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Recent Advances in Protein-Based Nano-Templates for Drug Delivery

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Abstract

Protein-based nanotemplates for drug delivery utilize naturally occurring self-assembled nanoparticles for various medical applications. They are ideal for drug delivery due to their biocompatibility and biodegradability coupled with low toxicity. The monodispersity (uniformed size), polyvalency and biodegradability push it to the frontier of drug delivery platforms. In this review, the recent strategic development of drug delivery is discussed with emphasis on protein-based platforms for drug delivery.

Keywords: *Viruses, Nanotechnology, Bioconjugation*.

Introduction

Beyond causing infectious diseases, viruses provide useful nanometer-sized templates. Viruses are naturally occurring nanoparticles with more than 2400 known viral species with extraordinarily diverse morphologies and biochemical composition.[1] Their diameters range from 20-500 nm, and their genomes from 3000 to 375000 nucleotides.[2] They can be either single- or double-stranded RNA or DNA genomes packaged into icosahedral or helical capsids that can sometimes be wrapped in a lipped envelope. The capsid protects the viral genome, carries it from cell to cell within the specific host organism and transmits it from infected to non-infected host. The virus particles are metastable, once within the susceptible and permissive host cell, the viral genome seizes the cellular biosynthetic machinery to produce the viral progeny.[3]

Drug delivery systems have been designed for various platforms including synthetic (silica, polymers, and gels) and natural nanoparticles (NP) (lipids, proteins, and oligosaccharides).[4] The most extensively investigated NPs regarding their use in drug delivery, bioimaging, in vivo biodistribution and toxicity are quantum dots (QDs).[5] QDs are semiconductor nanocrystals with a size range of ~2–100 nm with distinctive optical and electrical characteristics.[6] Protein-based nanomaterials are defined as naturally occurring self-assembled subunits of the same protein or a combination of proteins making up the whole particles structure. The recent rapid advances in protein engineering and nanotechnology are offering great promises to revolutionized nanomedicine and drug delivery. However, viruses, virus-like particles (VLPs) and

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other protein cage architectures provide alternative platforms for combined cell-targeted imaging and therapeutic delivery systems. Some examples of nanoplatforms that have been extensively studied are liposomes,[7] dendrimers,[8] micelles,[9] polyamide amine (PAMAM) dendrimer clusters,[10] poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles,[11] hydrogel dextran nanoparticles,[12] polysaccharide nanoparticles,[13] metal nanoshells,[14] amphiphilic core-shell nanoparticles,[15] viruses,[16] and virus-like particles (VLPs).[17] Each of these systems has its advantages and disadvantages, which will be discussed later. From a material science point of view, virus-based nanomaterials offer a broad range of naturally occurring platforms. A key characteristic feature of protein cage structures is their size uniformity (monodispersity), non-toxic biodegradability, the ability to functionalize at three well-defined interfaces (external, internal and inter-subunit) and their chemical/structural composition where each particle exhibits an extremely homogeneous structural composition. Multimeric protein structures comprised subunits repeats, which could be closed (cage) or extended (filamentous). Closed structures, referred to as protein cages henceforth, function as nanocontainers that encapsulate naturally biological cargo nucleic acid, storage (iron) or transport within the cellular environment. Some of the well-studied protein-based assemblies are shown in Figure 1.

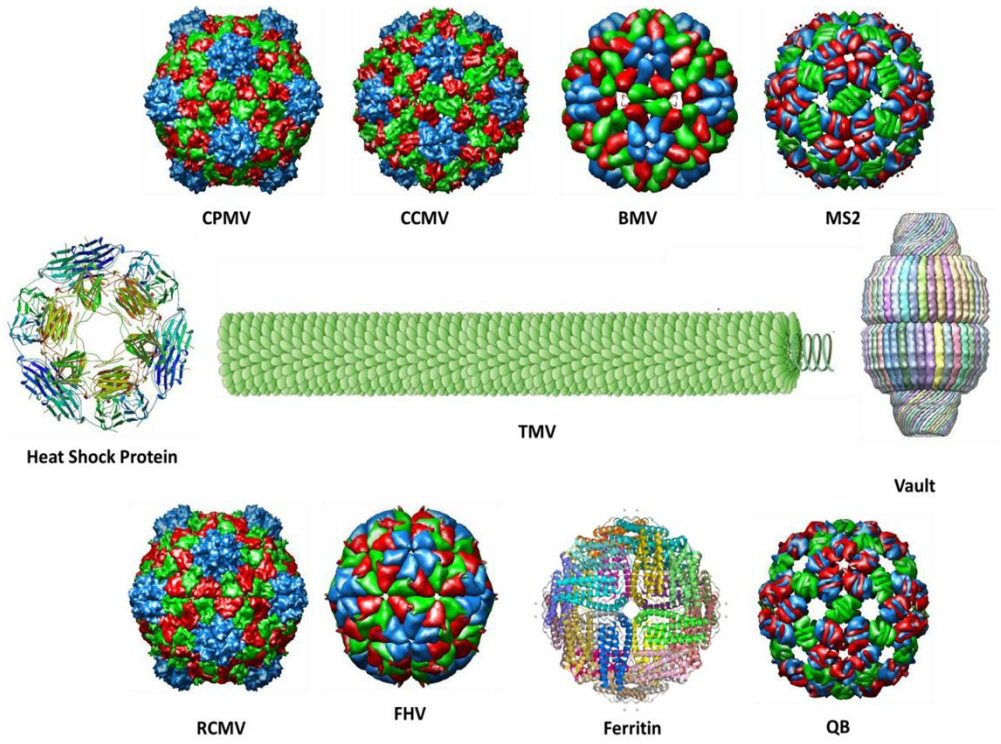

Figure 1: Library of few protein cages naturally present in nature such as Cowpea chlorotic mottle virus (CCMV; PDB ID: 1ZA7), Cowpea mosaic virus (CPMV, PDB:1NY7,) Red Clover Mottle Virus (RCMV; vdp 202) Heat shock protein (HSP; PDB ID: 1SHS), Ferritin (PDB ID: 1DAT) Flock House Virus (FHV, vdb 205), MS2 Bacteriophage(MS2; PDB ID: 2MS2), Brome Mosaic Virus (BMV; VDB: 1JS9), Vault protein (PDB: 4V60), Bacteriophage Qβ Capsid (QB; PDB: 1QBE), Tobacco mosaic virus (TMV; PDB: 3J06). All images were obtained from Protein Databank (http://www.rcsb.org/pdb) and VIPERdb as a database for icosahedral virus capsid structures (http://viperdb.scripps.edu/).

The diversity of such protein assemblies presents them as potentially useful building blocks for constructing novel new nanomaterials. The field of virology, in particular, resulted in a large scale of information ranging from the structure and assembly down to their atomic level. Also, proteins are known to be stimuli-responsive, that is, they respond to changes in the physical and chemical environment, which was exploited to design very responsive materials. Changes in pH, temperature, the presence of metals or other ligands, are among the most used factors that have been used to control the viral assembly/disassembly to enable loading viruses with valuable cargos. Protein assemblies have been isolated from organisms that live in extreme environments, such as in areas of high temperatures (hot springs isolated viruses, i.e., *Sulfolobus islandicus* Rod-shaped Virus-2).[18] In addition, the assemblies from peptides, DNA and lipids are also holding great potential in the field of nanoscience. The assembly of lipids into micelles and liposomes has been studied very well.[19] However, their assembly and the structure they are forming is quite different from those of proteins, peptides, and DNA; therefore, they will not be discussed further in this review.

Bionanoparticles

Bionanoparticles are nanomaterials created from biological building blocks; they can be used for biomedical applications such as drug delivery, gene therapy, vaccination, and bioimaging.[20] Protein cages are protein assemblies that form protein shell with a hollow interior cavity in which various biological or synthetic materials can be loaded within the cavity or conjugated to the interior/exterior capsid. Innovative use of protein cages has been through their use as size constrained vesicle for synthesizing inorganic and polymeric nanoparticles of highly monodisperse nature both in structure and composition.[21] A library of spherical protein cages derived mainly from viral capsids for internal mineralization (metal formation within the capsid) have been investigated. Reagents are entrapped within the virus capsid either through the disassembly of the protein cage and then the reassembly of the subunit in the presence of the reagents (drugs) or through a gating method where pores in the cage can be opened and closed allowing the diffusion of various materials within the virus capsids. These applications exploit the natural abilities within the viral capsid (electrostatic interaction with the molecules of interest with the amino acids within the assembled protein cage) without any further genetic or chemical modification to such assemblies while preserving particles integrity.[22]

VLPs consist of the virus capsid without the genetic material. For virons (whole viruses) the driving forces for self-assembly are multiple non-covalent interactions which lead to the formation of highly organized nanostructures of various sizes and shapes.[20] One of the major advantages of this type of nanomaterials is that they are

less expensive to produce than inorganic materials and can be easily produced in large quantities.[23]

Encapsulated chemicals within ferritin protein cage reported so far in the literature include: uranium,[24] copper,[25] manganese oxide,[26] vanadium,[27] beryllium,[28] cadmium, zinc, nickel, magnesium,[29] cobalt oxyhydroxide[30] as well as iron phosphate, iron arsenate, iron vanadate and iron molybdate.[31] Other molecules have also been encapsulated within ferritin such as desferrioxamine B complex,[32] Prussian blue[33] and doxorubicin, an anticancer drug.[34] Moreover, apo–ferritin protein is typically used for internal metal mineralization such as with cobalt oxide Co_3O_4 and Co(O)OH.[35] Mann and colleagues showed the formation of Fe_3O_4 (or γ–Fe_2O_3) under conditions of elevated temperature and pH.[36]

Ferritin comprises 24 subunits of two types (H and L) that self–assemble to form a hollow cage structure of 12 nm in diameter with hydrated iron (III) oxide encapsulated within the protein shell. In the native ferritin form, iron is stored within an 8 nm diameter cavity. The ferritin cage structure was first utilized in the biomineralization process.[37] Additionally, ferritin was developed as a delivery vehicle for the MRI contrast agent Gadolinium (Gd).[38] A variety of apo-ferritin types is commercially available and has been used for material encapsulation, e.g. the formation of Prussian blue in ferritin.[33]

Heat shock protein (Hsp), which originates from *Methanococcus jannaschii*, is another example of an extensively studied protein cage; it consists of 24 subunits that self-assemble into a cage with a 12 nm external diameter and 6.5 nm inner cavity.[39] Hsp differs from ferritin in having 3 nm pores that allow material exchange between the interior and the exterior environments.[40] Hsp has been utilized for the encapsulation and the release of antitumor drug doxorubicin.[22] Besides, Hsp was reported as MRI contrast agent with extremely efficient relaxivity.[41]

Vaults are another class of self–assembled cellular protein nanocapsules. They were named so as their morphology with many arches resembles that of cathedral ceilings.[42] Vaults are found in high copy number in many higher eukaryotes (including humans). Vaults are 13–MDa (million daltons) ribonucleoprotein particles with an internal volume of 5×10^7 Å^3. Vaults tend to have a hollow, barrel–like structure with two protruding caps and a thicker waist with overall dimensions of 420×420×750 Å (see Figure 1). Due to their large structure, vault particles can encapsulate and accommodate large molecules such as green fluorescent protein (GFP) and luciferase enzyme.[42]

Virus-based nanotemplates

The word virus is derived from Latin, and it means "poison." Viruses are defined as obligate intracellular parasites. Viruses can only replicate within a host cell that is susceptible and permissive.[43] Non-enveloped virus particles consist of two

components: one type or more of capsid proteins in addition to the viral genome.[44] Virus nanoparticles VNPs are considered as very stable macromolecular architects. Viruses are adapted to tolerate a broad range of environments (cellular and non-cellular), yet are sensitive enough to release their nucleic acid when they infect a susceptible cell to initiate the biosynthesis process.[45]

VNPs offer a significant advantage of morphological and structural uniformity, biocompatibility, safety and the amenability for genetic and chemical modification over nearly all synthetic counterpart NPs. Plant viruses are considered as non-pathogenic to animals and have been therefore utilized as clinical tools in nanomedicine. Particles can be obtained easily and rapidly using plants (> 1g/kg (1 gram per kilogram) of infected leaves).[46] Moreover, VNPs offer a broad range of templates ranging in size from ~10 nm to over a micron with a variety of distinctive shapes (most abundantly icosahedrons, spheres and tubular). Some examples of VNPs currently under research in nanoscience are shown in Figure 1.

Cowpea chlorotic mottle virus (CCMV)

Cowpea chlorotic mottle virus (CCMV) is a plant virus and a member of the bromovirus group of *Bromoviridae*.[47] CCMV consists of 180 identical coat protein subunits surrounding ssRNA genome. The protein subunits are arranged as 20 hexamers and 12 pentamers, forming an icosahedral shell with an external diameter of 28 nm (with T=3 triangulation symmetry).[48] 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 (Figure 2). CCMV can assemble into T=1 particles after the truncation of the N-terminus to generate particles 18 nm in diameter, or they can also assemble into pseudo T=2 particles with a 22 nm external diameter consisting of 60 and 120 capsid proteins, respectively.[49] The generated CCMV particles have reactive thiol functional groups present internally only on a single face of the capsid. It is believed that external cysteines will lead to virus aggregation through the formation of disulfide bonds. Surface modification to introduce external cysteines was further used to direct a 2-dimensional (2D), near single monolayer of capsids onto a gold-coated surface.[50] The dynamic structure of CCMV is one of its most attractive characteristics: CCMV undergoes a reversible pH-dependent swelling, resulting in the formation of 60 separate openings with diameters of 2 nm at the threefold axes.[47] This allows the use of CCMV particles as a drug carrier through simple incubation of the viral particles with the drug of interest.

Cowpea mosaic virus (CPMV)

CPMV is also another well-studied plant virus with a T=3 icosahedral symmetrical arrangement. CPMV particles have been extensively utilized as a scaffold for many novel materials using the accessible surface exposed amino acid side chains

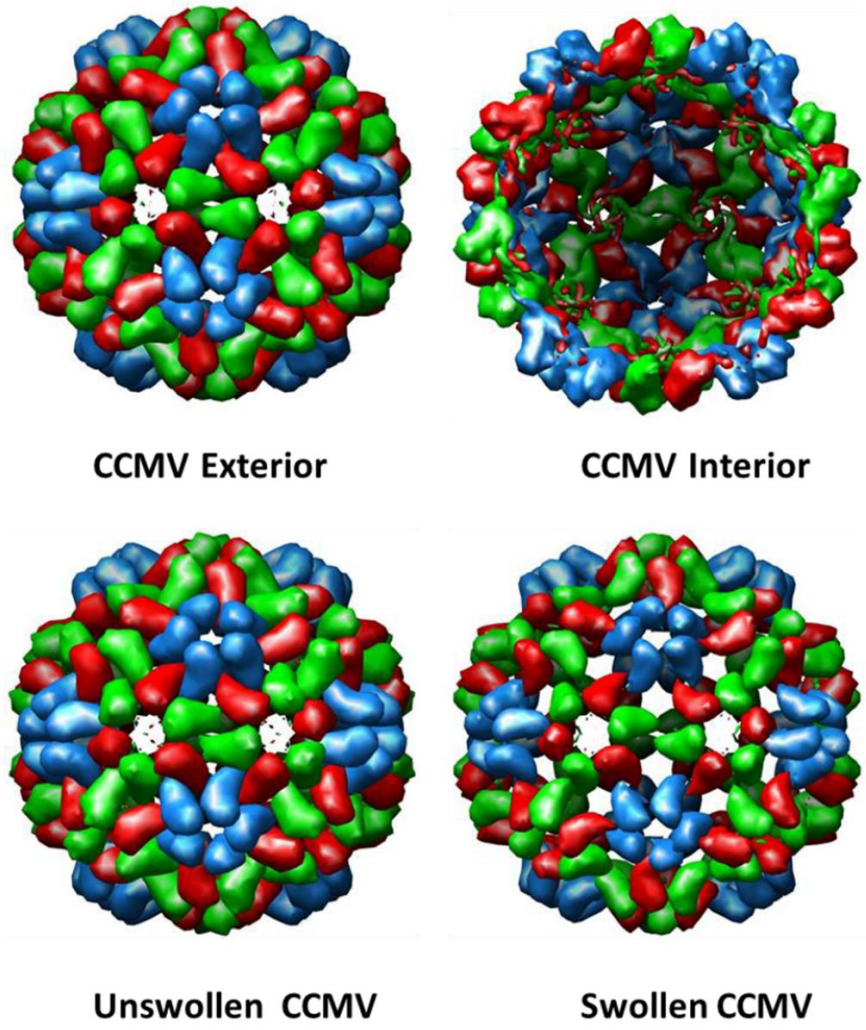

Figure 2: CCMV particles are showing the exterior and the interior of the capsid. In addition, it shows the conformational changes in the capsid in response to the environment: Unswollen conformation compatible with low pH and a swollen conformation induced by high pH. These images of CCMV were generated using PyMol on VIPERDB database.

On the exterior and the interior of the coat protein.[51] CPMV is a 30 nm particle made up of 60 identical asymmetric units packaged within a positive sense single strand RNA genome (+ ssRNA). CPMV particles are composed of two types of coat proteins (CP) referred to as large (L, made of 2 subunits B) and small (s, consisting of 1 subunit A) which form together with the asymmetric unit.[52b] The capsids are stable over a broad range of conditions including temperature (up to 50 °C), pH (3–11) and solvent effects (organic mixtures; dimethyl sulfoxide (DMSO) up to 50% v/v).[46, 52] The exterior surface of the CPMV capsid can be chemically addressed as exposed lysines,[53] cysteines,[54] tyrosine,[55] aspartic and glutamic acid[56] residues, naturally occurring or engineered by organic reagents.

Recent work has exploited Cu^{1} catalyzed “click” chemistry for selective attachment. The A subunits form the 12 pentameric positions while the B subunits form the icosahedral three-fold symmetrical positions. The presence of the two subunits offers an ability to modify the system at the five-fold, the three-fold or both positions in

the fully assembled virus particles by genetic or chemical modification of the A or B subunits. CPMV virus particles display on their multiple types of amino acids in which they could be chemically modified with various molecules of interest such as redox moieties, fluorescent dyes, metallic coatings, drug molecules (doxorubicin) and enzymes.[57]

CPMV has been functionalized with gadolinium-1,4,7,10-tetraazacyclododecane -1,4,7,10-tetraacetic acid (Gd[DOTA]) paramagnetic complexes for use as an MRI agent. It has been shown that Gd^{3+} ions can bind to CPMV nucleoprotein. Alternatively, Gd(DOTA) complexes can be attached via Cu-mediated azide–alkyne cycloaddition to different linkers conjugated to the CPMV surface exposed amino groups using carbodiimide chemistry and the reagents N-hydroxysuccinimide esters/1-ethyl- 3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (NHS/EDC).[58] Also, doxorubicin (Dox) moieties were conjugated to the surface exposed lysines and revealed an interesting slow degradation pattern with higher cell efficacy in comparison to the same dose of the free dox drug.[57a]

Red clover necrotic mosaic virus (RCNMW)

RCNMV is a plant virus belonging to the Dianthovirus genus and the family of Tombusviridae.[59] 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.[60] RCNMW can undergo a structural transition resulting in the formation of surface pores extending through the capsid. This is a direct response to cytoplasmic levels of divalent cations 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. This property was used for the encapsidation of preformed metallic nanoparticles labelled with oligonucleotides.[61] In addition, loading RCNMW virus with various therapeutics and utilizing chemical modification to impart targeting peptides was tested in HeLa cell lines (an immortal cell line derived from a cervical cancer patient called Henrietta Lacks) and proven to be specific.[62]

MS-2 Bacteriophage

MS2 is an RNA-containing bacteriophage (viruses that infect bacteria) of which the phage head displays an icosahedral symmetry with an average diameter of 27 nm.[63] The empty capsid contains 32 pores each of 1.8 nm in diameter which can be used for internal modification and loading the particles with various moieties. The spherical viral capsid comprises 180 identical protein monomers which can be expressed independently in bacteria by recombinant DNA (rDNA) technology and afterward be assembled to form VLPs. MS2 can be produced in a relatively high yield of ~ 30 mg/L from *E. coli* culture.[64]

Q-Beta (Qβ) bacteriophage

The bacteriophage Qβ is a member of the Leviviridae family, assembled into icosahedral capsids from 180 coat protein subunits around a central RNA genome.[65] Qβ is approximately 28 nm in diameter and closely related to the bacteriophage MS2. In particular, Qβ VLPs are very stable since these particles contain multiple intermonomer disulfide bonds arising from cysteine residues at positions 74 and 80 that are present near the 5- and 3-fold axes of symmetry. Each protein dimer is connected to the rest of the capsid by 4 disulfide bonds.[66]

Chemical modification of VNPs

Bioconjugation techniques have been widely adopted to modify the exterior surface of VNPs chemically. The bioconjugation technique relies on the presence of a natural amino acid that is accessible and can be easily modified with available chemical reagents. However, the development of bioorthogonal reactions to be used with virus modification is Cu (I)- catalyzed azide–alkyne cycloaddition (CuAAC) reaction. The required alkyne or azide groups are introduced on the VLPs by acylation of surface-exposed lysine residues using standard NHS-ester chemistry. Some 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 to target diseased cancer cell.[67] Folic acid (FA) has been widely used in targeted drug delivery to cancer cells. The uptake of FA into the cells is mediated by the folate receptor (FR). Binding of FA to FR initiates receptor-mediated endocytosis and internalization of FA.[68] While most normal cells express low levels of FR, the FR expression is elevated in cancer cells, e. g. cancer cells of the ovary, uterus, and mesothelium.[69]

Doxorubicin loaded HCRSV was conjugated with FA using the carbodiimide chemistry to the surface-exposed lysine moieties.[70] It was shown that approximately 360 FAs could be attached to one Dox-loaded VLP, corresponding to roughly two ligands per coat protein. Cell studies provided evidence that the folic acid conjugated VLPs were preferentially taken up by Ovarian Cancer type 3 (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 FA molecules per capsid.

In recent years, there have been an increasing interest 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] In addition, lactonic acid (LA) was utilized for the specific targeting of a rotavirus capsid viral-protein type 6 (VP6) to hepatocytes or hepatoma cells bearing asialoglycoprotein receptors (ASGPRs).[72] There are four reactive amino groups generated from surface lysine on each VP6 capsid protein (k118, k123, k125 and 145) which could potentially bind chemically to the carboxyl groups of LA. It was

shown that two LA molecules were eventually bound to each VP6 protein. This corresponds to 1560 LA per capsid, which greatly enhanced the receptor-mediated endocytosis as shown using human liver cancer cell line (Hep G2) cells.[73]

Genetic modification

The ability to manipulate the interactions of coat protein subunits at the interfaces in the capsid allows for some degree of control over the final capsid architecture with consequences on the template for material assembly. For example, the aspect ratio or the size of magnetic materials assembly within the interior cavity of the capsid can be controlled.[74] Advances in our understanding of the interactions between protein (asymmetric) subunits at the interfaces have imparted the ability to manipulate conditions to induce the self-assembly of controlled capsid architectures.[73b] These new architectures range from the *in vitro* assembly of viral capsids with varying numbers of subunits to non-native architectures of viral subunits that form tubes, sheets and shell structures containing thousands of protein subunits.[75]

In the 1960s, Bancroft *et al*. established the foundation of the systematic synthesis and controlled aggregation of viral protein subunits to form very distinct non-native structures.[75c] A mechanistic understanding of CCMV assembly has been gained by a combination of analyses of subunit genetic modifications in conjunction with controlled in vitro assembly conditions.[76] Genetic modification of the C and N termini ends required for capsid assembly led to the generation of an 18-nm diameter (60 subunits), a 24-nm diameter (120 subunits) or a 28-nm diameter (180 subunits) architecture.[49] In addition, it has been reported that the 24 amino acid peptide on the surface of the CPMV capsid is also responsible for the virus assembly.[77] This property, in particular, has been utilized in a study in which the capsid architecture-directing by nucleic acid has been synthetically mimicked by carboxyl terminated polyethylene glycol (PEG) attached to a well-defined Au nanoparticle.[78] In addition, DNA has also been shown to nucleate the assembly of tube-like structures composed of CCMV coat protein dimers.[79]

Genetic modification of VNPs is not only limited to imparting new functionalities on the virus capsid. They have been successfully utilized as vectors for gene delivery, in particular, the vector of retrovirus (dsRNA), lentivirus (HIV-1, HIV-2, Simian IV, Feline IV; dsRNA), adenovirus (Ad5-D24, CG870, Ad5-CD/TKrep; dsDNA) and adeno-associated virus (Parvovirus; ssDNA). Adenovirus vectors were developed and used for cancer gene therapy with induced significant anti-tumor activity when cytokines and chemokines were employed as therapeutic agents.[80] However, many clinical trials in which viral vectors were used have been terminated since the application of these vectors had induced unexpected adverse effects such as toxicities, immunogenicity, and oncogenicity.[81]

The ability to modify capsid subunits in defined locations, such as in the surface exposed loops, and their subsequent assembly into highly symmetrical capsid structures allow for incomparable control of ligands presentation on the exterior of the capsid. Not only VLPs can be used for the insertion of peptides or unnatural amino acids but also the wild-type. For example, the genetic/chemical modification of CCMV or TMV subunits results in the multivalent decoration of several hundred to thousands of copies of any genetically engineered functional group or ligand. These engineered functional groups can be used as attachment sites for ligand presentation on the exterior surface. For example, using activated fluorescein (maleimide or N-hydroxysuccinimide ester), quantitative labelling of these locations can be achieved without disrupting the overall cage architecture.[82]

Drug targeting by multivalency display

The strategies of drug delivery can be divided into two types of targeting strategies: passive and active.[83] Passive targeting combines a direct application that will inject drug molecules into tumors and a local application that will accumulate to tumors via enhanced permeability and retention (EPR) effect whereas effective targeting allows cancer cell-specific targeting through molecules such as antigenic agents, cell-surface sugar moieties, and receptors.[84]

Numerous approaches have been reported in the literature to controlling targeting and specificity that is based on the attachment of multiple copies of the targeting moieties which interact with the surface receptors on the diseased or targeted cells.[85] Targeting agents include but are not limited to antibodies, peptides, carbohydrates, glycopeptides with antibody therapy and targeting as the most promising agents in current cancer therapy.[86] Mammalian viruses present peptides or proteins with consistent spatial arrangements on their capsids. Therefore, the geometry of the targeting agents is necessary to mimic the effectiveness of viral-cell targeting. This type of spatial control is near impossible on the synthetic form of the nanoparticles. However, VLPs, dendrimers, and liposomes, as by their nature, are highly organized structure have more potential as they allow the spatial distribution of targeting agents.

Plant virus capsids as reaction vessels

The interior cavity of plant virus capsid architecture provides an ideal size-constrained environment where the interior surface can direct the attachment or nucleation of molecular or nanomaterials. Viruses package their viral nucleic acid within the capsid architecture, and our understanding of this process has been used to direct packaging of nonviral cargos such as drug molecules, dyes and small interference RNAs (siRNAs).[87]

Toxicity of VNPs

A major concern with all nanoparticles is their safety. Prior testing of the VNPs to evaluate their toxicities and biodistributions *in vivo* is vital. It has been reported that the size, shape, composition, surface chemistry and the physical properties of the NPs can influence their toxicological nature as well as deposition, clearance, and circulation within the body.[88] This would require a complete understanding of the circulation, clearance, blood half-life, stability, immunogenicity and the distribution of the VNPs in various organs. However, only a few extensively studied VNPs (CPMV and CCMV) have reached early stages of evaluating their toxicity and immunological properties *in vivo*.

Douglas and Young et al. assessed in 2007 two genetic mutations, CCMV S102C for microscopy studies and CCMV K42R for biodistribution studies.[89] 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 particles were rapidly (1 hour) detected after IV injection in the lungs, kidneys, and liver. However, their presence declined 24 hours after IV injection. For the biodistribution studies, CCMV particles were radiolabelled with ^{125}I and injected into both native 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 native and immunized mice. A high percentage of nanoparticles (57–73% ID) were excreted in 24 hours. A test on the mouse urine suggested that the ^{125}I-CCMV particles were degraded in vivo and excreted in components smaller than the capsid 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 big Immunoglobulin G (IgG) and Immunoglobulin M (IgM) response indicated that CCMV was immunogenic.

CPMV particles were found to be essentially safe and non-toxic, as was shown in a study by Finn and Manchester and their teams on the biodistribution, toxicity, and pathology of these particles *in vivo*.[90] Doses of 1, 10, and 100 mg/kg 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 suggest 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 could inhibit the induction of the anti-CPMV response.[91]

Although detailed pathological examination of most other VNPs has not been performed, the cellular uptake and cytotoxicity of several VLPs have been evaluated in vitro. It was concluded from an MTT (a yellow tetrazole, reduced to purple formazan in living cells)[92] cytotoxicity assay that the Hibiscus chlorotic ringspot virus (HCRSV) was not cytotoxic against the Lung Fibroblast Cells (CCL-186), human ovarian carcinoma cell line (OVCAR-3) and cellosaurus cell line (CNE-1) cells at concentrations up to 1 mg/mL.[70] Another study conducted by Koudelka et al.[93] showed that the uptake of CPMV by HeLa and Mouse cell line MFT-6 (vim^+) [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.

Advantages and limitations of VNPs

VNPs have some unique characteristics as compared to other protein-based materials. Their uniform capsid sizes allow for the loading of relatively even amounts of the delivery drug avoiding thus macromolecular aggregation of nanoparticles. Various VNPs offer different surfaces and a variety of chemical and biological structures for drug delivery.[51b,94] The naturally stable capsid in most physiological environments helps to protect drugs from enzymatic degradation.

VLPs and virosomes have made substantial progress in vaccine delivery because they are composed of viral components that retain the antigenicity of the parent virus. The major advantages of both systems over viral vectors include the ease of fabrication and the ability to scale up at a low cost. In addition, VLPs and virosomes can load many exogenous cargos such as siRNA, nucleic acids, peptides and/or proteins as well as antitumor drugs while sparing the beneficial traits of the parent viruses such as natural tropisms and modifiable surface properties.[95] Most studies of viral drug carriers, however, have been performed in vitro, and their *in vivo* efficacy has not been well established. Although the use of VLPs and virosomes has been approved for application in humans and their practical uses as drug carriers are very promising, the immunogenicity derived from viral components still exists, and further modifications are required to adapt VLPs and virosomes for drug delivery applications.[96]

Treating cancer with nanoparticles

Metastasis, the spread of cancer cells from a primary tumor to seed secondary tumors in distant organs, is one of the greatest challenges in cancer treatment

today.[97] For many patients, by the time cancer is detected, metastasis has already occurred. Over 80% of patients diagnosed with lung cancer, for example, present with metastatic disease.[97-98] Few patients with metastatic cancer are cured by surgical intervention across all cancer types, only one in five patients diagnosed with metastatic cancer will survive more than 5 years.[99] Different types of nanomaterials exhibit varying biodistribution, compatibility, degradation and circulations properties. No single parameter can be denoted as more important than the others for effective cancer treatment. Some recent studies have identified cytokines that are upregulated after the administration of positively charged nanoparticles.[100]

Nanoparticles with positive surface charge activate the classical complement pathway, and negatively charged particles activate the alternative known as lectin pathway.[101] It has been demonstrated that different degrees of pegylation (PEG) on nanoparticles surface affect the complement activation pathways; lower levels of PEG are associated with the classical pathway, while a higher degree of PEG is related to moderate activation of the lectin pathway.[102] In addition, particle size also has a role in this process; the larger the nanoparticle, the greater the extent of opsonization.[103] In many cases, adverse biological responses to nanoparticle administration, such as inflammation or complement activation, can be treated with pre-therapy or post-therapy medication.[104] In an attempt to improve the biocompatibility of nanoparticles *in vivo*, a hybrid biomimetic approach has been adopted. Some attempts included disguising the nanoparticles with a naturally derived erythrocyte membrane[105] as well as loading nanoparticles into stem cells, thereby evading reticuloendothelial system (RES) clearance and using natural pathway to target cancer.[106] An alternative approach uses biologically derived targeting moieties to deliver nanoparticles to specific tissue compartment.

Iron oxide nanoparticles (IOs) were decorated with dextran, which localizes within lymph nodes, have been investigated for the detection of prostate cancer in patients.[107] Targeted IOs, coated with RGD peptide, have been studied *in vivo* to image integrin αVβ3-positive tumor neovasculature189. NPs have been explored for targeting gadolinium-based contrast agents.

Conclusions

VNPs based on natural building blocks have become an emerging type of nanocarriers for targeted drug delivery. Viruses have the unique advantage of structural uniformity (monodispersity) 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 has 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.

Table 1: Plant virus capsids used as templates for nanomaterial in biomedical applications.

Virus	Shape	Decoration method	Ref.
Potato X virus (PVX) Filamentous		Genetic fusion to pIII, co-infection of bacteria with helper phage	[108, 109]
		PVX Nanoparticles for Subunit Vaccine Delivery	
		conjugated the Herceptin (Trastuzumab) monoclonal antibody for breast cancer	
CCMV	Icosahedral	Surface modifications	[110]
		Liquid crystals	[111]
		Nanowires/mineralization	[112]
		Coiled-coil interaction, pH-dependentm and co-encapsulation	[113]
		Polymer loading/encapsidation	[114]
		2D/3D Patterning/array formation	[115]
		Enzyme nanoreactor	[116]
		Imaging agents	[51d, 117]
		Chemical conjugation	[118]
		Targeting	[16, 119]
		Biodistribution	[120]
Bacteriophage Qβ	Icosahedral	Unnatural amino acids incorporation and azide–alkyne cycloaddition CuAAC ligation	[121]
Bacteriophage MS2	Icosahedral	Conjugation to an RNA packing sequence (pac site)	[122]
Brome mosaic virus (BMV)	Icosahedral	Au nanoparticles	[73]
		CdSe/ZnS semiconductor quantum	[123]
CPMV	Icosahedral	Chemical conjugation	[57, 123]
		Quantum dot decoration	[124]
		2D/3D Patterning/array formation	[125]
		Drug delivery platforms	[126]
		Imaging agents	[127]
		Biodistribution	[51a, 90]
Red clover necrotic mosaic virus (RCNMV)	Icosahedral	Au, $CoFe_2O_4$, and CdSe nanoparticles	[61]
		Doxorubicin/fluorophore infusion	[128]
Hibiscus chlorotic ringspot virus (HCRSV)		Polyacid loading	[129]

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