cancer imaging.

2.7. Other viruses

The tobacco mosaic virus (TMV) is a rod-like virus with a length of 300 nm and diameter of 18 nm. It can be purified from infected tobacco plants in kilogram quantities. TMV capsids are composed of 2130 identical protein subunits, which assemble around the viral RNA. The tubular capsid structure has a hollow core with a diameter of 4 nm. The structural chirality and inherent asymmetry of the virus make chemical or physical differentiation of one end of the helical rod possible [10]. The aspect ratio of TMV assemblies in vitro is determined by pH, ionic strength, and protein concentration. Due to its high stability in a broad range of pH's (3.5–9), at elevated temperature (up to 90 °C), and chemical environments, TMV could be chemically modified to a great extent on both its interior and exterior [42].

The turnip yellow mosaic virus (TYMV) is an icosahedral plant virus with a diameter of 28–30 nm, which can be isolated in gram quantities from turnip or Chinese cabbage [43]. It contains 180 chemically identical protein subunits of 20 kDa each, which self-assemble in T = 3 symmetry [44]. Empty capsids of TYMV can be isolated naturally from the host plant or generated artificially by manipulating the virus particles under pressure [45], basic environments [46], or repeated freeze–thaw cycles [47]. These methods may also induce holes in the capsid, making it possible to introduce guest materials into the interior. TYMV is also chemically (up to 50% organic solvent), pH (4–10), and temperature (4–60 °C) stable allowing for bioconjugation reaction. Chemo-selective modifications on native TYMV could be easily realized at the lysine residues and carboxylic acid groups. Studies on other icosahedral viruses such as the bromo mosaic virus (BMV) and the Canine parvovirus (CPV) [48] have also been reported. Several other plant viruses, such as the Tomato bushy stunt virus [49] and the Turnip crinkle virus [50], display similar properties as CCMV with respect to the reversible opening and closure of pores.

The insect nodavirus flock house virus (FHV) is a 34 nm particle containing 180 identical coat protein subunits. The exterior of the native FHV is not chemically reactive, thus genetic modifications were needed to introduce lysines on the capsid of the virus [51].

3. Packaging drug molecules into virus-like particles

3.1. Drug packaging based on supramolecular chemistry

Viruses generally package their genetic materials within the viral capsids. The principles governing the packaging of the viral RNA or DNA can also be utilized to package functional cargoes [52], based on simple supramolecular self-assembly and disassembly processes. The viruses that has been explored for material decoration in the interior include the cowpea chlorotic mottle virus (CCMV), the brome mosaic virus (BMV), the Simian virus (SV40), the red clover necrotic mosaic virus (RCNMV), the human polyomavirus JC virus, the Hibiscus chlorotic ringspot virus (HCRSV), and the alphaviruses. For most of these viruses, only "proof of principle" studies on their encapsulation based on self-assembly have been reported so far and these will be summarized here. However, the extension of these systems for the encapsulation of drug molecules can be envisaged.

CCMV has been studied quite extensively by several research groups with respect to its encapsulation of therapeutic and imaging agents for various applications. In the early studies, the opening and closure of the pores of the CCMV capsids under pH variations was used to nucleate inorganic mineralization reactions [53]. The interior surface of the native CCMV capsid is positively charged, which could be directly used for the encapsulation of negatively charged species. A straightforward way to entrap molecules inside CCMV is by altering the solution pH. CCMV coat proteins obtained by the disassembly of CCMV at higher pH (pH 7.5) could be assembled again by lowering the pH (pH 5) (Fig. 2). In the presence of a guest species this process could be applied for the encapsulation of compounds. Cornelissen et al. have reported on the entrapment of the enzyme horseradish peroxidase (HRP) for single enzyme studies [54]. In another paper the capsid proteins were incubated together with a negatively charged polymer, e. g. poly (styrene sulfonate) (PSS) leading to the formation of T = 1 particles [55]. This process could in principle be utilized for the encapsulation of negatively charged drug molecules for pharmaceutical applications.

However, the above methods are quite limited as they are only useful for the packaging of overall negatively charged compounds. Making use of a leucine zipper motif, the controlled encapsulation of multiple proteins was achieved [56], paving the way for the encapsulation of positively charged proteins inside CCMV. In this method, heterodimerization between the capsid protein provided with a positively charged leucine zipper K-coil and the fluorescence protein EGFP containing a negatively charged leucine zipper E-coil was achieved, resulting in the encapsulation of a desired number of EGFP molecules inside the capsid (Fig. 3).

Moving closer to real applications, recently a general way of loading virus nanocarriers has been reported based on CCMV [57]. In this method, CCMV capsids were loaded with polymer-DNA amphiphiles (Fig. 4). These amphiphilic biohybrid blockcopolymers aggregate into micelles with a hydrophobic core and a negatively charged DNA corona. The negatively charged micelles induce capsid formation, allowing the entrapment of a large number of small oligonucleotides (ODNs) as a constituent part of the micellar template. Furthermore, preloading of the micelles with hydrophobic entities (e.g. hydrophobic drugs) in the core or with hydrophilic entities by sequence-specific hybridization of DNA enables the encapsulation of various types of small molecules inside the viral capsids.

Making one step further in utilizing CCMV for drug delivery applications, Cornelissen et al. have recently described the encapsulation of a therapeutic agent, i.e. zinc phthalocyanine (ZnPc), into CCMV and its subsequent internalization into macrophage cells. Phthalocyanines (Pcs) are light-absorbing organic molecules that have been verified for its effectiveness in photodynamic therapy (PDT). PDT involves the generation of singlet oxygen molecules by a sensitizing group [58]. Porphyrins and phthalocyanines are commonly used
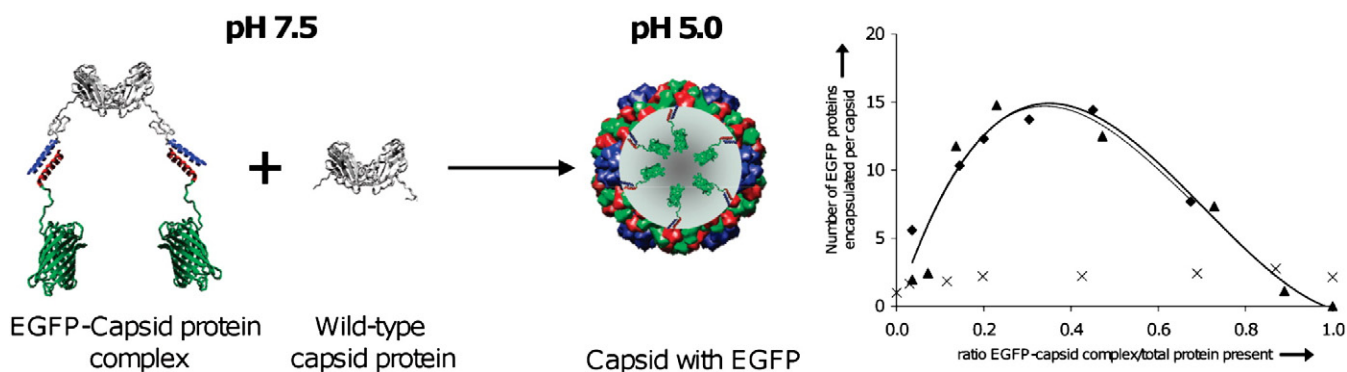
Fig. 3. Left: schematic representation of the encapsulation of enhanced green fluorescent protein (EGFP) into CCMV using the coiled-coil leucine zipper motif. The EGFP–capsid protein complex is mixed with wild-type capsid at pH 7.5 and subsequently dialyzed to pH 5.0 to induce capsid formation. Right: number of encapsulated EGFP proteins per capsid as a function of the EGFP–capsid complex/total protein ratio. Diamonds and triangles represent data points of duplicate experiments. Crosses represent negative control experiments in which unbound EGFP with a leucine zipper motif l was used instead of the EGFP–capsid protein complex. The thick line represents the polynomial trend line through the triangular-shaped data points and the thin line the polynomial trend line through the diamond-shaped data points. Reprinted with permission from [56]. Copyright 2009 American Chemical Society.

PDT agents due to their high extinction coefficients and efficient intersystem crossing to the triplet state [59]. By incubating the water-soluble ZnPc and CCMV capsid proteins at certain pH and salt concentrations, supramolecular stacks of ZnPc were formed inside CCMV (Fig. 5) [60]. CCMV $T = 1$ particles containing stacks of ZnPc were successfully internalized inside RAW 264.7 macrophage cells, which were subsequently killed upon red light irradiation. Although the current report is mainly focused on cell studies on macrophages, follow-up investigations in combination with cell-targeting strategies (vide infra) are expected to open possibilities for utilizing this system in cancer cell research.

In addition to directly making use of the positively charged interior of the CCMV cavity, the interior surface of the CCMV capsid could also be altered, through protein design and genetic engineering, to display a net negative charge, while maintaining its ability to assemble [61]. Based on this principle positively charged drugs could also be packaged inside the viral capsid.

Apart from CCMV, many other virus-based nanostructures have been studied for the encapsulation of nanoparticles based on simple self-assembly and disassembly processes. Dragnea et al. described

the incorporation of CdSe/ZnS semiconductor quantum dots (QDs) into BMV for the design of intracellular microscopic probes and vectors [62]. Efficient incorporations (~75%) were observed with HS-PEG-COOH coated QDs and DNA coated QDs. On the other hand, DHLA (dyhydroliponic acid) and lipid micelle-coated QDs could not induce the formation of the capsid. The HS-PEG-COOH coated QDs displayed the highest photostability to prolonged short-wavelength radiation and showed the highest ability in promoting virus capsid assembly. Qdot incorporation into viral capsids was also reported for the mammalian virus simian virus 40 (SV40) [63]. VP1, the major viral capsid protein of SV40 with an N-terminal polyhistidine tag (His-tag) was expressed in *E. coli* and the purified proteins were assembled together with QDs coated with mecaptoacetic acid (MAA) to give virus-like particles in high yield. It was shown that the QD encapsulated SV40 VLPs could be internalized into Vero cells, paving the way for the targeted delivery of nanoparticles.

Gold nanoparticles (GNPs) could also be encapsulated in VLPs in a similar way. The in vitro assembly of alphaviruses with functionalized GNPs has been reported [64]. Phosphane and DNA functionalized GNPs with sizes in the range of 8.3–27.4 nm were used as the

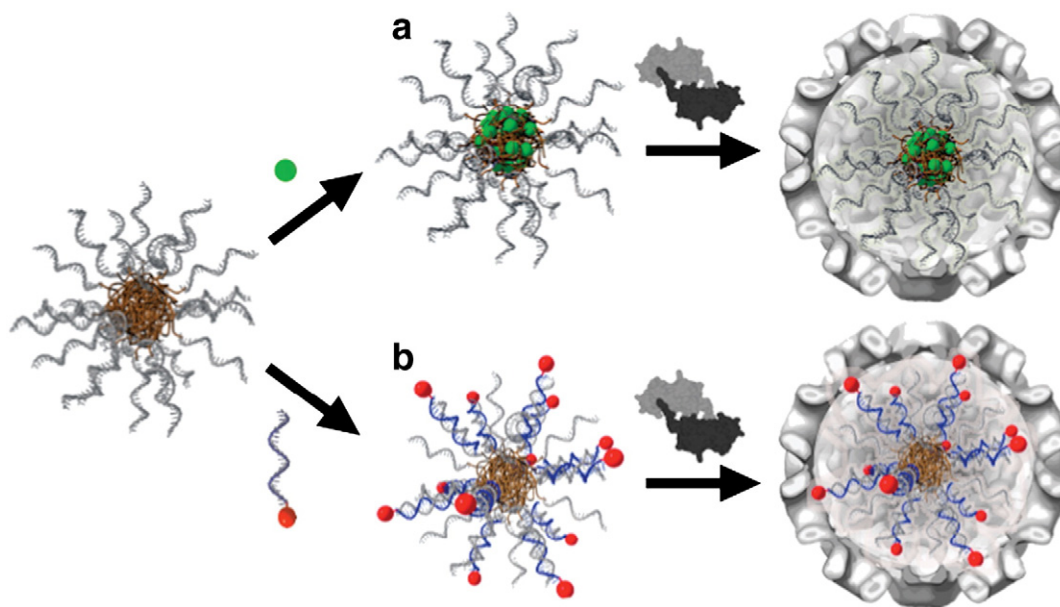**Fig. 4.** DNA micelle-templated virus-like particle formations. (A) Loading of hydrophobic molecules (green) into the core of the micelle. (B) Equipping the micelles with hydrophilic molecules (red) attached to complementary DNA strands by hybridization. CCMV coat protein molecules encapsulate the micelle by a simple mixing process at neutral pH. Reprinted with permission from [57]. Copyright 2010 American Chemical Society.
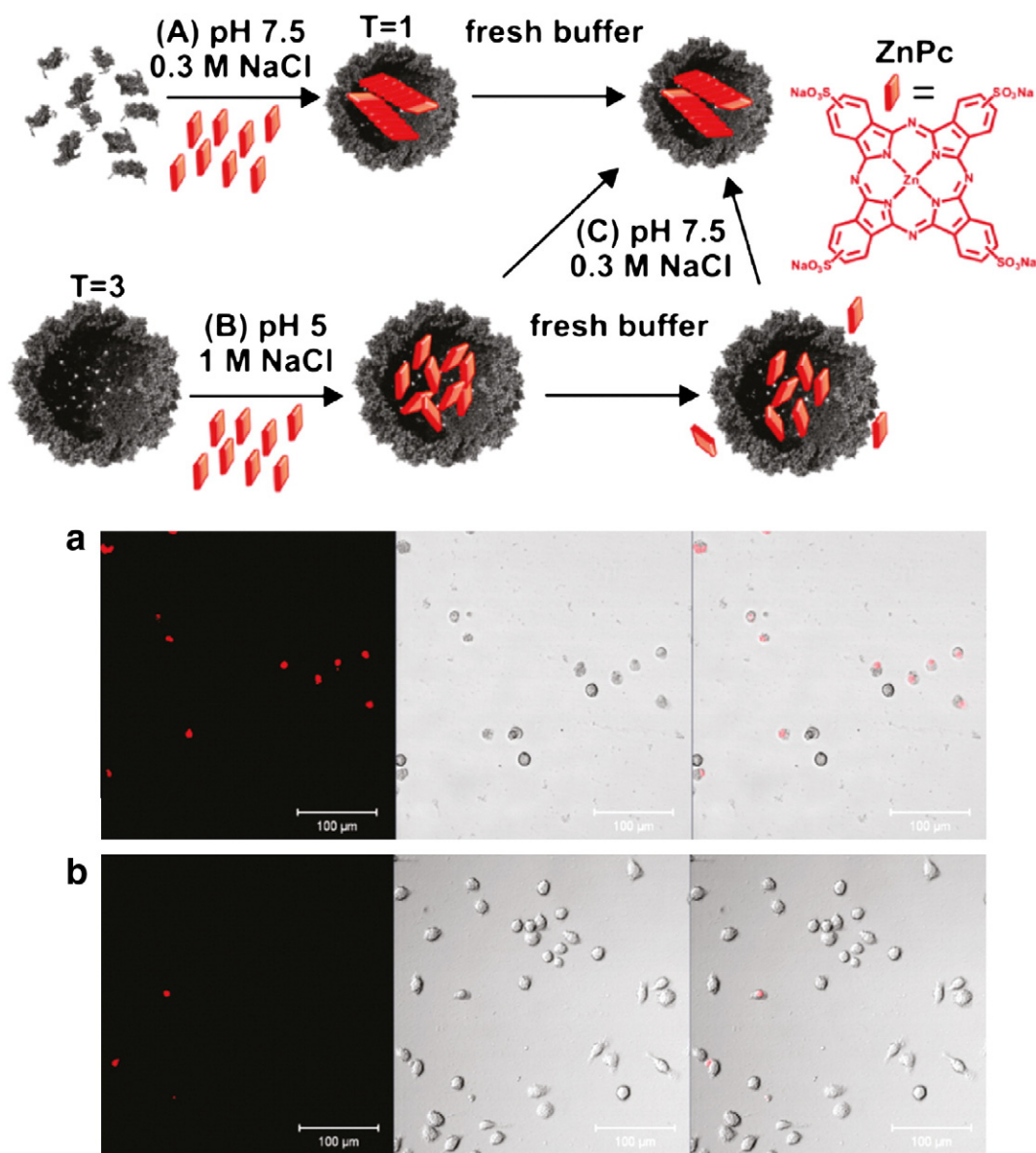
Fig. 5. The encapsulation of the photosensitizer ZnPc into CCMV and its application potential in photodynamic therapy (PDT): Top panel: schematic representation of the routes for the encapsulation of ZnPc stacks into CCMV VLPs. The length and arrangement of the stacks within the capsids in the cartoon are tentative. Lower panels: micrographs obtained when a culture of RAW 264.7 macrophage cells was irradiated with a mercury lamp (excitation BP wavelengths of 620/60 nm) for 20 min, (A) in the presence of ZnPc-loaded T = 1 capsids and (B) in their absence. Dead cells were stained with propidium iodide (PI), and they appear red in the fluorescence images. Left, fluorescence images (excitation at 543 nm, emission recorded with a LP 560 filter); middle, transmission images; right, overlap of the two images [60].

templates. It was shown that a maximum encapsulation yield of 62% could be achieved with cores of 18.7 nm in diameter. The GNP containing virus-like particles were shown to be more stable than empty capsids upon storage. As expected, charge neutralization was found to be necessary for the capsid formation.

In the case of RCNMV incorporation of nanoparticles requires a special procedure. The nanoparticles, e.g. GNPs (5–15 nm), QDs (CdSe, 3–5 nm), or magnetic material $CoFe_2O_4$ (4–15 nm) were first provided with an artificial RCNMV assembly motif [65]. This is due to the fact that the assembly of RCNMV coat proteins requires a genome RNA–RNA interaction prior to the CP assembly to form an inner scaffold (a T = 1 inner cage). The "origin of assembly" (OAS) tethered to the nanoparticles is a 20-base deoxyuridine-modified DNA oligonucleotide, which hybridizes with full length RNA-1 to form a functional artificial inner cage. This inner cage then templates the assembly of the coat proteins to form an enclosed protein cage. Nanoparticles presenting the OAS can nucleate the assembly of an

artificial VLP with sizes of ca. 32.8 nm, smaller than that of the native virus, which has a size of 36.6 nm. Only particles with sizes smaller than the critical size of 17 nm, i. e. the size of the inner cavity, can be encapsulated.

The Hibiscus chlorotic ringspot virus (HCRSV) has been loaded with polyanionic species such as polystyrenesulfonic acid and polyacrylic acid with an M_w threshold of about 13 kDa, yielding VLPs structurally similar to that of the native virus [66].

A pH-dependent drug release system making use of specific supramolecular interactions has been developed based on the human polyomavirus JC virus [67]. In this type of VLPs, the release mechanism is the change in pH and the drug is bound by making use of a hexahistidine motif (His_6). Two viral proteins, the major coat protein VP1 and the inner core protein VP2 are present in the JC VLPs. VP2 was used as an anchoring unit displaying His_6 tags inside the VLPs, which can offer specific and reversible attachment of drug molecules that contain a His_6 tag targeting nitrilotriacetic acid (NTA) segment.

The holes on the surface of JC VLPs are around 1 nm, allowing small drugs to pass through. As a proof of principle, a fluorescent small compound nitrilotriacetic acid-sulforhodamine (NTA-SR) was used as the guest molecule to test the feasibility of the encapsulation–release system. The controlled release of the guest dye was further examined using NIH3T3 cells. Petry et al. have also reported on the packaging of a small molecule dye, propidium iodide (PI), inside the JCV VP1–VLP [68] during the dissociation/re-association process. This suggests that VP1–VLP of JCV can also become a cell-specific drug delivery vehicle.

3.2. Drug loading by chemical attachment

Apart from simple disassembly/reassembly of the viral capsid, drug cargo can also be loaded through covalent attachment of drugs or their analogs to certain reactive moieties on the capsid proteins. Moreover, residues can also be site-specifically engineered into the capsid protein to provide reactive sites. For example, a cysteine residue genetically introduced into a subunit presents a reactive thiol group in the assembled capsid structure at all symmetry-related sites. Fig. 6 summarizes the general bioconjugation techniques that have been used for modifying VLPs. A few individual examples will be discussed below to highlight the current status in this field.

A general method for cargo attachment is by forming an amide bond between a carboxylate group and an amine group. One of the early examples was the general chemical modification method reported by Douglas and Young [69] in the case of CCMV. In this method, both small fluorescence molecules and peptides are attached to the exterior-surface exposed carboxylate or amine functional groups of CCMV. The carboxylate groups were first activated by reaction with 1-ethyl-3-(3-
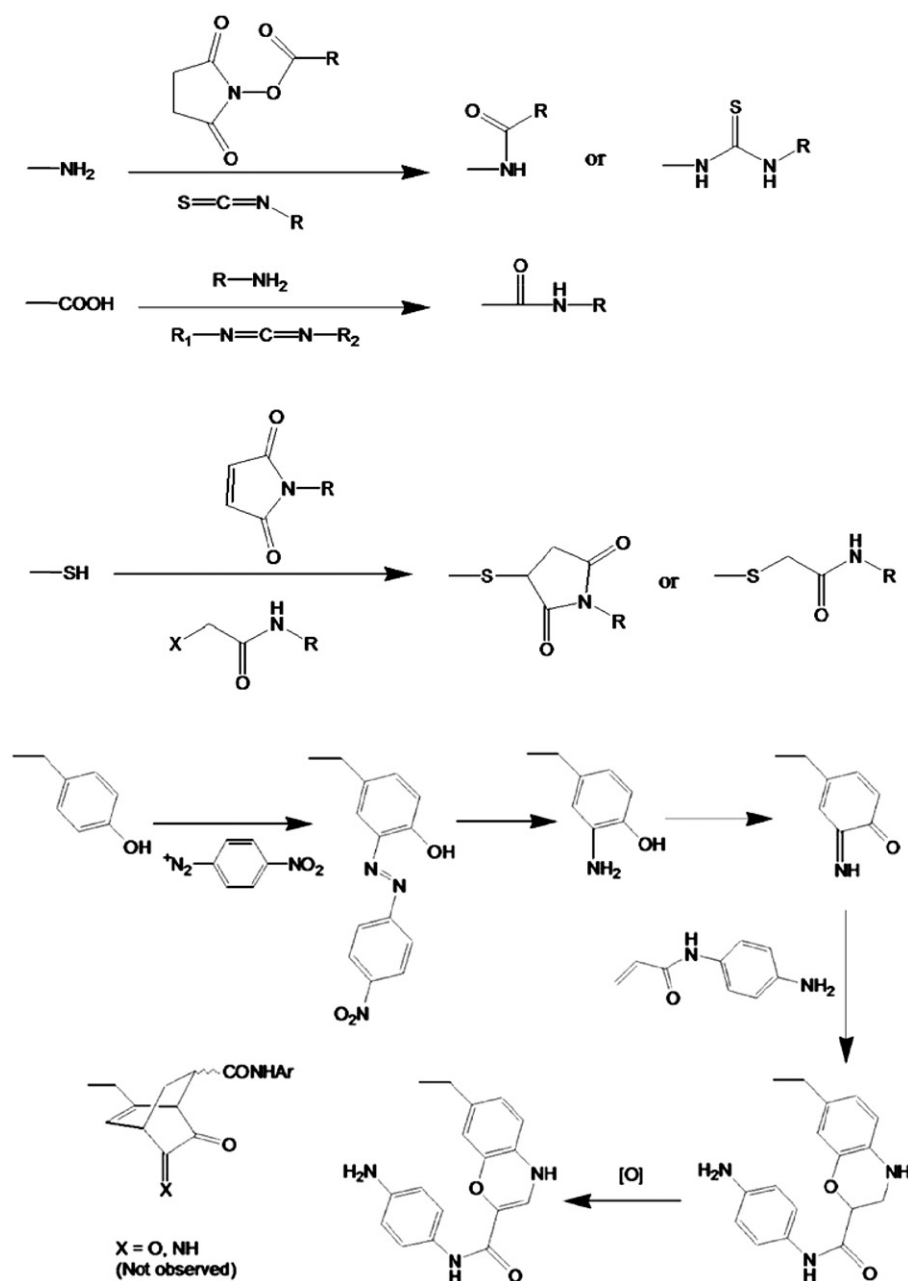
Fig. 6. Commonly used bioconjugation techniques for the covalent modification of VLPs: amino groups can be derivatized by reaction with N-hydroxysuccinimide esters or isothiocyanates; carboxylic acid groups can be activated using carbodiimides (usually 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, EDC) and reacted with amines; alkylation of thiol groups is possible, usually by a reaction with maleimide or bromo/iodo acetamides; the phenol groups on tyrosine residues may be modified with high efficiency and selectivity using diazonium-coupling reactions combined with a new hetero Diels–Alder reaction (adapted from Ref. [30]).

dimethyl-aminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to generate the succinimidyl esters, which were subsequently reacted with amine containing compounds. Based on the same reaction, surface exposed amine groups were derivatized using the succinimidyl esters of reactive carboxylic acid compounds. As mentioned, thiol groups could also be used as reactive sites. However, neither of the two native cysteines (59 and 108) in wild type CCMV is reactive. For this reason, CCMV was engineered using molecular biology techniques with reactive cysteines (R82C and A141C) and then reacted with the maleimide form of the targeted cargo. It was shown that using the above mentioned methods up to 540 amines, 560 carboxylic acids, and 100 thiol groups of CCMV could be labeled with fluorescent cargo. Moreover, the surface exposed cysteine residues could also be covalently attached to free thiols in peptides by an oxidative coupling reaction. In another application-oriented example [70], CCMV was first genetically modified by replacing lysine 42 with an arginine residue (CCMV/K42R). Subsequently, serines 102 and 130 were replaced by cysteines to give the modified species S102C/K42R and S130C/K42R. A ruthenium complex, which acts as a photosensitizer (PS) was modified with an iodoacetamide group and selectively reacted with the sulfhydryl groups of the surface exposed cysteines. The resulting PS–CCMV conjugates were then targeted into *S. aureus* cells and the photodynamic killing of these cells was evaluated.

In recent years there have been increasing interests in the use of fullerenes (C$_{60}$) for drug delivery and other applications in biomedicine. C$_{60}$ is a promising candidate to be used as a photosensitizer in cancer therapy and the treatment of inflammatory diseases [71,72]. However, the insoluble character of C$_{60}$ in water hinders its application potential. In order to solve this problem, Finn and Manchester et al. [73] studied the chemical attachment of C$_{60}$ to the VLPs of CPMV and Qβ. The VLPs were covalently decorated with C$_{60}$ in two ways: by using NHS chemistry with C$_{60}$ derivatives to form VLP–C$_{60}$ and by applying a Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAc) reaction to form VLP–PEG–C$_{60}$. It was shown that the click reaction was much more efficient in generating water-soluble and biocompatible conjugates. The cellular uptake of these conjugates was further investigated in HeLa cells, demonstrating that the internalization of these particles was not inhibited by the attachment of the C$_{60}$.

In addition to the above types of VLPs, the M13 bacteriophage has been chemically modified in a similar manner [74]. The modification of the amino groups of lysine residues and the carboxylate groups of aspartic acid and glutamic acid residues with fluorescent derivatives on the surface of M13 were achieved by the same NHS chemistry. In addition, the phenol groups on the tyrosine residues of M13 also showed reactivity toward chemical modification. 3-Ethynylbenzenediazonium was used for a diazonium coupling reaction with M13, resulting in an alkyne moiety linkage on its P8 protein. The alkyne functional group made it possible to efficiently conjugate a series of small molecules through the CuAAc reaction (vide infra).

Francis et al. have reported on the conjugation of the tyrosine residues on the interior surface of the MS2 bacteriophage [30]. It was shown that tyrosine 85 of the interior MS2 capsid surface can undergo rapid diazonium coupling with *p*-nitroaniline derivatives, including large dye molecules [75]. Up to 70 dye molecules could be coupled to the capsid in this way, which opens the possibility to include many drug cargo molecules. Furthermore, genetic modification rendered a unique reactive cysteine residue (C87) in the interior of the MS2 capsid, facilitating its functionalization with maleimide-containing reagents. In a more recent example of the group, up to 180 porphyrin maleimide molecules (Fig. 7, compound 1) [76] have been covalently attached to the interior surface of MS2. After directing their uptake by Jurkat leukemia T cells, a large number of cells were shown to be killed in only 20 min by the photodynamic effect of the porphyrin molecules.

In a similarly practical example, the anticancer drug doxorubicin (DOX) was covalently attached to the major structure protein VP6 of the rotavirus to form DOX–VP6 conjugates [77]. This was done via amide bond formation between the carboxyl groups of VP6 and the amine group of DOX. It was shown that most of the 6 carboxylate groups exposed on the exterior surface of each subunit and the 7 carboxylate groups located on the interior surface of VP6 were all chemically linked to DOX. The DOX–VP6 conjugates were subsequently assembled into VLPs and brought into cells [77]. The cytotoxicity of the conjugated DOX was compared to that of free DOX. This approach is very similar to the approach adopted by Douglas and Young [11] on the modification of the Hsp cage with DOX, which could be relevant in biomedical applications as the protein cages keep their cargo until the latter is directed to be released.
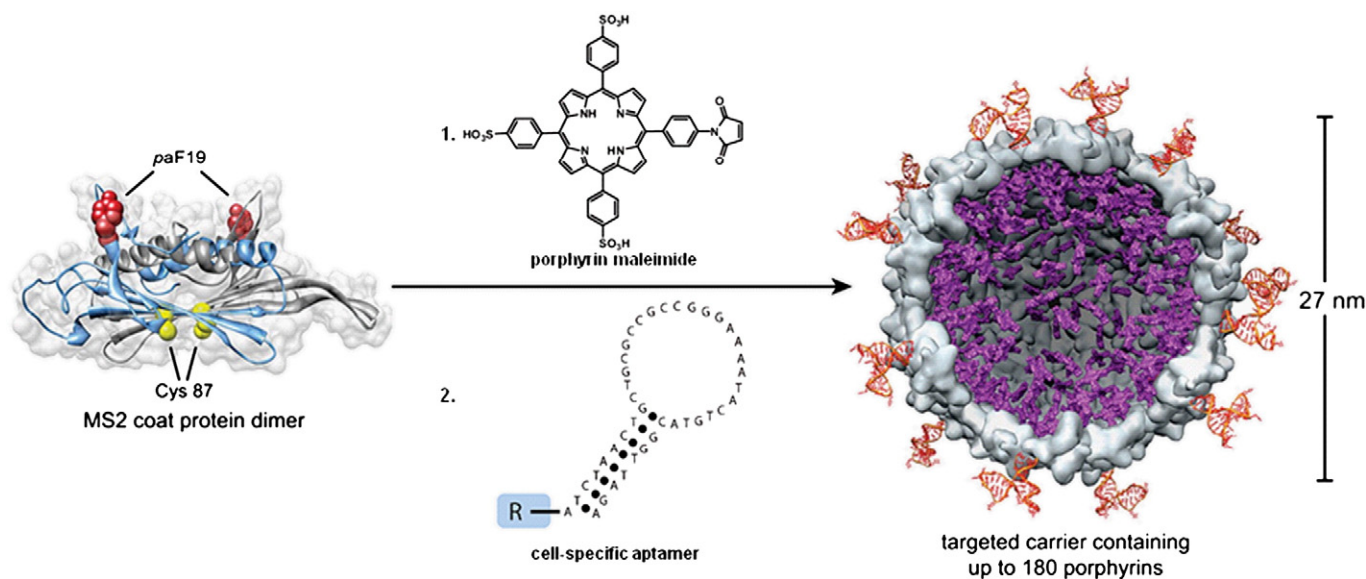

Fig. 7. Dual-surface modification of capsids for targeted delivery (adapted from Ref. [76]): 1. Covalent attachments of porphyrin moieties to the interior of bacteriophage MS2. Cysteine residues on the capsid interior were modified with a porphyrin maleimide derivative 1 (purple), enabling the system to be used in photodynamic therapy; 2. For the exterior surface modification, the aptamer is first modified with a phenylene diamine group. A T19paF mutation on the capsid allows for the attachment of the modified DNA to the exterior surface of MS2 by a NaIO$_4$-mediated oxidative coupling reaction (vide infra).
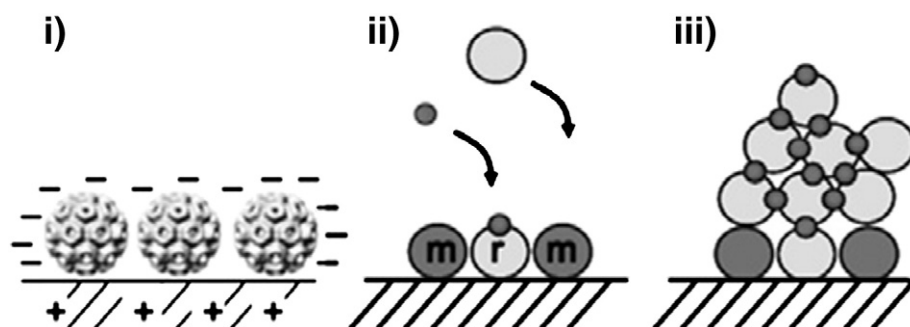
Fig. 8. (i) A base film of CCMV is formed from biotinylated CCMV (r) embedded in a matrix (m) of non-biotinylated CCMV; the base film is bound to a positively charged surface via electrostatic interactions. ii) Cycles of exposure of the film to streptavidin (small gray dots) followed by biotinylated, fluorescently labeled CCMV (light gray circles) are used to produce clusters on the surface (iii). Reproduced from Ref. [78].

The above mentioned tyrosine–diazonium reaction (see Fig. 6) was found to proceed with high levels of conversion for the functionalization of the exterior surface of TMV under a wide range of reaction conditions [42]. Subsequently, the ketone that was introduced on the protein surface was utilized for further conjugation of a series of alkoxyamines via oxime formation. In this two-step strategy over 2000 copies of the desired functionality could be covalently attached to the virus exterior. In the reported work, these functional sites were later used for the decoration of PEG to alter the solubility of the virus. In addition, the glutamate residues on the interior of TMV could also be modified through standard amide bond formation reaction by activation using EDC. It was found that the addition of excess hydroxy-benzotriazole (HOBT) could most effectively suppress by-products formation in this reaction.

3.3. Hierarchical architectures

In order to increase the drug loading capacity or to enhance the signal in imaging applications, structures of higher order have been introduced in virus-like particles, e.g. by utilizing the layer-by-layer (LBL) technique. In the example shown by Douglas and Young [78], both electrostatic and more specific interactions were used to direct the LBL assembly of CCMV. CCMV was first biotinylated via a bioconjugation reaction involving the amines on its lysine residues as discussed in Section 3.2. As shown in Fig. 8, a mixture of the native negatively charged CCMV and the biotinylated CCMV (ratio 9:1) were first adsorbed onto a positively charged substrate via electrostatic interactions, forming a base film. Subsequently, a core–shell structure was built up using the biotin receptor sites by exposing

the film alternating to streptavidin and biotinylated, fluorescently labeled CCMV. Cluster formation of fluorescently tagged CCMV on the base film produced an amplification of the fluorescent signal that corresponded well with predictions of the growth as determined by ATR-FTIR. This in vitro built hierarchical system is expected to be useful for further characterization of amplification processes that are relevant for in vivo applications and to increase the amount of cargo that can be loaded in VLP-based systems for specific drug delivery applications.

In another example by Mao et al. [79] structures of higher order were formed from ZnPc containing liposomes and the rod-like M13 bacteriophage. The latter bacteriophage was genetically engineered to display negatively charged peptides on the sidewall comprising ~2700 copies of ordered major coat proteins. These negative charges facilitated strong electrostatic interactions between the phage and the ZnPc loaded cationic liposomes (Fig. 9). The potential of this higher order structure for PDT applications was evaluated by measuring the production of singlet oxygen ($^1O_2$) and by studying the internalization of the ZnPc M13–liposome complex in SKBR-3 breast cancer cells. Since liposomes can encapsulate a variety of anti-cancer drugs and phage display can be used for presenting a targeting peptide on the structure, the phage–liposome complex approach has the potential to become a general strategy for the targeted delivery of drugs.

4. Drug targeting by multivalency display—in vitro studies

The goal of a targeted drug delivery system is to direct a drug to a diseased tissue or a specific body site and to have a prolonged
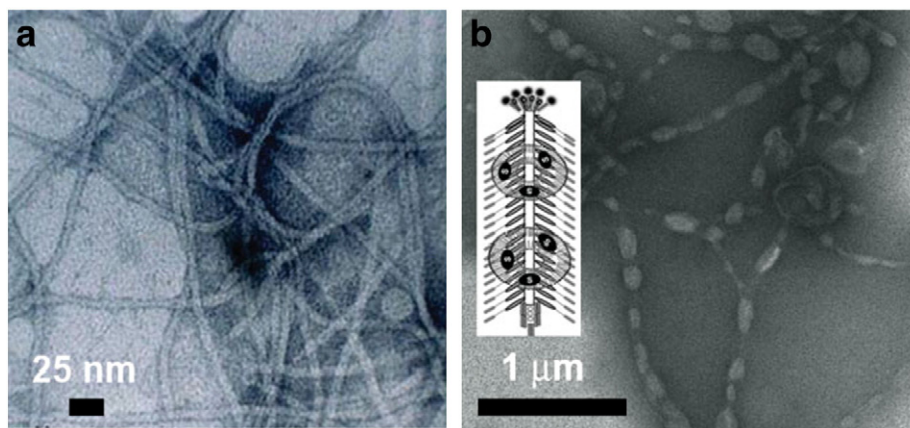
Fig. 9. TEM images of stained engineered phages a) before and b) 30 min after complexation with ZnPc-loaded liposomes. The formation of a beads-on-rod (BOR) structure is clearly visible as the liposomes are assembled on the surface of the phage to form the phage–liposome complex. Rodlike phages may be co-assembled in a head-to-tail format. The inset in (b) shows a cartoon (not to scale) of the liposomes–phage complex (the size of the liposome is actually much larger than the width of the phage, but smaller than the length of the phage). Reproduced from Ref. [79].

Fig. 10. Introduction of azides on the surface of VLP's by standard bioconjugation techniques, followed by coupling with alk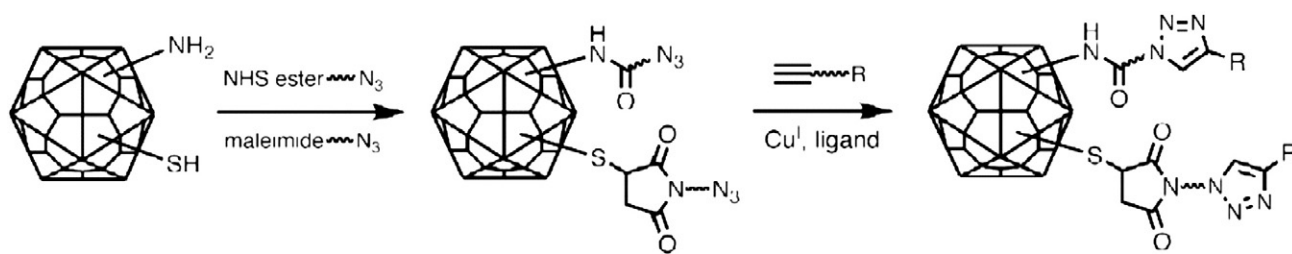ynes in the presence of a Cu (I) complex and a suitable ligand. Alkynes can be attached to the particle and then coupled to azides in the same way.

interaction with this tissue or site. Targeted delivery has a number of potential advantages including (i) reduction of harmful side effects due to controlled delivery to a particular cell type or tissue; (ii) the need of potentially decreased amounts of drug, decreased number of dosages, and possibly less invasive dosing; (iii) facilitation of drug administration of pharmaceuticals with short in vivo half-lives [80]. The targeting function of drug delivery vehicles can be accomplished by using appropriate receptor-binding domains and requires the linkage of these domains to drug carriers [81]. For virus nanocarriers there are generally two ways to achieve this, namely by chemically attaching the targeting domain (ligand) via bioconjugation to the exterior of the VLPs or by genetically inserting the sequence of a domain into the surface loops of the viral capsid protein.

4.1. Chemical modifications

The most straightforward way to chemically modify the exterior surfaces of virus nanocarriers is by bioconjugation techniques that have been routinely used in protein derivatization (see Section 3.2 of this review). However, in recent years bio-orthogonal reactions that involve functional groups that are inert to most biological molecules are gaining increased attention. One of the most successful bio-orthogonal reactions and most widely used with viruses is the Cu (I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction (Fig. 10). The required alkyne or azide groups are introduced on the VLP's by acylation of surface-exposed lysine residues using standard NHS ester chemistry. A number of cancer cell targeting ligands have been attached to different types of VLPs using the CuAAC reaction, including small molecules, antibodies, peptides, and proteins, as well as DNA aptamers.

Folic acid (FA) is a small molecule-ligand that has been widely used in targeted drug delivery to cancer cells. FA plays an essential role in human growth and development as well as in cell division

and DNA synthesis. Uptake of FA into cells is mediated by the folate receptor (FR). Binding of FA to FR initiates receptor mediated endocytosis and internalization of FA [82]. While most normal cells express low levels of FR, the FR expression is elevated in malignant cancer cells, e. g. cancer cells of ovary, uterus and mesothelium [83]. Dox loaded HCRSV was conjugated with folic acid by means of the carbodiimide coupling method using EDC and NHS [84], presumably to the surface-exposed lysine residues. It was shown that approximately 360 folic acids could be attached to one Dox-loaded virus-like particle, corresponding to roughly two ligands per coat protein. Cell studies provided evidence that the folic acid conjugated VLPs were preferentially taken up by OVCAR-3 cells. In a similar approach, an NHS ester of FA was conjugated to the surface lysine groups of CPMV, resulting in the attachment of 100 ± 10 folic acid molecules per capsid. However, in a model study with FR-expressing KB cells no measurable increase in the uptake of FA–CPMV was determined as compared to unmodified CPMV. As PEG conjugated CPMV's were shown to inhibit the natural uptake and targeting of CPMV, in a subsequent series of experiments a PEG–FA moiety was conjugated to CPMV through a CuAAC reaction between a CPMV–alkyne and an PEG–FA–azide. By controlling the density of PEG–FA on CPMV, approximately 60 ± 6 molecules could be linked to this virus. By comparing their targeting ability with PEG–CPMV, the specific interactions of PEG–FA–CPMV with tumor cells could be assessed and confirmed by flow cytometry [85].

Recently, lactobionic acid (LA) was used for the specific targeting of a rotavirus capsid VP6 to hepatocytes or hepatoma cells bearing asialoglycoprotein receptors (ASGPRs) [77]. There are four reactive amino groups on each VP6 capsid protein (k118, k123, k125 and 145), which could potentially bind chemically to the carboxyl groups of LA. Each VP6 viral capsid contains 780 capsid proteins. It was shown that two LA molecules were eventually bound to each VP6 protein. This corresponds to 1560 LA per capsid, which greatly
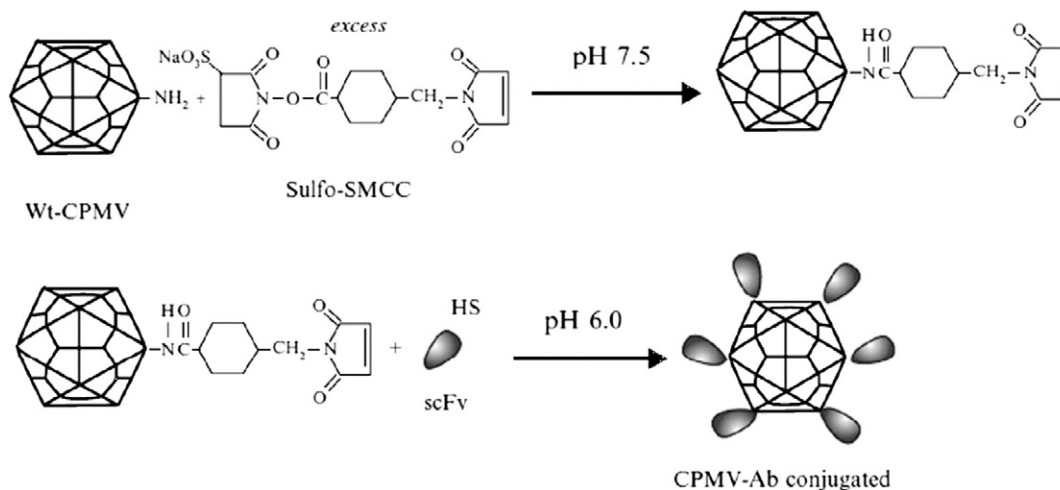

Fig. 11. Chemical conjugation of CPMV to scFv anti-CEA (adapted from [51]).

enhanced the receptor-mediated endocytosis as shown in the case of HepG2 cells.

Glycopolymes can be used for targeting purposes toward over expressed carbohydrate receptors in cancer cells. Finn et al. have reported on the attachment of a side chain neoglycopolymer with an alkyne chain end to an azide containing CPMV. The glycopolymer–CPMV was shown to have extraordinarily high binding affinities for lectins in the canonical hemagglutination assay [86].

Human holo-transferrin (Tfn) is an 80 kDa iron-carrier glycoprotein in vertebrates that is essential for iron homeostasis. It was used in a study in which proteins are applied as targeting elements. Tfn is specifically recognized by the Tfn receptor (TfnR), which is overexpressed on the surface of a variety of tumor cells and efficiently taken up by cells in the well-characterized clathrin-mediated endocytosis [87,88]. Tfn has been conjugated to CPMV [89] and Qβ [90]. In the latter case, the controlled derivatization of the sialic acid moieties on Tfn was applied, preserving the protein function and allowing for an efficient and controllable grafting of the protein to the surface of the VLP. It was shown that cellular uptake of the Qβ-Tfn particles was proportional to the ligand density, while the internalization was inhibited by equivalent concentrations of free Tfn.

Antibodies constitute another group of targeting proteins that could be chemically linked to VLPs. A single-chain (scFv) antibody

that recognizes the carcinoembryonic antigen (CEA), a cell-surface glycoprotein specifically overexpressed in a variety of tumor cells has been attached to CPMV in a way similar to the conjugation of Tfn [51]. CEA antibody (anti-CEA) was first expressed in bacteria; it possesses immunoglobin heavy and light chains that are connected via a flexible linker. A myc epitope sequence for detection and a cysteine residue for chemical conjugation was introduced at the C-terminus of the antibody. The anti-CEA attached CPMV VLPs were shown to bind specifically to HT-29 human tumor cells expressing CEA (Fig. 11).

An important strategy to improve cellular uptake of therapeutic molecules is the utilization of cell-penetrating peptides (CPPs). CPPs were first discovered as part of studies aimed at investigating the potency of several proteins to enter cells. Since then, CPPs constitute a very promising tool for the non-invasive cellular import of cargo and they have been successfully applied for in vitro and in vivo delivery of proteins and peptides, antisense oligonucleotides, siRNAs and other large particles to cells [91]. One of the CPPs that have been utilized in the delivery of VLPs is the tat peptide of the human immunodeficiency virus-1 (HIV-1). Tat enters the plasma membrane directly when applied extracellularly. The sequence [47]YGRKKRRQRRR[57] of the HIV-1 tat peptide is known to be responsible for the cellular uptake and is sufficient for the intracellular
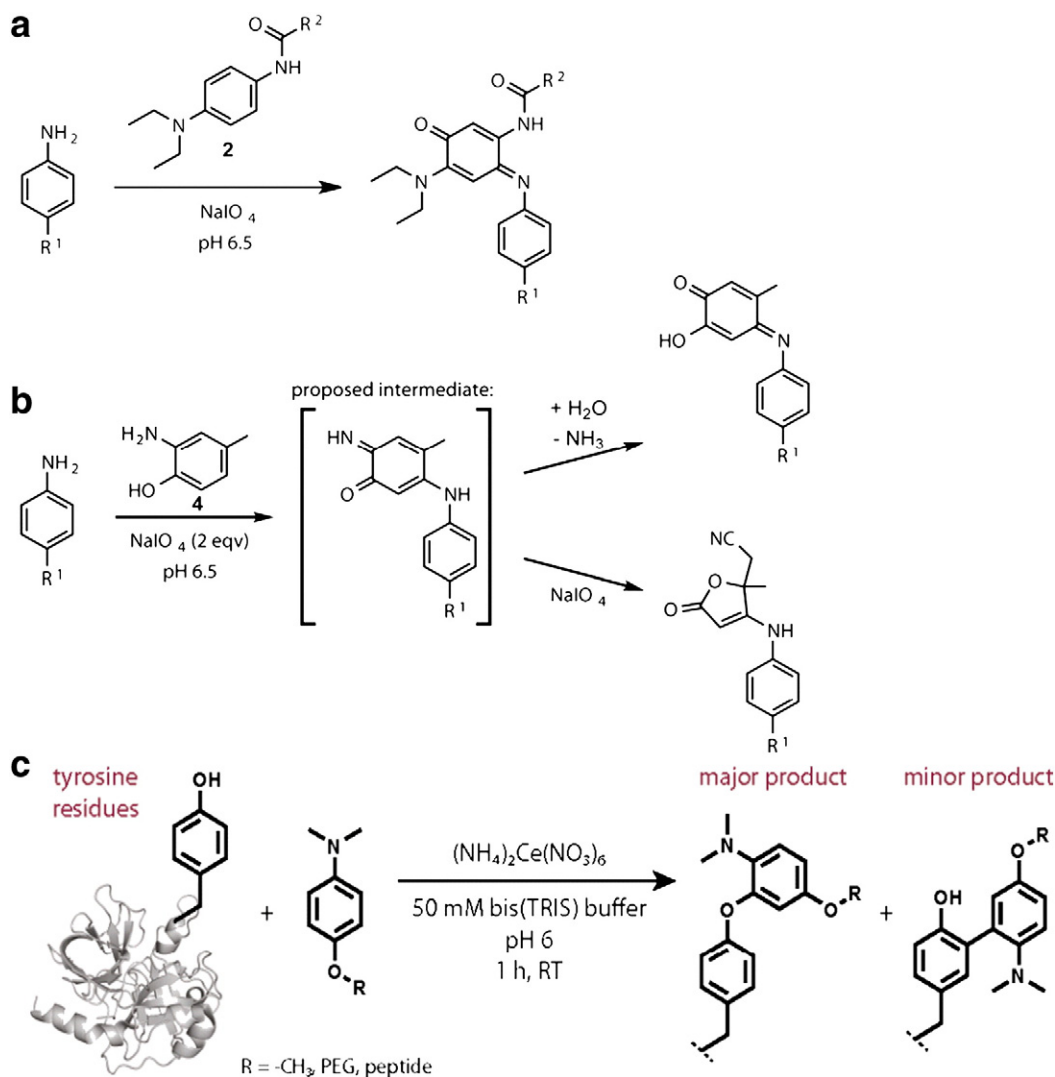
Fig. 12. New oxidative peptide conjugation reactions,: (a) coupling reactions between anilines and phenylene diamines, which occur within 30–60 min; (b) coupling reaction between anilines and aminophenols, which occur much faster to reach high levels of conversion in less than 2 min; (c) coupling reactions between tyrosines and anisidines using cerium (IV)- ammonium nitrate (CAN) as the oxidation agent. R, R^1 and R^2 can be CH_3, PEG or peptides. Reprinted with permission from [96,97]. Copyright 2011 American Chemical Society.

transduction and subcellular localization of the peptide [92,93]. Li et al. used a heterobifunctional crosslinker, sulfosuccinimidyl-4-(p-maleimidophenyl)-butyrate (sulfo-SMPB) to conjugate tat with the VLP of the bacteriophage MS2 [94] for the delivery of antisense RNAs. In this conjugation reaction, the cysteine residues on tat react with the amino groups of the MS2. The tat-MS2 VLPs were subsequently shown to be delivered to the Huh-7 cells. In another example, also based on MS2, an efficient oxidative method to attach peptides to aniline-containing amino acid side chains introduced into the virus capsids using stop condon suppression was described [95]. Three CPPs were chosen for attachment to MS2, the neuroblastoma and breast cancer cell line targeting sequence VPWMEPAYQRFLGGG, the matrix metalloproteinases binding peptide GGCTTHWGFTLCG, and a derivative of the kidney-targeting sequence CLSGRRVCG. The MS2 mutant with pAF at position T19 (MS2-pAF19) was used for the oxidative coupling reaction as depicted in Fig. 12. Very recently, the same research group reported on a more efficient version of this reaction involving addition of anilines to 0-aminophenols under similar oxidizing conditions (Fig. 12b). This new reaction could lead to protein conjugates with equivalent chemoselectivity to very high level of conversion in less than 2 min [96]. Moreover, a new bioconjugation strategy was developed using cerium (IV)- ammonium nitrate (CAN) as an oxidation reagent for the coupling of tyrosine and tryptophan residues to phenylene diamine and anisidine derivatives. As a proof of principle, PEG and an RGD-substituted anisidine derivative have been successfully attached to the exterior surface of the MS2 capsid [97].

Electrostatic and complementary biological interactions have been successfully utilized for the targeted delivery of CCMV into *S. aureus* cells. The cationic polymer poly-L-lysine (PLL), which has been shown to enhance the activity of photosensitizers (PS) in PDT (Section 3.2), was applied for the non-specific targeting of the net negatively charged cell walls of *S. aureus* cells. In this case, an interlayer of PLL was first adsorbed onto the cell walls, followed by exposures to the PS modified CCMV VLPs. In another experiment, complementary biological interactions between biotin and streptavidin have been used for selective targeting purposes. CCMV molecules provided with PS were first biotinylated by reaction with sulfosuccinimidyl-6′-(biotinamido)-6-hexanamido hexanoate. Subsequently, cells to be targeted were first coated with a biotinylated antibody, followed by reaction with streptavidin (StAv). Before reaction with biotinylated PS–CCMV, these cells were incubated with biotinylated antiprotein A (SpA) monoclonal antibody (anti-SpA mAb-B). The targeting procedure in which biotin–streptavidin interactions are used, was shown to be much more selective than the one in which just simple electrostatic interactions were applied [70].

Another particularly attractive and convenient ligand for targeting viral capsids is the DNA aptamer. DNA aptamers are well-defined, easily synthesized, and can be evolved to recognize a wide variety of epitopes using the SELEX procedure [98,99]. DNA aptamers have a higher stability than their RNA counterparts in vivo, which can be further enhanced by backbone substitutions [100]. The group of Francis has shown that multiple copies of an aptamer, targeting tyrosine kinase 7 (PTK7) receptors on Jurkat leukemia T cells (strand A), could be attached to the capsid of bacteriophage MS2 to achieve efficient and selective cell targeting [101]. An N, N-diethylphenylene diamine substituted DNA aptamer was linked to the paF group on the exterior surface of MS2 using sodium periodate, resulting in the attachment of circa 20 copies of the 41-nt aptamer to each capsid (Fig. 7, compound 2). These modified MS2 capsids, which were decorated on the inside with porphyrins, were used to selectively target and kill Jurkat T cells following a PDT procedure [76].

4.2. Genetic mutations

In addition to chemical modifications of viral capsids for multivalency display, strategies to achieve cell type specific targeting also include direct genetic modification of the viral coat proteins with receptor-binding domains or sequences. It should be mentioned that a discussion of the procedures to achieve one or more specific (reactive) amino acids in the viral capsid proteins by genetic mutations goes beyond the scope of this section. In one of the early examples, Wickman et al. [102] constructed a modified version of the adenovirus (Ad), which contains a heparin-binding domain for targeted gene delivery to heparin-containing cellular receptors. Later, Vigne et al. [103] showed that the insertion of an integrin-binding RGD sequence into the serotypically nonconserved loop of the hexon monomer extruding from the adenoviral capsid could facilitate its recognition at the cellular surface.

The targeting of CPMV and FHV VLPs to neuroblastoma tumor cells using specific peptides was also studied [51]. To this end the CPMV virus was genetically modified to display the neuropeptide Y (NPY) analog as a targeting ligand. The relatively large 36-residue NPY peptide was displayed in the βB–βC loop of CPMV. It was shown that the CPMV–NPY interacted specifically with SK-N-MC cells that overexpressed the Y1 receptor. FHV was found to be more amenable to display the NPY peptide. After insertion at the 205 site of FHV, the VLP assemblies were produced in high yields similar to wild type FHV–VLPs.

Böhm et al. [104] have reported on a general strategy for the binding of a targeting ligand to a viral capsid, simply by means of genetic fusion of a viral capsid protein with a WW domain. Surface loops of viral capsid proteins often show high sequence variability due to the influence of the immune system in viral life-cycles, which makes these loops susceptible to the insertion of foreign sequences. WW domains are small protein domains named after two conserved tryptophan residues which are essential for the maintenance of the native fold and for ligand binding [105]. In the reported work, recombinantly expressed polyomavirus-like particles were genetically modified by fusing the sequence of a WW domain into the β-turns of the outer capsid protein VP1. The capsid formation efficiency of these modified viral proteins was subsequently evaluated. It was shown that in one of the variants (VP1–WW150), the WW domain maintained its highly selective binding affinities for proline-rich ligands, allowing a short-term coupling of external ligands onto the surface of VLPs. With the help of a disulfide bridge formed between two cysteines introduced at positions 19 and 114, the assembly efficiency can reach values larger than 95%, similar to that of wild-type proteins.

Genetic modification of the M13 phage to display both targeting peptides and imaging agents has been reported by Chen et al. [106]. In this work, a chimeric M13 bearing an αv integrin binding peptide and a streptavidin-binding adaptor moiety was prepared. The modified M13 phage was shown to complex with streptavidin–QDs specifically targeted to tumors in vivo.

5. Toxicity and immunology—in vitro and in vivo studies

Before testing VLPs for real nanomedicinal applications, a thorough evaluation of their toxicities and biodistributions in vivo is necessary. The size, shape, composition, surface chemistry, and associated physical properties of nanoparticles can greatly influence their toxicological nature, as well as deposition and clearance from the body [107]. Thus a complete understanding of the circulation, clearance, blood half-life, stability, immunogenicity, and organ biodistribution of potentially useful VLPs is crucial for eventually realizing their biomedical value [108]. However, only for a few extensively-studied virus-based protein cage structures research has reached the stage of evaluating their in vivo toxicity and immunology properties. These virus platforms include CPMV and CCMV, whose biodistribution and toxicity studies will be discussed below.

The broad tissue distribution and rapid clearance of CCMV in vivo was reported by Douglas and Young et al. in 2007 [109]. Two genetic

mutations were evaluated, CCMV S102C for microscopy studies and CCMV K42R for biodistribution studies. Texas Red (TR)-labeled CCMV were intravenously (IV) injected into mice from which various tissues were subsequently harvested and evaluated by fluorescence microscopy. The non-cell targeted CCMV was rapidly, i.e. within 1 h after IV injection, found in the lungs, kidneys and liver, their presence declining 24 h after IV injection. For the biodistribution studies CCMV particles were radiolabeled with ^{125}I and injected into both naïve and immunized mice. After injection, the labeled CCMV rapidly dispersed throughout the mouse system freely and was not preferentially localized in any particular tissue or organ type except the brain in both naïve and immunized mice. A high percentage of nanoparticles (57–73% ID) were excreted in 24 h. A test on the mouse urine suggested that the ^{125}I-CCMV particles were degraded in vivo and excreted in components smaller than the cage protein subunits. The fast release of CCMV from the body could potentially lessen the detrimental side effects of dose molecules that do not interact with a targeted tissue and decrease excessive exposure to imaging agents. The production of a strong IgG and IgM response indicated that CCMV was immunogenic.

CPMV cages were found to be essentially safe and non-toxic, as was shown in a study by Finn and Manchester et al. [110] on the biodistribution, toxicity, and pathology of these particles in vivo. Doses of 1, 10, and 100 mg/kg bwt of CPMV VLP were intravenously inoculated in mice and no visibly concerned clinical signs were observed. These particles were also rapidly cleared from blood circulation within 30 min with an average half-life of 4–7 min in plasma. It was found that CPMV VLPs were primarily localized in the liver and to a lesser extent in the spleen, but with no associated toxicity observed in liver or other tissues. A mild leukopenia, which could be related to the presence of virus in circulation, was observed. CPMV was immunogenic as was shown by a strong evoked immune response in mice when higher doses were applied. The rapid clearance and liver-selective trafficking of CPMV VLPs suggests that specific targeting of this type of particles would require modification with immune masking agents such as PEG. It was also shown that a modest PEG coating can inhibit the induction of the anti-CPMV response [111] as well as reduce the VLP uptake in endothelial cells, liver and spleen [112,113]. In a study carried out by Raja et al., a standard ELISA protocol was used to monitor the immune response of mice to PEG decorated and non-decorated CPMV over a period of circa 4 months. The effectiveness of PEG decorated CPMV in suppressing primary antibody response in the initial 56-day period was evident. Similar to CPMV, FHV has also been administrated by the oral route and could be observed in the bloodstream and in a variety of tissues [51].

Although detailed pathological examination of most other viral particles has not been performed, the cellular uptake and cytotoxicity of several VLPs have been evaluated in vitro. It was concluded from an MTT cytotoxicity assay that the HCRSV was not cytotoxic against the CCL-186, OVCAR-3, and CNE-1 cells at concentrations up to 1 mg/mL [84]. A study carried out by Koudelka et al. [114] showed that the uptake of CPMV by HeLa and MFT-6 cells occurred rapidly via a specific endocytic process, involving the direct binding of the virus to vimentin within the enriched plasma membrane of human cells. In addition, CPMV was reported to be internalized by several subsets of antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages and B cells both in vitro and in vivo. However, CPMV–APC interactions need to be further studied toward the development of CPMV based vaccine systems [115].

Recent human clinical trials have revealed that rodlike phage particles will not induce obvious toxicity and immune response in human beings [15]. Recent animal studies also suggest that rodlike phages can carry drugs that are chemically tethered to its sidewall, penetrate the blood–brain barrier, and then deliver the drug to the brain [16,17].

6. Conclusions

Virus-like particles (VLPs) based on biological building blocks have become an emerging type of nanocarriers for targeted drug delivery. Viruses have the unique advantage of structural uniformity and their chemical and conformational structures can be produced precisely and in large quantities. The versatile hierarchical assembly of viral coat protein subunits provides a natural and easy way for drug packaging. A variety of viruses have been shown to be amenable to both chemical and genetic modifications of their inner cavities as well as their outer-surfaces, which facilitates the attachment of not only covalently-bond drug molecules but also various cell or tumor targeting ligands. VLP platforms can thus easily offer the requirements that are needed for a drug nanocarrier system, such as biocompatibility, solubility in water, and high uptake efficiency. Moreover, VLPs can be modified with polymers (e.g. PEG) to boost their half-life in the host by moderating their immunogenicity.

While in vitro studies on many different types of VLPs have already been carried out extensively, few in vivo studies for rapid clinical translation have been reported. Although initial results on CCMV and CPMV are quite promising, issues concerning the toxicity, bio-distribution, and immunology of different types of nanocarriers have to be fully evaluated. This information will provide the safety guidelines needed for the eventual utilization of these systems in therapeutic situations. Since VLPs from different origins differ in their stability, the shelf life of potential drug carriers should also be studied in order to obtain a comprehensive picture of the material in use.

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