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Plant virus nanoparticles for vaccine applications

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1 **Title Page**

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3 **Plant Virus Nanoparticles for Vaccine Applications**

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5

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19 **Running Title:**

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21 Plant-based Nano-vaccines

22

23 **Abstract**

24 In the rapidly evolving field of nanotechnology, plant virus nanoparticles (pVNPs) are emerging as
25 powerful tools in diverse applications ranging from biomedicine to materials science. The
26 proteinaceous structure of plant viruses allows the capsid structure to be modified by genetic
27 engineering and/or chemical conjugation with nanoscale precision. This means that pVNPs can be
28 engineered to display peptides and proteins on their external surface, including immunodominant
29 peptides derived from pathogens allowing pVNPs to be used for active immunization. In this
30 context, pVNPs are safer than VNPs derived from mammalian viruses because there is no risk of
31 infection or reversion to pathogenicity. Furthermore, pVNPs can be produced rapidly and
32 inexpensively in natural host plants or heterologous production platforms.

33 In this review, we discuss the use of pVNPs for the delivery of peptide antigens to the host immune
34 in pre-clinical studies with the final aim of promoting systemic immunity against the corresponding
35 pathogens. Furthermore, we described the versatility of plant viruses, with innate
36 immunostimulatory properties, in providing a huge natural resource of carriers that can be used to
37 develop the next generation of sustainable vaccines.

38

39

40 **Keywords:**

41 Molecular farming, CVP, eVLP, VLP, vaccine, peptide production

42

43 1. Introduction

44 Many different platforms have been developed in the emerging field of nanotechnology, ranging
45 from synthetic nanomaterials to naturally occurring bio-nanomaterials, the latter including protein
46 cages and viral nanoparticles (VNPs).

47 VNPs are nanomaterials based on viruses [1]. From a materials science perspective, VNPs are
48 interesting because they form self-assembling architectures that can easily be produced in milligram
49 quantities in the laboratory. These naturally occurring nanostructures have many unique properties,
50 including a quality-control system that guarantees that all the particles are monodisperse and nearly
51 identical in shape and size. These properties are difficult to achieve when producing synthetic
52 nanomaterials. Furthermore, VNPs-based nanomaterials are the result of the assembly of millions of
53 identical proteins, resulting in a highly defined and precise three dimensional structures, with
54 superior features as a scaffold than synthetic particles [2]. VNPs also tend to be symmetrical,
55 polyvalent, soluble in water and stable in aqueous buffers, which are attractive features for
56 biological applications [1].

57 Virus capsids occur naturally in many different sizes and shapes, including icosahedrons [3], tubes
58 [4] and filaments[5]. Therefore, VNPs can be designed with similarly diverse forms. However, one
59 common property shared by all capsids is their exceptional robustness, reflecting their natural
60 function to enclose and protect viral nucleic acids, thus increasing the resistance of VNPs to
61 extreme pH and temperatures. But because they are entirely composed of protein, VNPs are also
62 biodegradable and biocompatible. Furthermore, VNPs are suitable for scaled-up production and
63 industrial manufacturing, given their inexpensive production; in addition, in some cases ,VNPs can
64 be self-replicating, and this aspect can decrease even more their production expenses [2]. Together,
65 these features make VNPs one of the most advanced and versatile nanomaterials produced by
66 nature.

67 Although viral nanotechnology is a novel and emerging field, recombinant virus-based materials
68 have been used for nearly 50 years as vaccines and gene delivery vectors [6,7]. Several clinical
69 vaccines are based on VNPs, including the Human papillomavirus (HPV) vaccines Gardasil
70 produced by Merck and Cervarix produced by GSK [8]. Several gene therapies based on
71 Adenovirus, Adeno-associated virus and Lentivirus are undergoing clinical trials [9]. For the past
72 20 years, chemists, materials scientists and engineers have developed a range of methodologies that
73 can be applied to fine-tune and engineer VNPs for specific applications.

74 VNPs based on plant viruses (pVNPs) are particularly versatile because they pose no risk of
75 infection in mammals. In contrast, VNPs based on mammalian viruses have the potential to revert
76 to an infectious form. Experiments in mice have also shown that pVNPs have negligible toxicity.
77 The administration of up to 100 mg (10^{16} particles) per kg body weight has been demonstrated
78 without adverse effects, Cowpea Chlorotic Mottle Virus particles have been used for *in vivo* imaging
79 in a pre-clinical study, demonstrating their ability to spread in mice's organisms without leaving
80 trace after 24 hours [10]. Biomaterials derived from plant viruses can be delivered intravenously
81 and do not induce hemolysis or coagulation, and they are rapidly cleared from non-target organs
82 and tissues in a matter of hours [11].

83 The applications of pVNPs can be divided into four key fields: 1) vaccine development; 2)
84 engineering targeted imaging and/or therapeutic reagents; 3) data storage; and 4) the development
85 of films and arrays for applications ranging from electronics to tissue engineering.

86 This review focuses on the use of pVNPs for vaccine development, describing the main platforms
87 developed thus far and some examples of active immunization. However, the use of pVNP carriers
88 for the delivery of drugs and imaging reagents is also a rapidly developing field, particularly for the
89 diagnosis and treatment of cancer [1]. A groundbreaking example, in this framework, is the fusion
90 to the viral capsid of doxorubicin, a chemotherapeutic agent. The resulting pVNP displays both
91 immunotherapeutic and chemotherapeutic effects, resulting effective in the treatment of mice
92 melanoma [12]. The reader interested in this area should consult the extensive work of Steinmetz
93 and colleagues [12–15].

94 2. The Development of pVNPs-based platforms for displaying Subunit Vaccines

95 Unlike attenuated or killed pathogens, subunit vaccines comprise only a small antigenic component
96 of a target pathogen so there is no risk of reversion to pathogenicity. Subunit vaccine design
97 requires the selection of an immuno-dominant peptide that is able to induce a strong immune
98 response which at least partially protects against the pathogen from which the peptide was derived.
99 Subunit vaccines can be produced in many different expression systems, but one drawback is that
100 multiple doses are required combined with an efficient but nontoxic adjuvant to confer an
101 acceptable level protection. Poor immunogenicity has often limited the application of subunit
102 vaccines [16]. This probably reflects the incomplete folding and/or poor presentation of the antigen
103 subunits to the immune system. To enhance the immunogenicity of subunit vaccines, they can be
104 combined with stronger adjuvants [17] or displayed as a multivalent array on a larger carrier
105 molecule, such as a pVNP.

106 The structure of many plant viruses has been solved at high resolution thanks to a combination of
107 DNA sequence analysis and techniques such as X-ray crystallography and cryo-electron
108 microscopy (Table 1). Despite the large size of pVNPs, the availability of intricate structural
109 models means that regioselective modification is usually achievable and predictable. Viruses can
110 therefore function as nanoscale scaffolds allowing the multivalent attachment of functional ligands
111 at defined positions.

112 Because pVNPs are genetically encoded biomaterials they can be modified by genetic engineering.
113 This offers a clear advantage over any synthetic material because chemical modifications are not
114 100% efficient. The genetic insertion of heterologous coding sequences at defined positions within
115 the genes encoding virus coat proteins allows peptides with diverse biological functions to be
116 displayed on VNPs, usually on the external surface [18–20] but also occasionally on the surface of
117 an internal cavity or lumen [21].

118 Bioconjugation can be used instead of (or in combination with) genetic engineering to display not
119 only peptides but also entire complex proteins, organic molecules and polymers such as nucleic
120 acids and polyethylene glycol (PEG) on the VNP surface [22–24]. Bioconjugation protocols require
121 the presence of amino acids with reactive side chains such as lysine, cysteine or
122 aspartate/glutamate, which are accessible to *N*-hydroxysuccinimidyl (NHS) chemistry, Michael
123 addition to maleimides, and carbodiimide activation, respectively. This allows VNPs to be
124 functionalized with antibodies, oligonucleotides, peptides, proteins, carbohydrates, fluorescent
125 reagents and drugs. The bioconjugation with PEG, for example, has the aim to modulate the strong
126 immune response given from the pVNPs, in order to improve their bioavailability,
127 pharmacokinetics and to selectively target tissues [22,25]. Some viruses are also amenable to
128 physical modifications. For example, Tobacco mosaic virus (TMV) can be transformed from its
129 native rod-shape to a spherical form by increasing the temperature during particle assembly [26]. It
130 is know that the shape affects the behavior of the particles: for example, in tumor homing Potato
131 Virus X and Cowpea Mosaic Virus showed different profiles in bioaccumulation, with the former

132 showing a major penetration potential than the latter [27]. However, in both conformations, the
133 highly repeated structure appear to function as a Pathogen Associated Molecular Pattern, which
134 allow the design for new immunostimulation systems for the treatment of cancer [15].

135 3. The Production of VLPs and CVPs

136 The broad class of nanostructures known as pVNPs can be divided into two subclasses, namely
137 virus-like particles (VLPs) and chimeric virus particles (CVPs). VLPs do not carry any viral nucleic
138 acid and are therefore incapable of autonomous replication. They are produced by expressing the
139 modified coat protein of the virus in heterologous cells, resulting in the production of coat protein
140 molecules that self-assemble in to a VLP [28]. In contrast, CVPs function in much the same way as
141 the native virus (i.e. they remain replication-competent) but they carry additional genetic material
142 allowing them to display heterologous proteins or peptides [28]. VLPs rely on the ability of certain
143 viruses to self-assemble without any priming from the viral genetic material whereas CVPs exploit
144 both the virus coat protein and the genetic material. Therefore, VLPs can be produced in various
145 heterologous expression systems (including bacteria) but the titers depend on the efficiency of
146 expression, whereas CVPs must be produced in their natural plant hosts or a compatible species but
147 the titers are often very high due to the intrinsic replicative ability of the virus.

148 Whichever strategy is chosen, it is important to ensure that the pVNPs assemble correctly even
149 when the coat protein contains additional peptide sequences and that the peptides displayed on the
150 surface maintain their biological/immunological activity. This is critical when CVPs are used
151 because the production method involves replication in a host plant, and correct particle assembly is
152 required for infection and systemic spreading. One of the major drawbacks of the CVP approach is
153 the tendency for surface peptides to cause steric hindrance, thus inhibiting particle assembly [29].
154 The use of CVPs therefore has four inherent limitations. First, the characteristics of the foreign
155 peptide can sometimes affect the infectivity of the virus [30,31]. Second, the ability of the chimeric
156 virus to spread systemically in the whole plants can be inhibited by selectivity between the
157 recombinant virus RNA and the chimeric coat protein [32], and virus–host interactions [31,33].
158 Third, in most epitope presentation systems, chimeric coat proteins do not benefit the virus so
159 deletions that remove the insert are favored by natural selection [30], causing the CVP to revert to
160 wild-type after several cycles of infection [31]. Fourth, special structural features of the chimeric
161 coat protein can affect key domains required for assembly, preventing it from assembling into virus
162 particles [34]. One way to overcome the inhibitory effects of inserted peptide sequences is to
163 generate VNPs that combine some wild-type coat proteins and some fusion proteins, spreading the
164 displayed peptides further apart. This overcoat strategy has been particularly successful for
165 Potexviruses and is discussed in more detail below.

166 Plants offer an attractive system for the production of pVNP-based vaccines owing to their ability
167 to produce large quantities of the particles at low cost, the proper assembly of the particles
168 *in planta*, and the low-risk of introducing adventitious human pathogens during production [35]. In
169 this framework, the Alfalfa Mosaic Virus has been exploited as an antigen presenting system for the
170 rabies antigen and used as a candidate to boost the vaccination[36].

171 Plants do not require expensive fermentation facilities for biomass generation or the construction of
172 duplicate facilities for scaled-up production. Hence, plant biomass generation and upstream
173 processing capacity can be operated and scaled up in a flexible, cost-efficient manner that cannot be
174 easily matched by current fermentation-based technologies [37].

175 A further advantage of plant-based production systems is that edible plants can be engineered to
176 produce pVNPs, which can then be administered orally with minimal processing [38]. This novel

177 and cost-effective approach can be used to establish gut mucosal immunity by oral delivery. Plants
 178 can also be used to produce VLPs representing human viral pathogens for active immunization
 179 strategies. Examples include the use of plants to produce VLPs based on HPV and influenza virus at
 180 very high titers: chimeric Cucumber Mosaic Virus bearing an Hepatitis C antigen, for example has
 181 been proven to be a good candidate as an oral vaccine in rabbits [39]. This specific use of plants to
 182 produce VLP-based vaccines is beyond the scope of this review but it has been extensively
 183 described elsewhere [40,41].

184

185 4. Platforms for the production of pVNPs

186 Many different plant viruses have been considered as pVNP platforms, differing in terms of the host
 187 species, the format (VLP or CVP) and the number of peptides that can be displayed per particle
 188 (Table 1). Only a few species have been studied in detailed proof-of-concept experiments and five
 189 of the most prevalent are discussed below.

190 **Table 1:** Features of pVNPs for peptide display.

<i>pVNP</i>	<i>Structural Resolution</i>	<i>Target peptide insertion point</i>	<i>Number of peptides / pVNP</i>	<i>Host</i>	<i>CVP</i>	<i>VLP</i>	<i>References</i>
TBSV	2.9 Å	C-terminus	180	<i>Nicotiana benthamiana</i> Insect cell/baculovirus	X	X	[42–44]
CPMV	2.9 Å	βB- βC loop of the Small CP βC'-βC'' loop of the small coat protein	60	<i>Nicotiana benthamiana</i> <i>Pisum sativum</i> <i>Spinacea oleracea</i> <i>Vicia sativa</i> <i>Vigna unguiculata</i> <i>Vigna radiata</i>	X	X	[31,45,46]
CCMV	3.2 Å	C-terminus N-terminus In <i>P. pastoris</i> into an external loop	180 (Particles are formed by 90 homodimers)	<i>Pichia pastoris</i> <i>Escherichia coli</i>	X	X	[47–50]
PLRV	-	N-terminus	-	<i>Insect cells and baculovirus</i>	-	X	[51,52]
TMV	-	N-terminus C-terminus Surface loop aa 59-65	> 2100	<i>Nicotiana benthamiana</i> <i>Nicotiana excelsiana</i>	X	-	[53,54]
JGMV	-	N-terminus C-terminus	-	<i>Escherichia coli</i>	-	X	[55]
PVX	-	N- terminus	1300	<i>Nicotiana benthamiana</i>	-	-	[28,56,57]
PhMV	3.8 Å	Chemical conjugation to lysine or cysteine	Lys: 160-180 Cys: 40- 60	<i>Escherichia coli</i>	-	X	[58,59]
TuMV	-	N-terminus	Up to 2000 copies for the CVPs. For the VLPs it depends on the length of the formed particles	<i>Brassica juncea</i> <i>Nicotiana benthamiana</i> <i>Escherichia coli</i>	X	X	[60]

PapMV	2.7 Å	12 aa – 187 aa – C-terminus N-terminus N-terminus 12 aa before F13	For the VLPs it depends on the length of the formed particles	<i>Escherichia coli</i>	-	X	[61–66]
AltMV	13 Å	C-terminus	-	<i>Nicotiana benthamiana Portulaca grandiflora</i>	X	X	[67–69]
BaMV	5.6 Å	35 aa from the N-terminus	-	<i>Nicotiana benthamiana Chenopodium quinoa</i>	X	-	[70,71]
AIMV	-	N-terminus	-	<i>Nicotiana tabacum Nicotiana benthamiana</i>	X	-	[72–75]
CMV	3.2 Å	βH-βI loop	180	<i>Nicotiana benthamiana Nicotiana tabacum cv Xhanti</i>	X	X	[23,76–78]
CdMV	-	N-terminus C-terminus	-	<i>Escherichia coli</i>	-	X	[79]
AMCV	-	C-terminus	180	<i>Nicotiana benthamiana</i>	X	-	[80]
PPV	-	N-terminus	-	<i>Nicotiana clevelandii</i>	X	-	[81]
PVY	-	N-terminus	2000	<i>Escherichia coli</i>	-	X	[82]
CymRSV	-	-	280	<i>Nicotiana benthamiana</i>	-	X	[83]

191 **TBSV**: Tomato Bushy Stunt Virus; **CPMV**: Cowpea Mosaic Virus; **CCMV**: Cowpea Chlorotic Mottle Virus; **PLRV**:
192 Potato Leafroll Virus; **TMV**: Tobacco Mosaic Virus; **JGMV**: Johnson Grass Mosaic Virus; **PVX**: Potato Virus X;
193 **PhMV**: Physalis Mosaic Virus; **TuMV**: Turnip Mosaic Virus; **PapMV**: Papaya Mosaic Virus; **AltMV**: Alternanthera
194 Mosaic Virus; **BaMV**: Bamboo Mosaic Virus; **AIMV**: Alfalfa Mosaic Virus; **CMV**: Cucumber Mosaic Virus; **CdMV**:
195 Cardamom Mosaic Virus; **AMCV**: artichoke Mottled Crinkle Virus; **PPV**: Plum Pox Virus; **PVY**: Potato Virus Y;
196 **CymRSV**: Cymbidium Ringspot Virus.

197 4.1 Cowpea mosaic virus

198 Cowpea mosaic virus (CPMV) has been studied extensively for its biomedical applications in the
199 fields of cancer, cardiovascular medicine and vaccination [84,85]. CPMV has a bipartite RNA
200 genome and a 28-nm icosahedral capsid comprising 60 copies each of the large (L) and small (s)
201 coat proteins [86]. CPMV surface chemistry (inside and out) is well understood [87], allowing an
202 exquisite level of functionalization. Additionally, the large-scale production of pVNPs based on
203 CPMV can be achieved in its natural host (cowpea, *Vigna unguiculata*) and the model host tobacco
204 species *Nicotiana benthamiana*, which is highly scalable and economic.

205 4.2 Potato virus X

206 Potato virus X (PVX) is the type member of the Potexvirus genus. It has a positive-sense RNA
207 genome and forms filamentous and flexible rod-like particles measuring 515 nm in length and 13
208 nm in diameter, comprising 1270 identical 25-kDa coat protein subunits. The monopartite genome
209 has a 5' cap and 3' poly(A) tail and it contains five major open reading frames (ORFs) encoding
210 proteins for viral replication, movement and assembly. ORF5 encodes the coat protein, which is
211 also required for cell-to-cell and long-distance movement [88]. The N-terminal part of the coat
212 protein is exposed on the surface so most peptides displayed by PVX-based pVNPs have been

213 introduced by N-terminal fusion to the coat protein [89]. PVX naturally replicates in many
214 solanaceous species and *N. benthamiana* plants are particularly susceptible to mechanical
215 inoculation, producing milligram quantities of particles from 1 g of infected leaf material. Infectious
216 cDNA clones of PVX genomic RNA are available and genetic modification protocols have been
217 established for the production of CVPs. However, PVX coat protein subunits have not yet been
218 shown to assemble into filamentous VLPs in the absence of RNA either *in vivo* or *in vitro*. Whereas
219 genetic modification of PVX is well established, chemical bioconjugation methods are inefficient
220 (21–86% depending on the conjugation strategy and the size of the target molecule) [25, 26]. PVX
221 has been investigated for various applications ranging from vaccination to *in vitro* and *in vivo*
222 diagnosis [12, 27, 28]. PVX virion assembly is sensitive to steric hindrance, so the characteristics of
223 displayed peptides and their impact on the final structure have been studied in detail, revealing that
224 peptide sizes, pI and the presence of specific amino acids play crucial roles [30,93–95]. In order to
225 overcome size-limitation problems, an alternative ‘overcoat’ strategy was developed to improve the
226 stability of chimeric particles based on the use of the Foot and mouth disease virus (FMDV) 2A co-
227 translational dissociation sequence, allowing the simultaneous production of both wild-type coat
228 proteins and fusion proteins that can assemble into stable particles without steric hindrance at the
229 most effective wild-type: fusion protein ratio [96].

230 4.3 Tobacco mosaic virus

231 The TMV coat protein gene provides various possibilities for the insertion of heterologous
232 sequences. The N-terminus has been used successfully [20] but the C-terminus is a much more
233 common fusion site [97–99]. The latter can be achieved by fusion to threonine-158 at the extreme
234 C-terminus or insertion into the surface loop between serine-154 and glycine-155 [32]. Both
235 strategies produce viable and stable chimeric particles. It is unclear whether the four C-terminal
236 amino acids are required for the efficient systemic movement of recombinant viruses and the
237 assembly of chimeric particles. In infected tobacco plants, the viral coat protein may account for up
238 to 7% of total host cell protein and can be easily purified from plant tissue in the form of VNPs.
239 Several heterologous proteins and short peptides have been expressed in TMV as coat protein
240 fusions without affecting viral replication and assembly [101–105].

241 4.4 Bamboo mosaic virus

242 Bamboo mosaic virus (BaMV) is related to PVX and has a similar capsid and genome [102–105].
243 However, it has a broader host range, infecting both monocotyledonous and dicotyledonous plants
244 [101]. BaMV is not a pathogen in most crops and therefore may be ecologically safer for field use
245 than other pVNPs [101]. A novel transgenic cell-suspension culture system has recently been
246 developed, which continually produces self-replicating BaMV CVPs as well as coat proteins that
247 self-assemble into VLPs displaying target epitopes [106]. The size of the epitope presented by
248 BaMV-based CVPs is limited to 37 amino acids [70], and similar size constraints have been
249 reported for other viruses [97,107–109]. As for PVX, the FMDV 2A co-translational dissociation
250 sequence may therefore provide a solution to this challenge [93].

251 4.5 Papaya mosaic virus

252 Papaya mosaic virus (PapMV) is another Potexvirus, with a similar genome structure to PVX and
253 BaMV. It has a filamentous capsid, 500 nm in length and 14 nm in diameter. PapMV coat proteins
254 have been expressed in *E. coli* and showed the capacity of self-assemble into VLPs. The coat
255 protein has been separated from the genomic RNA by the degradation of purified virus particles
256 using acetic acid, thus facilitating *in vitro* assembly. The extracted coat protein formed a variety of
257 aggregates that included a disk-like helical structure, similar in architecture to the native virus

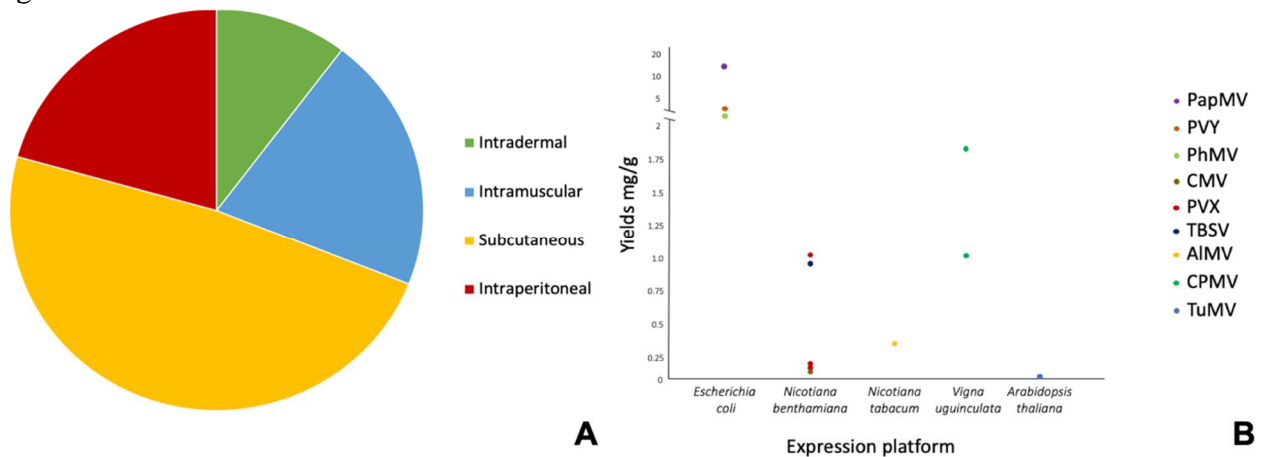
258 particle but comprising only 18–20 subunits. Furthermore, the addition of genomic RNA to the
 259 isolated disks triggered the *in vitro* assembly of long rod-shaped particles similar to the wild-type
 260 virus [67,110].

261 PapMV nanoparticles can be used as an adjuvant to improve the performance of vaccines [111] and
 262 cancer immunotherapy [112], or as a vaccine platform to trigger an immune response against specific
 263 peptide antigens [113]. In order to prevent long peptides interfering with the self-assembly of VLPs
 264 [49], a novel system was developed by coupling peptides to already self-assembled nanoparticles
 265 using the bacterial transpeptidase sortase A [62,100].

267 5. Development of pVNP Vaccines Displaying Antigenic Peptides

269 Many plant viruses have been used to display heterologous peptides for the development of vaccine
 270 candidates intended for active immunization. Some of these candidates have been tested *in vitro* and
 271 others have progressed to pre-clinical studies. The achievements of these investigations and the
 272 properties of the pVNPs and displayed peptides are summarized in Table 2. Interestingly, 10 of 36
 273 studies demonstrated that the ability of pVNPs to induce an immunological response against the
 274 target peptide conferred protection against the corresponding pathogen in subsequent challenge
 275 experiments. In some studies, pVNPs were even able to confer partial cross-protection against
 276 related pathogens [98].

277
 278
 279 Figure 1.



280
 281 **Figure 1.** The production of pVNPs for pre-clinical development. A: The yields of pVNPs achieved in different
 282 production platforms. B: Relative frequency of different administration routes for pVNPs in pre-clinical studies.

283
 284
 285 In most cases the administration route was parenteral with a high proportion of subcutaneous
 286 injections (Figure 1A). The pVNPs were always administered in two or three doses. The average
 287 yield of the pVNPs was strongly dependent on which virus was used and which target peptide was
 288 displayed, resulting in a broad distribution (Figure 1B). The yields of pVNPs are also dependent the
 289 position of the heterologous peptide on the viral surface, a property that also affects the
 290 immunological properties of the pVNP [114].

291
 292
 293 **Table 2:** Survey of pVNP *in vitro* tests and pre-clinical studies.

<i>pVNP</i>	<i>Target Peptide</i>	<i>Host</i>	<i>Pre-clinical and in vitro studies</i>	<i>References</i>
-------------	-----------------------	-------------	--	-------------------

TuMV	Human vascular endothelial growth factor receptor 3 (VEGFR-3)	<i>Arabidopsis thaliana</i>	Immunogenicity in mice	[60]
TMV	M2e epitope from influenza	<i>Nicotiana benthamiana</i>	Immunogenicity in vitro	[115]
TMV	Two peptides of CRPV	<i>Nicotiana tabacum</i> , <i>Nicotiana benthamiana</i>	Immunogenicity and challenging in rabbits	[98]
TMV	Peptide F20 (V141–P160 of FMDV VP1)	<i>Nicotiana tabacum</i> cv. <i>Samsun</i>	Immunogenicity in guinea pigs and swine and challenging	[107]
TMV	Peptide 9-14mer (TDAYNQKLSERRAN) of outer membrane (OM) protein F of <i>Pseudomonas aeruginosa</i>	<i>Nicotiana tabacum</i> cv. <i>Xanthi</i>	Immunogenicity and challenging in mice	[99]
TMV-U1	M2e epitope from influenza	<i>Nicotiana benthamiana</i>	Immunogenicity in BALB/c mice, virus challenging protection	[116]
BaMV	FMDV-VP1	<i>Nicotiana benthamiana</i> , <i>Chenopodium quinoa</i>	Immunogenicity in male and swine and challenging with the virus	[70]
BaMV	vvIBDV	<i>Nicotiana benthamiana</i> , <i>Chenopodium quinoa</i>	Immunogenicity, challenging with the virus and immunohistochemistry in chickens	[117]
PVX	HIV ELDKWA epitope from glycoprotein (gp) 41	<i>Nicotiana benthamiana</i>	immunogenicity in mice	[118]
PVX	HPV-16 L2 minor capsid protein (amino acids 108-120)	<i>Nicotiana benthamiana</i>	Immunogenicity in mice	[119]
PVX	H-2Db-restricted epitope ASNENMETM of influenza A virus nucleoprotein (NP)	<i>Nicotiana benthamiana</i>	Immunogenicity in C57BL/6J female mice	[28]
PVX	D2 peptide from <i>S. aureus</i> bronectin-binding protein (FnBP)	<i>Nicotiana benthamiana</i>	Immunogenicity in mice and rats	[120]
CPMV	D2 peptide from <i>S. aureus</i> bronectin-binding protein (FnBP)	<i>Nicotiana benthamiana</i>	Immunogenicity in mice and rats	[120]
CPMV	14 amino acid N1m-1A epitope from human rhinovirus 14 (HRV-14)	<i>Vigna unguiculata</i>	Immunogenicity	[114]
CPMV	Linear epitope from the VP2 capsid protein of mink enteritis virus (MEV)	<i>Vigna unguiculata</i>	Immunization of minks and challenging	[121]
CPMV	Peptide derived from the VP2 capsid protein of canine parvovirus (CPV)	<i>Vigna unguiculata</i>	Immunogenicity in dogs and challenging with the virus	[122]
CPMV	aa731- 752 of the gp41 of HIV-1 strain IIIB	<i>Vigna unguiculata</i>	immunogenicity in mice C57/BL6 (H-2b), BALB/c (H-2d) mice)	[123]
AMCV	HIV-1 2F5 epitope (2F5e)	<i>Nicotiana benthamiana</i>	ELISA	[80]
AIMV	Chimeric peptide representing rabies G (amino acids 253–275) and N (amino acids 404–418) proteins	<i>Nicotiana tabacum</i> cv. <i>Samsun</i>	Immunogenicity and challenging with the virus and Immunogenicity in humans of experimental plant virus-based rabies vaccine consumed in food	[72]
AIMV	V3 loop of the HIV-1 MN isolate (MNV3),	<i>Nicotiana benthamiana</i>	Immunogenicity in mice	[73]
AIMV	21-mer peptide representing amino acids 170-190 of the respiratory syncytial virus (RSV) G protein	<i>Nicotiana tabacum</i> cv. <i>Samsun</i>	immunogenicity in vivo in non-human primates and in vitro in human dendritic cells	[74]
AIMV	Small loop 15 amino acid epitope from domain 4 of the Bacillus anthracis protective antigen (PA-D4s).	<i>Nicotiana tabacum</i> cv. <i>Samsun</i>	Immunogenicity in Balb/c mice	[75]
PapMV	Hepatitis C virus (HCV) E2 epitope	<i>Escherichia coli</i>	Immunogenicity in C3H/HeJ and Balb/c mice and in humans	[63]
PapMV	M2e influenza epitope	<i>Escherichia coli</i>	Immunogenicity in BALB7c mice and challenging with the virus	[124]

PapMV	M2e influenza epitope	<i>Escherichia coli</i>	Immunogenicity in Balb/c mice	[64]
PapMV	HA11 peptide influenza peptide	<i>Escherichia coli</i>	Immunogenicity in Balb/c mice	[61]
TBSV	16 amino acid epitopes of ricin toxin A chain	Insect cells and <i>baculovirus</i>	Immunogenicity in Balb/c mice	[125]
TBSV	13-amino-acid peptide derived from the V3 loop of human immunodeficiency virus (HIV-1) glyco- protein 120 (gp120)	<i>Nicotiana benthamiana</i>	Immunogenicity in mice	[126]
JGMV	27 amino acid peptide A of the Japanese encephalitis virus (JEV)	<i>Escherichia coli</i>	Immunogenicity in FVB/J mice and challenge with the virus	[55]
PhMV	FMDV infection-related B-cell epitopes of NSPs 3A, 3B and 3D	<i>Escherichia coli</i>	Immunogenicity in cattle, buffalo, sheep, pig samples	[127]
CMV	17 amino acid (aa) neutralizing epitope of NDV's fusion (F) protein (aa 65– 81), 8 aa neutralizing epitope of the NDV hem-agglutinin-neuraminidase (HN) protein (aa 346– 353), and the same epitopes fused together in a tandem F–HN construct.	<i>Nicotiana benthamiana</i>	Immunogenicity in chickens	[77]
CMV	Peptide of HCV envelope protein E2 (R9 mimotope).	<i>Nicotiana tabacum cv. Xanthi</i>	Immunogenicity in rabbits, in vitro studies in humans	[76]
PVY	PreS1 or rubredoxin	<i>Escherichia coli</i>	Immunogenicity in Balb/c mice	[82]
CdMV	Kennedy peptide and the 2F5 and 4E10 epitopes of gp41 ofHIV.	<i>Escherichia coli</i>	Immunoreactivity with HIV patient sera	[79]
PPV	VP2 capsid protein of canine parvovirus (CPV)	<i>Nicotiana clevelandii</i>	Immunogenicity in mice and rabbits	[81]

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The dose of pVNP administered in each pre-clinical study strongly depended on the animal model, ranging from 0.5 to 400 µg/dose. In most cases, an adjuvant was also administered, with incomplete Freund's adjuvant and aluminum hydroxide the most widely used. The pre-clinical studies were mainly structured as a comparison between animals treated with pVNPs displaying the target peptide and a placebo group in which animals were treated with wild-type pVNPs lacking the displayed peptide. In a small number of cases, the experimental design also included a control group treated with the synthetic peptide. In these experiments, the quantity of peptide displayed by the pVNP was lower than the dose used for the synthetic peptide. Only one study included a control group in which the pVNPs were administered without an adjuvant [28]. This revealed that pVNPs administered with an adjuvant treatment achieved the greatest level of protection, and that pVNPs without adjuvant conferred the same protective effect as the synthetic peptide administered with the adjuvant. In other studies, the use of the synthetic peptide as a comparator was limited to indirect ELISA tests to detect the presence of specific antibodies [60,123]. Overall, these studies indicate that pVNPs can act as both as a carrier and an adjuvant, probably reflecting the particulate and repetitive nature of the virion [128].

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The particulate nature of VLPs and CVPs also allows them to induce potent T-cell responses by interacting with antigen-presenting cells (APCs), especially dendritic cells (DCs) [129]. Cytotoxic T cells do not recognize native antigens, but are activated by processed peptides derived therefrom presented in association with MHC class I molecules [130]. The best way to induce T-cell activation by vaccination is to mimic the process of a natural infection, including the recognition, uptake, and processing of particulate antigens, and the presentation of processed peptides to cytotoxic T cells in order to activate them and trigger their proliferation [131]. DCs can efficiently carry out these processes by (i) taking up antigens in the cytosol and presenting processed peptide

319 antigens on MHC class I molecules or receptors by cross-presentation, and (ii) by presenting the
320 peptides on MHC class II molecules as part of the classical antigen-processing pathway [131].
321 Thus, in addition to stimulating helper T cells, DCs can also stimulate the conversion of naive T
322 cells into cytotoxic T lymphocytes (CTLs), which subsequently eliminate intracellular pathogens or
323 cancer cells via the presentation of antigens on MHC class I molecules, effectively bridging innate
324 and acquired immunity. DCs preferentially take up particulate antigens by phagocytosis or
325 macropinocytosis if the particle diameter is 20–300 nm, the size range of most viruses [132].
326 Therefore, pVNPs are the ideal size for DC and macrophage uptake and antigen processing to
327 initiate antigen cross-presentation. Due to the high density of epitopes on their surface, the uptake
328 of a single pVNP feeds thousands of epitopes into the processing and presentation machinery of
329 APCs, further enhancing their ability to stimulate CTLs.

330 The presence of pVNPs can also directly activate B cells, leading to high antibody titers and long-
331 lasting B-cell memory, even in the absence of adjuvants. Like mammalian viruses, the quasi-
332 crystalline surface of pVNPs with arrays of repetitive epitopes is a prime target for B-cell
333 recognition [133] and can efficiently crosslink epitope-specific immunoglobulins on the cell
334 surface. Particulate antigens with repetitive epitopes spaced at 5–10-nm intervals may be unique to
335 microbial surfaces, and vertebrate B cells have therefore evolved to specifically recognize and
336 respond vigorously to these types of antigens [134]. The oligomerization of immunoglobulins in
337 this manner forms a strong activation signal that leads to B-cell proliferation and migration, the
338 upregulation of MHC class II molecules, T helper cell activation, IgM and IgG production and
339 secretion, and the generation of long-lasting memory B cells [133]. An advantage of CVPs over
340 VLPs is that the viral RNA may trigger Toll-like receptor 7 on antigen-presenting cells, hence
341 boosting the immune response like an adjuvant [135].

342 Another characteristic of pVNPs is that certain plant viruses (e.g. PapMV) are inherently highly
343 immunogenic, triggering a strong humoral response against the coat protein. However, pre-existing
344 antibodies recognizing virus coat do not appear to affect the ability of pVNPs to boost the humoral
345 response toward heterologous antigens displayed on their surface [124].

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347 In summary, pVNPs can be used to carry immunodominant peptides for active immunization
348 strategies, and their intrinsic properties, such as the ability to display arrays of antigens on a
349 scaffold with a repetitive structure, may help to promote their immunological efficacy.

351 6. Consent for Publication

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353 All authors read and approved the final manuscript.

354 7. Conflict of interest

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357 The authors declare that the research was conducted in the absence of any commercial or financial
358 relationships that could be construed as a potential conflict of interest.

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