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
Viral nanoparticles and new therapeutic approaches

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, revealed 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,
Viral nanoparticles and new therapeutic approaches

Table 1. Examples of viral nanoparticles used for biomedical applications

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

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 intracellular 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 pl value of the inserted peptide has a clear impact in the systemic movement of the virus. In fact, fused peptides with a pl 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 pl within the previously established permissible 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 in 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 noncovalent 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-S-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 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 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 labeling efficiency was verified by TEM, using gold coupled anti-biotin antibodies [47].

Non-covalent modifications

Intmolecular 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 monodispersity. 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

therapeutics. For this reason, a large number of exploitations in all of the biomedical and pharmacological applications later described have been found for bioconjugation.
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 tetrarmers 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 mononclonal 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].

Encapsulation

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 plasmid 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 monodispersion, 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 radio-labeled 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-
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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 peri-lymphoid zones, in the liver sinusoids, in the convoluted tubules of the kidney and in draining lymph nodes. 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 autoreactive cells 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 histocompatibility 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, PXV-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 VLPs 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 VNP{s} 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 VNP{s} in order to achieve tumor cells specific targeting. Interestingly, some VNP{s} 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 VNP{s}, was used to fluorescently label the particles to demonstrate the capability of in vitro binding and internalization. Fluorescence confocal microscopy revealed that CPV VNP{s} 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) VNP{s}, loaded with the chemotherapy agent doxorubicin, to the ovarian cancer derived cells OVCAR-3. Doxorubicin is an
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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 VNP s were chemically conjugated to FA using NHS ester chemistry (Figure 1c). Cellular uptake and cytotoxicity was tested on OVCAR-3 cells, which overexpress FR. VNP s 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 VNP s 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 VNP s 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, VNP s 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, VNP s have been used to generate: (i) new paramagnetic materials, typically based on gadolinium ions (Gd³⁺) 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) nanoparticles 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 VNP s. Divalent metal ions such as Ca²⁺ play an important role in the in vitro assembly of CCMV. 180 metal binding sites are present on the VNP s 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 Gd³⁺ and, the resulting VNP s of 28 nm in diameter, formed a monodispersed preparation with a binding capacity of 140 Gd³⁺ per VNP, making the complex an excellent potential candidate for MRI detection. However, in vivo, local high concentration of Ca²⁺ could compete with Gd³⁺ for VNP s binding, destabilizing the Gd³⁺ CCMV association with repercussions on toxicity due to the release of free Gd³⁺ [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 VNP s 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 VNP s. Commercial fluorescent dyes have been successfully conjugated to the VNP s 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. VNP s were distributed in all organs examined and in particular enabled the visualization of vasculature, intravital 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 dysfunctional 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. Spechtie 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

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[84] Mizuno M, Yoshida J, Colosi P and Kurtzmann G. Adeno-associated virus vector containing the herpes simplex virus thymidine kinase gene causes complete regression of intracerebrally implanted human gliomas in mice, in conjunc-


[86] Specthrie L, Bullitt E, Horiuchi K, Model P, Rus-
sel M and Makowski L. Construction of a micro-