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Original Article

Viral nanoparticles as macromolecular devices for new therapeutic and pharmaceutical approaches

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Abstract: Viral nanoparticles are molecular cages derived from the assembly of viral structural proteins. They bear several peculiar features as proper dimensions for nanoscale applications, size homogeneity, an intrinsic robustness, a large surface area to mass ratio and a defined, repetitive and symmetric macromolecular organization. A number of expression strategies, using various biological systems, efficiently enable the production of significant quantities of viral nanoparticles, which can be easily purified. Genetic engineering and *in vitro* chemical modification consent to manipulate of the outer and inner surface of these nanocages, allowing specific changes of the original physico-chemical and biological properties. Moreover, several studies have focused on the *in vitro* disassembly/reassembly and gating of viral nanoparticles, with the aim of encapsulating exogenous molecules inside and therefore improving their potential as containment delivery devices. These technological progresses have led research to a growing variety of applications in different fields such as biomedicine, pharmacology, separation science, catalytic chemistry, crop pest control and material science. In this review we will focus on the strategies used to modify the characteristics of viral nanoparticles and on their use in biomedicine and pharmacology.

Keywords: Nanoparticle, virus particle, virus like particle, protein cage, nanotechnology, nanodevice

Introduction

In nanotechnology, submicroscopic particles ranging from 1 to 100 nm in diameter are usually referred to as nanoparticles. In recent years there has been a great deal of development of nanoparticles of different size, chemical nature and structure. These characteristics influence the properties of nanoparticles, which have been investigated in order to tackle significant problems in different fields of application such as material science, separation and purification chemistry, electronics, sensors, communications, optics and many aspects of applied biology.

Nanoparticles can be developed from inorganic as well as organic compounds, or can be based on naturally occurring biomolecules. Biological nanoparticles mainly derive from the assembly of one or more protein subunits in defined macromolecular structures, although RNA based nanoparticles have also been described and investigated for the delivery of therapeutic molecules [1]. Although proteins such as ferritin and the small heat-shock protein, which are both individually able to self-assemble 24 subunits into spherical cages of 12 nm in diameter, have received particular attention [2, 3], virus and virus derived nanoparticles (VNPs) are considered the most promising protein cages. Viruses are designed by nature in great varieties; they are able to shield and transfer biomolecules throughout very different environments and to release their content under particular conditions.

Intact virus particles (VPs), able to replicate and infect their natural host system, have been extensively used especially as nano-scaffolds for the display of biomolecules, such as short peptides [4], entire proteins [5], nucleic acids [6], carbohydrates [7] and for capsid mediated drug delivery [8]. Genetic modifications of simple virions, consisting in many cases of one or just a few subunits, have led to the development of

chimeric virus particles (CVPs) with new biological properties, involved in immunogenicity, cooperative affinity, specificity and tropism (**Table 1**).

After purification, VPs can be subjected to *in vitro* chemical modifications for conjugation of small compounds as well as large biomolecules. Albeit with significant differences, according to the type of host system used, VPs can be economically produced in large quantities.

Many viral structural proteins, individually expressed from the relative coding sequences out of the context of their viral genome, are still able to self-assemble into organized macromolecular structures identical or similar to the cognate virion. These "empty shells", known as virus like particles (VLPs), lack viral nucleic acid and are therefore noninfectious. Recombinant gene expression has allowed the production of VLPs in different heterologous expression systems such as bacteria, yeast, mammalian and insect cells, whole plants and plant derived suspension cultures. Moreover, the possibility of synthetic gene design and construction has greatly expanded the utilization of VLPs that can be produced without the need of dealing with the native infectious agent. In fact, the structural protein coding sequence can be directly inserted in a particular expression cassette and moved to the desired biological production system.

As in the case of VPs, VLPs have also been subjected to genetic mutational approach and to chemical conjugation. Additionally, thanks to their empty inner core and to the possibility of *ex vivo* total or partial disassembly/reassembly, VLPs can be loaded to encapsulate molecules of different nature (**Table 1**).

VNPs engineering

Genetic modifications

Modification by design is a straightforward process due to the ease of manipulation of entire viral genomes or single coding sequences of viral structural proteins. Viruses used in VNP development are very well known in their genomic organization, sequence, expression strategies and virion structure. The understanding of the structural features of VNPs is very important to locate the precise position of the N - and C-termini of their subunits with respect to the particle organization and also to identify possible internal protein domains capable of sustaining genetic insertions. Depending on the goal, terminal or internal protein fusions can be utilized to introduce heterologous peptides, and in a few cases entire proteins, on the surface or inside the VNPs.

With regard to genetic modification, the hepatitis B core (HBc) VLPs are probably the best characterized. HBc is a 21 kDa protein that self-assembles into subviral nucleocapsid particles, which package the viral polymerase and pregenomic RNA during hepatitis B virus (HBV) infection. HBc monomers assemble into VLPs of 30 and 34 nm in diameter, composed of 180 or 240 subunits arranged with $T = 3$ or $T = 4$ icosahedral symmetry, respectively. Recombinant HBc or HBc fusions can be produced in virtually all known heterologous expression systems, including yeast [9], mammalian cell cultures [10], plants [11], *Xenopus laevis* oocytes [12] and bacteria such as *Escherichia coli* [13], *Bacillus subtilis* [14] and *Salmonella typhimurium* [15]. Structural studies of the HBc particles, reveled by electron cryomicroscopy and resolved by X-ray crystallography, together with computer predictions and empirical studies, led to the identification of three major sites for foreign insertions: the N- and C-termini of the protein and the internal major immunodominant region (MIR), which is located at the tip of the protruding spikes characteristic of the HBc VLPs. Structural data reveal that these regions do not participate in the intra and intermolecular interactions crucial for VLP assembly. N-terminus and MIR insertions have been employed for the display of foreign sequences on the outside of the particle. The N-terminus insertion site was the first to be investigated; it allows for a good level of antibody response against various inserted epitopes that can exceed 50 amino acids [16]. On the other hand, the MIR insertion site, spanning amino acid 76-81 of the HBc protein, is considered the insertion site of choice for foreign peptide display on the outer surface of VLPs. It can accommodate extraordinarily long insertions, as demonstrated by the fusion of the entire GFP (238 amino acids) and of 120 amino acid long immunogenic region of a hantavirus nucleocapsid protein [17, 18]. The precise structure of the basic C-terminus is not known, but it is expected from its nucleic acid binding function to face the inside of the particle [19, 20]. Interestingly,

Table 1. Examples of viral nanoparticles used for biomedical applications

VNP	Structural Informations	Type	Cited in Paragraph	Refs
Adenovirus	Spherical shape, 60-90 nm in diameter Several structural proteins, icosahedral symmetry	VP	Cancer treatment Nucleic acid delivery	[86] [83, 92-95]
Bacteriophage M13	Rod shape, 900 nm in length and 7 nm in diameter Several structural proteins, helical symmetry	VP	Non-covalent modifications Cancer treatment Nucleic acid delivery	[48] [8] [96]
Bacteriophage MS2	Spherical shape, 25 nm in diameter Single structural protein, 180 copies in icosahedral *T*=3 symmetry	VLP	Chemical conjugation Imaging Nucleic acid delivery	[44, 45] [90] [82, 84, 97]
Bacteriophage Qbeta	Spherical shape, 30 nm in diameter Single structural protein, 180 copies in icosahedral *T*=3 symmetry	VLP	Immune stimulation Cancer treatment	[75] [7]
Canine parvovirus (CPV)	Spherical shape, 26,4 nm in diameter Single structural protein, 60 copies in icosahedral *T*=1 symmetry	VLP	Cancer treatment	[63]
Cowpea chlorotic mottle virus (CCMV)	Spherical shape, 28 nm in diameter Single structural protein, 180 copies in icosahedral *T*=3 symmetry	VP	Encapsidation Imaging	[56] [89]
Cowpea mosaic virus (CPMV)	Spherical shape, 28 nm in diameter Two structural proteins, 60 copies each in icosahedral pseudo *T*=3 symmetry	VP	Chemical conjugation Immune stimulation Imaging	[6, 37, 40-43, 46] [85] [36]
Hepatitis B virus core (HBc)	Spherical shape, 30 and 34 nm in diameter Single structural protein, 180 or 240 copies in icosahedral *T*=3 and *T*=4 symmetry	VLP	Genetic modifications Immune stimulation	[9, 11, 15-18, 21] [75]
Hepatitis B virus L	Spherical shape, 50-500 nm in diameter Single structural protein, 110 copies embedded in a yeast ER derived vesicle	VLP	Immune stimulation Nucleic acid delivery	[77] [64]
Hibiscus chlorotic ringspot virus (HCRSV)	Spherical shape, 30 nm in diameter Single structural protein, 180 copies in icosahedral *T*=3 symmetry	VLP	Cancer treatment	[88]
Human papillomavirus (HPV) L1	Spherical shape, 55 nm in diameter Single structural protein, 360 copies in icosahedral *T*=7 symmetry	VLP	Encapsidation	[54, 55]
Norwalk virus (NV)	Spherical shape, 38 nm in diameter Single structural protein, 180 copies in icosahedral *T*=3 symmetry	VLP	Immune stimulation	[78]
Polyomavirus	Spherical shape, 46 nm in diameter Single structural protein, 360 copies in icosahedral *T*=7 symmetry	VLP	Genetic modifications Non-covalent modifications Encapsidation	[22, 23] [50, 51] [52]
Potato virus X (PVX)	Filamentous shape, 470-580 nm in length, 13 nm in diameter Single structural protein, 1300 copies in helical symmetry	VP	Genetic modifications Chemical conjugation Immune stimulation	[5, 27] [39] [80]
Red clover necrotic mosaic virus (RCNMV)	Spherical shape, 30 nm in diameter Single structural protein, 180 copies in icosahedral *T*=3 symmetry	VLP	Imaging	[91]
Sulfolobus islandicus rod-shaped virus 2 (SIRV2)	Rod shape, 900 nm in length and 23 nm in diameter Single structural protein in helical symmetry	VP	Chemical conjugation	[47]
Tobacco mosaic virus (TMV)	Rod shape, 300 nm in length and 18 nm in diameter Single structural protein, 2130 copies in helical symmetry	VP	Genetic modifications Chemical conjugation Non-covalent modification Immune stimulation	[28, 31-33] [38] [49] [81]

VNP, viral nanoparticle; VLP, virus like particle; VP, virus particle.

Beterams and colleagues [21] demonstrated that genetic substitution of the C-terminus of HBc can be employed to enclose an entire protein, the 17-kD nuclease of *Staphylococcus aureus*, inside the particle. This strategy allowed the packaging of up to 240 subunits of the enzyme that retained, at least partially, its original biological activity.

Another remarkable example of the use of genetic modification for the packaging of VNPs is given by the use of polyoma VLP. The virion of polyomavirus is composed of an outer shell, derived from the assembly of the major capsid protein VP1, and an inner core made of two minor structural proteins, VP2 and VP3. Polyoma VLPs can be obtained by single expression of VP1 [22]. Interestingly, the ability of VP2 to bind tightly and in precise positions in the inside of VP1 derived VLPs has been exploited. A conserved stretch of 47 amino acids of VP2 has been fused with GFP. The purified chimeric protein was able to assemble *in vitro* together with VP1, generating VLPs that enclose the foreign protein (**Figure 1a**). The amount of heterologous protein that can be encapsulated using this strategy is reproducible due to the fixed ratio of VP2 binding sites per VP1 VLPs. In addition, GFP fluorescence was employed for monitoring encapsulation efficiency, cellular uptake and intercellular distribution [23].

Strict general rules concerning genetic engineering strategies cannot be established; but different approaches need to be addressed on a case by case basis, often empirically, according to the particular VNP, the nature of the insertion sequence and the resulting position of the fusion peptide. However, some important issues can be preventively evaluated. Potential alteration of VNP surface charge and insert-dependent steric hindrance are indeed critical factors.

Variation of the surface charge of VPs could interfere with virion assembly, virus spread, particle aggregation, stability and biodistribution. Studies related to the modification of the surface charge of VNPs have been conducted mostly using VPs such as adenovirus (Ad) [24], bacteriophages [25], Cowpea mosaic virus (CPMV) [26], Potato virus X (PVX) [27] and Tobacco mosaic virus (TMV) [28]. In the case of PVX, a filamentous plant virus extensively used for CVP development in vaccinology, Lico and coauthors [27] have utilized a PVX mutant truncated at the N-terminus of the coat protein (CP). Although the mutant virus was able to produce infections indistinguishable from those induced by the wild type virus, it was shown that the fusion of heterologous peptides, of varying length and amino acidic composition, to the truncated N-terminus, did not always result in systemic infection, thus weakening its use from a production point of view. The effect of a vast plethora of peptides was investigated. They concluded that the pI value of the inserted peptide has a clear impact in the systemic movement of the virus. In fact, fused peptides with a pI value outside an experimentally defined range (from 5.24 to 9.18) significantly affected CVPs systemic movement; evidence also confirmed the fact that chimeras were infectivity restored, which brought back the peptide pI within the previously established permissive values. Surface charge can also play a crucial role for *in vivo* applications. The administration of engineered VNPs to animal model systems has revealed that, for example, modifications altering the surface charge of bacteriophage Qbeta particles have an influence in plasma clearance and tissue biodistribution [25]. Moreover, it has also been demonstrated that surface charge of CPMV has an important role in interaction and uptake processes of target cells [26].

Steric hindrance, originating from foreign sequence insertion, is another factor that should be taken into account, since it could prevent monomer self-assembly and, consequently, VNPs formation. A possible solution is the development of mosaic VNPs that are composed by wild type and genetically engineered CPs [5]. In the case of VLPs, they are typically generated by the co-expression of two proteins, for example the HBc and phage Qbeta derived mosaic VLPs [29, 30]. Nevertheless, especially considering CVPs, also other molecular approaches aimed to partially display heterologous sequences are available. One strategy is to modify the C-terminus of the CP gene so that the CP stop codon would function in a leaky fashion. This would cause most of the CP produced to be unfused, while a small portion would be fused to the peptide displayed on the surface [31-33]. Another strategy consists in employing the 2A peptide of the foot and mouth disease virus (FMDV). This sequence, inserted between the heterologous peptide coding sequence and the

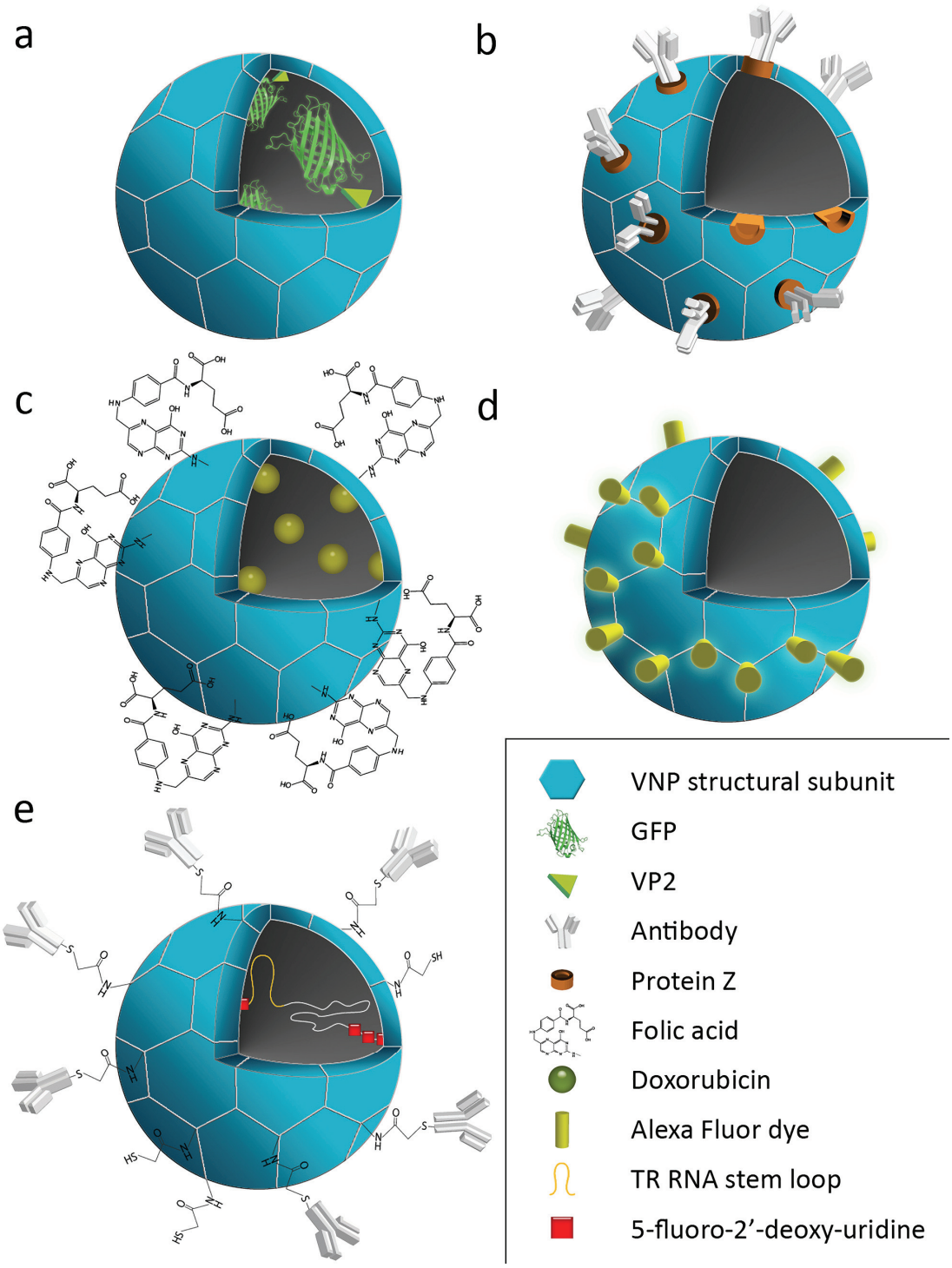

Figure 1. Schematic representation of selected VNPs modification strategies. (a) Genetic modification and selective encapsidation for preferential entrapping of proteic payload [23]; (b) Genetic and non-covalent modifications for outer surface display of antibodies [50]; (c) Chemical conjugation and encapsidation for outer surface display and entrapping of small molecules [88]; (d) Chemical conjugation for outer surface display of fluorophores [36]; (e) Chemical conjugation and selective encapsidation for outer surface display of antibodies and selective entrapping of nucleic acids [82]. VNP, viral nanoparticle; VP2, minor coat protein of polyomavirus; TR, translational operator of bacteriophage MS2.

CP gene, is able to produce, in different hosts and expression systems, a ribosomal skip during the translational procedure [34, 35]. The result is that only a small portion of the translational products will be a fusion between the heterologous sequence and the CP, while the majority will consist of the native CP, primary component of the mosaic particle, and the unfused foreign peptide that will not be part of the resulting VNPs.

Chemical conjugation

Functional groups contained in the side chain of native or genetically introduced amino acids of structural proteins forming the VNPs can be used for chemical conjugation. It has been shown that amines, thiol groups, carboxylic acids and side chains of tyrosines can react with activated conjugates. This strategy has been extensively adopted to covalently couple different molecules on specific positions of a variety of structurally diverse VNPs. As previously discussed for genetic VNP modification, particular attention is focused on the evaluation of the chemical characteristics of the binding molecule, since changes can have a remarkable effect on the overall resulting physicochemical and pharmacokinetical properties, generating particles different in size and surface charge.

The amine functional group lysine is a common target for conjugation. For example, CPMV contains 300 naturally occurring lysines that are solvent accessible on the surface of the virion. Of those, 150 are reactive and addressable as binding sites to display molecules very different in chemical nature such as fluorophores, peptides and nucleic acids [6, 36, 37]. Lysines have also been introduced by design, as in the case of TMV. Smith and coauthors employed a randomized library approach to introduce peptides having a reactive lysine in different amino acidic contexts at the externally located amino terminus of the TMV CP. An i*n vivo* library screening allowed selection and isolation of a chimeric virus which showed impressive recoveries, exceeding 7 g/Kg of infected tissue and well defined favorable purification properties. The introduced lysine, displayed on each CP monomer forming the VNP was accessible for chemical modification as demonstrated by complete biotinilation of the rod shaped particle [38]. *N*-hydroxysuccinimide (NHS) ester based chemistry has also been successfully employed to biotinilate lysine residues on the PVX and CPMV surface [39, 40]. In the latter case, each icosahedral virion was capable of exposing 60 biotin molecules per particle. So much interest on biotin is explained by its natural affinity for streptavidin (SA). As it will be later described, this property opens the way for further non-covalent modifications of the particles with molecules attached to avidin and hence using the biotin-avidin interaction as molecular bridge between the VNP and the molecule of interest. Lysine has also been the target of more sophisticated conjugation strategies, such as the copper catalyzed click chemistry, which have been successfully employed to conjugate a wide variety of compounds: from fluorophores up to large and complex biomolecules like carbohydrates and entire proteins [41, 42].

Thiol is the cysteine side chain, and is one of the most useful functional groups found in proteins. It can react with a large variety of organic reagents; for this reason it has been widely used for chemical derivatization [43, 44]. A notable example concerns the MS2 bacteriophage derived VLPs. MS2 CP contains two cysteines buried in the ternary structure of the CP and therefore inaccessible and unreactive. For this reason, once again, a genetic modification approach has been employed to introduce accessible cysteines on the surface of the VLPs. Several mutants were created by site directed mutagenesis of specific native amino acids of the CP, but just one substitution out of five was able to meet the required properties, demonstrating that even a single amino acid substitution can disrupt protein folding and stability with a high frequency. The selected mutant CP, in which the threonine 15 is replaced by a cysteine, was able to properly self-assemble into VLPs. However, particles were subjected to aggregation due to inter-particle formation of disulfide bridges, resulting in insolubility in water after purification, as shown by treatment with reducing agents. The accessibility of the new cysteine residues was addressed with different thiol specific chemical reagents. In particular, the use of fluorescein-5-maleimide resulted in green fluorescence of the mutant particles under UV light illumination [44].

Tyrosine residues of the MS2 CP have also been efficiently targeted. The predicted position of tyrosine 85 provides 180 modification sites in the inner side of each particle, opening the pos-

sibility of covalently loading the particles with drug cargo. The bacteriophage MS2 capsid shell has 32 pores, 1.8 nm wide in diameter, which provide access to the interior of the VNPs without the need for a complete disassembly. In this case, the particles were first emptied of their RNA by pH treatment. Subsequently, tyrosines were targeted with high efficiency and selectivity by a quick four-step procedure that introduced a highly reactive group for following bioconjugation [45].

Modification of exposed carboxylates arising from aspartic and glutamic acids has been also demonstrated. Several carboxylate groups on the outer surface of CPMV are present on both the S and L subunits. These functional groups were successfully used to bind a carboxylate-selective chemical dye. The derived conjugated VNPs were correctly assembled, monodispersed, showed no aggregation and were characterized by a UV/visible absorption distinctive of the chosen dye. In addition, denaturing SDS PAGE demonstrated that carboxylic acid groups were correctly addressed on both protein subunits [46].

Although the use of the previously described amino acid side chains is the most common strategy, carbohydrates can also be used for conjugation. In fact, many VNPs are glycosylated, displaying solvent exposed carbohydrates that, once oxidized, can react with a hydrazide resulting in a stable hydrazone linkage. In this way, biotin-hydrazide has been employed to covalently attach biotin on the surface of the archaeal *Sulfolobus islandicus* rod-shaped virus 2 (SIRV2); the VNPs were able to resist the harsh reaction conditions and labeling efficiency was verified by TEM, using gold coupled anti-biotin antibodies [47]. This strategy can offer a good alternative to more traditional methods. Obviously the conjugation density and the spatial distribution of the conjugated molecules are strictly dependent on the glycosylation status and pattern, typical of each kind of VNP.

Overall, chemical modifications offer a valid alternative to genetic engineering strategies for linking molecules, even very different in chemical nature, to viral nanoparticles. This approach can be used to functionalize the exterior as well as the interior surfaces, providing new antigenicity, specificity, tropism and physical properties, and allowing the contained delivery of therapeutics. For this reason, a large number of exploitations in all of the biomedical and pharmacological applications later described have been found for bioconjugation.

Non-covalent modifications

Intermolecular associations based on electrostatic attractions, hydrophobic bonds and molecular recognition interactions can also be used to engineer VNPs. This strategy usually requires a previous chemical or genetic modification of the particles to change their physicochemical properties or to provide a specific affinity to a molecule of interest. It has been used to associate large biomolecules on the outer and also inner side of VNPs. Electrostatic interactions have been used, for example, to create biological/organic hybrid nanoparticles. In fact, recently Ngweniform *et al.* [48] have described a biomimetic approach to stabilize liposomes, via the self-assembly of zinc phthalocyanine (ZnPc)-loaded liposomes on a genetically engineered M13 phage. ZnPc is a good candidate for photodynamic therapy, since illumination with the proper wavelength results in singlet oxygen formation. Genetic fusion of the coding sequence of a negatively charged peptide on the major CP allows multivalent electrostatic interactions with the cationic liposomes, leading to self-assembly and to immobilization of the liposomes on the phage particles. The authors report that the complex has an increased fluorescence intensity of the ZnPc in the lipid bilayer and speculate on an enhanced stability of the complex and on the possibility to engineer the minor CP for specific targeting of tumor cells.

As previously mentioned, one of the major exploitations of molecular recognition to decorate VNPs is based on the interaction between SA and biotin, which is one of the strongest non-covalent interactions known in nature. This particular strategy has allowed the display of very large proteins on the surface of VNPs that would be otherwise very difficult to produce, due to steric hindrance affecting assembly, yield and monodispersion. In the case of infectious VPs, the viral genome enlarged by the presence of the exogenous genetic material inserted, could represent a problem for genome packaging and virus replication. The possibility to uncouple the expression of the structural monomers forming the VNPs and the desired protein to display, could offer a solution. In this way, biotinilated

TMV rods have been externally decorated with the entire GFP genetically fused to SA. TMV and GFP-SA have been produced separately in tobacco plants; GFP-SA was able to correctly form the typical homotetramer necessary for biotin binding. Following biotin conjugation, the rod shaped VNPs were incubated with the GFP-SA tetramers resulting in the specific formation of a TMV/GFP complex. As expected, the interaction between the biotinilated TMV and the GFP-SA was very stable. Treatment of the complex at 60 °C in presence of SDS completely disassembled the particles, however, the biotin/SA bond remained intact [38].

Also, the well known molecular interaction between protein A and the heavy chain Fc region of immunoglobulins was exploited for post assembly, non-covalent decoration of VNPs. Again, the TMV was genetically modified, in this case to display a functional fragment of protein A as a C-terminal fusion to the CP on the surface. The CP-protein A fusion was expressed at a very high level in tobacco plant leaves (3 g/Kg) and was able to self-assemble into functional CVPs, which showed efficient binding to a recombinant monoclonal antibody, which was independently produced in tobacco leaves. It was estimated that one IgG molecule was bound to every third to fifth subunit of the particle. Although the proposed application of this engineered VNP concerns their use as immunoadsorbent devices for antibody purification, the same approach could be envisioned for functionalization of VNPs with immunoglobulins bearing different desired specificities of therapeutic interest [49]. In fact, a similar strategy was used to couple antibodies to polyoma VLPs. Protein Z, an engineered antibody binding domain derived from the protein A of *Staphylococcus aureus*, was genetically inserted into an internal loop of the polyoma VP1 monomer and the resulting *in vitro* assembled VP1-Z derived VLPs were able to bind and therefore display antibodies on their surface (**Figure 1b**). Individual incubation of the genetically engineered VLPs with two humanized antibodies, the anticarcino embryogenic antigen and Herceptin, indicated that almost every protein Z displayed on the VNP could bind one antibody molecule [50].

Interestingly, molecular recognition has also been applied to modify the inner core of polyoma VLPs. In this case a WW domain from the mouse forming binding protein 11 (FBP11) was used as an N-terminal fusion of VP1. WW domains, named after two conserved tryptophan residues, bind prolin-rich ligands with a specific consensus motif (PPLP). They are particularly suited for this strategy since they are among the smallest known protein-protein interaction domains. The WW-VP1 fusion proteins retained their native structure and ability to assemble *in vitro* into VLPs. In this way, using a PPLP tagged GFP, it was possible to specifically and efficiently encapsidate 260 molecules of the fluorescent protein inside the VNPs [51].

Encapsidation

The primary function of virions is to encapsidate, transport and release nucleic acids, and in some cases proteins, among different environments. Many aspects of viral assembly have been addressed and physicochemical conditions for *in vitro* disassembly/reassembly and for the reversible opening of virion gated pores have been defined, setting the ground for loading VNPs with extra non viral cargo. Two techniques used for loading VNPs with nucleic acids, low molecular weight substances and proteins are the assembly in the presence of high concentrations of foreign molecules [52] and the application of pH shock [53].

DNA entrapped by *in vitro* association has been achieved using different VNPs. An example concerns the human papillomavirus (HPV). The virus capsid is primarily composed of a structural protein denoted L1, which alone is capable of self-assembly into VLPs when expressed in heterologous systems. Conditions for quantitative disassembly/reassembly are well defined; modification of the ionic strength and of the reducing conditions allows to selectively and reversibly establish the process *in vitro* [54]. Reassociation in the presence of DNA constructs leads to the packaging of foreign nucleic acids inside the particles. In fact, insect cells that produced HPV VLPs could be loaded with an expression plasmid vector carrying either a gene for the GFP or β-galactosidase during *in vitro* disassembly/reassembly. Efficiency evaluation estimated that between 1/3 and 1/6 of the VNPs contained the plamid DNA [55].

The inner core of icosahedral viruses can also be reached by switchable pH dependent gating. Dynamic structural transition, induced by de-

fined chemical switches, occurs in many virions and has been used to manipulate different VNPs. In this respect, Cowpea chlorotic mottle virus (CCMV) is one of the mostly investigated. In a pivotal work, Douglas and colleagues [56] demonstrated that the CCMV capsid can undergo a pH and metal ion dependent reversible structural transition that modifies the permissive state of the 60 pores present on the capsid. Under swollen conditions (pH > 6.5) the interior of the virus is exposed and free molecular exchange is possible. At pH < 6.5 the VNP is in the unswollen state and no exchange of large molecules with the external environment is possible. The successful exploitation of this process led to the use of different icosahedral VNPs for mineralization and encapsidation of payload into the particle inner core.

Future development of this approach will rely on other strategies. Modifications of the inner surface can provide new affinity properties for specific capture of the foreign molecules, increasing loading efficiency.

VNPs applications

Historically, the first and currently the most advanced application of VNPs concerns vaccine development. Several VLPs produced in heterologous systems have been tested for their ability to generate an immune response which can be used for prophylactic and therapeutic purposes. This kind of subunit recombinant vaccine is unable to be replicated, but still exhibits the same structural immunological determinants of the virus they are derived from. For this reason they are considered a safer alternative compared to traditional vaccines based on killed or attenuated viruses, which still bear the theoretical risk of viral replication. Several preclinical studies have underlined the ability of VLPs to generate a neutralizing protective response against various infectious agents. Several VLP based vaccines are now at different stages of clinical development and the only recombinant vaccines currently marketed are based on VLPs derived from HBV and HPV [57-59]. In addition, VNPs have been used as scaffolds for the display of non-native epitopes on their surface, creating a general strategy for the development of vaccines independently of the specific immunological properties of the protein cage.

Also, one of the highest expectations in the development of new platforms for drug delivery is to combine the possibility to confine the therapeutic agent together with the delivery and release to specific organs or cellular subtypes. The peculiar features of VNPs, and the possibility to use in combination with different modification strategies on the same structure, makes them an ideal candidate for this purpose. Technologies for multivalent display and cargo upload are well established and designing new specificity by surface display of ligands has been shown to be effective in redirecting the delivery of VNPs to target cell types. A growing number of peptidic molecules specific for targeting different tissues and cellular physiological conditions are available [60-62]. Small proteic ligands, full antibodies, single chain variable fragments and nanobodies can be linked on the surface of VNPs to specifically direct their action. Moreover, many VNPs still retain their natural tropism and, for this reason, some animal virus-derived VNPs have been successfully used for specific targeting *in vivo* [63, 64]. In this respect, the main applications concern the delivery of chemotherapeutic agents for cancer treatment, of nucleic acid constructs for gene therapy and antisense technology, and of various compounds for molecular imaging.

Multi-functionality, as a result of heterologous display, can be also beneficial in the clustering of different ligands on the surface of VNPs, opening the possibility of their employment in multiple binding of cooperative receptors, where affinity is increased by the collective ligand/receptor interactions [65].

Although the advantages of VNPs, compared to other types of nanoparticles, include biodegradability, biocompatibility and monodispertion, an evaluation of potential toxicity and biodistribution of targeted and non-targeted particles for in *vivo* application must be addressed. A deeper understanding of different VNP based platforms such as circulation, clearance rates, blood half life, stability, immunogenicity and organ distribution is needed to get these novel molecular devices to the market. In this respect, Kaiser *et al.* [66] have tested the biodistribution and clearance of fluorescently labeled and radiolabeled CCMVs upon intravenous (i.v.) injection into mice. The authors evaluated the quantitative biodistribution and blood clearance at 1 and 24 h from administration. Also, the immu-

nogenicity and its role in subsequent administrations was assessed. Their conclusions pointed out that CCMV was broadly distributed and able to move through tissues, VNPs were detected in circulatory and lymphatic endothelium, in pulmonary epithelial surfaces, in the lung interstitium, in the splenic outer capsule and perilymphoid zones, in the liver sinusoids, in the convoluted tubules of the kidney and in draining lymphnodes. CCMV were almost completely eliminated from animals by 24 h post injection.

Also CPMV particles have been investigated in this respect, both qualitatively and quantitatively. After i.v. injection in mice, 90% of the CPMV particles localized to the liver. Toxicity, evaluated by necropsy, hematology and tissue histopathology, was not observed in liver or other tissues. Even when very high dosage was used, no visible adverse clinical signs were observed. CPMV particles were rapidly cleared from blood circulation within 30 minutes with an average half-life of 4–7 minutes in plasma [67, 68].

Immune stimulation

VNPs hold a great potential in prophylactic and therapeutic vaccines as well as in immune-drug development. A wide variety of VNPs have shown promising results in animal models and, in some cases, in clinical studies. They have been successful in the establishment of a protective immunity against infectious agents and also in the induction of autoantibodies for the neutralization of proteins related to non-communicable chronic diseases [69, 70]. The size of VNPs has been shown to be favorable for their uptake by antigen presenting cells (APCs); their ordered, dense and repetitive structures are able to activate innate immunity through pattern recognition and stimulate a potent humoral immunity; their propensity for being uptaken by dendritic cells (DCs) with the subsequent transport to lymph nodes is the base for a proper T-cell response development. In addition, DCs were reported to be able to cross-present VNP derived peptides on molecules of both major hystocompatibility complex (MHCs) molecules [71, 72].

VLPs are commonly developed to stimulate an immune response against the cognate virus they are derived from, often with the aim of eliciting both humoral and cell mediated immunity. However, VNPs have been also extensively used as platforms for the presentation of foreign epitopes (chimeric VLPs and CVPs), uncoupling the specific immune determinants from the molecular display scaffold. As their organic counterparts, a VNPs also offer the possibility of co-delivery of immunopotentiators to direct and tune the desired immune response [73-75]. All these properties make VLPs a superb immunogen that, in some cases, does not appear to require the use of adjuvants to achieve an effective immune stimulation, virtually combining the advantages distinctive of recombinant subunit and whole virus vaccines into one system.

Although VNP vaccine production is usually less expensive compared to live attenuated and killed vaccine, manufacturing costs are still in some cases too high, undermining their utilization, especially when vaccination aimed toward developing countries and mass immunization programs are considered. In this respect, plant based production systems could play a crucial role. The production of correctly assembled VLPs in different plant systems has been widely reported (see [76] for a review on the topic). Different plant-produced VLPs have been shown to retain antigenicity and immunogenicity and, for example in the case of HBV surface antigen (HBsAg) VLPs, their oral administration to humans has shown a specific IgG production that could be correlated to protection levels [77]. Plants have proven to be an excellent production platform for Norwalk virus (NV) derived VLPs, which were produced in tobacco leaves, potato tubers and tomato fruits. The use of a transient viral-based expression system showed that the expression of the NV VLPs in *Nicotiana benthamiana* was at considerable levels (0.86 mg/g of leaf fresh weight). In this case, immunogenicity of the plant-purified VLPs was tested in mice upon oral administration, and a clear specific humoral and mucosal antibody response in the gut, as well as at distant mucosal surfaces, was detected. The amplitude of the immune response was dose-dependent and NV VLPs were immunogenic even without the use of the cholera toxin (CT) adjuvant, although the groups dosed with equal amounts of antigen co-administered with CT showed significantly stronger responses than those lacking the adjuvant [78].

Many plant viruses have been used as epitope

display platforms. They can be produced very efficiently in terms of speed and quantity, up to levels of g per Kg of leaf fresh weight in a one week time period. Although different from VLPs, CVPs still enclose their genome, they are unable to replicate in vertebrates and, interestingly, there is increasing evidence that suggests the viral genome could play an additional role in stimulating the immune response. In fact, almost the totality of plant VPs used as epitope display systems harbor a single stranded RNA genome that may interact with the intracellular toll-like receptors (TLRs) 7/8, activating downstream pathways important for DC maturation [79]. Specific T cell response elicited by peptides delivered by CVPs has been reported. For example, recently, PVX-based CVPs were designed to display, on their surface, a MHC class I restricted nonapeptide derived from the influenza A virus nucleoprotein as gene fusion to the virus CP coding sequence. CVPs were administered subcutaneously to mice at different dosages with and without the incomplete Freund's *adjuvant (IFA)* to evaluate their ability to induce the activation of MHC class I-restricted epitope-specific immune responses. Detection and enumeration of antigen specific T cells *ex vivo* by IFN-γ ELIspot assay determined a clear and durable induction of IFN-γ-producing CD8+ T cells. Moreover, at low doses, the activation was successful without the need of an adjuvant, highlighting the intrinsic adjuvancy of CVPs [80]. A strong T-cell activation was also achieved using a TMV based system exposing two well characterized T cell epitopes, known to provide protection against tumor challenge in mice: the p15e melanoma epitope and the Ova epitope. Both peptides were exposed on the surface of the VNPs by means of genetic modification and chemical conjugation. Cellular immune response activation was demonstrated by IFN-γ secretion. Notably, *in vivo* T cell function was shown via protection in respective tumor challenge survival mice models [81].

Cancer treatment

Therapeutic advantages regarding drug containment, characteristic of such molecular devices, are particularly obvious in cancer treatment. Conventional small chemotherapeutic agents, which are commonly nonspecifically administered, affect pathological and normal cells with consequences concerning general toxicity and therapeutic potential of the drug. Several strategies have been investigated using different nanoparticles that exploit passive targeting, derived from the enhanced permeability and retention effect of the vasculature surrounding tumors, and more interestingly also by active targeting due to specific interaction to cellular receptors. The combination of drug containment and active targeting using VNPs can offer a very promising approach to prolong drug systemic circulation lifetime, decrease dosage, enhance drug uptake, co-delivery of chemo-sensitizing agents with major beneficial outcomes such as decreased toxicity, reduced chemoresistance and enhanced therapeutic index.

Several ligands including antibodies [82] , antibody derivatives [83], transferrin (Tf) [84], folic acid (FA) [85], oligopeptides [86] and oligosaccharides [7] can be displayed in a biologically functional form on VNPs surface in order to achieve tumor cells specific targeting. Interestingly, some VNPs can naturally target cancer cells. For example, the canine parvovirus (CPV) binds the Tf receptor (TfR), and this specific binding is able to mediate the virus internalization into the intracellular environment. The circulatory iron carrier Tf is greatly demanded during proliferation and the surface cellular receptor TfR is significantly upregulated in cancer cells [87]. CPV derived VLPs, produced in insect cells, retain TfR binding properties. Chemical modification, consisting in derivatization of lysines exposed on the surface of the CPV based VNPs, was used to fluorescently label the particles to demonstrate the capability of *in vitro* binding and internalization. Fluorescence confocal microscopy revealed that CPV VNPs were internalized by endocytosis following TfR interaction into HeLa, HT-29 and MDA-MB231 tumor cell lines, which are known to over-express TfR, while no internalization was detected in TfR low expressing cells [63].

FA, involved in cell division and DNA synthesis, plays a crucial role in cellular metabolism. Its uptake is mediated by its specific interaction with a cell surface receptor, the folate receptor (FR). FR expression on cells is upregulated during cellular activation and proliferation and is thus particularly high on cells of several human tumors. In a notable work, Ren *et al.* [88] used this evidence to direct Hibiscus chlorotic ring spot virus (HCRSV) VNPs, loaded with the chemotherapy agent doxorubicin, to the ovarian cancer derived cells OVCAR-3. Doxorubicin is an

anthracycline antibiotic used to treat different kinds of cancer, including ovarian cancer. Drug entrapment was performed in the presence of doxorubicin by *in vitro* assembly of purified CPs. Subsequently, drug loaded VNPs were chemically conjugated to FA using NHS ester chemistry (**Figure 1c**). Cellular uptake and cytotoxicity was tested on OVCAR-3 cells, which overexpress FR. VNPs displaying FA mediated a significantly higher uptake of doxorubicin, which resulted in higher cytotoxicity compared to doxorubicin loaded particles not exposing FA and to free doxorubicin.

Recently, chemical, genetic and non-covalent modifications were employed to achieve tumor targeted, capsid mediated drug delivery. Hygromycin was successfully conjugated on the surface of functionalized filamentous M13 phage derived VNPs using the carboxyl side chain of amino acids present on the g8p major CP. In addition, the g3p minor *cp* gene was genetically modified by the insertion of the protein A derived Z domain coding sequence, in order to provide IgG binding capability by molecular recognition. The chimeric phage, conjugated with hygromycin, was complexed with the chFRP5 mAb *in vitro* for targeted delivery to ErbB2 overexpressing human breast adenocarcinoma SKBR3 cells. By using a cell killing assay, an impressive potency improvement by the use of these VNPs was shown, resulting in more than 1000 folds over free hygromycin administration [8].

Imaging

The same principle of multifunctionality, consisting of the specific delivery of desired compounds, can be exploited for diagnostic purposes such as *in vivo* and *in vitro* imaging. Non-invasive imaging has a huge potential for the early detection and subsequent timely treatment of diseases. It is also able to retrieve important information on anatomopathological structures, inflammation, angiogenic progression and diffusion. New nano-materials are rapidly evolving to identify and image molecular events in the body in real time and to recognize diseases at cellular level. Although much attention has been focused on non-biological nanoparticles, VNPs are gaining interest in the delivery of contrast agents, due to their potential in overcoming problems related to stability, bioavailability, toxicity, engineering flexibility and production cost. In particular, VNPs have been used to generate: (i) new paramagnetic materials, typically based on gadolinium ions (Gd^{3+}) and iron oxides for MRI contrast detection [89, 90], (ii) contrast devices based on heavy metals, such as gold and bismuth for computed tomography detection [91] and (iii) nano-fluorescent probes that can be visualized by excitation with an appropriate light source coupled to capture emitted photons by optical detectors [36].

Paramagnetic nanoparticles have been successfully generated using CCMV and MS2 VNPs. Divalent metal ions such as Ca^{2+} play an important role in the *in vitro* assembly of CCMV. 180 metal binding sites are present on the VNPs at the interface between subunits, and their removal at neutral pH promotes the phenomenon of viral particle gating. This property has been used to successfully incorporate Gd^{3+} and, the resulting VNPs of 28 nm in diameter, formed a monodispersed preparation with a binding capacity of 140 Gd^{3+} per VNP, making the complex an excellent potential candidate for MRI detection. However, *in vivo*, local high concentration of Ca^{2+} could compete with Gd^{3+} for VNPs binding, destabilizing the Gd^{3+} CCMV association with repercussions on toxicity due to the release of free Gd^{3+} [89]. This problem could be potentially overcome using Magnevist, a strong chelate of gadolinium, most commonly used as an MRI contrast agent. Activated Magnevist was conjugated on MS2 VNPs using surface exposed lysines, resulting in the incorporation of 500 gadolinium chelates per VNP [90].

A remarkable example of intravital vascular imaging is the use of fluorophore-conjugated CPMV VNPs. Commercial fluorescent dyes have been successfully conjugated to the VNPs up to a density of 120 dyes per particle, providing a fluorescence intensity significantly higher than conventional vascular imaging (**Figure 1d**). The monodispersed preparation was injected in adult mice and, in living chick and mouse embryos. VNPs were distributed in all organs examined and in particular enabled the visualization of vasculature, intravitally and in fixed tissues. Detection was possible in the smallest capillaries, overcoming inadequate tissue penetration seen from common fluorescent agents and thus allowing resolution of vascular structures in several organs such as kidney, heart and liver for at least 72h with no visible deleterious effects. As

demonstrated by injection and detection in chick embryos, the labeled CPMVs were internalized by vascular endothelial cells through active endocytosis and were not detected outside the endothelial layer. This property is particularly beneficial to map areas involved in neovascularization. In fact, upon injection with dye -labeled CPMV, it was possible to visualize vessels entering and exiting the tumor in a well-established chick angiogenesis model [36].

Nucleic acid delivery

The possibility of delivering gene expression constructs directly to patients to replace the activity of a nonfunctional or disfunctional protein, holds a great potential in the battle against genetic diseases and cancer. On the other hand, there is a necessity to down-regulate the production of specific proteins believed to be involved in the development of a pathologic state, in its progression and/or resistance to therapy. Antisense technology is a field currently being explored, with the purpose to design specific nucleic acids that inhibit their expression upon hybridization to the corresponding mRNA sequence.

In the last thirty years, the development of viral based vectors for nucleic acid delivery has played a crucial role in the improvement of this approach. The fundamental challenge originates from the charged chemical nature of nucleic acids which makes naked molecules very unstable in physiologic environments and extremely inefficient in crossing biological barriers (such as cell membranes). Non-targeted viral-based systems have been employed, taking advantage of different natural circulatory and targeting properties specific of viruses. More recently, engineered VNPs have been developed to specifically deliver constructs to particular cell types.
The ideal gene therapy vector system should be administered by a noninvasive route, target only the desired cells and express a therapeutic amount of transgene products for a defined period of time. In the history of gene delivery, adenoviral vectors represent one of the first and the most used platforms, due to specific features such as low pathogenicity in humans, relative broad range of tissues able to transduce and the abilities to infect both dividing and nondividing cells [92-95].

Viral natural tropism has been exploited using HBV derived L nanoparticles. When heterologously expressed in yeast, 110 L protein monomers, embedded in the endoplasmic reticulum membrane, form organized and monodispersed vesicles of 80 nm in diameter. This singular feature has allowed the development of a novel loading strategy based on simple electroporation of DNA plasmids. The N-terminal amino acid residues of the L protein is displayed on the surface of L particles and functions as a specific ligand for receptor binding on human hepatocytes. Studies using a GFP reporter gene construct *in vivo* and *ex vivo* confirmed the high transfection efficiency and specificity of the system. Also, the human F9 gene, coding for the clotting factor IX (hFIX), was inserted in the context of an expression cassette and incorporated into the VNPs. A mouse xenograft model of hepatic tumor was employed to test the functionality of the VNPs. Plasma hFIX levels were observed initially on the seventh day after i.v. injection and regularly detected for at least 1 month after administration. Plasmatic concentration of hFIX obtained *in vivo* were estimated be sufficient to convert severe hemophilia to moderate hemophilia in humans [64].

More commonly, the delivery of nucleic acids using VLPs employs the encapsidation of exogenous gene constructs, as an alternative to VP-based strategies that generally make use of insertion in the vector genomic context. In this perspective, preferential entrapping and consequently enriched encapsidation is beneficial to the efficiency of the whole process. Specthrie and coauthors [96] used the packaging signal of the filamentous phage M13 for this purpose. The exogenous DNA material was designed to contain selected elements of the M13 intergenic region, indispensable for binding the viral structural proteins and naturally used by the phage to pack its genome inside the virions. Specific encapsidation during VNPs assembly in the host cell system was successful, as revealed by sequence analysis of the genetic material extracted from the purified particles.

Another example of preferential packaging of nucleic acids is offered by the MS2 VNPs. Proper self-assembly of MS2 bacteriophage CP in icosahedral particles has been long investigated, as well as its sequence specific interaction with the genomic RNA during capsid formation. A 19 nt long translational operator (TR)

RNA stem loop sequence contained in the MS2 genome mediates its encapsidation during virion assembly by binding CP dimers. This specific interaction has been used to direct selective loading of exogenous material, in particular to exploit antisense technology against acute myelogenous leukemia. In fact, antisense oligonucleotides (ODNs), designed on the p120 nucleolar gene and fused to the TR RNA sequence, have been loaded inside MS2 VLPs, subsequently conjugated on the outer surface with Tf. The resulting particles carrying the DNA/RNA hybrid oligonucleotides were used to treat a promyelocytic leukemia cell line. The short fragments of single-stranded DNA induced a specific down-regulation of gene expression by interaction with complementary cellular mRNAs, resulting in apoptosis and tumor killing. In addition, to examine whether encapsidation within the MS2 shell was able to improve cell delivery, the uptake of radiolabeled ODNs was monitored and compared with the administration of free radio oligonucleotides. At all concentrations the MS2 particles containing the TR-ODN and exposing Tf resulted in greater internalization of the label [84].

Another successful application of MS2 VLPs has been the development of the antisense RNA delivery system. Packaging of therapeutic nucleic acid was obtained by simple coexpression of two plasmids in *E.coli*: one encoding the CP and the other for an antisense RNA, containing the modified TR stem-loop sequence of the MS2 genome. After isolation, the particles were chemically conjugated to the human immunodeficiency virus-1 (HIV-1) Tat protein transduction domain (PTD) and tested for their ability to enter the plasma membrane when applied extracellularly. Cells of the human hepatoma cell line Huh -7, containing the hepatitis C virus (HCV) luciferase reporter system, were used to analyze the cellular uptake of the MS2 VLPs and the correlated therapeutic effects. The antisense RNAs, designed on the 5'-untranslated region (UTR) and on the internal ribosome entry site (IRES) of the HCV genome, were delivered into the cell by the MS2 based VNPs. Interestingly, reporter luciferase activity showed a clear dose dependent inhibition of HCV expression [97].

In addition to gene function replacement and antisense therapy, nucleic acid delivery can also be used for chemotherapeutic aims. The nucleotide analogue 5-fluorouridine (5fU) has been often chosen for this purpose due to its well-known features. The incorporation of 5-fluoro-2'-deoxy-uridine (5fdU) as 5' and 3' extension of the 19 nt long TR RNA stem loop sequence combined the possibility to mediate its specific loading into empty MS2 particles with the exploitation of this nucleotide analogue toxic nature. Furthermore, VNPs were decorated by covalent attachment of monoclonal antibodies directed against a cell surface antigen, which is associated with human breast tumors (**Figure 1e**). The cytotoxicity of such 'smart bombs' was tested on ZR-75-1 breast carcinoma cells, which express the DF3 surface antigen at high levels, and on Caov-3 cells as a negative control. At lower concentrations, results showed a higher toxicity of the targeted VNPs compared to naked 5fdU containing oligonucleotides. Very little difference was detected when VNPs packaged with the oligonucleotide, but not exposing the mAb, were tested on Caov-3 cells. Both observations support the idea that the engineered particles enhanced the toxicity of the oligonucleotide by protecting it from degradation and by increasing the level of specificity and uptake [82].

Conclusions

The immense potential of VNP technology is just beginning to be fully understood and developed. A vast plethora of VNPs different in size, shape and structural properties are available and the number of academic and industrial research groups committed to a truly multidisciplinary approach for the improvement of this technology is steadily growing. In addition, the enormous flexibility of VNPs concerning structural/functional molecular engineering, their ease of production/purification and the numerous possibilities of application in different fields make them one of the most promising tools of synthetic biology for biomedical applications. A much wider exploitation of their potential of combining the ability to functionalize the exterior surface with the capacity to encapsulate a bioactive molecule or a contrast agent will be crucial to develop successful and affordable new methods for prevention, treatment and diagnosis of diseases. Future directions will also need to focus on a deeper understanding of the *in vivo* behavior of VNPs. Assessment of biodistribution, plasma stability, pharmacodynamics and toxicity will be necessary in order to achieve safe, effective and marketable products.

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References

[1] Li L, Liu J, Diao Z, Shu D, Guo P and Shen G. Evaluation of specific delivery of chimeric phi29 pRNA/siRNA nanoparticles to multiple tumor cells. Mol Biosyst 2009; 5: 1361-1368.

[2] Yamashita I, Iwahori K and Kumagai S. Ferritin in the field of nanodevices. Biochim Biophys Acta 2010; doi:10.1016/j.bbagen.2010.03.005.

[3] Flenniken ML, Liepold LO, Crowley BE, Willits DA, Young MJ and Douglas T. Selective attachment and release of a chemotherapeutic agent from the interior of a protein cage architecture. Chem Commun (Camb) 2005; 447-449.

[4] Donini M, Lico C, Baschieri S, Conti S, Magliani W, Polonelli L and Benvenuto E. Production of an engineered killer peptide in Nicotiana benthamiana by using a potato virus X expression system. Appl Environ Microbiol 2005; 71: 6360-6367.

[5] Cruz SS, Chapman S, Roberts AG, Roberts IM, Prior DA and Oparka KJ. Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. Proc Natl Acad Sci U S A 1996; 93: 6286-6290.

[6] Strable E, Johnson JE and Finn MG. Natural Nanochemical Building Blocks: Icosahedral Virus Particles Organized by Attached Oligonucleotides. Nano Letters 2004; 4: 1385-1389.

[7] Kaltgrad E, O'Reilly MK, Liao L, Han S, Paulson JC and Finn MG. On-virus construction of polyvalent glycan ligands for cell-surface receptors. J Am Chem Soc 2008; 130: 4578-4579.

[8] Bar H, Yacoby I and Benhar I. Killing cancer cells by targeted drug-carrying phage nanomedicines. BMC Biotechnol 2008; 8: 37.

[9] Miyanohara A, Imamura T, Araki M, Sugawara K, Ohtomo N and Matsubara K. Expression of hepatitis B virus core antigen gene in Saccharomyces cerevisiae: synthesis of two polypeptides translated from different initiation codons. J Virol 1986; 59: 176-180.

[10] Hirschman SZ, Price P, Garfinkel E, Christman J and Acs G. Expression of cloned hepatitis B virus DNA in human cell cultures. Proc Natl Acad Sci U S A 1980; 77: 5507-5511.

[11] Huang Z, Santi L, LePore K, Kilbourne J, Arntzen CJ and Mason HS. Rapid, high-level production of hepatitis B core antigen in plant leaf and its immunogenicity in mice. Vaccine 2006; 24: 2506-2513.

[12] Standring DN, Ou JH, Masiarz FR and Rutter WJ. A signal peptide encoded within the precore region of hepatitis B virus directs the secretion of a heterogeneous population of e antigens in Xenopus oocytes. Proc Natl Acad Sci U S A 1988; 85: 8405-8409.

[13] Burrell CJ, Mackay P, Greenaway PJ, Hofschneider PH and Murray K. Expression in Escherichia coli of hepatitis B virus DNA sequences cloned in plasmid pBR322. Nature 1979; 279: 43-47.

[14] Hardy K, Stahl S and Kupper H. Production in B. subtilis of hepatitis B core antigen and a major antigen of foot and mouth disease virus. Nature 1981; 293: 481-483.

[15] Schodel F, Milich DR and Will H. Hepatitis B virus nucleocapsid/pre-S2 fusion proteins expressed in attenuated Salmonella for oral vaccination. J Immunol 1990; 145: 4317-4321.

[16] Koletzki D, Lundkvist A, Sjolander KB, Gelderblom HR, Niedrig M, Meisel H, Kruger DH and Ulrich R. Puumala (PUU) hantavirus strain differences and insertion positions in the hepatitis B virus core antigen influence B-cell immunogenicity and protective potential of core-derived particles. Virology 2000; 276: 364-375.

[17] Kratz PA, Bottcher B and Nassal M. Native display of complete foreign protein domains on the surface of hepatitis B virus capsids. Proc Natl Acad Sci U S A 1999; 96: 1915-1920.

[18] Koletzki D, Biel SS, Meisel H, Nugel E, Gelderblom HR, Kruger DH and Ulrich R. HBV core particles allow the insertion and surface exposure of the entire potentially protective region of Puumala hantavirus nucleocapsid protein. Biol Chem 1999; 380: 325-333.

[19] Bottcher B, Wynne SA and Crowther RA. Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. Nature 1997; 386: 88-91.

[20] Zlotnick A, Cheng N, Stahl SJ, Conway JF, Steven AC and Wingfield PT. Localization of the C terminus of the assembly domain of hepatitis B virus capsid protein: implications for morphogenesis and organization of encapsidated RNA. Proc Natl Acad Sci U S A 1997; 94: 9556-9561.

[21] Beterams G, Bottcher B and Nassal M. Packaging of up to 240 subunits of a 17 kDa nuclease into the interior of recombinant hepatitis B virus capsids. FEBS Lett 2000; 481: 169-176.

[22] Montross L, Watkins S, Moreland RB, Mamon H, Caspar DL and Garcea RL. Nuclear assembly of polyomavirus capsids in insect cells expressing the major capsid protein VP1. J Virol 1991; 65: 4991-4998.

[23] Abbing A, Blaschke UK, Grein S, Kretschmar M, Stark CM, Thies MJ, Walter J, Weigand M, Woith DC, Hess J and Reiser CO. Efficient intracellular delivery of a protein and a low molecular weight substance via recombinant polyomavirus-like particles. J Biol Chem 2004; 279: 27410-27421.
[24] Li QG, Lindman K and Wadell G. Hydropathic characteristics of adenovirus hexons. Arch Virol 1997; 142: 1307-1322.
[25] Prasuhn DE, Jr., Singh P, Strable E, Brown S, Manchester M and Finn MG. Plasma clearance of bacteriophage Qbeta particles as a function of surface charge. J Am Chem Soc 2008; 130: 1328-1334.
[26] Zhang Y, Yang M, Portney NG, Cui D, Budak G, Ozbay E, Ozkan M and Ozkan CS. Zeta potential: a surface electrical characteristic to probe the interaction of nanoparticles with normal and cancer human breast epithelial cells. Biomed Microdevices 2008; 10: 321-328.
[27] Lico C, Capuano F, Renzone G, Donini M, Marusic C, Scaloni A, Benvenuto E and Baschieri S. Peptide display on Potato virus X: molecular features of the coat protein-fused peptide affecting cell-to-cell and phloem movement of chimeric virus particles. J Gen Virol 2006; 87: 3103-3112.
[28] Bendahmane M, Koo M, Karrer E and Beachy RN. Display of epitopes on the surface of tobacco mosaic virus: impact of charge and isoelectric point of the epitope on virus-host interactions. J Mol Biol 1999; 290: 9-20.
[29] Kazaks A, Borisova G, Cvetkova S, Kovalevska L, Ose V, Sominskaya I, Pumpens P, Skrastina D and Dislers A. Mosaic hepatitis B virus core particles presenting the complete preS sequence of the viral envelope on their surface. J Gen Virol 2004; 85: 2665-2670.
[30] Brown SD, Fiedler JD and Finn MG. Assembly of hybrid bacteriophage Qbeta virus-like particles. Biochemistry 2009; 48: 11155-11157.
[31] Hamamoto H, Sugiyama Y, Nakagawa N, Hashida E, Matsunaga Y, Takemoto S, Watanabe Y and Okada Y. A new tobacco mosaic virus vector and its use for the systemic production of angiotensin-I-converting enzyme inhibitor in transgenic tobacco and tomato. Biotechnology (N Y) 1993; 11: 930-932.
[32] Sugiyama Y, Hamamoto H, Takemoto S, Watanabe Y and Okada Y. Systemic production of foreign peptides on the particle surface of tobacco mosaic virus. FEBS Lett 1995; 359: 247-250.
[33] Borovsky D, Rabindran S, Dawson WO, Powell CA, Iannotti DA, Morris TJ, Shabanowitz J, Hunt DF, DeBondt HL and DeLoof A. Expression of Aedes trypsin-modulating oostatic factor on the virion of TMV: A potential larvicide. Proc Natl Acad Sci U S A 2006; 103: 18963-18968.
[34] Donnelly ML, Luke G, Mehrotra A, Li X, Hughes LE, Gani D and Ryan MD. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. J Gen Virol 2001; 82: 1013-1025.
[35] de Felipe P and Izquierdo M. Construction and characterization of pentacistronic retrovirus vectors. J Gen Virol 2003; 84: 1281-1285.
[36] Lewis JD, Destito G, Zijlstra A, Gonzalez MJ, Quigley JP, Manchester M and Stuhlmann H. Viral nanoparticles as tools for intravital vascular imaging. Nat Med 2006; 12: 354-360.
[37] Chatterji A, Ochoa W, Shamieh L, Salakian SP, Wong SM, Clinton G, Ghosh P, Lin T and Johnson JE. Chemical conjugation of heterologous proteins on the surface of Cowpea mosaic virus. Bioconjug Chem 2004; 15: 807-813.
[38] Smith ML, Lindbo JA, Dillard-Telm S, Brosio PM, Lasnik AB, McCormick AA, Nguyen LV and Palmer KE. Modified tobacco mosaic virus particles as scaffolds for display of protein antigens for vaccine applications. Virology 2006; 348: 475-488.
[39] Steinmetz NF, Mertens ME, Taurog RE, Johnson JE, Commandeur U, Fischer R and Manchester M. Potato virus X as a novel platform for potential biomedical applications. Nano Lett 2010; 10: 305-312.
[40] Wang Q, Kaltgrad E, Lin T, Johnson JE and Finn MG. Natural supramolecular building blocks. Wild-type cowpea mosaic virus. Chem Biol 2002; 9: 805-811.
[41] Wang Q, Chan TR, Hilgraf R, Fokin VV, Sharpless KB and Finn MG. Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. J Am Chem Soc 2003; 125: 3192-3193.
[42] Sen Gupta S, Kuzelka J, Singh P, Lewis WG, Manchester M and Finn MG. Accelerated bioorthogonal conjugation: a practical method for the ligation of diverse functional molecules to a polyvalent virus scaffold. Bioconjug Chem 2005; 16: 1572-1579.
[43] Steinmetz NF, Evans DJ and Lomonossoff GP. Chemical introduction of reactive thiols into a viral nanoscaffold: a method that avoids virus aggregation. Chembiochem 2007; 8: 1131-1136.
[44] Peabody DS. A Viral Platform for Chemical Modification and Multivalent Display. J Nanobiotechnology 2003; 1: 5.
[45] Hooker JM, Kovacs EW and Francis MB. Interior surface modification of bacteriophage MS2. J Am Chem Soc 2004; 126: 3718-3719.
[46] Steinmetz NF, Lomonossoff GP and Evans DJ. Cowpea mosaic virus for material fabrication: addressable carboxylate groups on a programmable nanoscaffold. Langmuir 2006; 22: 3488-3490.
[47] Steinmetz NF, Bize A, Findlay KC, Lomonossoff GP, Manchester M, Evans DJ and Prangishvili D. Site-specific and Spatially Controlled Addressability of a New Viral Nanobuilding Block: Sulfolobus islandicus Rod-shaped Virus 2. Adv

Funct Mater 2008; 18: 3478-3486.
[48] Ngweniform P, Abbineni G, Cao B and Mao C. Self-assembly of drug-loaded liposomes on genetically engineered target-recognizing M13 phage: a novel nanocarrier for targeted drug delivery. Small 2009; 5: 1963-1969.
[49] Werner S, Marillonnet S, Hause G, Klimyuk V and Gleba Y. Immunoabsorbent nanoparticles based on a tobamovirus displaying protein A. Proc Natl Acad Sci U S A 2006; 103: 17678-17683.
[50] Gleiter S and Lilie H. Coupling of antibodies via protein Z on modified polyoma virus-like particles. Protein Sci 2001; 10: 434-444.
[51] Schmidt U, Gunther C, Rudolph R and Bohm G. Protein and peptide delivery via engineered polyomavirus-like particles. FASEB J 2001; 15: 1646-1648.
[52] Goldmann C, Stolte N, Nisslein T, Hunsmann G, Luke W and Petry H. Packaging of small molecules into VP1-virus-like particles of the human polyomavirus JC virus. J Virol Methods 2000; 90: 85-90.
[53] Ren Y, Wong SM and Lim LY. In vitro-reassembled plant virus-like particles for loading of polyacids. J Gen Virol 2006; 87: 2749-2754.
[54] McCarthy MP, White WI, Palmer-Hill F, Koenig S and Suzich JA. Quantitative disassembly and reassembly of human papillomavirus type 11 viruslike particles in vitro. J Virol 1998; 72: 32-41.
[55] Touze A and Coursaget P. In vitro gene transfer using human papillomavirus-like particles. Nucleic Acids Res 1998; 26: 1317-1323.
[56] Douglas T and Young M. Host-guest encapsulation of materials by assembled virus protein cages. Nature 1998; 393: 152-155.
[57] McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ and Hilleman MR. Human hepatitis B vaccine from recombinant yeast. Nature 1984; 307: 178-180.
[58] Villa LL, Costa RL, Petta CA, Andrade RP, Ault KA, Giuliano AR, Wheeler CM, Koutsky LA, Malm C, Lehtinen M, Skjeldestad FE, Olsson SE, Steinwall M, Brown DR, Kurman RJ, Ronnett BM, Stoler MH, Ferenczy A, Harper DM, Tamms GM, Yu J, Lupinacci L, Railkar R, Taddeo FJ, Jansen KU, Esser MT, Sings HL, Saah AJ and Barr E. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncol 2005; 6: 271-278.
[59] Harper DM, Franco EL, Wheeler C, Ferris DG, Jenkins D, Schuind A, Zahaf T, Innis B, Naud P, De Carvalho NS, Roteli-Martins CM, Teixeira J, Blatter MM, Korn AP, Quint W and Dubin G. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet 2004; 364: 1757-1765.
[60] Temming K, Schiffelers RM, Molema G and Kok RJ. RGD-based strategies for selective delivery of therapeutics and imaging agents to the tumour vasculature. Drug Resist Updat 2005; 8: 381-402.
[61] Van Bockstaele F, Holz JB and Revets H. The development of nanobodies for therapeutic applications. Curr Opin Investig Drugs 2009; 10: 1212-1224.
[62] Shadidi M and Sioud M. Selective targeting of cancer cells using synthetic peptides. Drug Resist Updat 2003; 6: 363-371.
[63] Singh P, Destito G, Schneemann A and Manchester M. Canine parvovirus-like particles, a novel nanomaterial for tumor targeting. J Nanobiotechnology 2006; 4: 2.
[64] Yamada T, Iwasaki Y, Tada H, Iwabuki H, Chuah MK, VandenDriessche T, Fukuda H, Kondo A, Ueda M, Seno M, Tanizawa K and Kuroda S. Nanoparticles for the delivery of genes and drugs to human hepatocytes. Nat Biotechnol 2003; 21: 885-890.
[65] Mammen M, Choi S-K and Whitesides GM. Polyvalent Interactions in Biological Systems: Implications for Design and Use of Multivalent Ligands and Inhibitors. Angewandte Chemie International Edition 1998; 37: 2754-2794.
[66] Kaiser CR, Flenniken ML, Gillitzer E, Harmsen AL, Harmsen AG, Jutila MA, Douglas T and Young MJ. Biodistribution studies of protein cage nanoparticles demonstrate broad tissue distribution and rapid clearance in vivo. Int J Nanomedicine 2007; 2: 715-733.
[67] Rae CS, Khor IW, Wang Q, Destito G, Gonzalez MJ, Singh P, Thomas DM, Estrada MN, Powell E, Finn MG and Manchester M. Systemic trafficking of plant virus nanoparticles in mice via the oral route. Virology 2005; 343: 224-235.
[68] Singh P, Prasuhn D, Yeh RM, Destito G, Rae CS, Osborn K, Finn MG and Manchester M. Biodistribution, toxicity and pathology of cowpea mosaic virus nanoparticles in vivo. J Control Release 2007; 120: 41-50.
[69] Jennings GT and Bachmann MF. Designing recombinant vaccines with viral properties: a rational approach to more effective vaccines. Curr Mol Med 2007; 7: 143-155.
[70] Jennings GT and Bachmann MF. Immunodrugs: therapeutic VLP-based vaccines for chronic diseases. Annu Rev Pharmacol Toxicol 2009; 49: 303-326.
[71] Storni T and Bachmann MF. Loading of MHC class I and II presentation pathways by exogenous antigens: a quantitative in vivo comparison. J Immunol 2004; 172: 6129-6135.
[72] Kurts C, Robinson BW and Knolle PA. Cross-priming in health and disease. Nat Rev Immunol 2010; 10: 403-414.
[73] Malyala P, O'Hagan DT and Singh M. Enhancing the therapeutic efficacy of CpG oligonucleotides using biodegradable microparticles. Adv Drug Deliv Rev 2009; 61: 218-225.
[74] Chikh G, de Jong SD, Sekirov L, Raney SG, Ka-

zem M, Wilson KD, Cullis PR, Dutz JP and Tam YK. Synthetic methylated CpG ODNs are potent in vivo adjuvants when delivered in liposomal nanoparticles. Int Immunol 2009; 21: 757-767.
[75] Storni T, Ruedl C, Schwarz K, Schwendener RA, Renner WA and Bachmann MF. Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. J Immunol 2004; 172: 1777-1785.
[76] Santi L, Huang Z and Mason H. Virus-like particles production in green plants. Methods 2006; 40: 66-76.
[77] Thanavala Y, Mahoney M, Pal S, Scott A, Richter L, Natarajan N, Goodwin P, Arntzen CJ and Mason HS. Immunogenicity in humans of an edible vaccine for hepatitis B. Proc Natl Acad Sci U S A 2005; 102: 3378-3382.
[78] Santi L, Batchelor L, Huang Z, Hjelm B, Kilbourne J, Arntzen CJ, Chen Q and Mason HS. An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles. Vaccine 2008; 26: 1846-1854.
[79] Baum A and Garcia-Sastre A. Induction of type I interferon by RNA viruses: cellular receptors and their substrates. Amino Acids 2010; 38: 1283-1299.
[80] Lico C, Mancini C, Italiani P, Betti C, Boraschi D, Benvenuto E and Baschieri S. Plant-produced potato virus X chimeric particles displaying an influenza virus-derived peptide activate specific CD8+ T cells in mice. Vaccine 2009; 27: 5069-5076.
[81] McCormick AA, Corbo TA, Wykoff-Clary S, Nguyen LV, Smith ML, Palmer KE and Pogue GP. TMV-peptide fusion vaccines induce cell-mediated immune responses and tumor protection in two murine models. Vaccine 2006; 24: 6414-6423.
[82] Brown WL, Mastico RA, Wu M, Heal KG, Adams CJ, Murray JB, Simpson JC, Lord JM, Taylor-Robinson AW and Stockley PG. RNA bacteriophage capsid-mediated drug delivery and epitope presentation. Intervirology 2002; 45: 371-380.
[83] van Beusechem VW, Grill J, Mastenbroek DC, Wickham TJ, Roelvink PW, Haisma HJ, Lamfers ML, Dirven CM, Pinedo HM and Gerritsen WR. Efficient and selective gene transfer into primary human brain tumors by using single-chain antibody-targeted adenoviral vectors with native tropism abolished. J Virol 2002; 76: 2753-2762.
[84] Wu M, Sherwin T, Brown WL and Stockley PG. Delivery of antisense oligonucleotides to leukemia cells by RNA bacteriophage capsids. Nanomedicine 2005; 1: 67-76.
[85] Destito G, Yeh R, Rae CS, Finn MG and Manchester M. Folic acid-mediated targeting of cowpea mosaic virus particles to tumor cells. Chem Biol 2007; 14: 1152-1162.
[86] Dirven CM, Grill J, Lamfers ML, Van der Valk P, Leonhart AM, Van Beusechem VW, Haisma HJ, Pinedo HM, Curiel DT, Vandertop WP and Gerritsen WR. Gene therapy for meningioma: improved gene delivery with targeted adenoviruses. J Neurosurg 2002; 97: 441-449.
[87] Gomme PT, McCann KB and Bertolini J. Transferrin: structure, function and potential therapeutic actions. Drug Discov Today 2005; 10: 267-273.
[88] Ren Y, Wong SM and Lim LY. Folic acid-conjugated protein cages of a plant virus: a novel delivery platform for doxorubicin. Bioconjug Chem 2007; 18: 836-843.
[89] Allen M, Bulte JW, Liepold L, Basu G, Zywicke HA, Frank JA, Young M and Douglas T. Paramagnetic viral nanoparticles as potential high-relaxivity magnetic resonance contrast agents. Magn Reson Med 2005; 54: 807-812.
[90] Anderson EA, Isaacman S, Peabody DS, Wang EY, Canary JW and Kirshenbaum K. Viral nanoparticles donning a paramagnetic coat: conjugation of MRI contrast agents to the MS2 capsid. Nano Lett 2006; 6: 1160-1164.
[91] Loo L, Guenther RH, Basnayake VR, Lommel SA and Franzen S. Controlled encapsidation of gold nanoparticles by a viral protein shell. J Am Chem Soc 2006; 128: 4502-4503.
[92] Kay MA, Holterman AX, Meuse L, Gown A, Ochs HD, Linsley PS and Wilson CB. Long-term hepatic adenovirus-mediated gene expression in mice following CTLA4Ig administration. Nat Genet 1995; 11: 191-197.
[93] Lieber A, Vrancken Peeters MJ, Meuse L, Fausto N, Perkins J and Kay MA. Adenovirus-mediated urokinase gene transfer induces liver regeneration and allows for efficient retrovirus transduction of hepatocytes in vivo. Proc Natl Acad Sci U S A 1995; 92: 6210-6214.
[94] Mizuno M, Yoshida J, Colosi P and Kurtzman G. Adeno-associated virus vector containing the herpes simplex virus thymidine kinase gene causes complete regression of intracerebrally implanted human gliomas in mice, in conjunction with ganciclovir administration. Jpn J Cancer Res 1998; 89: 76-80.
[95] Nguyen DM, Spitz FR, Yen N, Cristiano RJ and Roth JA. Gene therapy for lung cancer: enhancement of tumor suppression by a combination of sequential systemic cisplatin and adenovirus-mediated p53 gene transfer. J Thorac Cardiovasc Surg 1996; 112: 1372-1376; discussion 1376-1377.
[96] Specthrie L, Bullitt E, Horiuchi K, Model P, Russel M and Makowski L. Construction of a microphage variant of filamentous bacteriophage. J Mol Biol 1992; 228: 720-724.
[97] Wei B, Wei Y, Zhang K, Wang J, Xu R, Zhan S, Lin G, Wang W, Liu M, Wang L, Zhang R and Li J. Development of an antisense RNA delivery system using conjugates of the MS2 bacteriophage capsids and HIV-1 TAT cell-penetrating peptide. Biomed Pharmacother 2009; 63: 313-318.