VIRUS-LIKE PARTICLES: MODELS FOR ASSEMBLY STUDIES AND FOREIGN EPITOPE CARRIERS

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INTRODUCTION

Virus-like particles (VLPs) formed by the structural elements of viruses have received considerable attention over the past two decades. The number of reports on newly obtained VLPs has grown proportionally with the systems developed for the expression of these particles. When expressed in a suitable heterologous system, viral structural proteins involved in capsid or envelope formation often self-assemble into VLPs in the absence of other viral components usually required for virus assembly, such as multiple structural or non-structural proteins and viral genomes. Protein-protein interactions in VLPs are relatively strong and can result in the formation of stable structures. In recent years several experiments have been reported that may help answer questions regarding the requirements for VLP formation. Knowledge on the assembly process of VLPs is crucial to define the usefulness of such particles for the presentation of their own or foreign epitopes as carriers for transiently expressed proteins as a means of vaccine production. The aim of the present review is to outline recent achievements in two important fields of research brought about by the availability of VLPs produced in a foreign host. These are 1) the requirements for VLP assembly, and 2) the use of VLPs as carriers for foreign epitopes. To date, reviews in these areas have mainly focused on results obtained with a specific virus genus or family of viruses [1-5] and the reader is advised to refer to these reviews for complementary information.

I. REQUIREMENTS FOR VLP ASSEMBLY

Most viruses and VLPs have either a helical or an icosahedral symmetry and are composed of a single layer of viral proteins. Others are double-layered, possessing an outer and an inner layer, whereas others sitll are triple-layered. Some additionally possess an envelope. Virus assembly involves bringing together many protein subunits, packaging the genome and for some viruses ultimately maturing them into an infective particle. The assembly pathway is difficult to establish because polymerization occurs rapidly, yet with high fidelity and generally without the accumulation of intermediates [6]. However, assembly is not always a single high-order reaction and can include a cascade of low-order, intermediate reactions [7]. One of the ways of trapping intermediates at different stages of assembly is by mutagenesis of the structural protein followed by expression in a suitable heterologous system.

VLPs serve as excellent model systems to study assembly of macromolecular complexes. The pathway of VLP assembly can differ depending on the nature of the stabilizing interactions such as protein-protein, protein-nucleic acid, and metal ion or disulfide bond-mediated interactions. In more complex viruses, assembly can also be mediated by proteolytic maturation of viral proteins, by expression of scaffolding elements or by cell membranes, which make up viral envelopes. The available recent data on the assembly and structure of VLPs are discussed in the following sections and summarized in Table 1.

A. *Protein requirements for the assembly of spherical VLPs*

For a number of eukaryotic viruses, structural proteins alone are competent to assemble into VLPs even when expressed in heterologous systems without any of the remaining viral components. Structural requirements for the formation and stability of VLPs are carried by one, two or more viral proteins and correspond to their different domains. To investigate the main driving forces responsible for VLP formation, the assembly of several viral structural proteins was studied.

Norwalk virus (NV; family *Caliciviridae*), is unique among animal viruses in possessing a single structural capsid protein, and hence shares the structural simplicity of plant viruses. Expression of the capsid protein (VP1) in heterologous systems results in the formation of empty VLPs [8-10]. A detailed study of the ability of a set of deletion mutants to assemble into VLPs revealed the regions involved in capsid formation. The NV VP1 contains two domains, a shell (S) domain at the N-terminus, and a protruding (P) domain at the C-terminus in which two subdomains (P1, P2) can be distinguished. The S domain alone can initiate VLP assembly whereas the P domain controls the stability and size of the particle when expressed in insect cells [11]. Similar results were obtained for the VLPs of Venezuelan equine encephalitis virus (*Togaviridae* family) expressed in mammalian cells in which mutations ablating VLP assembly are located in the N-terminal and P1 domains [12]. For NV VLPs it was also demonstrated that a minor structural protein VP2 may be encapsidated by the particles when co-expressed in insect cells and cell free systems [13, 14]. This small basic protein increases the expression and stability of VP1 protecting it also from disassembly and protease degradation [15]. VP1 of another member of the *Caliciviridae* family, the Snow Mountain norovirus expressed by a recombinant baculovirus also self-assembles into VLPs: assembly analyses of the wild type and mutant VLPs expressing VP1 indicate that a single histidine residue at position 91 (S domain) is crucial for VLP formation [16].

The VP1, VP2, VP3 and VP4 polypeptides of Junonia coenia densovirus (family *Parvoviridae*) expressed by multiple overlapping translational initiation events at in-frame AUG codons and composing the viral capsid can assemble into VLPs when expressed independently by recombinant baculovirus in insect cells. No significant differences are observed between VLPs composed of any of the four polypeptides. VP4 alone is sufficient to form VLPs and different ratios of the wild-type nucleocapsid mixtures of VP2, VP3 and VP4 can assemble into indistinguishable VLPs [17].

The capsid of Adeno-associated virus 2 (family *Parvoviridae*) is composed of three proteins, VP1, VP2 and VP3 of which only the last two are necessary for capsid formation. VP2 and VP3 expressed in insect cells generate VLPs regardless of the ratio of the expressed proteins [18]. In contrast, the Cowpea mosaic comovirus large (L) and small (S) coat proteins (CPs) assemble into VLPs only if both capsid components are expressed from the same transcription unit i.e., a single plasmid expressing both proteins L and S in equimolar amounts from separate promoters [19, 20].

The Rice dwarf reovirus (RDV) icosahedral particle contains at least six structural proteins, P1, P2, P3, P5, P7 and P8 [21]. The structural requirements leading to the formation of VLPs are carried by P3 and P8. Co-expression in transgenic rice plants of P3 and P8, but not expression of P8 alone, results in the formation of double-shelled VLPs similar to native RDV particles. Hence, these two proteins appear sufficient for the structural integrity of the VLPs in plants [22]. Double-shelled VLPs were also observed upon co-expression or mixing of P3 and P8 *in vitro*. The structural protein P7 expressed in a similar manner was located inside the VLPs [23]. Expression of the structural protein P3 in insect cells resulted in the formation of single-shelled core-like particles (CLPs). Self-assembly of P3 CLPs is driven by the amino-terminus of P3 and proteins with serial amino-terminal deletions form particles with gradually decreased stability. After removing the 52 amino-terminal residues, VLPs are no longer formed [24].

The Physalis mottle tymovirus (PhMV) CP can assemble into VLPs when expressed in *Escherichia coli*. Up to 30 amino acids can be deleted from the N-terminus of the CP without

hampering assembly. Moreover, addition of 41 amino acid residues to the N-terminus of the wild type CP does not alter VLP formation. Comparison of the crystal structure of the empty T=3 VLPs with that of native T=3 virus shows that the N-terminus provides the structural dynamism required for virus disassembly [25, 26]. In contrast, deletion of one, five or ten residues from the C-terminus alters VLP formation, and deletion of the C-terminal asparagine residue produces unstable VLPs that disassemble to form discrete intermediates [27, 28]. Similarly in Polyomavirus (family *Polyomaviridae*), truncation of the C-terminus of VP1, the major structural protein, results in the formation of pentameric capsomers but not of VLPs, suggesting that the C-terminus is involved in specific association of the capsomers [29].

In contrast to the majority of members of the *Alfamovirus* genus, most strains of Alfalfa mosaic virus (AlMV) are composed of baciliform particles. Expression of the AlMV CP in *E. coli* produces T=1 icosahedral VLPs in the absence of viral RNA [30]. Analysis of the crystal structure of the VLPs suggests that dimers are formed by interaction between the N- and C-termini. Deletion of the last 18 amino acids or introduction of point mutations into the C-terminus of the CP results in failure to form dimers or VLPs. Thus the CP dimer is the minimum building block of the VLP [31].

In insect cells, the Potato leafroll polerovirus (PLRV) CP is unable to assemble into VLPs when expressed without modifications. This can be overcome by addition of 6 histidines at the N-terminus; the resulting icosahedral VLPs co-sediment in sucrose gradients with PLRV particles suggesting that the VLPs contain RNA [32].

A well-established *in vitro* assembly system for Sindbis alphavirus (SINV) CLPs was used to explain the role of a coiled coil structure designated helix I spanning residues 38 to 55 of the viral capsid protein. As the analysis of mutants showed, three conserved leucine residues L38, L45 and L52 are crucial for the function of helix I, a leucine zipper-type determinant of CLP assembly [33]. Assembly studies of chimeric capsid proteins with helix I substituted by the unrelated coiled coil of the yeast transcription activator GCN4 showed a similar ability to form CLPs as have the wild type proteins. Electron microscopy indicated that the chimeric particles were not as spherical as those observed for the unmodified particles and could be influenced by the difference in length between helix 1 and the helix of GCN4 [34].

B. *Protein requirements for the assembly of rod-shaped VLPs*

Basically two types of rod-shaped viruses exist, the rigid rod-shaped viruses such as the tobamoviruses (ex. Tobacco mosaic tobamovirus; TMV) and the flexible filamentous viruses such as the potyviruses (ex. Johnsongrass mosaic potyvirus; JGMV). In both cases, the capsids generally consist of a single protein species.

The CP of TMV has long served as model in various kinds of structural studies. Depending on the conditions (pH, temperature, ionic strength, protein concentration) the TMV CP forms several types of specific ordered aggregates in solution [35]. Native TMV CP expressed in *E. coli* forms nonhelical, stacked-disc aggregates after dialysis against pH 5 buffer and is inactive for *in vitro* assembly with TMV RNA. However, when co-expressed *in vivo* with foreign single-stranded RNAs (up to about 2 kb) containing the TMV origin-of-assembly sequence, high yields of helical VLPs of the predicted length are obtained [36]. A model was proposed for the assembly of TMV or VLPs containing foreign RNA in which 20S

protohelix CP aggregates with single-stranded RNA transcripts (including the origin-of-assembly sequence) are the starting point for the formation of virions or VLPs.

cDNA copies of the Indian Peanut clump virus (genus *Pecluvirus*) CP gene were expressed in *E. coli* and transgenic *Nicotiana benthamiana* plants giving rise of rhod-shaped VLPs. Depending on the source of extracts examined (either from *E. coli* or from *N. benthamiana*), VLPs of different lengths between 30 to 120 nm were identified. Most of them were about 30 nm long and immunocapture RT-PCR experiments confirmed that the VLPs contained the mRNA of the expressed CP gene. Such results suggest the presence of an origin of assembly motif or a specific sequence in the mRNA [37].

Systems in which JGMV CP or its mutated versions are expressed and assembled into VLPs provide a means of identifying regions of the protein crucial for filamentous particle assembly. Mutations of highly conserved amino acids in the third and fifth helices based on the anticipated structural model preclude VLP assembly [38]. Site-directed mutagenesis of two charged residues previously proposed to represent a pair in the construction of a salt bridge crucial for the assembly process of the virus suggests that these two residues are required for assembly but not necessarily as a pair in a common salt bridge [39]. Expression of the CP of Pepper vein banding potyvirus (PVBV) resulted in the formation of flexuous rods of heterogenous lengths encapsidating the viral RNA [40]. *In vitro* assembly studies of the overexpressed and purified CP showed that VLPs of PVBV are formed via a ring-like intermediate. By modifying the pH, ionic strength, proteinase treatment or by progressive deletions at the N- and C-terminus of the recombinant CP it was demonstrated that these residues contribute to protein-protein interactions involved in the early stages of VLP assembly. Basing on the results obtained a working model for flexuous virus assembly was proposed [41].

C. *Role of viral scaffolding proteins*

Scaffolding proteins play an essential structural role in ensuring correct assembly for large viruses. They have been described for various virus systems and are found in most double-stranded DNA phages [42].

Herpes simplex virus (family *Herpesviridae*) capsids are composed of an outer icosahedral shell of four proteins (VP23, VP5, VP26 and VP19C) and an inner core of three proteins (VP21, VP24 and VP22a), of which principally VP22a serves as core scaffolding protein in particle assembly [43]. The scaffolding protein is required for the correct assembly of the outer shell of the capids. Co-expression of all seven components in insect cells results in the formation of VLPs indistinguishable from native particles. VLP assembly does not take place in the absence of VP23, VP5 or VP19C, whereas lack of VP26 is without effect on VLP formation. Partial and deformed capsid shells are formed in the absence of VP21, VP24 and VP22a [44]. The C-terminal 25 amino acids of VP22a are cleaved after assembly or when complexed with one or more capsid shell proteins [45]. However, expression of the C-terminally truncated VP22a leads to aberrant, incomplete capsid structures suggesting a role of the C-terminal region in the formation of sealed VLPs [43, 46]. In addition, the concentration of the scaffolding protein VP22a affects the structure of the nascent procapsids [47].

In the case of the triple-layered *Rotavirus* particles such as those of the Simian rotavirus and consisting of the four major structural proteins VP2, VP4, VP6 and VP7, as well the two

minor proteins VP1 and VP3 responsible for initial transcription of the genome, different variations of VLPs are formed depending on the protein composition [48]. VP2 by itself forms CLPs and in particular its N-terminus is responsible for binding and encapsidation of VP1 and VP3 [49]. A detailed analysis showed that VP2 with any N-terminal truncation between amino acids 1 and 25 fails to bind VP1, yet§ that VP2 lacking the first 92 amino acids is still able to form single- and double-layered VLPs [50]. VP2/VP6 interactions are sufficient to form double-layered VLPs, while addition of the other major capsid components VP4 and VP7 leads to the formation of triple-layered particles resembling native rotavirus particles. The Bovine rotavirus VP2 also can serve as a scaffold for the assembly of the other capsid proteins originating from the human virus [48]. Based on the innermost core VP2, components of the second (VP6) and third (VP7) layer may be derived from viruses of different serotypes thus forming heterotypic (chimeric) particles [51, 52, 53].

Bluetongue virus (BTV; family *Reoviridae*) as well other orbiviruses possess four major structural proteins VP2, VP3, VP5 and VP7; they also possess three minor structural proteins VP1, VP4 and VP6 that have RNA-transcriptase and RNA-modifying properties. VP3 and VP7 are sufficient to form single-shelled CLPs in a baculovirus expression system [54]. Deletion, point mutation and domain switching analyses of the two proteins VP3 and VP7 helped to identify certain sequences that are essential for the formation of CLPs [55]. Simultaneous co-expression of all the major structural proteins resulted in the assembly of double-shelled VLPs [56, 57]. Identification of regions in the inner scaffold proteins VP3 and VP7 involved in CLP formation [58, 57], has made it possible to assemble VLPs using capsid components from different orbiviruses. The nature of the interactions between the innermost VP3 and the intermediate VP7 layer were studied by analysis of VLP assembly of VP7 single or multiple site mutants of VP7. It was clearly shown that both the position and the nature of single residues are critical for the formation of VLPs with VP3 and VP7 [59].

Assembly studies of the Infectious bursal disease virus (family *Birnaviridae*) structural proteins expressed in a heterologous system showed that formation of VLPs depends on coexpression of VP3 and pV2, an immature precursor of VP2. It was also demonstrated that the 71 C-terminal amino acids of pVP2 are essential for the establishment of VP2-VP3 interactions [60]. A critical role in VLP formation is played by the C-terminal oligomerization domain of the VP3 scaffold protein: a product lacking the 13 C-terminal residues as a result of proteolysis was unable to form VLPs. Prevention from protease activity by coexpression of VP1 which binds to the C-terminus of VP3 led to efficient VLP formation [61]. The C-terminal residue of VP3, glutamic acid, controls the assembly process. Deletion or replacement of the 5 terminal amino acids, or fusion with small proteins such as the green fluorescent protein (GFP) or truncated ovalbumin, as well as deletion of the C-terminal glutamic acid residue promoted VLP formation suggesting that assembly requires screening of the negative charges at the C terminus of the scaffold protein VP3 [62].

D. *Protein-nucleic acid interactions*

The main role of the various viral structural proteins is protection of the viral genetic material from degradation and ~~the~~ formation of a relative durable carrier/delivery system. Coevolution of viral genomes with their own structural elements has also led to interactions in which nucleic acids are the main driving force for VLP formation.

Hepatitis C flavivirus (HCV; family *Flaviviridae*) VLPs have been detected in various samples of infectious sera as well as in bacterial, insect, yeast and other eukaryotic cell

expression systems [63-66]. However, attempts to obtain sufficient amounts of VLPs failed in most cases when using the core and envelope (E1 and E2) protein genes in the absence of other viral components that might enhance the assembly process. The first report on structural proteins assembled into VLPs with a bilayer envelope indicated the requirement of at least part of the 5' untranslated region of the viral genome [67]. HCV CLP formation depends on the highly basic N-terminal 120 [68] or 124 [69] amino acids of the core protein and on oligonucleotides corresponding to the 5' untranslated region that harbours predicted secondary structural elements [69]. Assembly of symmetrical nucleocapsid-like particles was highly dependent on the formation of secondary structures within the RNA molecule that was co-incubated with the 124 amino acids of the core protein. Unfolded RNA obtained by treatment with EDTA could not lead to core formation.

The role of the N-terminal domain of the Sesbania mosaic sobemovirus (SeMV) CP in the assembly of VLPs was elucidated from the analysis of VLPs formed from wild type or N-terminally deleted CPs. When expressed in *E. coli*, the recombinant CP as well as a CP mutant whose first 22 amino acids had been deleted, self-assembled similarly to native virion. When 36 residues comprising the basic arginine-rich motif were removed, only T=1 and pseudo T=2 particles were observed. Hence, the arginine-rich motif can act as a molecular switch in T=3 assembly [70]. Deletion of the N-terminal 65 residues resulted in the formation of a T=1 particle only. Comparison of the crystal structure of this T=1 particle with native SeMV showed that the major structural difference was in the icosahedral dimers [71, 72]. Interestingly, all the VLPs encapsidated 23S rRNA or its degraded fragments emphasizing the importance of RNA-protein interactions during the formation of these VLPs.

The role of RNA in viral assembly has been demonstrated in Brome mosaic bromovirus (BMV). The 3' terminal tRNA-like structure (TLS) is necessary for capsid assembly, since BMV genomic RNA lacking the 3' TLS failed to assemble into mature capsids in the presence of CP. However, VLPs were formed when tRNAs or short RNAs containing the TLS were included, although these additional RNAs were not encapsidated with the TLS-deprived BMV genomic RNA. Such tRNAs and TLS acted as chaperones in VLPs assembly [73].

The Flock house nodavirus (FHV) capsid protein cleavage product CP-alpha deleted of the N-terminal 50 residues containing positively charged amino acids failed to assemble into VLPs, whereas removal of the first 31 residues resulted in heterogeneous particles with heterologous RNA [74-76]. When expressed in the presence of replicating FHV RNAs, uniform particles were formed suggesting that particle polymorphism was imposed by the type of RNA selected for packaging [74]. Similarly expression of the capsid protein of a fish nodavirus, Malabaricus grouper nervous necrosis virus in insect cells, resulted in VLP formations that were similar in size and geometry to native virus and were packed by random cellular RNAs [78].

Nucleic acids play an important role in the assembly of alphaviruses such as SINV and Ross River virus (family *Togaviridae*). The purified capsid proteins expressed in *E. coli* could form core-like particles in the presence of single-stranded but not double-stranded nucleic acids [79]. It was shown that the predominantly monomeric CP rapidly forms CLPs of regular size and shape following the addition of an appropriate nucleic acid substrate such as viral RNA, yeast tRNA or single stranded DNA. Further nucleic acid-dependent cross-linking experiments of the nucleocapsid protein suggests assembly models involving nucleic acid-bound dimers of the capsid protein in early steps of the CLP assembly pathway [80]. Analyses of dimerization and *in vitro* assembly of CLPs from truncated and mutant CPs of SINV

support the proposed model of assembly and suggest a possible role for the N-terminal region of the protein in bridging the space between two monomers and stabilizing the interaction of the intercapsomer dimer [81].

The purified retrovirus Gag proteins or Gag protein fragments of Rous sarcoma virus (RSV) are capable of assembling into VLPs *in vitro* in the presence of RNA [82]. Soluble Gag protein missing its N- and C-terminal domains was used to study the requirements of VLP formation. The results indicated that VLPs were formed independently of the length or type of nucleic acid used and that the ratio of protein mass to nucleic acid mass in purified particles was constant. Furthermore it was shown that the dimerization process of Gag molecules is crucial for *in vitro* formation of VLPs. This process may be driven by nucleic acids interacting with the nucleic acid binding domain of Gag proteins or of foreign dimerization domains replacing the functional site of protein-nucleic acid interaction [83], [84]. Finally a model was proposed in which two adjacent Gag molecules bound on a stretch of nucleic acid can dimerize and expose a surface hidden in monomers, which allows formation of spherical shells [85].

E. *Cell membranes*

Enveloped viruses form their proteolipid envelope by budding through cell membranes, and particle formation is concomitant with budding. Two mechanisms can be distinguished in the assembly of such viruses. The first mechanism is nucleocapsid dependent. The nucleocapsid of retroviruses is sufficient for budding of VLPs from the cell membrane, whereas togaviruses require both nucleocapsid and envelope proteins. The second mechanism is nucleocapsid independent and budding occurs by formation of capsidless envelopes.

The native Pr57gag precursor of Simian immunodeficiency virus (SIV; family *Retroviridae*) when expressed and myristoylated in a baculovirus system assembles into 100-120 nm VLPs that bud from the cell membrane into the environment [86]. Similarly, the unprocessed Gag precursor (Pr55gag) of Human immunodeficiency retrovirus 1 (HIV-1) forms VLPs. Deletion of the C-terminus together with the finger domain of the Gag precursor abolishes assembly of VLPs, whereas membrane targeting and evagination are still possible [87]. Expression of the entire Gag-Pol protein in mammalian cells by way of a Vaccinia virus expression vector leads to the formation of immature and mature retrovirus-like particles budding from the cell surface [88], whereas expression of Gag in yeast cells does not lead to budding unless the yeast cell wall has been removed, forming spheroplasts [89]. Unique among retroviruses, transient expression of the Env protein of Simian foamy virus in BHK-21 cells leads to efficient budding and formation of VLPs that appear normal in size and morphology. This finding and other similarities align foamy viruses with Hepatitis B virus despite all the significant structural differences that exist between viruses of these families [90]. Rubella virus (family *Togaviridae*), forms VLPs by a budding mechanism, which involves coordinated expression in mammalian cells of the capsid protein and the two envelope glycoproteins E1 and E2 [91-93]. Each glycoprotein contains two domains, the transmembrane and the cytoplasmic domain, required in early and late steps of the assembly pathway respectively. E2 acts as a support recruiting newly synthesized E1 molecules. The E2 transmembrane domain contains a Golgi retention signal and its cytoplasmic domain is required for interaction with the capsid proteins when VLPs are secreted [94].

Unlike retrovirus requirements for VLP formation, coronaviruses assemble their viral envelopes into VLPs independently of the nucleocapsid. Two proteins, the viral membrane

glycoprotein (M) and the envelope protein (E), are required for assembly of coronavirus VLPs resembling native virions [95]. The E protein occurs in trace amounts in VLPs whereas the M glycoprotein that spans the membrane bilayer three times is abundant. Detailed mutation analyses and co-assembly of mutated M protein with assembly-competent (wild-type) M protein demonstrated that transmembrane domains play a crucial role in homotypic interactions between particular M proteins [96, 97]. A detailed study of VLP formation driven by the M and E proteins of Infectious bronchitis virus (IBV; family *Coronaviridae*) showed that proteins correctly targeted to the Golgi complex require their cytoplasmic tails to assemble into VLPs. [98]. To study the major driving forces in budding of Influenza virus (family *Orthomyxoviridae*) VLPs a number of plasmids expressing the viral structural components in COS-1 cells were constructed. Co-transformation experiments followed by identification of VLPs by biochemical assays, immunoblotting and electron microscopy indicated that extracellular membranous spikeless particles similar in size and shape to those of true virions were formed in cell cultures expressing exclusively the viral matrix protein M1. Therefore, the M1 protein has all the structural information required to induce efficient formation of VLPs that bud from the cell membranes [99]. Similarly, the human parainfluenza virus type 1 (hPIV-1; family *Paramyxoviridae*) matrix protein M alone, when expressed from a plasmid in mammalian cells, induced budding of VLPs from the plasma membrane. Furthermore, co-expression of the nucleoprotein, which only leads to the formation of intracellular nucleocapsid-like structures, resulted in budding of VLPs enclosing the nucleocapsid-like structures. This observation suggests that the M protein of hPIV-1 has the ability to induce the formation of VLPs and to incorporate nucleocapsid-like structures into these vesicles [100].

The viral protein 40 (VP40) of Ebola virus and Marburg virus (members of the *Filoviridae* family) appears to be equivalent to the matrix protein of other viruses. Ebola virus VP40 was widely studied to elucidate its function and clarify its role in the formation of membrane-bound particles when expressed in mammalian cells [101, 102]. The expression of VP40 in the absence of any other Ebola virus proteins leads to budding and formation of characteristic filamentous spikeless VLPs. The central role of VP40 in this process is supported by the fact that co-expression of the viral glycoprotein (GP) which is found in pleomorphic particles budding from the plasma membrane when expressed alone, leads to incorporation of the GP into filamentous VLPs [103]. Such a mechanism of VP40-GP-membrane interaction seems to be common to all the members of the *Filoviridae* family. Co-expression of the GP and matrix protein of Marburg virus in mammalian cells also results in formation of filamentous VLPs [104].

The requirements for budding of the paramyxovirus Simian virus 5 (SV-5) VLPs seems to be more complex. The matrix protein expressed in mammalian cells was not sufficient to direct vesicle budding and was not secreted from the cell. Only the M protein in the form of VLPs was efficiently secreted when expressed with one of the two viral glycoproteins, hemagglutinin-neuramidase (HN) and fusion protein (F), together with the nucleocapsid protein (NP) [105]. Detailed analyses of deletion mutants of particular VLP components suggest that multiple viral components are important for the budding process of SV-5.

F. *Disulfide bonds and/or metal ions*

As it was shown for several viruses, not only the viral structural components are important in VLP formation. Experiments on assembly and disassembly of VLPs indicated, that the stability of such particles depends often on the ionic or reducing conditions in which

the assembling occurs. Formation of disulfide bonds between interacting domains of viral structural proteins or binding of divalent ions that may link corresponding elements often leads to conformational alteration of the VLP structure and can set a new stage of the particle.

The icosahedral polyomaviruses Murine polyomavirus, Simian virus 40 (SV40), Human BK polyomavirus and JC polyomavirus (JCV) possess a capsid consisting of three proteins VP1, VP2 and VP3, of which VP1 is the major structural protein. When overexpressed in bacteria, VP1 forms pentameric capsomers and assembles into VLPs stabilized at low ionic strengths [106]. In contrast, when expressed in the cytoplasm of insect cells VP1 is transported to the nucleus and forms VLPs [107, 108] that may be disrupted into pentameric capsomers by EDTA or DTT [108]. This indicates that the minor capsid proteins are not essential for VLP formation and implies a role for disulfide bonds and/or metal ions.

When the assembled VP1 capsids of JCV were treated with chelating and reducing agents, they dissociated into capsomers that retained the ability to reassemble [108]. Substitutions of the calcium ion binding residues of SV40 VP1 capsid protein resulted in the formation of tubular particles as well as VLPs with increased stability in the absence of calcium ions suggesting that appropriate affinities of calcium ion binding are important for assembly of the capsid. Cysteine mutations revealed that the disulfide linkage stabilizes the calcium ions and hence the stability of the particles of SV40 [109]. Recombinant capsids assembled upon expression of the major structural protein VP1 of JCV form disulfide linkages that stabilize dimeric and trimeric interactions. Such VLPs remained intact even after treatment with DTT, and disassembly occurred only after treatment with EGTA. When the capsids were treated with EGTA without reducing the disulfide bonds they failed to disassemble, suggesting that the disulfide bonds play a vital role in maintaining the capsid structure by protecting the calcium ions from chelation [110]. In the icosahedral SeMV, chelation of calcium ions by the addition of EGTA led to swelling of the particles. Two aspartate residues are involved in coordination of the calcium ions. When both were mutated to asparagine, assembly was severely affected and resulted in heterogenous particles of low sedimentation value were formed [111].

The role of calcium ions in the formation of SV40 VLPs was demonstrated in a series of *in vitro* assembly experiments [112]. The VP1 protein obtained as pentamers was exposed to various reconstruction buffers and the results clearly showed that in absence of $CaCl_2$ only tiny particles were formed. Enrichment of the assembly buffer with calcium, cadmium or manganese ions whose ionic radii are similar, resulted in VLP formation. This supports the notion that appropriate divalent ions must bind to calcium binding pockets of VP1 before the protein can assemble into larger particles. Interestingly, VP1 pentamers could also form long tubular particles with similarity to those of rod shaped viruses.

Expression of the major late 1 (L1) structural protein using the prokaryotic, baculovirus, yeast or mammalian systems results in the self-assembly of Human papillomavirus (HPV; family *Papillomaviridae*) VLPs. Coexpression of the minor late 2 (L2) structural protein increases the efficiency of particle formation but is not strictly required. Reduction of intermolecular disulfide bonds by DTT led to disassembly of VLPs into L1 capsomers to which L2 remained associated, indicating that disulfide bond formation could be essential for capsid assembly [113]. Further experiments demonstrated that mutation of only two highly conserved cysteine residues in L1 leads to the formation of monomers. It has also been suggested that trimerization of L1 is indispensable for the stabilization of intercapsomeric contacts in papillomavirus VLPs [114]. Expression of L1 from two different serotypes

resulted in VLPs with covalent disulfide bonds between these two types of proteins. This may provide a new tool to induce antibodies against multiple HPV types [115].

G. *The role of proteolytic maturation in VLP formation*

Development of mature virus particles is the final step of an infection event. To reach this moment, viruses adopted a series of gene expression strategies allowing them the control of multiplication. Expression of immature precursor structural components of viral envelopes followed by their processing seems to be an effective strategy in controlling the completion of the viral particle.

When the FHV CP precursor CP-alpha was expressed in insect cells, precursor VLPs were formed. Maturation by autocatalytic cleavage led to polypeptide chains beta and gamma that form particles indistinguishable from authentic FHV. Alteration of proteolytic cleavage by mutations resulted in defective VLPs some of which possessed unusual structural features. Particles with the N363D mutation were fragile and broke into half-shells. Those with the N363A mutation displayed a distinct hole [75].

The pre-CP of Thosea asigna virus (TaV; family *Tetraviridae)* is cleaved at two positions to produce the L and S CPs as well as a predicted third non-structural protein [116]. In insect cells, VLPs were only formed when the L and S fragments were expressed from the same promoter, indicating that cleavage between the L and S CPs is an essential step in TaV capsid assembly [117].

The rotavirus (family *Reoviridae*) VP6 expressed as a free protein in plants using the Potato potexvirus X- (PVX) based vector forms only paracrystalline sheets and tubes. Expression of VP6 fused to the PVX CP yields flexuous rods containing a heterologous surface overcoat. Interestingly, in plants, such presentation of VP6 on the surface of the flexuous rod, followed by proteolytic cleavage, resulted in the formation of some icosahedral VP6 rotavirus-like particles indicating a possible role of maturation in VLP assembly [118]. The engineering of a morphogenesis switch to control a particular type of capsid protein assembly was reported for Infectious bursal disease virus (family *Birnaviridae)* [119]. The expression of the pVP2-VP4-VP3 segment in insect cells resulted mainly in the formation of tubular structures composed of pVP2, the precursor of VP2. Expression of pVP2 alone resulted in the formation of isometric particles smaller than the VLPs. When the entire GFP was fused to the C-terminus of VP3 of the pVP2-VP4-VP3 segment, a large number of VLPs could be visualized by electron microscopy. This is the first report in which addition of a foreign protein to the inner VP3 capsid protein triggered VLP assembly.

H. *Host cell factors supporting VLP formation*

In most cases, the formation of VLPs depends on viral structural or functional elements that are expressed during the infection stage. Yet, co-evolution of viruses with their hosts has resulted in cellular factors enhancing or controlling the assembly of VLPs. As a consequence, the host cell is frequently an important player in defining the efficiency of virus and VLP production.

It was found that expression of the RSV Gag pmrotein in insect cells failed to produce VLPs even after artificial introduction of a myristoylation signal that was lacking from the N-terminus of the protein, whereas deletion of the proteinase domain from the Gag protein

restored VLP formation. The same Gag protein expressed in mammalian and avian cells was fully competent for assembly into VLPs without any modifications. It has been speculated that a chaperone activity in vertebrate cells may cause proper folding of the proteinase-containing Gag protein and that this activity is absent or quantitatively insufficient in insect cells [120].

Similarly, Human T-cell leukemia virus type I (HTLV-I) Gag and Gag-pro polyproteins showed differential budding efficiencies depending on the cell system used for their expression. The $Pr53^{Gag}$ polyprotein accumulated at the plasma membrane in insect cells and was unable to be released as immature VLPs. In contrast, in human 293 cells Gag particles were released into the medium as enveloped VLPs. Such cell type-dependent assembly strongly suggests involvement of an unknown mammalian host factor in the final stages of VLP assembly [121].

As mentioned previously, expression of the HIV-1 Gag-Pol protein resulted in VLP assembly and release from human cells and yeast spheroplasts, suggesting that no other viral factor is required. Using insect cells and baculovirus vectors for the expression of the Gag protein, only VLPs representing the immature form of the virus were obtained. Expression of Gag-Pol constructs in insect cells abolished VLP formation. Even intermediate levels of proteinase activity obtained by mutations did not result in mature VLPs [122]. This indicates that specific cell factors are required for the maturation of Gag protein and VLP synthesis of HIV-1.

It was demonstrated that retroviral Gag proteins contain sequences termed late (L) domains that facilitate the final stages of VLP budding from the plasma membranes. L domains of HIV-1 and Ebola virus encode small sequence motifs that recruit two human cell factors, Tsg101 a tumor susceptibility gene and a component of the vesicular sorting machinery, and Nedd4 an ubiquitin ligase [123-125]. These interactions are crucial for the budding process during VLP assembly. It was shown, that Nedd4 and Tsg101 might play complementary roles by recruiting cellular factors of two independent pathways to the budding site at the plasma membrane.

II. VLPs AS EPITOPE CARRIERS AND FOREIGN ANTIGEN PRESENTATION SYSTEMS

Although it is known that chemically synthesized peptides are capable of eliciting the production of protective antibodies against the antigens from which they derive, the peptides by themselves present a low immunogenicity level. Antigenicity can be increased by conjugating/fusing the peptides to carrier proteins, thereby extending their lifetime and presenting them in a manner in which they will be better recognized by the immune system. Hence the attractive prospect of presenting antigenic epitopes on the surface of viruses or VLPs and of using these chimeric constructs for vaccination and gene therapy.

The potential use of viruses as epitope presentation systems has had some success in recent years, but this strategy has largely been supplanted by the use of VLPs in place of viruses. This new approach has been facilitated by the development of various systems such as bacterial, yeast, insect and mammalian cells in which VLPs can be produced and assembled [126]. VLPs are generally easy to produce on a large scale, and are non-infectious. Two classes of VLPs can be considered, 1) non-chimeric VLPs, or 2) chimeric VLPs harboring a given peptide in an appropriate VLP context. Crucially, the use of VLPs for

epitope presentation depends on precise knowledge of the detailed structure of the VLPs, so as to evaluate the region of the viral protein into which the foreign sequence is to be introduced. Moreover, the size of the insert [127, 128], the site of insertion, the mode of presentation together with the presence of short flanking sequences, and the expression system employed can all contribute to dramatically affect the immunological response [129, 130].

Antigenic epitopes presented on the surface of VLPs can be taken up by antigen-presenting cells. The peptides presented on the surface of VLPs are exposed on MHC class I molecules at the cell surface. This primes the T cell response, either against the viral particle-forming protein itself or against the additional peptide sequence fused to the VLP. Recent achievements in the development of VLPs as epitope carriers are summarized here and in Table 2.

The full-sized capsid of parvoviruses is composed of three proteins whose major structural protein V2 by itself is able to form stable VLPs [131, 132]. The specific three dimensional structure of the Canine or Porcine parvovirus (CPV and PPV, respectively) VP2, with its four loops between eight-stranded antiparallel β-barrel motifs, appears to be a particularly suitable site for the insertion of epitopes to produce VLPs as carriers of molecules for antigen delivery. Two regions of VP2 dispensable for capsid formation, the N-terminus which is directed towards the inside of the VLP, and loop 2 which is partially presented on the surface of the capsid, proved suitable sites for foreign epitope insertion in antigen presentation when expressed in insect cells [133-136]. To elucidate events related to CPV infection, fluorescent VLPs were developed. The insertion of the enhanced GFP at the N-terminus of VP2 did not alter assembly of the VLPs. Using these chimeric particles their entry process into mammalian cells was followed, and intracellular trafficking could be observed [137]. Inserting into the N-terminus of the PPV VP2, a CD8+ cytotoxic T lymphocyte (CTL) epitope from Lymphocytic choriomenengitis virus (LCMV; family *Arenaviridae*), produced a readily self-assembling PPV:VLP-LCMV when expressed in insect cells with a morphology closely related to that of PPV:VLP. Such particles elicited a strong CD8+ class I-restricted CTL response and totally protected mice against lethal chorimenengitis [133]

Biologically active SIV envelope proteins were incorporated on the surface of SIV-derived VLPs. Purified VLPs plus mucosal cholera toxin (CT), a well-known mucosal adjuvant, were used for intranasal immunization of mice. CT could efficiently be replaced by its non-toxic B subunit (CTB) either free or conjugated to the VLP , the conjugated CTB being more efficient than the free CTB [138]. The antigen response was dose-dependent and high levels of serum IgG antibody production was achieved. Antibody production was detected at the mucosal surface and increased numbers of MHC I-restricted peptide-specific T cells were detected. Other experiments have been directed towards developing other approaches than the use of CTB to enhance the immunogenicity of SIV or Simian/human immunodeficiency virus (SHIV) VLPs. Since Influenza virus induces strong immune responses by systemic as well as mucosal administration, it was investigated whether the influenza virus surface glycoprotein hemagglutinin (HA) would enhance immunogenicity of SHIV [139]. Chimeric HA/SHIV VLPs indeed increased the humoral and cellular immune responses in the systemic and mucosal compartments.

To develop an experimental vaccine against HPV-induced tumors, fragments of the HPV16-E7 oncoprotein were expressed on three proteins capable of VLP formation: the Hepatitis B virus (HBV; family *Hepadnaviridae*) surface (HBs) and core (HBc) antigens, and

the RNA phage fr coat protein. Only the HBV chimeras carrying the HBs and HBc assembled into VLPs. Nevertheless, analysis of the IgG isotype profile from immunized mice showed that the epitopes carried by all three viral proteins induced efficient antibody response to the antigens delivered. [140].

The icosahedral nucleocapsid of HBV is composed of a single core protein that can form VLPs in the absence of its genome. The C-terminus of the protein can be replaced by the 17 kDa nuclease of *Staphylococcus aureus*, also leading to VLPs. The foreign epitope is internally located in the chimeric VLPs, yet retains its nuclease activity [141]. A highly appropriate central location in the HBV VLP was chosen to introduce GFP (238 amino acids). The hybrid protein formed fluorescent particles, the GFP domains being exposed to the surface, and humoral response was elicited against GFP [142].

Advantage has also been taken of the availability of the HBV core to improve immunogenicity of protein fragments derived from Influenza virus [143] and malaria [144]. More recently [145], HBV core fusions were produced in *E. coli* by introducing the 120 N-terminal amino acids of the N protein of one of three hantaviruses into an internal major immunodominant region of the HBV core. This region is preferred over the N- or the C-terminal region as insertion site for epitope presentation in HBV because of the surface exposure of the inserts that result in high immunogenicity. In all cases, chimeric core particles were formed. Cryo-electron microscopy revealed that the chimeric cores present a fuzzier outline with spikes emerging from the shell than the original HBV cores. The chimeric core particles elicited a strong N protein-specific antibody response in two mouse strains.

BTV forms icosahedral particles composed of two or three concentric shells (see above: C. Role of scaffolding proteins). The outer and inner core are composed of VP7 and VP3 respectively as well as three minor proteins. The outer shell is composed of VP2 and VP5. CLPs are assembled in insect cells using the recombinant baculovirus system. Such particles have been used as foreign epitope carriers of peptide sequences from the Rabies virus glycoprotein [146] or the Hepatitis B virus preS2 region [147] that were introduced at the N terminus of the VP7 protein. Moreover, the BTV non-structural protein NS1 of unknown function encoded by the RNA segment M6 when expressed in insect cells, produces tubular structures with a helical conformation. These structures have been successfully used for epitope presentation. Introducing sequences (ranging from amino acids 44 to 116) from the *Colstridium difficile* toxin A, the HBV preS2 region, or the entire bovine leukemia virus p15 protein at the C-terminus of NS1 produced highly immunogenic tubules, in which the foreign epitope was exposed on the surface [128] Similar results were obtained when a peptide derived from VP1 of foot-and-mouth disease virus was used as epitope, or a peptide derived from the Influenza virus HA protein [148]. The results demonstrate that the purified tubules elicit both humoral and cell-mediated responses.

Over-expressing part of open reading frame (ORF) 2 of Hepatitis E virus (HEV; family *Calicivirus*), in a baculovirus expression system produces VLPs similar to mature HEV although slightly smaller. Chimeric constructs in which a B-cell epitope was introduced into various insertion sites in the protein derived from ORF2 were used as oral vaccines. The C-terminus of ORF2 was the only appropriate site for insertion of the foreign epitope; it induced specific IgG and IgA to the epitope and to the VLP in intestinal fluids [149].

To study of the formation and release of Influenza virus VLPs from the surface of insect cells a quadruple baculovirus recombinant expressing simultaneously the viral

hemagglutinin (HA), neuramidase (NA), matrix M1 and M2 proteins was produced. Immunogold labelling and electron microscopy examination indicated that HA and NA were exposed on the surface of VLPs. Furthermore, using the same expression system, replacing HA by the G protein of Vesicular stomatitis virus or by a hybrid containing the cytoplasmic tail and transmembrane domain of HA and the ectodomain of the G protein, resulted in the formation of chimeric VLPs [150]. Such system may serve as a convenient tool for the development of novel vaccines.

VLPs of the flexible filamentous JGMV also yield highly ordered aggregates forming particles in which vaccine subunits are presented. Short peptides or large antigens of foreign epitopes such as the merozoite surface antigen of malaria and/or the luteinizing hormone releasing hormone fused to, or replacing, the N- or C-termini of the CP, were highly immunogenic in mice and rabbits without adjuvant [151]. Moreover, the JGMV CP expressed in a recombinant Vaccina virus to sufficiently high levels, led to the formation of potyviral VLPs in mammalian cells [152]. Synthesis of such self-adjuvanting molecules carrying foreign epitopes without the need of protein purification before vaccination would be of immense value.

As discussed above (Section C. Role of scaffolding proteins) in Rotavirus VLP assembly, the amino terminus of VP2 of double-layered VLPs composed of VP2 and VP6 is dispensable for the formation of such particles. The GFP protein was introduced at the N-terminus of VP2 as model insert [153]. VP2 thus modified assembled correctly into VLPs when co-expressed with VP6, and the GFP molecules were present inside the core at the 5-fold vertices. Such chimeric particles enhanced by the presence of the outer capsid layer VP4 and VP7 were able to penetrate mammalian cells demonstrating their usefulness in macromolecule delivery into living cells.

The major coat protein VP1 of Polyoma virus was shown to be competent in forming VLPs when it presented a foreign protein such as the *E. coli*-derived dihydrofolate reductase on its surface [154]. Further engineering of the surface of polyomavirus VLPs by modifying the natural binding site of VP1 to mammalian cells resulted in particles that could serve as epitope-presenting systems i.e. for tumor specific antibody fragments [155]. Evaluation of this system showed that it resulted in VLPs with the ability of cell type specific gene transfer whose main role was played by a tumor specific antibody Fv fragment which allows highly specific attachment and cell entry of the chimeric VLPs [156]. Such cell entry was demonstrated for a variant of VP1 fused to protein Z, which is an engineered antibody-binding domain of protein A from *Staphylococcus aureus*; in this case, antibodies directed against the receptor tyrosine kinase ErbB2 allowed specific transduction of mammalian ErbB2-positive cells with plasmids encoding eGFP or beta-galactosidase [157].

III. CONCLUDING REMARKS

VLP technology appears to be a rapidly advancing domain of molecular and structural biology. Extensive progress in VLP studies was achieved ever since the insect-cell-based protein production system was developed. This baculovirus expression system has many advantages for the synthesis of viral structural proteins resulting in the formation of VLPs. It allows production of large amounts of correctly folded proteins also providing cell membranes that can serve as structural elements for enveloped viruses. These features give us the opportunity to gain insights in the interactions and requirements accompanying VLP formation that are similar to the assembly events occurring in mammalian cells. Other

encouraging elements are the ability to easily scale-up the system, and the simplicity of purification of the assembled VLPs.

To date aspects of VLP technology are mostly concentrated on three areas. The first is focused on the characterization and extension of our knowledge on viral structural elements participating in the assembly process of particular VLPs. The main efforts are directed towards solving the structure of VLPs and defining the rules managing their formation. The second, concentrates mostly on designing of chimeric VLPs in particular those that can expose foreign epitopes on their surface. Particular attention is given to the development of new VLP-based vaccines. The third direction focuses basically on engineering of effective carry/delivery systems, new vehicles useful in gene therapy [158] and virus trafficking studies.

Structural characterization of VLPs is mainly performed by advanced EM techniques with the help of computational analyses, and focuses on the comparison of VLPs composed of different numbers and combinations of structural proteins [159]. The use of biochemical methods provides not only details on individual viral structural components, but also answers questions regarding the structural basis of assembly, packaging and the interactions of VLPs with host components. Intensive studies are aimed at characterizing the minimal requirements for VLP formation and on prospects of modifying the original proteins without hampering the natural ability of these proteins to assemble into highly organized macromolecules. As a consequence, modified structural proteins appropriate for assembly of multipurpose chimeric VLPs can be designed. Our growing knowledge of the rules directing VLP assembly makes it possible to engineer particles composed of fusion proteins carrying viral structural domains and foreign epitopes. It has been shown that such modifications can lead to the formation of VLPs that are highly effective as subunit vaccines and can mimic the overall structure of virus particles without containing the viral genetic material. The use of VLPs as novel vaccines and immunogens has recently been widely reviewed [126, 160].

The growing number of VLPs carrying foreign protein fragments on their surface and studies on the successful assembly of these chimeric molecules is a promising avenue towards the development of a new technology in which the newly designed VLPs will be directed to particular mammalian cell types of by exposing specific binding domains. The progress made in modeling the surface of VLPs makes them to date the best candidates for the design of delivery systems that can efficiently reach their targets.

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