The True Story and Advantages of RNA Phage Capsids as Nanotools

Paul Pumpens\textsuperscript{a} Regina Renhofa\textsuperscript{a} Andris Dishlers\textsuperscript{a} Tatjana Kozlovsk\textup{a} Velta Ose\textsuperscript{a} Peter Pushko\textsuperscript{c} Kaspars Tars\textsuperscript{a,b} Elmars Grens\textsuperscript{a} Martin F. Bachmann\textsuperscript{d,e}

\textsuperscript{a}Latvian Biomedical Research and Study Centre, and \textsuperscript{b}Faculty of Biology, Department of Molecular Biology, University of Latvia, Riga, Latvia; \textsuperscript{c}Medigen Inc., Frederick, Md., USA; \textsuperscript{d}Jenner Institute, University of Oxford, Oxford, UK; \textsuperscript{e}University Institute of Immunology, University of Bern, Inselspital, Bern, Switzerland

Key Words
RNA phages · Coat proteins · Virus-like particles · Self-assembly · 3D structure · Vaccines · Immunology · Nanocontainers · Drug delivery · Viral nanotechnology

Abstract
RNA phages are often used as prototypes for modern recombinant virus-like particle (VLP) technologies. Icosahedral RNA phage VLPs can be formed from coat proteins (CPs) and are efficiently produced in bacteria and yeast. Both genetic fusion and chemical coupling have been successfully used for the production of numerous chimeras based on RNA phage VLPs. In this review, we describe advances in RNA phage VLP technology along with the history of the \textit{Leviviridae} family, including its taxonomical organization, genomic structure, and important role in the development of molecular biology. Comparative 3D structures of different RNA phage VLPs are used to explain the level of VLP tolerance to foreign elements displayed on VLP surfaces. We also summarize data that demonstrate the ability of CPs to tolerate different organic (peptides, oligonucleotides, and carbohydrates) and inorganic (metal ions) compounds either chemically coupled or noncovalently added to the outer and/or inner surfaces of VLPs. Finally, we present lists of nanotechnological RNA phage VLP applications, such as experimental vaccines constructed by genetic fusion and chemical coupling methodologies, nanocontainers for targeted drug delivery, and bioimaging tools.

Family of RNA Phages

Taxonomy
Pili-specific RNA phages, currently the most promising virus-like particle (VLP) carriers, are nonenveloped, spherical viruses with $T = 3$ icosahedral symmetry and diameters ranging from approximately 28 to 30 nm. RNA phage particles are composed of 178 chemically identical coat protein (CP) molecules, or 89 CP dimers, and one copy of maturation, or A, protein, which replaces a single CP dimer. The phage’s monopartite, positive-sense, single-stranded (plus-ssRNA) genome is approximately 4 kb in size and serves as messenger RNA for the synthesis of the capsid-forming CP as well as three other viral proteins: the maturation, replicase, and lysis proteins (fig. 1).

The first \textit{Escherichia coli}-infecting RNA phages that played an important role in VLP technology were identified in the early 1960s and included the f2 [1], MS2 [2],...
R17 [3], fr [4], M12 [5], and Qβ [6] phages. The early history and basic properties of RNA phages were reported in detail in two books [7, 8] and in one original review [9].

Later, other RNA phages that became more common as VLP carriers were described, including the E. coli phages SP, FI [10] and GA [11], the Caulobacter crescentus phage φCB5 [12], the Pseudomonas aeruginosa phage PP7 [13], the broad host range, P-pili-specific phage PRR1 [14], and the Acinetobacter phage AP205 [15, 16].

According to the most current ICTV taxonomy release [17], RNA phages are members of the family Leviridae. This family has not been assigned to any higher viral order and comprises two genera: Levivirus, which includes the species BZ13 (first mentioned in Inokuchi et al. [18]) and MS2, and Allolevivirus, which includes the species FI and Qβ. Other common RNA phages were mentioned in earlier ICTV taxonomy releases until 1998.

According to current NCBI Taxonomy browser information [19], the Levivirus genus consists of the BZ13 and MS2 species. The BZ13 species includes the phages GA and JP34, among others, as subspecies, while the MS2 species includes the phages f2, fr, M12, and R17, among others. The Levivirus genus also includes some unclassified members such as the Acinetobacter phage AP205 and the Pseudomonas phage PP7.

Based on the NCBI Taxonomy classification of the Allolevivirus genus, the FI and Qβ species include FI group subspecies (including phages SP, TW19, and TW28, among others) and Qβ group subspecies (including phages MX1 and ST, among others). Some species, including the Caulobacter phage φCB5 and P-pili-specific phages, predominantly the Pseudomonas phage PRR1, remain in the NCBI classification as unclassified Leviridae members.

Sero- and Genogroups

Generally, the current classification is based on serological typing [20] and has divided RNA phages into four serogroups, namely, serogroups I to IV [for a detailed discussion, see 21]. This classification scheme has been confirmed by studies of the template specificity of RNA phage replicases [22], genetic analysis [23, 24], and physicochemical data, e.g. by resistance to high hydrostatic pressure [25]. The RNA phages MS2, GA, Qβ, and SP are typically recognized as reference strains for the sero- and genogroups I, II, III, and IV, respectively. Therefore, group I and II members belong to the Levivirus genus, while serogroup III and IV members belong to the Allolevivirus genus. Ecological and wastewater studies have revealed that RNA phages from groups II and III are associated with human waste, whereas group I and IV members are predominantly associated with animal waste [21]. However, this distribution is not absolute and requires further refinement [26, 27].

The genomic structures of the Levivirus and Allolevivirus genera members demonstrate some differences (fig. 1). In addition to the standard maturation protein, CP, and replicase subunit, the Allolevivirus genome encodes a C-terminally extended CP known as the minor A1 protein, which appears as a result of ribosomal read-through of a leaky opal termination codon of the CP gene [28] and is essential for the formation of viable Qβ particles in vivo [29–31]. The A1 protein is incorporated in 3–10 copies per virion, or in 12 copies in accordance with a recent study [32], it is required for infection, but its precise function is not known [for more references, see 8, 29–33]. A recent electron microscopy visualization of foreign epitopes carried by A1 protein within infectious Qβ particles showed that the A1 protein molecules are occupying corners of the Qβ icosahedron [32]. The lysis protein forms pores in the cellular membrane, leading to activation of autolysins and, eventually, cell lysis [34].

Drawbacks of Classification

The current NCBI classification comprising two genera as well as four serogroups are adequate in the case of coliphages; however, attempts to assign phages from other hosts to Levivirus, Allolevivirus, or ‘unclassified’ members have been rather artificial and lack solid rationale aside from historical considerations. In our opinion, a new classification system based solely on sequence similarities should be employed. For example, Levivirus and Allolevivirus genera could be left only for coliphages, and new genera could be introduced based on sequence similarities among conserved replicase protein sequences.

Unique Scientific Role

RNA phages were the first classic models used in early molecular biology and are considered ‘instrumental in the making of molecular biology’ [35]. They provided the scientific community with purified RNA and markedly contributed to the decryption of the genetic code, the understanding of RNA translation and replication mechanisms, and the elucidation of virus–host interactions and the self-regulation of biological systems [for details and references, see 7–9, 35]. The RNA phage MS2 was the first organism with a fully sequenced genome [36]. The capsids of the RNA phages R17 and F2 were among the first observed virions with resolved icosahedral symmetry.
[37], after the classical work by Caspar and Klug [38] on the structural analysis of plant viruses. RNA phages have also presented substantial background for studies on phylogeny and genome evolution [33]. Furthermore, RNA phages paved the way for antisense-based gene therapy via the generation of the so-called ‘mRNA-interfering complementary RNA (miRNA) immune system’ for the prevention of phage SP proliferation [39, 40]. RNA phages, especially the phage MS2, have also markedly contributed to ecological and disinfection studies via their use in the development of numerous physical and chemical methods for genome inactivation, from early attempts [41] to recent systematic studies [42]. The RNA phages MS2, GA, Qβ, Fi, SP, and PP7 are still efficiently used as surrogate models for the control of viral contamination in food production and storage, in industrial and clinical applications, and on health care personnel [for two recent examples, see 43, 44], in addition to serving as viral and microbial source tracking materials in wastewater [for references and discussion, see 45]. It is broadly accepted that RNA phages are fully adequate surrogates for human enteroviruses in studies of virus contamination [46, 47]. RNA phages are also often used as internal controls of extraction/amplification efficiency in modern RT-PCR kits for the surveillance of emerging pathogens, including the Ebola virus [48].

**CP as a Repressor**

RNA phages were the first examples of an ‘operon’ mechanism of gene regulation by ‘self’ proteins [49]. This mechanism can be described as a full-cycle regulated biological system, where gene regulation is performed by two phage proteins: CP and replicase [50, 51]. The CPs of most RNA phages have been shown to repress translation of the replicase gene by binding to an RNA hairpin as an operator at the start site of the replicase gene [7–9]. The ability of RNA phage CP to recognize the corresponding operator stem-loop led to the development of an efficient methodology based on the tethering of CP to

---

**Fig. 1.** Genomic structure of RNA phages that have been used in viral nanotechnology applications. **a** Locations of the CP genes (colored in dark pink) within the genomes of RNA phages. The genomes are shown to scale and are in alphabetical order. AP and A2 are maturation proteins. RP = Replicase; L = lysis protein. The Qβ genome does not encode L protein. The A1 extension of the Qβ CP is indicated in light pink. The numbering used for CP aa residues is indicated for each species. The data are compiled from the NCBI taxonomy browser for the *Leviviridae* family [19]. The experimentally determined lengths of phage CPs are always one aa residue shorter than the actual proteins because the N-terminal methionine is cleaved off in infected *E. coli* cells. This explains the discrepancies in CP numbering in different published works. **b** The phenogram of the CP aa sequences obtained by Clustal V alignment in the MegAlign program from DNASTAR Lasergene.
CP-operator-tagged RNAs. Using this technique, mRNAs that are tagged with the operator sequence are highly specifically indistinguishable by CP, which can be fused to fluorescent or other functional probes. This CP-operator tethering methodology enables imaging of the processing, export, localization, translation, and degradation of operator-tagged mRNA in living cells (see the review by Lampasona and Czaplinski [52] and a recent protocol from Bensidoun et al. [53] as an example). Furthermore, the tethering technique allows affinity purification of the desired RNA-protein complexes [54]. The tethering methodology mostly exploits the CP-operator composition from phage MS2, although PP7-based methodology, including the use of simultaneous MS2 composition from phage MS2, although PP7-based tethering methodology mostly exploits the CP-operator [55].

Levivirus

The early development of the tethering approach described. A phage R17-based technique was also used in the early development of the tethering approach [56]. It is noteworthy that the tethering technique has recently been applied to further develop the highly productive CRISPR-Cas9 technology [57].

Expression and Structural Investigations

Expression of CP Genes

The expression of RNA phage CP genes in E. coli led to the high-level production of correctly self-assembled icosahedral capsids that were morphologically and immunologically indistinguishable from virions in the case of MS2 [58, 59] and fr [60] of group I, JP34, an intermediate between groups I and II [61], GA of group II [62], Qβ of group III [63], SP of group IV [64], the unclassified Leviviridae members PP7 [65, 66] and AP205 [67, 68], and the unclassified Leviviridae phages PRR1 [69] and qCB5 [70]. It is noteworthy that not only E. coli cells but also the E. coli-based cell-free translation system can be used for efficient in vitro production of MS2 VLPs [71, 72].

Coinfection with two phages led to the production of mixed particles only in the case of the closely related phages MS2 and fr from the serogroup I and not in the case of the more distantly related phages fr and GA; however, the reassembly of recombinant fr and GA CP dimers in vitro allowed the generation of the mixed particles in both spherical and rod-like configurations [73].

Highly efficient production of VLPs was also achieved in the yeast species Saccharomyces cerevisiae and Pichia pastoris for phages MS2 [74], Qβ [75], GA [76], fr, AP205, PP7, and qCB5 [77]. Attempts to prepare RNA phage SP VLPs in S. cerevisiae and P. pastoris were unsuccessful [77].

3D Structures of Phage Capsids

As mentioned above, the RNA phages R17 and f2 played a unique role in revealing the virion’s icosahedral symmetry [37]. The place of RNA phages in the global history of viral architecture was presented in a recent review [78]. Early electron microscopy studies demonstrated clear paracrystalline arrays of virions in E. coli cells infected with the RNA phages f2 [79], μ2 [80], and R17 [81]. Similar paracrystalline structures were later found in E. coli cells expressing the CP gene and producing VLPs; for example, see the electron micrograph of a slice of a cell filled with phage Qβ VLPs in Pumpens and Grens [82].

Fine 3D structures of the most typical RNA phage representatives have been resolved by X-ray crystallography (fig. 2) and were found to be very similar, despite the marked diversity in the primary structures of their CPs (fig. 1). The first 3D structure resolved was for MS2 virions. At the time, this structure showed no similarity to any other known viruses or proteins of any origin. The MS2 virion structure was first determined at a resolution of 3.3 Å [83–85] and then refined to a resolution of 2.8 Å [86]. Next, the crystal structure of an MS2 capsid with amino acid (aa) exchanges in the FG loop was resolved [87]. Historically, the first phage MS2 crystals and preliminary X-ray examination data were obtained in Walter Fiers’ lab in 1979 [88].

According to the 3D structure of MS2, the 180 CP subunits of the virion are arranged in dimers as initial building blocks and form a lattice with a T = 3 triangulation number (fig. 2). The CP subunit consists of a five-stranded β-sheet facing the inside of the particle and a hairpin and two α-helices on the outside.

The structure of a recombinant capsid of the RNA phage fr was determined by X-ray crystallography at a resolution of 3.5 Å and was shown to be identical to the protein shell of the native virus [89, 90]. This was followed by determination of the structure of the GA phage, which showed some structural differences compared to MS2 and fr phage/VLPs, especially in the N- and C-terminal regions [91]. The structures of virions and recombinant capsids of the Qβ phage were resolved at a resolution of 3.5 Å [92, 93]. These structures differed from previously determined RNA phage structures by the presence of stabilizing disulfide bonds on each side of the flexible FG loop, which covalently links CP dimers. A comparison with the structure of the related phage MS2 shows that although the fold of the Qβ CP is very similar, the details of the protein-protein interactions are completely different [93].

RNA Phage Capsids as Nanotools

3D Structures of Phage Capsids

As mentioned above, the RNA phages R17 and f2 played a unique role in revealing the virion’s icosahedral symmetry [37]. The place of RNA phages in the global history of viral architecture was presented in a recent review [78]. Early electron microscopy studies demonstrated clear paracrystalline arrays of virions in E. coli cells infected with the RNA phages f2 [79], μ2 [80], and R17 [81]. Similar paracrystalline structures were later found in E. coli cells expressing the CP gene and producing VLPs; for example, see the electron micrograph of a slice of a cell filled with phage Qβ VLPs in Pumpens and Grens [82].

Fine 3D structures of the most typical RNA phage representatives have been resolved by X-ray crystallography (fig. 2) and were found to be very similar, despite the marked diversity in the primary structures of their CPs (fig. 1). The first 3D structure resolved was for MS2 virions. At the time, this structure showed no similarity to any other known viruses or proteins of any origin. The MS2 virion structure was first determined at a resolution of 3.3 Å [83–85] and then refined to a resolution of 2.8 Å [86]. Next, the crystal structure of an MS2 capsid with amino acid (aa) exchanges in the FG loop was resolved [87]. Historically, the first phage MS2 crystals and preliminary X-ray examination data were obtained in Walter Fiers’ lab in 1979 [88].

According to the 3D structure of MS2, the 180 CP subunits of the virion are arranged in dimers as initial building blocks and form a lattice with a T = 3 triangulation number (fig. 2). The CP subunit consists of a five-stranded β-sheet facing the inside of the particle and a hairpin and two α-helices on the outside.

The structure of a recombinant capsid of the RNA phage fr was determined by X-ray crystallography at a resolution of 3.5 Å and was shown to be identical to the protein shell of the native virus [89, 90]. This was followed by determination of the structure of the GA phage, which showed some structural differences compared to MS2 and fr phage/VLPs, especially in the N- and C-terminal regions [91]. The structures of virions and recombinant capsids of the Qβ phage were resolved at a resolution of 3.5 Å [92, 93]. These structures differed from previously determined RNA phage structures by the presence of stabilizing disulfide bonds on each side of the flexible FG loop, which covalently links CP dimers. A comparison with the structure of the related phage MS2 shows that although the fold of the Qβ CP is very similar, the details of the protein-protein interactions are completely different [93].

Intervirology 2016;59:74–110
DOI: 10.1159/000449503
Fig. 2. Crystal structures of RNA phages that have been used as VLP carriers. The structures are presented in alphabetical order with their protein data bank IDs shown in parentheses and the outer diameters indicated for each species. The AB loops are exposed on the full-capsid surfaces. Also shown are the corresponding trimeric asymmetric units with the indicated N- and C-termi-ni. The outer surface is oriented towards the reader. The AB loops are indicated by arrows. The Qβ AB loops are distinguished by Lys14 residues, which are indicated by the shaded areas. The CP chains A, B and C are indicated in red, green, and blue, respectively. The structural data are compiled from the VIPERdb (http://viperdb.scripps.edu) database [389] and were visualized using Chimera software [390].
The crystal structure of the *P. aeruginosa* phage PP7 was resolved at a resolution of 3.7 Å [94, 95]. As in the case of the MS2 and Qβ CPs, the RNA recognition site on the PP7 CP was determined [96]. Recently, a detailed biophysical study of PP7 virions, including the effects of pH and salt concentration on the charge transition from net-positive to net-negative, was undertaken [97]. The crystal structure of another *Pseudomonas* phage, PRR1, was resolved at a resolution of 3.5 Å and exhibited a binding site for a calcium ion close to the quasi-3-fold axis [69].

The crystal structure of a very distant RNA phage, the *Caulobacter* phage φCB5, was resolved at 3.6 Å, and the structure of a φCB5 VLP was resolved at 2.9 Å [70]. The structures appeared to be nearly identical, with some differences in the average density of RNA. Unlike in other phages, φCB5 capsids are significantly stabilized by calcium ions, similarly to some plant viruses [98]. Disassembly of these capsids occurs when the calcium ions are chelated with EDTA and/or there is a reduction in the surrounding salt concentration. Another unique feature of φCB5 is the involvement of RNA bases in the stabilization of its interdimer contacts [70].

Recently, the crystal structure of a phage AP205 dimer was solved at a resolution of 1.7 Å and then fitted into a 6.6-Å resolution cryo-EM map [99]. The structure of the AP205 CP dimer can be regarded as a circular permutant relative to the structures of MS2 and other family members (fig. 3). This feature is made possible by the fact that

---

**Fig. 3.** Differences in CP structures between the AP205 and MS2 phages. The overall folds of the CP dimers (top) are similar for both phages. The CP monomers are shown in rainbow coloring, from the blue N-terminus to the red C-terminus. From the more schematic picture of secondary structure elements (middle), it is obvious that AP205 has N- and C-termini that are located in roughly the same place as the AB loops from MS2. As a result, the N- and C-termini in AP205 are well exposed on the capsid surface (blue and red, respectively), similarly to the AB loops (black) in MS2.
the N-terminus of one monomer in the dimer is in close proximity to the C-terminus of the other monomer. Compared to MS2 and other phages with known structures, the AP205 CP is missing one beta strand in its N-terminus, but it has an extra beta strand in its C-terminus. However, when considered from a 3D perspective, the position of the beta strand is essentially the same in both cases. As a consequence, AP205 has N- and C-termini in the same locations as those occupied by surface-exposed AB loops in other phage capsids. It is notable that the N- and C-termini in other phages are not well exposed on the surface and are clustered around the quasi-3-fold axes. This explains the previous observations that, in contrast to other phages, the AP205 CP can tolerate long additions at its N- and C-termini without compromising capsid assembly [67].

In addition to the structures of self-assembled RNA phage capsids, the crystal structures of unassembled mutant CP dimers of MS2 [100] and GA [62] have also been resolved. These structures showed only minor differences in comparison to their self-assembled counterparts. Surprisingly, the MS2 CP sustained a genetic fusion, resulting in a duplicated CP that folded normally and functioned as a translational repressor due to its physical proximity to the N- and C-termini of the CP [101].

Furthermore, the crystal structure of an icosahedral MS2 capsid that was assembled from covalently joined dimers, or so-called single-chain dimers, was resolved at 4.7 Å [102]. The structure resembled the wild-type (wt) virion except for the intersubunit linker regions, but a fraction of the capsids was unstable in phosphate buffer because of assembly defects [102]. Moreover, the organization of MS2 single-chain dimers into crystals may have resulted in an arrangement of subunits that corresponds to T = 3 icosahedral particles [103]. In this case, the arrangement of dimers is somewhat similar to that in normal T = 3 icosahedral particles, except that four FG loops interact near the 4-fold axis of symmetry on an icosahedron rather than five FG loops interacting near the 5-fold axis of symmetry on an icosahedron. However, when MS2 CP dimers are not crystallized in the F cubic crystal form, they are assembled into T = 3 icosahedral capsids that are indistinguishable from the wt particles [103].

**Structural Basis of RNA Recognition**

X-ray crystallography led to a breakthrough in understanding the protein-RNA interactions that occur during translational repression and genome encapsidation. This breakthrough was particularly apparent after the first crystal structure of a complex of recombinant MS2 capsids with the 19-nucleotide RNA operator was resolved at 2.7 Å [104–106]. The residues responsible for the protein-RNA interactions were localized by analysis of aa exchanges at positions 45 and 59 [107–109]. Furthermore, numerous other mutations responsible for altering the specificity [110] and efficiency [111] of translational operator complexes were identified. The crystal structures of MS2 VLPs complexed with RNA aptamers, which differ in secondary structure from wt RNA [112–114] or involve the presence of 2‘-deoxy-2-aminopurine at the critical –10 position [115], have also been resolved.

Structures of CPs in complex with operator RNA fragments have also been solved for PRR1 [116], PP7 [117], and Qβ [118]. The CP dimers formed by the RNA phages studied to date all recognize a stem-loop sequence around the replicase start codon. This CP-RNA interaction serves as a mechanism for translational repression and self-genome recognition during virion assembly. Although the overall binding mode of the stem-loop to CP is similar in all the studied cases, the details are surprisingly different among different viruses. A number of nucleotides form sequence-specific and sequence-unspecific interactions with CPs. For CP recognition, the most important nucleotides are two adenosines, one located in the loop region and another in a stem bulge. In MS2, PP7 and Qβ, these adenosines form quite different interactions with the CP dimer (fig. 4). It should be noted that we have failed to identify analogous CP-RNA interactions in the more distantly related phages AP205 and φCB5 [K. Tars, unpub. observations], suggesting that mechanisms of genome recognition and translational repression may differ significantly among distant Leviviridae family members.

Electron cryomicroscopy studies have shown that, in addition to the operator, many other RNA sequences in the MS2 genome are able to bind to the CP dimer [119]. Such studies have allowed 3D visualization of icosahedrally averaged genomic MS2 RNA at a resolution of 9 Å [120]. Recently, direct evidence for packaging signal-mediated assembly of the MS2 phage was presented based on cross-linking studies of peptides and oligonucleotides at the interfaces between the capsid proteins and the genomic RNA of this phage [121, 122]. Remarkably, the same CP-RNA and maturation protein-RNA interfaces were identified in every viral particle.

**The A Protein**

Particles of RNA phages contain a single copy of a maturation protein, also known as the A protein, which binds to genomic RNA and is absolutely necessary for the infectivity of phages by attachment to bacterial pili. Therefore,
the A protein must be exposed at both the inner and outer surfaces of the CP shell. Because the capsids of ssRNA phages contain holes at the 5-fold and 3-fold axes of symmetry that are large enough for the diffusion of RNA fragments, it was long believed that the A protein binds in proximity to these axes. However, it was recently shown in cryo-EM studies that the A protein actually replaces a single CP dimer \cite{123, 124}; as such, the virion actually contains 178 CP monomers.

Comparative investigations of MS2 VLPs and virions containing a copy of the A protein by SANS (small-angle neutron scattering) revealed some differences, particularly the presence of ‘thin’ (preinfection) and ‘thick’ (postinfection) capsids, which are not seen by crystallography \cite{125}. The role of the A protein during virus assembly may involve the accumulation of tension that is later used to eject the genomic RNA and the A protein into a host cell.

**Intrinsic Antigenicity and Immunogenicity**

Species-specific virus-neutralizing antibody responses to RNA phages were studied in the late 1960s \cite{126–129}. The MS2 phage was characterized as a T cell-dependent antigen that may also function as a T cell-independent antigen when used in high doses \cite{130}, with high efficacy in inducing T cell response \cite{131}. However, no B cell epitopes have been mapped for virus-neutralizing antibodies on RNA phage capsids. General observations have shown the conformational nature of the B cell epitopes \cite[our unpubl. data]{129}. It was found that only a synthetic MS2 CP peptide spanning from positions 89 to 108 is capable of inhibiting phage neutralization by specific antiserum and of inducing the production of virus-neutralizing antibodies in rabbits \cite{132} and guinea pigs \cite{133}. Chemical conjugation of an N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) adjuvant to the 89–108 peptide also markedly increased the induction of virus-neutralizing

---

**Fig. 4.** Different binding modes of operator stem-loop sequences to CP dimers from MS2, Qβ, and PP7. For the operator stem-loop sequences, adenosine, which is required for the binding of loop nucleotides, is shown in red, while bulged adenosine is shown in blue. The replicase gene initiation codon is boxed.
antibodies in rabbits [134]. Although proper CP epitopes have not been mapped, the epitope-recognizing complementarity-determining region (CDR) peptides from a virus-neutralizing monoclonal antibody specific for phages fr, MS2, and GA were described and documented for their capability to neutralize the abovementioned phages [135]. Recently, CDRs from thermostable single-domain anti-MS2 antibodies obtained by the immunization of llamas were sequenced [136].

RNA Phage Coats as a VLP Carrier

Genetic Fusion as a First Step in the Development of RNA Phage VLP Carriers

Recombinant capsids of fr [137, 138] and MS2 [139] were the first VLP carriers proposed for the presentation of foreign immunological epitopes on their surfaces by genetic fusion. To identify appropriate CP regions, oligonucleotide linkers encoding short aa sequences and containing convenient restriction sites were inserted into different regions of the fr CP [140]. Remarkably, this work was based on computer predictions of the spatial structure of the fr CP and was conducted before the first crystal structure of an RNA phage, namely that of the homologous MS2 capsid, had been resolved [83–86]. Recombinant fr CPs containing 2- to 12-aa-long additions to their N- or C-termini or insertions at position 50 in the RNA-binding region were capable of self-assembly, but this was not the case at positions 97–111 in the αA-helix [140]. The majority of other fr CP mutants demonstrated reduced self-assembly capabilities and formed either CP dimers (aa exchanges at residues 2, 10, 63, or 129) or both dimer and capsid structures (residue 2 or 69) [141]. The FG loop of the fr CP was also recognized as a potential target for insertions/replacements and was initially modified by a 4-aa-long deletion [142]. The deletion variant retained the ability to form capsids, although they displayed significantly reduced thermal stability. Furthermore, the 3D structures of the mutant capsids revealed that the modified loops were disordered near the 5-fold axis of symmetry and were too short to interact with each other [142]. Because of the high importance of the FG loop in capsid stability, further development of chimeras based on the implementation of FG loops was not pursued.

Vectors were constructed for the insertion of foreign sequences into codon position 2 of the fr CP-coding sequence in all possible reading frames [137]. When the preS1 epitope DPAPFR from hepatitis B virus (HBV) was inserted as a marker at positions 2, 10, and 129, it appeared in all cases on the particle surface [137]. Attempts to introduce the 40-aa-long V3 loop from the human immunodeficiency virus 1 (HIV-1) gp120 protein into the N-terminus, at positions 10, 12, and 15, or into the FG loop led to unassembled products [K. Tars, unpubl. data].

Attempts to use the N-terminal β-hairpin exposed at the surface of the capsid, namely aa residues 15/16, allowed for the production of MS2 capsids bearing a number of different peptide sequences up to 24 aa residues in length [139]. Foreign epitopes exposed on these chimeras were found to be immunogenic in mice [139]. Mutational mapping revealed residues that are responsible for inter- and intramolecular contacts and therefore for the thermal stability of MS2 capsids [143].

The addition of the Flag octapeptide to the N-terminus or its insertion into the N-terminal β-hairpin of the MS2 CP prevented self-assembly and proper folding, respectively. However, genetic fusion of the Flag to a duplicated CP-encoding sequence resulted in the synthesis of a protein considerably more tolerant to the structural perturbations and mostly corrected the defects accompanying Flag peptide insertion [144]. The putative protective epitope T1, spanning 24 aa in length and derived from the immunodominant liver stage antigen-1 of the malaria parasite Plasmodium falciparum, was inserted at the tip of the N-terminal β-hairpin (between positions 15 and 16) of the MS2 CP [145].

fr VLPs have shown unusually high capacities as vectors, particularly when the addition of long segments of hamster polyomavirus VP1 to the N-terminus of the fr CP did not prevent VLP self-assembly [146]. When discovered, these findings markedly enhanced interest in using RNA phage coats as potent vaccine candidates.

However, the relatively low tolerance of fr- and MS2-based VLP vectors to long foreign insertions represents a clear limitation of these models. For example, the fr CP failed to form VLPs following the insertion of long HBV preS1 sequences [147]. To overcome this difficulty, Qβ protein A1, namely the C-terminal extension of the Qβ CP within the A1 protein, has been proposed as a site for foreign insertions [148, 149]. Potentially, the 195-aa extension of the Qβ CP could be considered an ideal target for insertions. This region was found to contain elements that typically protrude as spike-like structures on the particle surface. Furthermore, the self-assembly capabilities of capsids with mutually exchanged extensions of the Qβ and SP CPs were confirmed experimentally [64]. A following mathematical prediction showed possible colin-
earity between the Qβ CP extension and the surface-located HBV preS sequence [148]. It is noteworthy that the crystal structure of the read-through domain from the Qβ A1 protein was recently determined at a resolution of 1.8 Å and revealed a fold that is unique among all proteins found in the protein data bank [150].

Qβ CP proteins with additions at their C-termini failed to form particles in most cases. However, such additions were incorporated into particles in the presence of a wt ‘helper’ CP to form mixed, or mosaic, particles. Mosaic VLPs differ therefore from chimeric VLPs which are built from identical recombinant CP molecules, without any helper molecules. Such mosaic particles were constructed by either enhancing the level of UGA suppression in the presence of overexpressed suppressor tRNA [151] or by exchanging the UGA stop codon to a GGA sense codon and expressing the extended and helper forms of the Qβ CP from two separate genes. These genes were located either on the same plasmid or on two separate plasmids with different antibiotic resistance genes. Potential insertion sites were mapped by insertion of the 5-aa preS1 DPAFR model epitope and the 39-aa-long HIV-1 gp120 V3 loop [149]. After enhancing UGA suppression, mosaic particles were detected, but the proportion of A1 to helper CP in these particles dropped from 48 to 14% as the length of the A1 extension increased [152]. A model insertion of the preS1 epitope DPAFR located on the particle surface produced specific antigenicity and immunogenicity in mice [152]. The antibody response to the preS1 epitope was higher for self-assembled Qβ-preS1 VLPs than for a nonassembled Qβ-preS1 variant [153]. When the Qβ CP was modified to carry long HBV preS insertions (full-length preS, preS1, or preS2 alone) instead of the A1 extension, mosaic particles formed that had surface-exposed preS, but regular VLPs did not form without the presence of the Qβ CP as a helper [I. Cielen and R. Renhofa, unpubl. data].

On Qβ CP itself, the residues responsible for RNA recognition have been mapped [154, 155]. This was helpful for the development of packaging and gene transfer technologies based on the Qβ phage model. The ability of the RNA phage CP to package RNA in vivo [156] demonstrated the potential of RNA phages as gene delivery vectors (see Nonvaccine Applications).

Surprisingly, icosahedral Qβ VLPs have been converted into rods after modification of the FG loop structure [157]. As mentioned above, the appearance of alternate VLP forms of RNA phages was further confirmed by the presence of rod-like structures in the case of the coassembly of phage fr and GA CPs [73].

RNA Phage Capsids as Nanotools

Chemical Coupling to Further Develop VLP Technology

Chemical coupling of foreign oligopeptides to the surface of VLPs was developed as an alternative method to the genetic fusion of epitope-encoding sequences. Chemical coupling was initially applied to RNA phage Qβ VLPs in 2002 [167] by using an approach initially developed for another broadly used recombinant VLP, HBc (HBV core antigen) [167, 168]. Model oligopeptides containing a free cysteine residue at the N-terminus were coupled to an exposed lysine residue on the Qβ VLP surface using the hetero-bifunctional cross-linker maleimidobenzoic acid sulfosuccinimidyl ester. The modified VLPs showed efficient induction of oligopeptide-specific antibodies in mice [167]. This chemical coupling approach initiated the development of a panel of experimental therapeutic vaccines (see early reviews by Bachmann and Dyer [169] and Dyer et al. [170], and a recent review by Bachmann and Jennings [171]). By following the lysine-cysteine oligopeptide coupling methodology, an experimental West Nile virus vaccine was constructed on an AP205 VLP platform [68].

Another chemical coupling approach was developed based on the rational design of modified MS2 VLPs that displayed a reactive thiol on the VLP surface as a result of a T15C substitution in the MS2 CP [172, 173]. Cysteines are among the most useful functional groups found in proteins as they can bind a variety of metals and react with a large collection of organic reagents, and are therefore obvious targets for protein modification [172]. Two cys-
tein residues that are present in the wt MS2 CP are internally located and therefore relatively unreactive. Thiolated MS2 VLPs were chemically modified with fluorescein-5′-maleimide to create the first fluorescent nanoparticles [173]. Additional functionalization was achieved in a cell-free protein synthesis platform by the production of MS2 and Qβ VLPs with surface-exposed methionine analogues (azidohomoalanine and homopropargylglycine) containing azide and alkyne side chains [174]. Such VLPs can be used for one-step, direct conjugation schemes to display multiple ligands of interest. Using such technology, proteins including an antibody fragment and granulocyte-macrophage colony-stimulating factor, as well as nucleic acids and poly(ethylene glycol) chains, were displayed on the VLP surface using Cu(I) catalyzed click chemistry [174]. Surface functionalization methodology has not only been applied to the VLP surface but also to the interiors of MS2 VLPs by modification of tyrosine residues via a recently developed hetero-Diels-Alder bioconjugation reaction [175].

The preparation of histidine-tagged MS2 VLPs by the introduction of a His6 linker between CP codons 15 and 16 to simplify the purification of VLP-covered RNAs is another example of VLP surface functionalization [176]. Moreover, a set of advanced His6-tagged Qβ VLPs was generated, and their ability to complex metal-derivatized compounds was confirmed [177, 178]. In parallel, VLP vector capacity for chemical coupling was broadened by the introduction of azide- or alkyne-containing unnatural amino acids, which was achieved by expression of the Qβ CP gene in a methionine auxotrophic strain of E. coli [179].

A highly specific approach for the further development of VLP vectors was achieved by the asymmetrization of Qβ VLPs after the introduction of a single copy of the maturation protein A2, which allowed the production of VLPs with a single unique modification [180].

**Fig. 5.** Electron micrographs of negatively stained AP205 virions (a), recombinant AP205 VLPs (b), chimeric AP205 VLPs carrying 151 aa residues of human interleukin-1β at the C-terminus (c), GA virions (d), recombinant GA VLPs (e), and chimeric GA VLPs carrying a 61-aa-long ZHER2.34 affibody at the C-terminus (f). VLPs are purified from the appropriate gene-expressing E. coli cells. For electron microscopy, the grids with the adsorbed particles were stained with aqueous solutions of 1% uranyl acetate (pH 4.5) or 2% phosphotungstic acid (pH 7.0) and examined with JEM-100C or JEM-1230 electron microscopes (Jeol Ltd., Tokyo, Japan) at 100 kV. Well-ordered knobs are clearly visible on the surfaces of the chimeric VLPs.
Recently, a novel plug-and-display system was established for modular RNA phage VLP functionalization via further decoration of VLPs with peptides of interest [181, 182]. The decoration of VLPs is based on the so-called bacterial superglue approach, namely on the ability of a peptide (SpyTag) and a protein (SpyCatcher) to form spontaneous covalent isopeptide bonds between lysine and aspartic acid residues under physiological conditions [183]. The SpyTag and SpyCatcher are split units of the Streptococcus pyogenes fibronectin-binding protein FbaB and can form a highly stable amide bond by an irreversible reaction that occurs within minutes [184]. The SpyTag or SpyCatcher sequences were genetically fused to the N- and/or C-terminus of the AP205 CP. After mixing modified AP205 VLPs with the correspondingly linked peptides, the quantitative covalent coupling of the peptides to the VLPs was observed [181, 182].

**Stability of VLPs**

Efforts to improve the stability of natural RNA phage VLPs started with the development of a methodology that allowed the screening of bacteria for the synthesis of mutant MS2 CPs with altered assembly properties [185] and the selection of D11N variant CPs that formed virions more stable than the wt CP [186]. The introduction of interdisulfide bonds into the 5-fold axis of symmetry to cross-link MS2 VLPs improved their thermal stability to the level of that seen in Qβ VLPs, which possess natural intersubunit disulfide bonds [187]. In contrast, cross-linking at the 3-fold axis of symmetry resulted in variant CPs that were unable to self-assemble [187]. The development of an E. coli-based cell-free protein synthesis system opened a direct avenue for studying the role of disulfide bond formation in the stability of mutant MS2 VLPs in comparison to Qβ and HBc VLPs [188]. Through the construction of a set of Qβ CP mutants, it was found that disulfide linkages are the most important stabilizing elements in VLPs and that interdimer interactions are less important than intradimer interactions for Qβ VLP assembly [189].

Elucidation of the thermal stability of foreign epitope-carrying VLPs, such as the chimeric MS2 VLPs formed by single-chain dimers, in comparison to natural disulfide cross-linked PP7 VLPs [190] is of great importance for the further development of RNA phage-based nanotechnology. The genetic fusions of two copies of the MS2 [159], PP7 [191], and GA or Qβ [I. Cielens and A. Strods, unpubl. data] CPs resulted in a self-assembly-competent single-chain dimer that not only increased thermodynamic stability but also considerably improved tolerance to foreign insertions in the AB-loop. The resultant correctly assembled VLPs encapsidated mostly their ‘own’ CP-encoding mRNAs.

**Vaccines and Vaccine Candidates**

Vaccines represent the most advanced field of the RNA phage VLP applications due to the excellent and well-established scaffold properties and structural tolerance to the decoration by foreign immunogenic sequences. Such decoration can be performed both genetically and chemically, and the VLP scaffold may provide foreign epitopes with a strong T cell response. Moreover, RNA phage VLPs can serve as nanocontainers that can encapsulate specific adjuvants, such as immunostimulatory oligodeoxynucleotides or CpGs, as TLR7 ligands [192]. RNA phage VLPs can also be packaged with single-stranded or double-stranded RNA fragments as TLR7 and TLR3 ligands, respectively [193]. Moreover, recombinant RNA phage VLPs contain encapsulated bacterial RNA, which may act as an adjuvant. Reviews on vaccine applications include articles discussing RNA phage VLPs among other VLP candidates [82, 169–171, 194–202] and articles focused either on RNA phage VLPs in general [203] or on the use of specific RNA phage species, such as MS2 VLPs, as vaccines [204].

**Genetic Fusions**

Table 1 contains a detailed list of the vaccine candidates that have been constructed from RNA phage VLPs using genetic fusion methodology. First, the success of an experimental human papilloma virus (HPV) vaccine based on PP7 single-chain-dimer VLPs [191, 205–209] developed in preclinical studies [210] must be mentioned. A similar HPV vaccine candidate was constructed on MS2 single-chain-dimer VLPs and tested in preclinical studies [210, 211]. Both PP7 and MS2 VLP-based vaccines were immunogenic, but the MS2-L2 VLPs induced a broader HPV-neutralizing antibody response. This is likely because of the structural context of L2 display on the VLPs, since L2 was displayed on the AB-loop of the PP7 CP, but at the N-terminus of the MS2 CP [191]. A review on HPV vaccine candidates, including RNA phage VLP-based vaccines, was recently published [212]. A malaria vaccine based on MS2 VLPs has also been reported as very promising [213, 214]. As to the selection of optimal VLP carriers for such purposes, AP205 VLPs have demonstrated a high capacity and tolerance to foreign insertions [67]. Moreover, the AP205 VLPs represent good candidates for the construction of mosaic VLPs [215].
<table>
<thead>
<tr>
<th>Vaccine target</th>
<th>Source of epitope</th>
<th>Epitope length, aa&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Position of insertion or addition</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AP205</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acquired</td>
<td>Coreceptor CCR5, ECL2 loops: mini-loop</td>
<td>12, 33</td>
<td>C-terminus</td>
<td>VLPs carrying mini-loops are formed with GTAGGGSG, but not with GSG linker. VLPs carrying full-length loops are formed with both linkers</td>
<td>Cielens and Renhofa, unpubl. data</td>
</tr>
<tr>
<td>immunodeficiency: human immunodeficiency virus (HIV)</td>
<td>Coreceptor CXCR4, mini-loop: CNVSEADDRTYC</td>
<td>12</td>
<td>C-terminus</td>
<td>VLPs carrying mini-loops are formed with GTAGGGSG, but not with GSG linker</td>
<td>Cielens and Renhofa, unpubl. data</td>
</tr>
<tr>
<td></td>
<td>Coreceptor CXCR4, as 1-39</td>
<td>39</td>
<td>N-terminus</td>
<td>The epitope is definitely displayed on the VLP surface</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Net protein, aa 66–100 and 132–151</td>
<td>55</td>
<td>C-terminus</td>
<td>The Net T cell epitopes are displayed on the VLP surface</td>
<td>67</td>
</tr>
<tr>
<td><strong>Autoimmune arthritis</strong></td>
<td>Interleukin-1β, human</td>
<td>17 kDa</td>
<td>C-terminus</td>
<td>Mosaic particles are formed by: (i) suppression of C-terminal amber or opal codons or (ii) coexpression of the chimeric gene with the helper CP gene. The GSG or GSGG linkers were used</td>
<td>Jansons et al., unpubl. data</td>
</tr>
<tr>
<td></td>
<td>Interleukin-1α, murine</td>
<td>157, 152</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Interleukin-1β, murine and human</td>
<td>154</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cancer, prostate</strong></td>
<td>Gonadotropin releasing hormone (GnRH), aa 1-10</td>
<td>10</td>
<td>N-terminus, C-terminus</td>
<td>The VLPs carrying C-terminal fusion induce a strong antibody response that inhibits GnRH function in vivo</td>
<td>Cielens and Renhofa, unpubl. data</td>
</tr>
<tr>
<td><strong>Chikungunya virus</strong></td>
<td>Glycoprotein E1, the virus-neutralizing domain III (DIII); glycoprotein E2, full-length, domain A, domain B, domains A+B</td>
<td>84, 361, 131, 60, 298</td>
<td>C-terminus</td>
<td>Mosaic particles formed by suppression of the C-terminal amber codons or (ii) coexpression of the chimeric gene with the helper CP gene. The GSG or GSGG linkers were used</td>
<td>Cielens and Renhofa, unpubl. data</td>
</tr>
<tr>
<td><strong>HBV</strong></td>
<td>preS1, aa 21–47</td>
<td>27</td>
<td>N-terminus</td>
<td>The SGTAGGGSGS linker was more preferable for the outcome than the SGG linker</td>
<td></td>
</tr>
<tr>
<td></td>
<td>preS1, aa 21–47, 20–58, 20–119</td>
<td>27, 39, 100</td>
<td>C-terminus</td>
<td>The GTAGGGSG linker was used</td>
<td></td>
</tr>
<tr>
<td><strong>Hepatitis C virus</strong></td>
<td>E2 protein, genotype 1a, HVR sequence, aa 384–411; a ‘consensus’ HVR sequence</td>
<td>28, 31</td>
<td>N-terminus</td>
<td>The SGTAGGGSGS linker was used in both cases</td>
<td></td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td>Angiotensin II, aa 1–8</td>
<td>8</td>
<td>N-terminus, C-terminus</td>
<td>Both variants display angiotensin II epitope on the VLP surface</td>
<td>67</td>
</tr>
<tr>
<td><strong>Influenza virus</strong></td>
<td>M2e protein, N-terminal ectodomain, consensus sequence, aa 2–24</td>
<td>23</td>
<td>N-terminus</td>
<td>Strong M2-specific antibody response was achieved upon immunization in mice: protection of 100% of mice from a lethal influenza infection</td>
<td>67, 295</td>
</tr>
<tr>
<td><strong>Lymphocytic choriomeningitis virus</strong></td>
<td>Glycoprotein, peptide p33: KAVYNFATM</td>
<td>9</td>
<td>C-terminus</td>
<td>Chimeric VLPs were formed also when different linkers and another epitope variant KAVYNFATMA have been used</td>
<td>Cielens and Renhofa, unpubl. data</td>
</tr>
<tr>
<td><strong>Obesity</strong></td>
<td>Ghrelin, aa 24–31: GSSFLSPE</td>
<td>8</td>
<td></td>
<td>The SGTAGGGGS linker was more preferable for the outcome than the SGG linker</td>
<td>Cielens and Renhofa, unpubl. data</td>
</tr>
<tr>
<td></td>
<td>Gastric inhibitory peptide (GIP), aa 1–15 of mature GIP (42 aa): YAEGTFISDYSIAMD</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Salmonella typhi</strong></td>
<td>Outer membrane protein, D2 peptide, aa 266–280</td>
<td>15</td>
<td>N-terminus, C-terminus</td>
<td>The epitope is displayed on the VLP surface</td>
<td>67</td>
</tr>
<tr>
<td><strong>West Nile virus</strong></td>
<td>Glycoprotein E, the virus-neutralizing domain III (DIII), aa 296–406</td>
<td>111</td>
<td>C-terminus</td>
<td>Mosaic particles. Immunization of mice resulted in the induction of IgG2 isotype anti-DIII antibodies</td>
<td>215</td>
</tr>
<tr>
<td><strong>fr</strong></td>
<td>Hamster polyomavirus</td>
<td>VP1, aa 364–384, 351–374, 351–384, 333–384</td>
<td>N-terminus</td>
<td>Induction of anti-VP1 antibodies in rabbits and mice</td>
<td>146</td>
</tr>
</tbody>
</table>
### Table 1 (continued)

<table>
<thead>
<tr>
<th>Vaccine target</th>
<th>Source of epitope</th>
<th>Epitope length, aa&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Position of insertion or addition</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GA</strong></td>
<td>Coreceptor CCR5, ECL1 loop</td>
<td>14</td>
<td>FG loop</td>
<td>A shorter insertion without Cys residue</td>
<td>GYAAQQDFGNTTG did not result in the self-assembled VLPs</td>
</tr>
<tr>
<td></td>
<td>YAAQDDFGNTMCQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coreceptor CCR5, ECL2a loop</td>
<td>9</td>
<td>AB loop, aa 14/15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>QKEGLHYTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coreceptor CCR5, N-terminus, aa 1 – 27</td>
<td>27, 31</td>
<td>N-terminus of the second CP copy</td>
<td>Mosaic particles with the GA CP as a helper were formed in both cases</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coreceptor CXCR4, aa 1 – 40</td>
<td>40</td>
<td>N-terminus</td>
<td>Four variants with different linkers and surrounding sequences formed VLPs</td>
<td></td>
</tr>
<tr>
<td><strong>Influenza virus</strong></td>
<td>M2e protein, N-terminal ectodomain consensus sequence, aa 2 – 24 with C-terminally added G residue</td>
<td>24</td>
<td>N-terminus, C-terminus</td>
<td>The GSGS (GSRS) and GSG linkers were used for N-terminal and C-terminal insertions, respectively</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2e protein, N-terminal ectodomain consensus sequence, aa 2 – 24 with C-terminally added G residue</td>
<td>24</td>
<td>N-terminus of the first CP copy of the CP single-chain dimer</td>
<td>The GSG linker was used</td>
<td></td>
</tr>
<tr>
<td><strong>S. typhi</strong></td>
<td>Outer membrane protein, D2 peptide, aa 266 – 280</td>
<td>15</td>
<td>N-terminus</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MS2</strong></td>
<td>Cancer, ovarian</td>
<td>A peptide mimicking the cancer-associated antigen 125 (CA125/MUC16)</td>
<td>10</td>
<td>AB loop of the CP single-chain dimer, aa '15/14'</td>
<td>The MS2-DISGNTNTRSA VLPs induced murine antibodies that cross-reacted with CA125 from ovarian cancer cells Preoperative ovarian cancer patient plasma was assessed</td>
</tr>
<tr>
<td><strong>Cholesterol lowering</strong></td>
<td>Proprotein convertase subtilisin/kexin type 9 (PCSK9), aa 153 – 163, 188 – 200, 208 – 222, 368 – 381</td>
<td>11, 13, 17, 14</td>
<td>N-terminus of the CP single-chain dimer</td>
<td>There were not as dramatic reductions in total cholesterol in mice immunized with recombinant MS2-PCSK9 VLPs, in comparison to chimeric Qβ VLPs (table 2)</td>
<td></td>
</tr>
<tr>
<td><strong>Foot and mouth disease virus</strong> (FMDV)</td>
<td>VP1, FMDV O/ OZK, aa 141 – 160</td>
<td>20</td>
<td>AB loop</td>
<td>The effective immune response in mice and protection of guinea pigs and swine against FMDV were achieved</td>
<td></td>
</tr>
<tr>
<td><strong>HIV</strong></td>
<td>gp120 protein, V3 loop</td>
<td>10</td>
<td>AB loop of the CP single-chain dimer, aa '15/14'</td>
<td>The V3 insertion disrupted self-assembly, but was tolerated by the CP fusion into the single-chain dimer. High immunogenicity in mice. The ability to pack their own mRNA was demonstrated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coreceptor CCR5, ECL2 loop</td>
<td>10</td>
<td></td>
<td>The ECL2 insertion disrupted self-assembly, but was tolerated by the CP fusion into the single-chain dimer. High immunogenicity in mice. Ability to pack their own mRNA</td>
<td></td>
</tr>
<tr>
<td><strong>HPV</strong></td>
<td>L1 protein</td>
<td>20</td>
<td>AB loop, aa '15/14'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L2 protein</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L2 protein of HPV16, HPV18, HPV31, aa 17 – 31</td>
<td>15</td>
<td>N-terminus and AB loop of the CP single-chain dimer</td>
<td>Two different epitopes were displayed on the same particle The strong protection of mice from genital infection with HPV pseudoviruses representing 11 diverse HPV types was demonstrated. Preclinical studies are ongoing</td>
<td></td>
</tr>
<tr>
<td><strong>Influenza virus</strong></td>
<td>Hemagglutinin, epitope</td>
<td>9</td>
<td>AB loop, aa '15/14'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>YPYDVDPDYA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2e protein, conserved epitope</td>
<td>9</td>
<td>AB loop of the CP single-chain dimer, aa '15/14'</td>
<td>The scalable purification protocol for the potential veterinary vaccine application was elaborated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EVETPRINE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Malaria</strong></td>
<td><em>Plasmodium falciparum</em>, liver stage antigen-1 (LSA-1), T1 epitope</td>
<td>24</td>
<td>AB loop, aa '15/14'</td>
<td>The LSA-1-carrying VLPs stimulated a type 1-polarized response, with significant upregulation of interferon-γ, a finding which corroborates naturally acquired resistance to liver stage malaria</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. falciparum</em>, RH5 protein, a peptide SAIEKPTV mimicking a linear epitope</td>
<td>8</td>
<td>AB loop of the CP single-chain dimer, aa '15/14'</td>
<td>The chimeric VLPs elicited antibodies that inhibit parasite invasion and could form the basis of an effective vaccine against malaria</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. falciparum</em>, blood stage antigen AMA1 (apical membrane antigen-1)</td>
<td>10</td>
<td></td>
<td>The mimotope identified by VLP-peptide display induced murine antibodies that cross-reacted with AMA1</td>
<td></td>
</tr>
</tbody>
</table>
Table 1 (continued)

<table>
<thead>
<tr>
<th>Vaccine target</th>
<th>Source of epitope</th>
<th>Epitope length, aa (^1)</th>
<th>Position of insertion or addition</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP7 Flag peptide</td>
<td>DYKDDDDK</td>
<td>8</td>
<td>AB loop of the CP and of the CP single-chain dimer, aa 11/12</td>
<td>The VLPs were highly immunogenic in mice and packaged their own mRNA</td>
<td>191</td>
</tr>
<tr>
<td>HIV gp120 protein, V3 loop, a peptide</td>
<td>IQRGPGRAPV</td>
<td>10</td>
<td></td>
<td>The VLPs were highly immunogenic in mice and packaged their own mRNA</td>
<td>191</td>
</tr>
<tr>
<td>HPV L2 protein of HPV16, aa 17–31: QLYKTCKQAGTCPPD</td>
<td></td>
<td>15</td>
<td></td>
<td></td>
<td>191, 206</td>
</tr>
<tr>
<td>HPV L2 protein of HPV1, 5, 6, 11, 16, 18, 45, 58, aa 17–31</td>
<td></td>
<td>15</td>
<td>AB loop of the CP single-chain dimer, aa 11/12</td>
<td>Mice immunized with the mixture of eight L2 VLPs were strongly protected from genital challenge with pseudovirions representing 8 diverse HPV types. The anti-L2 antibodies persisted over 18 months and vaccinated mice retained protection over a year after immunization. For the first time in the generation of chimeric VLPs, RNA was removed from the VLP inside by alkaline treatment, as it has been performed before on nonchimeric MS2 VLPs [175]</td>
<td>205, 207</td>
</tr>
<tr>
<td>HPV L2 protein of HPV16, aa 17–31, 35–50, 51–65, 65–79, 65–85, Consensus 65–85 (for 65–85 variant)</td>
<td></td>
<td>21 (for 65–85 variant)</td>
<td>Insertion of HPV16 L2 aa 35–50 and aa 51–65 was compatible with VLP assembly, but insertion of aa 65–79 was not. The VLPs displaying the 65–85 consensus peptide of high-risk HPV types induced murine sera that neutralized heterologous high-risk HPV pseudovirions</td>
<td></td>
<td>208</td>
</tr>
<tr>
<td>HPV L2 protein of HPV1, HPV16, HPV18, aa 17–31</td>
<td></td>
<td>15</td>
<td>N-terminus and AB loop of the CP single-chain dimer</td>
<td>Two different epitopes were displayed on the same particle. The strong protection of mice from genital infection with HPV pseudoviruses representing 11 diverse HPV types was detected</td>
<td>209</td>
</tr>
<tr>
<td>Qb FMDV VP1 protein, G-H loop peptide</td>
<td></td>
<td>14</td>
<td>C-terminally added to the shortened A1 protein gene within the viable Qb genome</td>
<td>A replication-competent hybrid phage that efficiently displayed the FMDV peptide was achieved. The surface-localized FMDV VP1 G-H loop cross-reacted with the anti-FMDV monoclonal antibody SD6 and was found by electron microscopy to decorate the corners of the Qb icosahedral shell. The hybrid phages induced polyclonal antibodies in guinea pigs with good affinity to both FMDV and hybrid Qb-G-H loop</td>
<td>32</td>
</tr>
<tr>
<td>HBV preS1 epitope: 31-DPAFR-35 31-DPAFRA-36</td>
<td></td>
<td>5, 6</td>
<td>A1 protein, C-terminal extension, aa 72/73, after aa 3, 6, 13, 19; as well as instead of the C-terminal extension</td>
<td>Mosaic particles were formed by A1-derived chimeras and Qb CP helper via: (i) suppression of leaky UGA stop codon of the CP gene and (ii) simultaneous expression of CP helper and A1-derived genes obtained after the changing of CP-terminating UGA to strong UAA stop codon or sense GGA codon, respectively. The proportion of A1-extended to short CP in mosaic particles varied from 48 to 14% after increase of the length of A1 extension. The preS1 epitope ensured specific immunogenicity in mice</td>
<td>148, 149, 152</td>
</tr>
<tr>
<td></td>
<td>Full-length preS1 and preS2; preS1, aa 20–47, 20–58, or 31–58</td>
<td>28–163</td>
<td>A1 protein, C-terminal extension, aa 18 or after leaky termination codon instead of the C-terminal extension</td>
<td>Mosaic particles were formed by: (i) suppression of terminal amber or opal codons or (ii) coexpression of the chimeric gene with the CP helper gene. In the second case, it was necessary to place the chimeric gene first within the plasmid</td>
<td>Cielens and Renhofa, unpubl. data</td>
</tr>
<tr>
<td>HIV gp120 protein, V3 loop, aa 299–337</td>
<td></td>
<td>39</td>
<td>A1 protein, C-terminal extension, aa 72/73; after aa 19; instead of the C-terminal extension</td>
<td>Mosaic particles were formed</td>
<td>148, 149</td>
</tr>
</tbody>
</table>

\(^1\) When a precise number of aa residues is difficult to assess, the size of the epitopes is expressed in kDa.
### Table 2. Vaccines and vaccine candidates constructed on RNA phage VLPs by chemical coupling methodology

<table>
<thead>
<tr>
<th>Vaccine target</th>
<th>Source of epitope</th>
<th>Epitope length, aa&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Coupling site</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP205 Asthma/allergy</td>
<td>IL-5, murine</td>
<td>33 kDa</td>
<td>SpyTag or SpyCatcher: genetically fused to the N-terminus and/or C-terminus</td>
<td>The VLP display led to the efficient breaking of self-tolerance</td>
<td>182</td>
</tr>
<tr>
<td>Cancer</td>
<td>Human telomerase reverse transcriptase, the mutant Telo epitope biotin-GAHIVMV-DAYKPTREARPALLTSRLRFIPK</td>
<td>30</td>
<td>SpyCatcher genetically fused to the N-terminus</td>
<td>SpyCatcher is a genetically encoded protein designed to spontaneously form a covalent bond to its peptide-partner SpyTag carrying desired epitope</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td>Human epidermal growth factor receptor (EGFR) from glioblastoma, fusion junction epitope LEEKGNVYVTDHGAGHIVMV-DAYKPTK-biotin</td>
<td>27</td>
<td>SpyTag or SpyCatcher: genetically fused to the N-terminus and/or C-terminus</td>
<td>The VLP display led to the efficient breaking of self-tolerance</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>Murine proteins involved in cancer (CTLA-4, PD-L1, Survivin and HER2)</td>
<td>kDa: 15</td>
<td>SpyTag or SpyCatcher: genetically fused to the N-terminus and/or C-terminus</td>
<td>The gp41 peptides were coupled to AP205 VLPs through a bifunctional cross-linker SMPH. The chimeric VLPs elicited high titers of gp41-specific antibodies. 1, 2, or 3 peptide copies were coupled to each AP205 subunit</td>
<td>301</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>PCSK9, murine</td>
<td>84 kDa</td>
<td>Lysine residues</td>
<td>Chimeric VLPs were packaged by CpG and induced effective CTL response in mice</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>Human immunodeficiency virus (HIV)</td>
<td>Six peptides covering the α-helical regions of gp41: 3 – 13, 3 – 17, 3 – 20, 3 – 24, P1, P8, provided with a C-terminal Cys residue</td>
<td>12, 16, 19, 23, 40, 66</td>
<td>The gp41 peptides were coupled to AP205 VLPs through a bifunctional cross-linker SMPH. The chimeric VLPs elicited high titers of gp41-specific antibodies. 1, 2, or 3 peptide copies were coupled to each AP205 subunit</td>
<td>301</td>
</tr>
<tr>
<td></td>
<td>Lymphocytic choriomeningitis virus (LCMV)</td>
<td>Glycoprotein, the p33 peptide KAVYNFATM</td>
<td>9</td>
<td>Lysine residues</td>
<td>Chimeric VLPs were packaged by CpG and induced effective CTL response in mice</td>
</tr>
<tr>
<td></td>
<td>Malaria</td>
<td>P. falciparum, membrane protein 1 (PfEMP1) containing the Complex lysine and cysteine-rich inter-domain region (CIDR)</td>
<td>27 kDa</td>
<td>SpyCatcher genetically fused to the N-terminus</td>
<td>A pair of SpyTag and SpyCatcher as a functional unit were used (see above). Injecting SpyCatcher-VLPs decorated with a malarial antigen efficiently induced antibody responses after only a single immunization</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P. falciparum, Pf623 protein</td>
<td>kDa: 53, 32, 118, 40</td>
<td>SpyTag or SpyCatcher: genetically fused to the N-terminus and/or C-terminus</td>
<td>The Pf623 and VAR2CSA vaccines showed efficacy that is comparable with the efficacy of the best existing analogues</td>
</tr>
<tr>
<td></td>
<td>Salmonella typhi</td>
<td>Outer membrane protein, D2 peptide, aa 266 – 280: TSNSGNPSTSYGFAN with N-terminal CGG linker</td>
<td>15</td>
<td>Lysine residues</td>
<td>The vaccine preparations of 13, 56, 94, 142, and 293 peptides per VLP were used. A phenomenon of carrier induced epitopic suppression could be overcome by high coupling densities, repeated injections and/or higher doses of conjugate vaccine</td>
</tr>
<tr>
<td></td>
<td>Tuberculosis</td>
<td>Mycobacterium tuberculosis, Ag85A protein</td>
<td>48 kDa</td>
<td>SpyTag or SpyCatcher: genetically fused to the N-terminus and/or C-terminus</td>
<td>Domain III was engineered to comprise a His&lt;sub&gt;4&lt;/sub&gt; tag and a Cys-containing linker at its C-terminus. The antibodies induced in mice were able to neutralize virus in vitro and provided partial protection from a challenge with a lethal dose of WNV</td>
</tr>
<tr>
<td></td>
<td>West Nile virus (WNV)</td>
<td>Glycoprotein E, the virus-neutralizing domain III (DIII), aa 582 – 696 of the WNV polyprotein precursor</td>
<td>115</td>
<td>Lysine residues</td>
<td>Domain III was engineered to comprise a His&lt;sub&gt;4&lt;/sub&gt; tag and a Cys-containing linker at its C-terminus. The antibodies induced in mice were able to neutralize virus in vitro and provided partial protection from a challenge with a lethal dose of WNV</td>
</tr>
<tr>
<td>Q&lt;sup&gt;β&lt;/sup&gt; Acquired immunodeficiency: feline immunodeficiency virus (FIV)</td>
<td>Transmembrane (TM) glycoprotein, a peptide containing tryptophan-rich motif, aa 767 – 786: LQKWEDWYGWIGNIPQYLKG</td>
<td>20</td>
<td>Lysine residues</td>
<td>Immunized cats developed antibodies that reacted with the epitope, but failed to recognize whole FIV. The coupling efficiency was never higher than 20%</td>
<td>302</td>
</tr>
<tr>
<td></td>
<td>Acquired immunodeficiency: HIV</td>
<td>Coreceptor CCR5, N-terminal ECL domain, circularized</td>
<td>20</td>
<td></td>
<td>Immunized mice and rabbits generated antibodies that recognized native CCR5 and inhibited entry of pseudotype viruses bearing envelope glycoproteins from diverse primary strains in vitro</td>
</tr>
</tbody>
</table>
### Table 2 (continued)

<table>
<thead>
<tr>
<th>Vaccine target</th>
<th>Source of epitope</th>
<th>Epitope length, aa&lt;sup&gt;l&lt;/sup&gt;</th>
<th>Coupling site</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acquired immunodeficiency: HIV/simian immunodeficiency virus (SIV)</td>
<td>Coreceptor CCR5, macaque, ECL1, N-terminal aa MDYQVSSPTYDDYTFEPC, ECL2, a cyclic peptide, aa 168 – 177 DRSQREGLHFTYG linked through an DG dipetide spacer</td>
<td>20, 10</td>
<td>Lysine residues</td>
<td>Immunization of mice and rats induced anti-CCR5 antibodies that recognized native CCR5 and inhibited SIV infection in vitro. Equal amounts of both constructs were mixed to formulate the vaccine that was protective in macaques. The coupling efficiency reached 90 EC1 or 270 ECL2 peptides per particle</td>
<td>305, 306</td>
</tr>
<tr>
<td>Allergy</td>
<td>Cysteine protease, the major fcal allergen of the house dust mite D. pteronyssinus, Der p 1 peptide, aa 117 – 133: CGTYPNANKIREALQTHSA</td>
<td>21</td>
<td>–</td>
<td>The vaccine was administered without adjuvants and found safe and immunogenic in humans after evaluation of different doses and routes of immunization</td>
<td>307</td>
</tr>
<tr>
<td>Allergy, cat</td>
<td>Fel d 1 protein, major cat allergen, a covalent dimer of chain 2 and chain 1 of Fel d 1 spaced by a 15 aa-linker (GGGGS)x3 and added to the coding sequence for LEHHHHHHGGC at the C-terminus</td>
<td>23 kDa</td>
<td>–</td>
<td>A single vaccination by Qb-Fel d 1 was sufficient to induce protection against type I allergic reactions in mice. Moreover, Qb-Fel d 1 did not induce degranulation of basophils derived from human volunteers with cat allergies. The coupling density was 40 %, or 70 covalent Fel d 1 dimers per VLP</td>
<td>308</td>
</tr>
<tr>
<td>Allergy, rhinoconjunctivitis, rhinitis</td>
<td>A-type CpG (QbG10) was encapsulated as a TLR9 ligand. Mixed with house dust mite (HDM) extract</td>
<td>–</td>
<td>No coupling</td>
<td>A phase I study. All patients achieved practically complete alleviation of allergy symptoms after 10 weeks of immunotherapy</td>
<td>217</td>
</tr>
<tr>
<td>Allergy, asthma</td>
<td>CYT003-QbG10: CpG QbG10 was encapsulated</td>
<td>–</td>
<td>–</td>
<td>A phase IIb study. Treatment with high-dose CYT003-QbG10 improved disease symptoms. The QbG10 content was approximately 20% of the total mass, corresponding to ~60 molecules per VLP</td>
<td>218, 219</td>
</tr>
<tr>
<td>Allergy, asthma and rhinitis</td>
<td>Two IgE peptides, different loops of the C3 domain: ADSNPRGVSAYLSRPSPGGC and YQRVTHPHLPRALMRS</td>
<td>20, 16</td>
<td>Lysine residues</td>
<td>The vaccine induced high titers of anti-human IgE antibodies by preclinical studies in mice</td>
<td>309</td>
</tr>
<tr>
<td>Allergy to red meat</td>
<td>α-1, 3-galactosyl transferase, Galα3LN epitope. For conjugation, α-Gal trisaccharide and glucose were converted to their respective alkene derivatives. Each alkene was attached by a two-step procedure in which the protein nanoparticle was first acylated with an azide-terminated N-hydroxysuccinimide ester and then acylated with an azide-terminated which the protein nanoparticle was first acylated with an azide-terminated N-hydroxysuccinimide ester and then acylated with an azide-terminated</td>
<td>Carbohydrates are conjugated</td>
<td>Search for the presence of α-Gal-containing epitopes in the saliva of Amblyomma sculptum. Bites from the A. sculptum tick may be associated with the allergenic reactions to red meat in Brazil</td>
<td>310</td>
<td></td>
</tr>
<tr>
<td>Alzheimer disease</td>
<td>Aβ peptide (1 – 9)-GGC: DAEFRHDSGGGC</td>
<td>12</td>
<td>–</td>
<td>Qb-Aβ VLPs elicited anti-Aβ antibody responses at low doses and without the use of adjuvants</td>
<td>311</td>
</tr>
<tr>
<td></td>
<td>Aβ peptide: N-terminal Aβ(1 – 9) or C-terminal Aβ (28 – 40)</td>
<td>12, 16</td>
<td>–</td>
<td>Both of these immunogens produced significant antibody titers without use of additional adjuvants and reduced Aβ levels when tissues were examined 8 months after the first inoculation</td>
<td>312</td>
</tr>
<tr>
<td></td>
<td>CAD106 vaccine: Aβ peptide 1 – 6 DAEFRH plus a GGC spacer</td>
<td>9</td>
<td>–</td>
<td>CAD106 avoided activation of Aβ-specific T cells and was efficacious in reducing the amyloid accumulation in transgenic mice without evidence of unwanted side effects and is currently being tested in patients in a phase II study. Each VLP contained ~350 – 550 Aβ effects and is currently being tested in patients in a phase II study. Each VLP contained ~350 – 550</td>
<td>313</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>IL-1α, full length, containing aa linker at C-terminus</td>
<td>17 kDa</td>
<td>–</td>
<td>Immunization of mice reduced both the inflammatory reaction in the plaque as well as plaque progression</td>
<td>314</td>
</tr>
<tr>
<td>Autoimmune arthritis</td>
<td>IL-1α, murine, aa 117 – 270 of IL-1α precursor IL-1β, murine, aa 119 – 269 of IL-1β precursor, both provided with aa linkers at C-termini</td>
<td>17 kDa</td>
<td>–</td>
<td>Immunization of mice elicited a rapid and long-lasting autoantibody response. In the collagen-induced arthritis model, both vaccines strongly protected mice from inflammation and degradation of bone and cartilage. In the T and B cell-independent collagen Ab transfer model, immunization with the IL-1β vaccine strongly protected from arthritis, whereas immunization with the IL-1α vaccine had no effect. The coupling efficiency was about 20% in the case of IL-1α and 28% in the case of IL-1β, or 36 molecules of IL-1α or 50 molecules of IL-1β per VLP</td>
<td>315, 316</td>
</tr>
</tbody>
</table>

DOI: 10.1159/000449503

Intervirology 2016;59:74–110

Pumpens/Renhofa/Dishlers/Kozlovska/Ose/Pushko/Tars/Grens/Bachmann
### Table 2 (continued)

<table>
<thead>
<tr>
<th>Vaccine target</th>
<th>Source of epitope</th>
<th>Epitope length, aa&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Coupling site</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoimmune arthritis, encephalomyelitis and myocarditis</td>
<td>IL-17, murine, aa 26–158, with a C-terminal linker GGGGGC</td>
<td>32 kDa</td>
<td>Lysine residues</td>
<td>Immunization induced high levels of autoantibodies in mice and was effective in ameliorating disease symptoms in animal models of autoimmunity. The coupling efficiency was about 10%, or 18 IL-17 homodimers per VLP.</td>
<td>317–319</td>
</tr>
<tr>
<td>Cancer, induction of antitumor antibodies</td>
<td>Tumor-associated carbohydrate antigens (TACAs): monomorphic Tn antigen (GalNAc-α-O-Ser/Thr) that is overexpressed on the surface of a variety of cancer cells including breast, colon, and prostate cancer, and is involved in aggressive growth and lymphatic metastasis of cancers</td>
<td>Carbohydrates are conjugated Lysine residues and N-terminus</td>
<td>The antibodies generated in mice were highly selective toward Tn antigens and reacted strongly with the native Tn antigens on human leukemia cells</td>
<td>235</td>
<td></td>
</tr>
<tr>
<td>Cholesterol lowering</td>
<td>Proprotein convertase subtilisin/kexin type 9 (PCSK9), human, aa 68–76, 153–163, 207–223</td>
<td>9, 11, 17</td>
<td>Lysine residues</td>
<td>Vaccination of mice and macaques led to significant reductions in total cholesterol, free cholesterol, phospholipids, and triglycerides (table 1)</td>
<td>296</td>
</tr>
<tr>
<td>Chronic inflammatory disorders: rheumatoid arthritis, psoriasis, Crohn’s disease</td>
<td>TNF-α, murine, aa 80–235 of the TM form, aa 4–23 peptide: CCGGSDKQNSDKKPVPVHVANHQE</td>
<td>156, 20</td>
<td>Lysine residues</td>
<td>Immunization by Qβ-TNFα(4–23) showed a potential to become an effective and safe therapy against inflammatory disorders. The coupling efficiency was about 60 TNFα 1–156 or ~340 TNFα 4–23 molecules per VLP</td>
<td>320</td>
</tr>
<tr>
<td>Chronic inflammatory illnesses: type 2 diabetes mellitus</td>
<td>IL-1β, murine, rhesus monkey and human, two muteins: mIL-1β(D143K), mIL-1β (D143K) with strongly reduced inflammatory activity</td>
<td>153</td>
<td>Lysine residues</td>
<td>As a potential therapy for the prevention and long-term treatment of type 2 diabetes, the vaccine showed good tolerability in mice and nonhuman primates and induced long-lasting, but reversible, IL-1β-neutralizing antibody titers, improved glucose tolerance, and enhanced insulin secretion in a mouse model of diet-induced diabetes. The preclinical study was followed by a phase 1/II clinical trial in patients with type 2 diabetes mellitus using the human version of the vaccine hIL1β(Qβ) and the neutralizing IL-1β-specific antibody response was registered. The epitope density was about 0.5 IL-1β molecules per Qβ monomer corresponding to a total of 90 IL-1β molecules per Qβ VLP</td>
<td>321, 322</td>
</tr>
<tr>
<td>Eosinophilia</td>
<td>Recombinant IL-5; recombinant eotaxin, both murine</td>
<td>17 and 8 kDa</td>
<td>Lysine residues</td>
<td>Both vaccines overcame self-tolerance and induced high antibody titers against the corresponding self-molecules in mice. Immunization with either of the two vaccines reduced eosinophilic inflammation of the lung in an ovalbumin based mouse model of allergic airway inflammation. The coupling efficiency was about 47 or 15% Qβ monomers cross-linked to rIL-5 or r-eotaxin, respectively, or about 80–90 rIL-5 and 25–30 r-eotaxin molecules per VLP</td>
<td>323</td>
</tr>
<tr>
<td>Hen egg lysozyme (HEL), as a model for overcoming self-tolerance</td>
<td>Full-length</td>
<td>129</td>
<td>Lysine residues</td>
<td>HEL-conjugated VLPs were generated by linking biotinylated HEL to biotinylated VLPs using streptavidin. The Qβ-HEL VLPs elicited high-titer IgG responses against HEL in tolerant sHEL transgenic mice in the absence of exogenous adjuvants. Therefore, the presentation of HEL onto Qβ VLPs allowed overcoming the effects of anergy in the HEL mouse model.</td>
<td>324</td>
</tr>
<tr>
<td>HPV</td>
<td>L2 protein, N-terminal HPV16 peptides: aa 34–52, 49–71, 65–85, 108–120, consensus 65–85 peptide</td>
<td>19, 23, 21, 13</td>
<td>Lysine residues</td>
<td>Although all VLP-displayed peptides elicited high-titer anti-peptide antibody responses, only a 65–85 peptide-induced response strongly neutralized HPV16 pseudovirion infection. To overcome HPV type specificity, a consensus 65–85 peptide was introduced and broad neutralizing activity against all of the HPV types tested was achieved</td>
<td>208</td>
</tr>
<tr>
<td>Hypertension</td>
<td>CYT006-AngQβ vaccine: angiotensin II-derived peptide CGGDRVYIHPF where CGG is a linker</td>
<td>11</td>
<td>Lysine residues</td>
<td>Successful preclinical trials in mice and rats and phase I and IIa clinical trials were performed</td>
<td>325, 326</td>
</tr>
</tbody>
</table>

RNA Phage Capsids as Nanotools

Intervirology 2016;59:74–110

DOI: 10.1159/000449503
<table>
<thead>
<tr>
<th>Vaccine target</th>
<th>Source of epitope</th>
<th>Epitope length, aa$^1$</th>
<th>Coupling site</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, diabetic nephropathy, atherosclerosis</td>
<td>ATRQβ-001 vaccine: ATR-001 peptide CAFHYEQ corresponding to an epitope of the ECL2 of human AT1R, a G-protein coupling receptor</td>
<td>8</td>
<td>Lysine residues</td>
<td>A successful preclinical trial: the ATRQβ-001 vaccine decreased the blood pressure of Ang II-induced hypertensive mice and spontaneously hypertensive rats. The vaccine provided a promising method to treat diabetic nephropathy in rats and atherosclerosis in mice</td>
<td>327 – 329</td>
</tr>
<tr>
<td>Inflammatory hyperalgesia: potential long-term therapy for chronic pain</td>
<td>Nerve growth factor (NGF), murine, aa 19 – 241 of pro-NGFβ and an additional 9 aa extension at the C-terminus comprising aHis tag and GGC sequence</td>
<td>223</td>
<td></td>
<td>Vaccination with NGFQβ substantially reduced hyperalgesia in collagen-induced arthritis or postinjection of rynoso A, two models of inflammatory pain in mice. The coupling efficiency was about 60 NGF molecules per VLP</td>
<td>330</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>M2 protein, N-terminal extracellular domain</td>
<td>23</td>
<td></td>
<td>Influnca virus M2 protein, N-terminal extracellular domain 23 Intranasal immunization of mice with Qβ VLP-M2</td>
<td>226</td>
</tr>
<tr>
<td></td>
<td>Hemagglutinin, globular head domain (gH1), A/California/07/2009 (H1N1) strain, aa 49 - 325 and C-terminal extension GGGCG</td>
<td>281</td>
<td></td>
<td>Vaccination with Qβ VLPs substantially reduced the blood pressure of Ang II-induced hypertensive mice and spontaneously hypertensive rats. The vaccine provided a promising method to treat diabetic nephropathy in rats and atherosclerosis in mice</td>
<td>331 - 333</td>
</tr>
<tr>
<td>LCMV</td>
<td>Glycoprotein, peptides: p33, KAVYNNFATM; p33, GLNGPDIYKYVQKSVFED; p33-gp61, C6SLKAVYNNFATMGLNGPDIYKG-VYQKSVIF with a GGC linker added to the C-terminus</td>
<td>9, 20, 32</td>
<td></td>
<td>Vaccination with LCMV peptides played crucial role in the studies of the CTL induction by the CpG packaging</td>
<td>222 – 229</td>
</tr>
<tr>
<td>Malaria</td>
<td>Plasmodium falciparum, CSP, an almost full-length CSP consisting of 19 NANP and 3 NVDP repeats and the majority of the N- and C-terminal regions (residues 281tyr-1271val, linked to 207pro-383ser)</td>
<td>45 kDa</td>
<td></td>
<td>By immunization of mice, the Q8-CSP induced higher anti-NANP repeat titers, higher levels of cytotoxic IgG2b/c antibodies and a trend towards higher protection against transgenic parasite challenge as compared to soluble CSP formulated in the same adjuvant. The coupling efficiency was about 12 – 25%, or an average of ~ 30 CSP molecules per VLP</td>
<td>334</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Mel-QβG10 vaccine: Melan-A/Mart-1 A27L variant peptide CGGHHSYTTIAELAGIGILTV Packaging with QβG10 CpG: GGGGGGGGGGGGACGATCGTGCGGGGGGGGGGG</td>
<td>20</td>
<td></td>
<td>Promising results were obtained by phase I and IIa clinical trials on stage II–IV melanoma patients. The coupling density reached 160 peptide copies per VLP</td>
<td>335 – 337</td>
</tr>
<tr>
<td>Nicotine addiction</td>
<td>CYT002-Nic-Qβ vaccine, or NIC002 (formerly known as Nicotine-Qβ) or Nic-Qβ: nicotine was covalently coupled to Qβ VLPs via a succiniminate linker</td>
<td>15</td>
<td></td>
<td>Preclinical studies in mice and successful phase I and II clinical trials were performed. Stable vaccine formulations enabling storage were developed. An experimental comparison to a NIC-CRM vaccine by Pfizer was achieved. The coupling density was about 3.25 nicotine molecules per CP monomer, or 585 nicotine molecules per VLP</td>
<td>231 – 234, 338</td>
</tr>
<tr>
<td>Obesity</td>
<td>Q8-GIP vaccine: gastric inhibitory peptide (GIP, also known as glucose-dependent insulinoergic polypeptide), aa 1 – 15 YAEGPISDYNAAD of mature GIP (42 aa) with C-terminally added linker GC</td>
<td>15</td>
<td></td>
<td>Immunization of mice with Q8-GIP vaccine induced high titers of specific antibodies and efficiently reduced body weight gain in animals fed a high-fat diet. Moreover, increased weight loss was observed in obese mice vaccinated with VLP-GIP. The coupling density was about 1.6 – 2.2 GIP molecules per CP monomer</td>
<td>339</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>Qβ-TRANCE/RANKL vaccine: TNF-related activation-induced cytokine (TRANCE), also known as receptor activator of NF-κB ligand (RANKL), aa 158 – 316 (extracellular domain) of the mature form of murine TRANCE/RANKL with a Cys-linker and His tag</td>
<td>159</td>
<td></td>
<td>Immunization of mice with Q8-TRANCE/RANKL VLPs overcame the natural tolerance of the immune system toward self-proteins and produced high levels of specific antibodies without the addition of any adjuvant. Serum antibodies of immunized mice neutralized TRANCE/RANKL activity in vitro and were highly active in preventing bone loss in a mouse model of osteoporosis. The coupling density was about 14%, indicating that about one of seven CP monomers was covalently attached to a peptide. As TRANCE/RANKL forms a trimer, ~25 C-TRANCE trimers were displayed per VLP</td>
<td>340</td>
</tr>
</tbody>
</table>
Chemical Coupling

The chemical coupling approach was validated by an impressive line of experimental therapeutic vaccines [169, 171]. The idea of therapeutic vaccines is based on the assumption that VLP carriers can present surface-displayed self-antigens and to augment their ability to overcome the natural tolerance of the immune system toward self-proteins and to induce high levels of specific autoantibodies [216]. Initially, this approach was planned as a method to replace host-specific monoclonal antibodies in the treatment of acute and chronic diseases, starting with noninfectious diseases [169]. Therefore, the transition from passive administration of monoclonal antibodies to active vaccination against self-antigens was a logical step in drug development, focusing on affordable medicines and broader patient acceptance and regulatory compliance. The induction of autoantibodies might be beneficial under certain physiological conditions in order to remove unwanted excess of a particular self-antigen, such as angiotensin in the case of hypertension. Table 2 presents a detailed list of predominantly therapeutic vaccines and demonstrates the validity of this assumption.

We included the well-known Qβ VLP-based allergy vaccine CYT003-QbG10 that contains an encapsulated CpG sequence, so-called QbG10, in table 2, although it does not carry any attached epitope [217–221]. However, this potentially successful vaccine initially originated from epitope-coupling methodology. Moreover, we included RNA phage VLPs carrying model epitope p33, which is derived from the lymphocytic choriomeningitis virus glycoprotein [222–229], and epitope D2, which is derived from Salmonella [230]. Although these are not actual vaccine candidates, the model epitopes have played a central role in the elucidation of the fine immunological mechanisms that govern responses to chimeric VLPs.

The display of small antigens, such as nicotine [231–234] or carbohydrate moieties [235, 236], on RNA phage VLP surfaces is a novel approach that enabled the generation of strong immunological responses against non-peptide antigens and paved the way for the development of experimental vaccines against nicotine addiction and cancer, respectively. We recommend a recent review on novel vaccines constructed on RNA phage VLPs [237] as well as special reviews on vaccines against allergies [238–240], Alzheimer disease [241], hypertension [242–244], influenza [245, 246], malaria [247], and nicotine addiction [248–252].

Nonvaccine Applications

Attention is currently focused on the use of virus-based nanoparticles as potential scaffolds for novel biomaterials and as subjects for nanoscale engineering applications involving exposure to various chemical compounds [253–256].

Drug Delivery by Nanocontainers

Table 3 summarizes RNA phage VLP-based experimental approaches that could be classified as VLP packaging and targeting methodologies. Historically, the idea of encapsulation/targeting by RNA phage VLPs appeared in the early 1990s, only a few years after the vaccine/epitope display approaches described above. A first attempt at RNA phage nanocontainer-targeted drug delivery involved, first of all, encapsulation of a deglycosylated ricin A chain coupled to an RNA operator stem-loop and decoration of the MS2 VLPs by transferrin [257–259]. Such structures, called ‘synthetic virions’ by the authors, demonstrated high toxicity to leukemia cells carrying transferrin receptor. A detailed review of these and similar pi-
Table 3. The RNA phage VLPs as models for nanocontainer packaging and decoration with addressing/targeting/delivery purposes

<table>
<thead>
<tr>
<th>Interior: packaged by</th>
<th>Exterior: decorated by</th>
<th>Addressed to</th>
<th>Supposed to treat</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP205</td>
<td>HBV, full-length preS1, aa 13–119</td>
<td>Eukaryotic cell lines HepG2, HeLa, Jurkat, Namalwa, and BHK-21</td>
<td>HBV infection</td>
<td>The uptake took place, but seemed cell line unspecific and not highly efficient</td>
<td>341</td>
</tr>
<tr>
<td>fr</td>
<td>IgG-binding Z domain at the C-terminus of the CP coexpressed with native CP as a helper</td>
<td>Rather unspecified targeting</td>
<td>Potentially broad applications in diagnostics</td>
<td>This is a first example of the generation of mosaic VLPs carrying the IgG-binding Z domain that could be targeted to antibodies displayed on the cell surface and used in diagnostics</td>
<td>Jansons and Sominskaya, unpubl. data</td>
</tr>
<tr>
<td>GA</td>
<td>GA operator-specified mRNA in vivo</td>
<td>No decoration</td>
<td>No cell targeting</td>
<td>Broad spectrum of potential targets</td>
<td>Packaging of different mRNAs encoding GA coat protein, ENA-78, and GFP in vivo in yeast S. cerevisiae</td>
</tr>
<tr>
<td>GA</td>
<td>HIV-Tat (48–60) or WNV E protein DIII domain</td>
<td>Human peripheral blood mononuclear cells (PBMCs)</td>
<td>PBMC-derived failure</td>
<td>Production of mosaic VLPs that are packaged by mRNAs encoding IL2 or GFP was achieved in vivo in yeast S. cerevisiae</td>
<td>343</td>
</tr>
<tr>
<td>MS2 operator-specified mRNA in vivo</td>
<td>No decoration</td>
<td>No cell targeting</td>
<td>Broad spectrum of potential targets</td>
<td>Introduction of single (S87N, K55N, R43K) and double (S87N + K55N and S87N + R43K) aa exchanges into the GA CP allowed self-assembly and packaging of MS2 operator-carrying mRNAs in vivo in yeast S. cerevisiae</td>
<td>344</td>
</tr>
<tr>
<td>No specific packaging</td>
<td>ZHER2:342 affibody, 61 aa in length</td>
<td>HER2 receptor on cancer cells</td>
<td>Diagnostics/therapy</td>
<td>Ability of GA-ZHER2:342-VLPs to recognize selectively and enter the HER2 receptor-bearing cells was demonstrated. Electron microscopy of this construction is presented in figure 5</td>
<td>Strods and Renhofa, unpubl. data</td>
</tr>
<tr>
<td>No specific packaging</td>
<td>Stromal cell-derived factor (SDF1), aa 1–41 and 1–19</td>
<td>CXCR4 receptor on leukocytes</td>
<td>Diagnostics/therapy</td>
<td>Insertions at the N-terminus of the first or second single-chain dimer copy were performed. Some members of the family recognized specific cells</td>
<td></td>
</tr>
<tr>
<td>MS2</td>
<td>Transferrin, anti-MS2 antibodies, anti-DF3 antibodies</td>
<td>Cells of the immune system; breast carcinoma and leukemia cells</td>
<td>Broad spectrum of potential targets</td>
<td>Classical attempt to use RNA operator as a carrier of the desired material</td>
<td>257, 258</td>
</tr>
<tr>
<td>Antisense oligodeoxynucleotides (ODNs) targeted to human nucleolar protein p120 mRNA</td>
<td>Covalent decoration with transferrin on the VLP surface</td>
<td>Promyelocytic leukemia cell line</td>
<td>Acute myelogenous leukemia</td>
<td>The ODNs were synthesized as covalent extensions to the 19 nt long operator sequence</td>
<td>345</td>
</tr>
<tr>
<td>50–70 fluorescent molecules conjugated to the interior of VLPs</td>
<td>180 PEG-2000 or PEG-5000 chains on the exterior of VLPs</td>
<td>Tumor cells</td>
<td>Solid tumors</td>
<td>An early attempt to construct an addressed nanocontainer for the potential delivery of therapeutic cargo</td>
<td>262</td>
</tr>
<tr>
<td>Fluorescent dye conjugated to the VLP interior</td>
<td>A 41-nt operator-containing sequence covalently bound to the VLP surface</td>
<td>Tyrosine kinase receptor on the Jurkat T cells</td>
<td>Leukemia</td>
<td>Colocalization experiments using confocal microscopy indicated that the operator-labeled capsids were endocytosed and trafficked to lysosomes for degradation that could allow the targeted drug delivery of acid-labile prodrug</td>
<td>265</td>
</tr>
<tr>
<td>Antisense RNA against the 5’-UTR and IRES of HCV</td>
<td>HIV-1 Tat cell-penetrating peptide conjugated chemically to VLPs</td>
<td>Hub-7 cells containing an HCV reporter system</td>
<td>Chronic HCV</td>
<td>The packaged antisense RNA showed an inhibitory effect on the translation of HCV genome</td>
<td>346</td>
</tr>
<tr>
<td>Fluorescent dyes as donor chromophores</td>
<td>Zinc porphyrins capable of electron transfer</td>
<td>No cell targeting</td>
<td>No definite target disease specified</td>
<td>Specific positioning allowed energy transfer and sensitization of the porphyrin at previously unusable wavelengths, as demonstrated by the system’s ability to effect a photocatalytic reduction reaction at multiple excitation wavelengths</td>
<td>266</td>
</tr>
</tbody>
</table>
### Table 3 (continued)

<table>
<thead>
<tr>
<th>Interior: packaged by</th>
<th>Exterior: decorated by</th>
<th>Addressed to</th>
<th>Supposed to treat</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coupling of 180 porphyrins capable of generating cytotoxic singlet oxygen upon illumination</td>
<td>~20 copies of a Jurkat-specific aptamer</td>
<td>Jurkat leukemia T cells</td>
<td>Leukemia</td>
<td>The doubly modified VLPs were able to target and kill Jurkat cells selectively even when mixed with erythrocytes</td>
<td>267</td>
</tr>
<tr>
<td>Nanoparticles, chemotherapeutic drugs, siRNA cocktails, and protein toxins</td>
<td>SP94 peptide that binds human hepatocellular carcinoma (HCC) cells</td>
<td>Hep3B cells</td>
<td>HCC</td>
<td>The targeted VLPs loaded with doxorubicin, cisplatin, and 5-fluorouracil selectively killed the HCC cell line, Hep3B. Encapsulation of a siRNA cocktail induced growth arrest and apoptosis of Hep3B cells. Loading of VLPs with ricin toxin A-chain killed the entire population of Hep3B cells</td>
<td>268</td>
</tr>
<tr>
<td>Micro-RNA: pre-miR 146a</td>
<td>Particles conjugated with HIV-1 Tat(47–57) peptide</td>
<td>Human PBMCs</td>
<td>Systemic lupus erythematosus, osteocondensation</td>
<td>Restoring the loss of miR-146a was effective in eliminating the production of autoantibodies</td>
<td>347 - 349</td>
</tr>
<tr>
<td>RNA conjugate encompassing a siRNA and the operator sequence</td>
<td>Covalent attachment of human transferrin</td>
<td>HeLa cells</td>
<td>Potentially broad applications</td>
<td>The VLPs entered cells via receptor-mediated endocytosis and produced siRNA effects better than by traditional lipid transfection route</td>
<td>350</td>
</tr>
<tr>
<td>Gold nanoparticles</td>
<td>Alexa Fluor 488 (AF 488) labelled DNA strands</td>
<td>No cell targeting</td>
<td>No definite target disease specified</td>
<td>The VLP architecture by placing the dye at distances of 3, 12, and 24 bp from the surface of VLPs bearing 10-nm gold nanoparticles allowed the rapid exploration of many variables involved in metal-controlled fluorescence</td>
<td>351</td>
</tr>
<tr>
<td>Porphyrin: loaded with approximately 250 cationic porphyrins through a novel assembly packaging mechanism</td>
<td>Cancer cells targeting nucleic acid aptamers via chemical conjugation</td>
<td>MCF-7 human breast cancer cells</td>
<td>Breast cancer upon photoactivation</td>
<td>The MCF-7 cells incubated with targeted, porphyrin-loaded virus capsids exhibited cell death. The strategy offered an approach for efficient targeted delivery of photoactive compounds for site-specific photodynamic cancer therapy</td>
<td>352</td>
</tr>
<tr>
<td>Various reporters to be used by fluorescence-based flow cytometry, confocal microscopy, and mass cytometry</td>
<td>Antibodies using a rapid oxidative coupling strategy</td>
<td>Antibody-capsid conjugates are targeting extracellular receptors on human breast cancer cell lines</td>
<td>Breast cancer</td>
<td>The broad set of conjugates with various reporters on the interior of VLPs may lead to many clinically relevant applications, including drug delivery and in vivo diagnostics</td>
<td>353</td>
</tr>
<tr>
<td>Long noncoding RNA: MEG3 RNA</td>
<td>GE11, a dodecapeptide YHWYGYTPQNY, ligand of epidermal growth factor receptor (EGFR), chemically coupled</td>
<td>EGFR</td>
<td>HCC cell line</td>
<td>The targeted delivery was dependent on clathrin mediated endocytosis and MEG3 RNA suppressed tumor growth mainly via increasing the expression of p53 and its downstream gene GDF15, but decreasing the expression of MDM2</td>
<td>354</td>
</tr>
<tr>
<td>LacZ RNA fused to RNA operator</td>
<td>No decoration</td>
<td>No cell targeting</td>
<td>Potentially broad applications</td>
<td>This is a classical attempt to ensure operator-specific packaging in vivo</td>
<td>156</td>
</tr>
<tr>
<td>MS2 operator sequence linked to the human growth hormone mRNA for in vivo packaging in S. cerevisiae</td>
<td>No decoration</td>
<td>No cell targeting</td>
<td>Potentially broad applications</td>
<td>This is a sort of application belonging to the ‘armored RNA’ technology. Functionality of packaged mRNA was confirmed by translation of mRNAs purified from VLPs</td>
<td>74</td>
</tr>
<tr>
<td>Taxol</td>
<td>No decoration</td>
<td>No cell targeting</td>
<td>Potentially broad applications</td>
<td>This is a first attempt to encapsulate enzymes where the encapsulated enzyme had the same K(m) value and a slightly lower k(cat) value than the free enzyme</td>
<td>356</td>
</tr>
<tr>
<td>HIV-1 gag mRNAs (1544 bases) produced in S. cerevisiae</td>
<td>No decoration</td>
<td>No cell targeting</td>
<td>Potentially broad applications</td>
<td>This is a classical attempt to ensure operator-specific packaging in vivo</td>
<td>156</td>
</tr>
<tr>
<td>Alkaline phosphatase tagged with a 16 aa peptide</td>
<td>No decoration</td>
<td>No cell targeting</td>
<td>Potentially broad applications</td>
<td>This is a classical attempt to ensure operator-specific packaging in vivo</td>
<td>156</td>
</tr>
</tbody>
</table>

**RNA Phage Capsids as Nanotools**

Intervirology 2016;59:74–110
DOI: 10.1159/000449503

95
mRNA joined to 19-nt operator/aptamer

Macrophages

Prostate cancer

The packaged mRNA ensured strong humoral and cellular immune responses and protected mice as a therapeutic vaccine against prostate cancer

No specific packaging

Tumor cell-specific peptides

Tumor cells

Solid tumors

Three peptides known to target specific tissues: (i) neuroblastoma and breast cancer cell lines, (ii) matrix metalloproteinases, (iii) kidney were chosen as attachment models

Conjugation of azide- and alkyne-containing proteins (an antibody fragment and the granulocyte-macrophage colony stimulating factor), nucleic acids, and PEG

Broad spectrum of potential targeting

Tumors and other possible targets

This is a universal approach based on the inclusion of surface exposed methionine analogues (azidohomoalanine and homopropargylglycine) containing azide and alkyne side chains by cell-free synthesis technology

Qβ

CpG ODNs

With and without specific addressing

Cells of immune system

Potentially broad applications

This is a classic example of the ODN encapsulation for the broad clinical applications as prophylactic and/or therapeutic vaccines

~60 Alexa Fluor 568 fluorophores per VLP

Fullerene C60 and PEG

HeLa cell line

Cancer

This approach overcame the insolubility of C60 in water and opened the door for the applications in photoactivated tumor therapy

Metalloporphyrin derivative for photodynamic therapy

Glycan ligand for specific targeting of cells bearing the CD22 receptor

CD22 receptor

This approach benefited from the presence of the strong targeting function and the delivery of a high local concentration of singlet oxygen-generating payload

Aptamers embedded in a longer RNA sequence with the Qβ CP operator

No decoration

No cell targeting

Potentially broad applications in therapy

The VLPs ensured the delivery of the encapsulated aptamers that were protected from degradation and retained ability to bind their small-molecule ligands

Positively charged synthetic polymer by atom transfer radical polymerization (ATRP) methodology

No definite target disease specified

Potentially broad applications in therapy and imaging diagnostics

This is a robust method for removing encapsidated RNA from VLPs and the use of the empty interior space for site-specific, ‘graft-from’ ATRP reactions

Proteins: 25-kDa N-terminal aspartate dipeptidase, peptidase E, 62-kDa firefly luciferase (Luc), a thermostable mutant of Luc

No specific packaging

No specific targeting

Variety of cells

Potentially broad applications

Bioconjugations of Qβ VLPs derivatized with azide moieties at surface lysine residues were performed. Complete derivatization of more than 600 reactive sites per particle was achieved

Glycans used as substrates for glycosyltransferase reactions to build di- and trisaccharides

Cognate receptors on the appropriate beads and cells

Potentially broad applications

The elaborated methodology provided a convenient and powerful way to prepare complex carbohydrate ligands for clustered receptors

Cationic aa motifs by genetic engineering or chemical conjugation

Inhibiting the anticoagulant action of heparin

Clinical overdose of heparin

The polycationic motifs displayed on the mutated Qβ VLPs acted as heparin antagonists

IgG-binding Z domain at the C-terminus of the CP coexpressed with native CP as a helper

Rather unspecified targeting

Potentially broad applications in diagnostics

This presents generation of mosaic VLPs carrying the IgG-binding Z domain that could be targeted to antibodies displayed on the cell surface or used in diagnostics
oneering experiments was published in 2002 [260]. Another detailed review devoted to anticancer perspectives of nanoparticles filled with siRNA was recently published [261].

The next step in the development of VLP packaging/targeting technology included the chemical coupling of putative cargo and targeting molecules to the outer and inner surfaces of VLPs, respectively [175, 262–267]. It has been demonstrated that MS2 VLPs can be conjugated to peptides recognizing human hepatocellular carcinoma cells and can be loaded with vastly different types of cargo, including low molecular weight chemotherapeutic drugs, siRNA cocktails, protein toxins and nanoparticles, resulting in the selective killing of target cells [268]. The packaging of Qβ VLPs with immunostimulatory CpG sequences led not only to the development of potential al-

\[ \text{Table 3 (continued)} \]

<table>
<thead>
<tr>
<th>Interior: packaged by</th>
<th>Exterior: decorated by</th>
<th>Addressed to</th>
<th>Supposed to treat</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>No specific packaging</td>
<td>Oligomannosides that are modelling the 'glycan shield' of HIV envelope</td>
<td>No cell targeting</td>
<td>HIV/AIDS as a model</td>
<td>The oligomannose clusters were recognized by monoclonal anti-HIV antibody, but did not induce antibodies against the HIV epitopes by immunization of rabbits</td>
<td>372</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Transferrin receptors</td>
<td>Potentially broad applications in diagnostics and therapy</td>
<td>This approach allowed cellular internalization of chimeric VLPs through clathrin-mediated endocytosis</td>
<td></td>
<td>373</td>
</tr>
<tr>
<td>DNA</td>
<td>No cell targeting</td>
<td>Potentially broad applications in diagnostics and therapy</td>
<td>A noncompact lattice was created by DNA-programmed crystallization using surface-modified Qβ VLPs and gold nanoparticles, engineered to have similar effective radii.</td>
<td></td>
<td>374</td>
</tr>
<tr>
<td>Poly(2-oxazoline)</td>
<td>No cell targeting</td>
<td>Potentially broad applications in diagnostics and therapy</td>
<td>This showed that the size and content of VLP-polymer constructs could be controlled by changing the polymer chain length and attachment density. The system is universal because of the convenient click chemistry applications</td>
<td></td>
<td>375</td>
</tr>
<tr>
<td>Conjugation of azide- and alkyne-containing proteins</td>
<td>Broad spectrum of potential targeting</td>
<td>Tumors and other possible targets</td>
<td>See above, the same for MS2</td>
<td></td>
<td>174</td>
</tr>
<tr>
<td>Modification via the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction</td>
<td>No definite cell targeting specified</td>
<td>Potentially broad applications in therapy and imaging diagnostics</td>
<td>This is a methodology to use VLPs as multivalent macroinitiators for ATRP</td>
<td></td>
<td>376</td>
</tr>
<tr>
<td>Human EGF as a C-terminal fusion to the Qβ CP</td>
<td>EGF receptor</td>
<td>Therapy based on interactions with cells overexpressing their cognate receptor</td>
<td>Mosaic particles with an approximately 5–12 EGF molecules on the VLP surface were obtained. The particles were found to be amenable to bioconjugation by standard methods as well as high-fidelity CuAAC reaction</td>
<td></td>
<td>377</td>
</tr>
<tr>
<td>Sulfate groups that elicit heparin-like anticoagulant activity</td>
<td>No cell targeting</td>
<td>Blood coagulation – as heparin-like drugs</td>
<td>Following conversion of VLP surface lysine groups to alkynes, the sulfated ligands were attached to the VLP via CuAAC. 3–6 attachment points per CP monomer were modified via CuAAC. The sulfated VLPs were able to perturb coagulation</td>
<td></td>
<td>378, 379</td>
</tr>
<tr>
<td>Polynorbornene block copolymers</td>
<td>No cell targeting</td>
<td>Potentially broad applications in therapy and imaging diagnostics</td>
<td>Poly(norbornene-PEG)-b-poly(norbornene anhydride) of three molecular masses: 5, 10, and 15 kDa were added to lysine residues</td>
<td></td>
<td>380</td>
</tr>
</tbody>
</table>

φCb5

Gold nanoparticles, tRNA, diphtheria toxin, mRNA, CpG

No decoration

No cell targeting

Potentially broad applications in therapy

The ease with which the φCb5 CP dimers can be purified and reassembled into VLPs makes them attractive for the internal packaging of nanomaterials and the chemical coupling of peptides | 77 |

The data are presented in alphabetical order of the phage model. For each model, the studies are ordered chronologically, but at first presenting studies describing both packaging and decoration, then data with packaging only, and then data with decoration only.
The VLPs were decorated with gadolinium complexes using the CuI-mediated Qβ agent. Radiochemical purity higher than 90% was obtained. PP7 control, nontargeted VLP-based nanoagents capsids bound to fibrin, exhibiting higher signal-to-background ratios than capsids enabled optical detection of binding to fibrin clots. The targeted ∼90 copies of a fibrin-targeting peptide to the exterior of each protein shell. The resulting signal amplification facilitated the detection of sensor at 0.7 pM, the lowest to that date for any molecular imaging agent used in magnetic resonance. The combination of the use of a long-lifetime Ru(II) metal-ligand complexes with blue light-emitting diode (LED) as the modulated light source. The results showed that RuBDC can be useful for studying rotational diffusion of biological macromolecules.

The VLPs were modified using an oxidative coupling reaction, conjugating fluorobenzaldehyde functional group was first attached to interior tyrosine residues through a fluorobenzaldehyde through a multistep bioconjugation strategy. An aldehyde functional group was first attached to interior tyrosine residues through a diazonium coupling reaction. The aldehyde was further elaborated to an alloxazine functional group, which was then condensed with [19F] fluorobenzaldehyde.

The MS2 phage was labeled with the succinimidyl ester of [Ru(2,2′-bipyridine)2(dicarbboxy-2,2′-bipyridine)]2+ (RuBDC), which is a very photostable probe that possesses favorable photophysical properties, including a long lifetime, high quantum yield, large Stokes’ shift, and highly polarized emission. The RuBDC luminescence attacks lysine residues.

The biodistribution and circulation properties of the VLP-based PET imaging agents were investigated carefully in order to realize the potential of such agents for the future use in in vivo applications.

The VLPs were used as a specific tracer of P. aeruginosa infection in mice for contrast agents based on original VLPs and VLPs with mutated surface aa residues.

Approximately 1.5 × 10^6 xenon MRI sensor molecules were incorporated in the interior of an MS2 VLPs, conferring multivalency and other properties of the VLP to the sensor molecule.

The circulation lifetime, plasma clearance, and distribution in major organs were studied in mice for contrast agents based on original VLPs and VLPs with mutated surface as residues.

The PET imaging characteristics were improved by the usage of PEG chains added to MS2 VLPs. The MS2- and MS2-PEG VLPs possessing interior DOTA chelators and labeled with 64Cu were compared by injecting intravenously into mice possessing tumor xenografts.

The phage capsids sequestering the Gd-chelates on the interior surface (attached through tyrosine residues) not only provided higher relaxivities than their exterior functionalized counterparts (which relied on lysine modification) but also exhibited improved water solubility and capsid stability. There are strong advantages to using the internal surface for contrast agent attachment, leaving the exterior surface available for the installation of tissue targeting groups.

The phage capsids were sequenced and modified independently through the appropriate choice of reagents.

The order of data presentation is the same as in table 1.

The use of imaging agents in combination with RNA phage VLPs has contributed to the high-resolution and noninvasive visualization of these particles, as well as to the potential treatment of diseases [254]. The RNA phase VLP-based applications that have been developed as imaging technologies are compiled in table 4. The first studies on the generation of nanoparticles for magnetic resonance imaging applications and the first comparisons of interior versus exterior cargo strategies appeared in the

Table 4. The bioimaging agents on the basis of RNA phage VLPs and virions

<table>
<thead>
<tr>
<th>Contrast agent</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS2 phage was labeled with the succinimidyl ester of [Ru(2,2′-bipyridine)2(dicarbboxy-2,2′-bipyridine)]2+ (RuBDC), which is a very photostable probe that possesses favorable photophysical properties, including a long lifetime, high quantum yield, large Stokes’ shift, and highly polarized emission. The RuBDC luminescence attacks lysine residues.</td>
<td></td>
<td>381</td>
</tr>
<tr>
<td>The VLPs were modified using an oxidative coupling reaction, conjugating fluorobenzaldehyde functional group was first attached to interior tyrosine residues through a diazonium coupling reaction. The aldehyde was further elaborated to an alloxazine functional group, which was then condensed with [19F] fluorobenzaldehyde.</td>
<td></td>
<td>383</td>
</tr>
<tr>
<td>The VLPs were used as a specific tracer of P. aeruginosa infection in mice for contrast agents based on original VLPs and VLPs with mutated surface aa residues.</td>
<td></td>
<td>386</td>
</tr>
<tr>
<td>The VLPs were decorated with gadolinium complexes using the CuI-mediated Qβ agent. Radiochemical purity higher than 90% was obtained. PP7 control, nontargeted VLP-based nanoagents capsids bound to fibrin, exhibiting higher signal-to-background ratios than capsids enabled optical detection of binding to fibrin clots. The targeted ∼90 copies of a fibrin-targeting peptide to the exterior of each protein shell. The resulting signal amplification facilitated the detection of sensor at 0.7 pM, the lowest to that date for any molecular imaging agent used in magnetic resonance. The combination of the use of a long-lifetime Ru(II) metal-ligand complexes with blue light-emitting diode (LED) as the modulated light source. The results showed that RuBDC can be useful for studying rotational diffusion of biological macromolecules.</td>
<td></td>
<td>387</td>
</tr>
<tr>
<td>The phage capsids sequestering the Gd-chelates on the interior surface (attached through tyrosine residues) not only provided higher relaxivities than their exterior functionalized counterparts (which relied on lysine modification) but also exhibited improved water solubility and capsid stability. There are strong advantages to using the internal surface for contrast agent attachment, leaving the exterior surface available for the installation of tissue targeting groups.</td>
<td></td>
<td>388</td>
</tr>
</tbody>
</table>

The order of data presentation is the same as in table 1.

The use of imaging agents in combination with RNA phage VLPs has contributed to the high-resolution and noninvasive visualization of these particles, as well as to the potential treatment of diseases [254]. The RNA phase VLP-based applications that have been developed as imaging technologies are compiled in table 4. The first studies on the generation of nanoparticles for magnetic resonance imaging applications and the first comparisons of interior versus exterior cargo strategies appeared in the
following these reports, MS2 VLPs were loaded with positron emission tomography radiolabels [273]. To date, MS2 phages and VLPs have played a leading role in bioimaging studies.

**Armored Polynucleotides for Diagnostic Applications**

The ‘armored’ nucleic acids are useful as noninfectious, easily available reagents for the quality control in the diagnosis of pathogenic viruses like HCV (hepatitis C virus). As an example of ‘armored RNA’ technology, MS2 VLPs were made to perform in vivo encapsidation of a desired RNA, which was accomplished by including the MS2 operator sequence on the RNA molecule to enable its packaging. The armored RNA was therefore protected from RNase digestion [274]. Recently, the creation of armored dsDNA using HBV and HPV particles was accomplished on the basis of previous work using MS2 VLPs [275]. Armored RNAs have multiple uses, such as for the detection of HCV, severe acute respiratory syndrome coronavirus, and influenza virus RNA [276] or for the creation of unique RNA molecules harboring both tRNA and mRNA functions [277]. Perspectives on the production and practical use of MS2 VLPs for routine diagnostics including food quality control were discussed in a recent review [278].

**Future Perspectives**

The next milestones for the development of the RNA phage VLP field are presented in figure 6. The major tendency provides the combination of both (i) decoration of the VLP scaffolds with molecules of interest and (ii) packaging of foreign material into VLP nanocontainers.

**Vaccine Design**

Vaccines will remain a classic area for the application of RNA phage VLPs [279]. For example, clear progress in vaccine formulation and storage was achieved through a MS2 VLP-based papillomavirus vaccine [280]. RNA phage CP properties can be used in combination with other possible vaccine carriers, such as retroviral platforms, with a special goal to improve RNA packaging and delivery [281].

**Peptide Display**

MS2 VLPs [160] and Qβ phage [32] offer favorable alternatives to filamentous bacteriophages for the display of immunologically active peptides. Recently, a tumor-associated antigen was identified using MS2 peptide-display technology [282], confirming the importance of such a methodology for future studies.

**Nanomachines**

RNA phage VLPs are regarded as potential components of future nanomachines, which is a general term for a machine ranging in size from 1 nm to 1 μm. Perspectives on the revolutionary nanoscale engineering of RNA phage VLPs as natural prefabricated scaffolds to contain molecules in precisely defined arrays were described in a recent review [253]. The future of VLP-derived materials [for chemical strategies and details of VLP bioconjugation technologies, see 283] is quite impressive because the number of methods that can be used to change both the interior and exterior surfaces of capsids by the incorporation of organic and inorganic compounds is unlimited. Further development of VLPs with defined antigenic and immunogenic properties, as well as VLPs with improved...
packaging and targeting capabilities, will create novel viral nanotechnology applications and is expected to produce nanomachines with rationally designed characteristics. For example, an interesting new approach is exemplified by the use of DNA as a scaffold to arrange MS2 VLPs into one-dimensional arrays with precise nanoscale positioning [284]. Such bioinspired plasmatic nanostructures may provide a flexible design base for manipulating photonic excitation and photoemission [285].

Search for New RNA Phage Vectors

The clear differences that exist in RNA phage VLP structures, stabilities, and reconstruction capacities have provoked extensive studies aimed at identifying novel members of the *Leviviridae* family. Out of such efforts, 19 new genomes of icosahedral RNA phages [286], genomes from the phages M [287], C-1 and HgalI [288], DL52 and DL54 [289], from a so-called JS-like group [290] and of the phages EC and MB [291], have been described.

A recent survey of metagenomic databases revealed 158 partial single-stranded RNA phage genome sequences belonging to about 120 distinct phylotypes [292]. Sixty-six of the genomes contained a putative open reading frame predicted to be the CP [292]. Novel RNA phage sequences were present in samples collected from a range of ecological niches worldwide, including invertebrates and extreme microbial sediment, demonstrating that they are more widely distributed than previously recognized [292]. These genomes are expected to undergo studies of CP gene expression as well as elucidation of their 3D capsid structures, and will potentially serve in new applications.

Structural Studies

3D structural investigations have revealed unexpected capabilities of RNA phage capsids, such as their ability to transform into \( T = 1 \) [77] or rod-like [157] structures. Moreover, such studies have contributed worthwhile information towards understanding protein folding and virus assembly [293]. Additional important directions for future studies involve the elucidation of VLP surface properties in the context of the presence of internal RNA, as reported previously [294], and/or the external display of foreign sequences of different origins, such as oligonucleotides, peptides, sugars, and metal ions. Such studies would be helpful for the prediction of VLP characteristics in the context of aqueous and nonaqueous media, as well as under different biological conditions, which should facilitate the use of these particles as vaccines and/or gene therapy tools.

Acknowledgements

We thank Dr. Maija Bundule, Dr. Indulis Cielens, Dr. Dzidra Dreilina, Dr. Juris Jansons, Dr. Andris Kazaks, Dr. Janis Klovins, Dr. Janis Rummiekis, Dr. Dace Skrastina, Dr. Irina Sominskaya, Dr. Arnis Strods, Dr. Alexander Tsimanis, Dr. Inara Akopjana (Riga) for their contributions and sharing of unpublished data. We are grateful to Prof. Dr. Rüdiger Schmitt (Regensburg) for the first N-terminal aa sequence of the AP205 CP and to Prof. Dr. Wolfram H. Gerlich (Giessen) for his critical reading of the manuscript and helpful comments.

References

RNA Phage Capsids as Nanotools

Intervirology 2016;59:74–110
DOI: 10.1159/000445903
64 Priano C, Arora R, Butke J, Mills DR: A com-

102

1164.

86 Golmohammadi R, Valegård K, Fridborg K, Unge T: The

1992;24:235–244.

87 Stonehouse NJ, Vagleård K, Golomhammi-


102


58 Remaut E, Waele PD, Marmenout A, Stans-

209.

95 Tars K, Fridborg K, Bundule M, Liljas L: The
crystal structure of bacteriophage GA at 3.7 Å resolution. J Mol


110.

463x382

Pseudomonas aeruginosa

230x544

the major groups of


335:3:

Valegård K, Liljas L: The refined structure of bacterio-

from yeast Saccharomyces cerevisiae and Pichia pastoris. Acta

287:452–455.

59 Kastelein RA, Berkhout B, Overbeek GP, van


98.

391:354

95:3:


4138–4144.

408x411

1996;24:2352–2359.


325:22507–22513.

408x259

1164.

408x202

102


408x382


408x401


408x430

Pseudomonas aeruginosa:


102

391:354

95:3:


59 Kastelein RA, Berkhout B, Overbeek GP, van

246:279–290.

2164.

54.70.40.11 - 10/3/2017 3:57:22 PM

391:354

95:3:


246:279–290.

391:354

95:3:


246:279–290.

391:354

95:3:


246:279–290.

391:354

95:3:


246:279–290.

391:354

95:3:


246:279–290.
102 Plevka P, Tars K, Liljas L: Structure and stabi-
licity of icosahedral particles of a covariant coaceti
103 Plevka P, Tars K, Liljas L: Crystal packing of a bacteriophage MS2 coat protein mutant corre-

104 Valegård K, Murray JB, Stockley PG, Stone-
105 Stockley PG, Stonehouse NJ, Murray JB, Stockley K: Probing sequence-specific RNA recog-
106 Valegård K, Murray JB, Stonehouse NJ, van den Worm S, Stockley PG, Liljas L: The three-di-
107 Lago H, Fonseca SA, Murray JB, Stonehouse NJ, Stockley PG: Dissecting the key recogni-
108 van den Worm SH, Stonehouse NJ, Valegård K, Murray JB, Walton C, Fridborg K, Stock-
ley PG, Liljas L: Crystal structures of MS2 coat protein mutants in complex with wild-
109 Peabody DS, Chakerian A: Asymmetric con-
version of bacteriophage MS2 coat protein to its RNA binding specificity of a translational ope-

110 Horn WT, Convery MA, Stonehouse NJ, Adams CJ, Liljas L, Phillips SE, Stockley PG: The crystal structure of a high affinity RNA stem-loop complexed with the bacteri-
111 Lim F, Peabody DS, Chakerian A: PRR1 coat protein binding to its RNA translational ope-
114 Koning R, van den Worm S, Plaisier JB, van Duin J, Pieter Abrahams J, Koerten H: Visual-
117 Stockley PG, White RJ, Dykeman E, Man-
field I, Rolfssoo O, Patel N, Bingham R, Barker A, Wroblewski E, Chandler-Bostock R, Wei Siu EU, Ranson NA, Tuma R, Twarock R: Bacteriophage MS2 genomic RNA en-
119 Koning RJ, Gomez-Blanco J, Akopian I, Vargas J, Kazaks A, Tars K, Carazo JM, Kos-
121 Rowlands DT Jr: Precipitation and neutral-
122 Stockley PG, White SJ, Dykeman E, Mann-
127 Arnon R, Sela M, Paramit C, Chedd I: Anti-
viral response elicited by a completely syn-
129 Liu JI, Zabetakis D, Goldman ER, Anderson GP: Selection and evaluation of single do-
130 Borisova G., Bundule M, Grinstein E, Dreii-

133 Arnon R, Sela M, Paramit C, Chedd I: Anti-
viral response elicited by a completely syn-
135 Liu JI, Zabetakis D, Goldman ER, Anderson GP: Selection and evaluation of single do-
136 Borisova G., Bundule M, Grinstein E, Dreii-

138 Gren EJ, Pumpen P: Recombinant viral caps-
139 Mastico RA, Talbot SJ, Stockley PG: Multi-
ple presentation of foreign peptides on the surface of an RNA-free spherical bacterio-
141 Pushko P, Kozlovskaia T, Sominskaya I, Brede A, Stankevica E, Ose V, Pumpens P, Grens E: Analysis of RNA phage fr coat pro-
tein assembly by insertion, deletion and sub-

RNA Phage Capsids as Nanotools

Intervirology 2016;59:74–110
DOI: 10.1159/000449503


156 Smith MT, Varner CT, Bush DB, Bundy BC: Incorporation of the A2 protein to pro-


DOI: 10.1159/000449503

Intervirology 2016:59:74–110

104 Pumpens/Renhof/Dishlers/Kozlovskia/Ose/Pushko/Tars/Grens/Bachmann


247 Reyes-Sandoval A, Bachmann MF: Plasmodium vivax malaria vaccines: why are we where we are? Hum Vaccin Immunother 2013;9:2558–2565.


RNA Phage Capsids as Nanotools

Intervirology 2016;59:74–110

107
RNA Phage Capsids as Nanotools

Intervirology 2016;59:74–110
DOI: 10.1159/000449503


Downloaded by: 54.70.40.11 - 10/3/2017 3:57:22 PM