European Journal of Pharmaceutical Sciences 189 (2023) 106558

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Viral protein-based nanoparticles (part 2): Pharmaceutical applications

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ARTICLE INFO

Keywords:
Virus-like particles
Virosomes
Vaccines
Immunostimulation
Drug delivery
Diagnostics

ABSTRACT

Viral protein nanoparticles (ViP NPs) such as virus-like particles and virosomes are structures halfway between viruses and synthetic nanoparticles. The biological nature of ViP NPs endows them with the biocompatibility, biodegradability, and functional properties that many synthetic nanoparticles lack. At the same time, the absence of a viral genome avoids the safety concerns of viruses. Such characteristics of ViP NPs offer a myriad of opportunities for theirapplication at several points across disease development: from prophylaxis to diagnosis and treatment. ViP NPs present remarkable immunostimulant properties, and thus the vaccination field has benefited the most from these platforms capable of overcoming the limitations of both traditional and subunit vaccines. This was reflected in the marketing authorization of several VLP- and virosome-based vaccines. Besides, ViP NPs inherit the ability of viruses to deliver their cargo to target cells. Because of that, ViP NPs are promising candidates as vectors for drug and gene delivery, and for diagnostic applications. In this review, we analyze the pharmaceutical applications of ViP NPs, describing the products that are commercially available or under clinical evaluation, but also the advances that scientists are making toward the implementation of ViP NPs in other areas of major pharmaceutical interest.

1. Introduction

In the 1960s, an unknown antigen was detected in the serum of an Australian individual during a study of hemophilia patients. Although it was initially linked to the development of leukemia, it was eventually connected to an infection with the hepatitis B virus (HBV). Electron microscopy and immunofluorescence techniques finally revealed that the so-called "Australian antigen" was actually a surface antigen of the HBV, which formed structures similar to viral capsids, but smaller and devoid of genetic material (Blumberg and Alter, 1965; Blumberg, 1977). This finding is considered the first evidence of the existence of virus-like particles (VLPs) (Yan et al., 2015).

Since then, many other VLPs have been identified and synthesized. VLPs are defined as supramolecular assemblies of proteins that mimic viral structure but lack genetic material, so they are non-infective (Jennings and Bachmann, 2008). VLPs are either prepared by expressing complete viruses and removing the viral genome, or by direct expression of viral capsid proteins from a vector. They are usually rod-shaped or icosahedral and their size varies between 20 and 200 nanometers in diameter (Jennings and Bachmann, 2008; Dai et al., 2018). Depending on the parental virus, VLPs can be enveloped or not. Non-enveloped VLPs can be simple viral capsids formed by one or two major proteins, such as human papillomavirus (HPV)-VLPs, or complex viral capsids made up of several protein layers, such as rotavirus VLPs (Shirbaghaee and Bolhassani, 2016; Fuenmayor et al., 2017). In enveloped VLPs, such as human immunodeficiency virus-1 (HIV-1)-VLPs, the viral capsid is surrounded by a lipid bilayer from the host cell membrane containing glycoprotein spikes (Fuenmayor et al., 2017).

When comparing VLPs to synthetic nanoparticles, the latter are easier to produce since they do not require expression systems and do not need complex downstream processing (Plummer and Manchester, 2011). However, alternatives such as the production of VLPs in edible plants may overcome this limitation (Biemelt et al., 2003). Regarding immunogenicity, VLPs typically exhibit greater immune stimulation compared to synthetic nanoparticles. This result is undesirable when VLPs are intended as delivery vehicles, but strategies have been developed to make "stealth" VLPs (Chen et al., 2016). Finally, although

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https://doi.org/10.1016/j.ejps.2023.106558
Received 17 February 2023; Received in revised form 10 July 2023; Accepted 4 August 2023
Available online 9 August 2023

List of abbreviations

AIT	Allergen-specific immunotherapy
APC	Antigen-presenting cell
CCMV	Cowpea chlorotic mottle virus
CED	Convection-enhanced delivery
CpG	Cytosine-phosphate-guanosine oligodeoxynucleotides
CSP	Circumsporozoite protein
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
DODAC	N,N-dioleyl-N,N-dimethylammonium chloride
DOSPER	1,3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamide
DOTAP	1,2-dioleoyl-3-trimethylammonium propane
DOX	Doxorubicin
EMA	European Medicines Agency
EMHV	Erythro-magneto-hemagglutinin virosome
FDA	Food and Drug Administration
HA	Hemagglutinin
HBcAg	Hepatitis B core antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
IRIV	Immunopotentiating reconstituted influenza virosome
MERS-CoV	Middle East respiratory syndrome coronavirus
MHC	Major histocompatibility complex
miRNA	micro-RNA
MPL	3-O-desacyl-4′-monophosphoryl lipid A
MRI	Magnetic resonance imaging
NA	Neuraminidase
NIR	Near-infrared
PEG	Polyethyleneglycol
PET	Positron emission tomography
RBD	Receptor-binding domain
SARS-CoV	Severe acute respiratory syndrome coronavirus
siRNA	Small interference RNA
SV40	Simian virus 40
TAA	Tumor-associated antigen
TAT	Transactivator of transcription
TLR	Toll-like receptor
TMV	Tobacco mosaic virus
tPA	Tissue plasminogen activator
ViP NP	Viral protein nanoparticle
VLP	Virus-like particle
WHO	World Health Organization

synthetic nanoparticles can be as safe as VLPs, their in vivo elimination is generally less favorable (Plummer and Manchester, 2011).

A decade after the discovery of VLPs, Almeida et al. succeeded at including hemagglutinin (HA) and neuraminidase (NA) in a liposome, creating what they called a virosome (Almeida et al., 1975). Virosomes are lipid/viral protein nanometric structures with spherical shape and a diameter of around 60 to 200 nanometers (van der Velden et al., 2022). They are prepared by solubilizing native enveloped viruses, removing the nucleocapsid, to finish with the reconstitution of the viral envelope proteins into a liposome (Fig. 1) (Huckriede et al., 2003; Asadi and Gholami, 2021; Mak and Saunders, 2006).

While virosomes share a similar structure with liposomes, their distinct viral glycoprotein coating sets them apart significantly in terms of functionality. The incorporation of antigens into liposomes is challenging and, when achieved, adjuvants are required to elicit strong immune responses (Kersten and Crommelin, 2003). Both requirements are avoided when developing a virosomal vaccine. Moreover, virosomes have a big advantage when endosomal escape is needed (see Section 3.1).

Both VLPs and virosomes are highly versatile, where active/functional agents can be incorporated either in their coating layers or in their inner cavity (Fig. 1). Examples of such agents are ligands, drugs, antigenic sequences, contrast agents, and nucleic acids (van der Velden et al., 2022; Asadi and Gholami, 2021; Koudelka et al., 2015; Diaz et al., 2018; Ding et al., 2018). A powerful strategy is to encapsulate the therapeutic agent inside the viral nanoparticle cavity, where it is protected from degradation, while its surface is functionalized with a targeting moiety for a specific cell type (Thong et al., 2019). Other strategies like the attachment of polymers to the surface of VLPs or virosomes can avoid undesirable immunogenicity (Farkas et al., 2013).

Besides, these viral protein nanoparticles (ViP NPs; i.e., a term we use to refer collectively to VLPs and virosomes) show lower toxicity and less safety concerns than other biological particles (Yan et al., 2015; Asadi and Gholami, 2021). They can be administered through a variety of parenteral routes, and the possibilities offered by their transmucosal administration are currently being investigated (Mohsen et al., 2020; De Bernardis et al., 2015; Huang et al., 2005; Sedlik et al., 1999). Once in the circulation, ViP NPs are completely degraded in a matter of days (Kaiser et al., 2007). In a previous review paper, we have comprehensively covered these structural, toxicological, and biopharmaceutical issues. We also analyzed the manufacturing process and the formulation strategies (Mellid-Carballal et al., 2023).

In this comprehensive review, we focus on medical applications of ViP NPs and the functional characteristics that confer them competitive advantages. Specifically, we delve into the utilization of VLPs and virosomes in the fields of immunology and immunotherapy, shedding light on their clinical use and emerging applications. Furthermore, we analyze ongoing research endeavors investigating the potential use of ViP NPs in other domains, including drug/gene delivery and diagnostics. Finally, we address pivotal mechanistic inquiries, such as the intrinsic immunogenic properties of ViP NPs, their targeting and intracellular trafficking capabilities, and the effective loading of contrast agents within these biomedical platforms.

2. ViP NPs for vaccination

Until the 80s, vaccines were based on inactivated or attenuated viruses (Mohsen et al., 2017). Although they showed excellent immunogenicity, safety issues and difficulties in mass production limited their use (Mohsen et al., 2017; Mohsen et al., 2020). The discovery in the 70s that a single viral protein could be enough to induce immunostimulation (Roldão et al., 2010) laid the foundations for the development of subunit vaccines, which are safer but less immunogenic than conventional vaccines.

Nucleic acid vaccines are easier to produce than conventional and subunit vaccines. However, the requirement for an optimal delivery system limited their development (Liu, 2019; Ho et al., 2021). Some DNA vaccines are available in the veterinary field (Chang et al., 2007; Salonius et al., 2007; McLean and Lobetti, 2015), but none are for human use (Ho et al., 2021). mRNA vaccines are a new technology that has demonstrated great value when rapid development is mandatory, as occurred with the COVID-19 pandemic (Dolgin, 2021).

Nanoparticle-based vaccines induce excellent immune responses due to their polyvalent surfaces (Bachmann and Jennings, 2010; Zabel et al., 2013). Their nanometric size allows them to freely drain to the lymph nodes (Fifis et al., 2004), where they are taken up by antigen-presenting cells (APCs) (Manolova et al., 2008). Although nanoparticles have an adjuvant effect by themselves, they can also be combined or conjugated

to other adjuvants to increase their immunogenicity. Moreover, they are safe and easy to produce (Gomes et al., 2017). Nanoparticle-based vaccines include liposomes, lipid nanoparticles, cationic polymers, viral vectors, and ViP NPs. VLPs and virosomes are the only biological nanoparticle-type antigenic structures that have been approved as vaccines for human use (Gomes et al., 2017). The current diversity of vaccine structures is visually depicted in Fig. 2.

2.1. Immunostimulation mechanisms of ViP NPs

2.1.1. VLPs

VLPs display antigens in a similar conformation to the natural virus. The repetitive pattern of antigens on the VLP surface (multivalent presentation) enhances the activation of B cells by crosslinking multiple B-cell receptors. Therefore, VLPs induce stronger humoral responses than subunit vaccines (Shirbaghaee and Bolhassani, 2016; Donaldson et al., 2018), even in the absence of adjuvants (Yan et al., 2015; Mohsen et al., 2017; Mohsen et al., 2020; Donaldson et al., 2018; Jeong and Seong, 2017). In addition, their size range is optimal for draining to the lymph nodes, where they can be efficiently taken up by dendritic cells (Yan et al., 2015; Mohsen et al., 2017; Jeong and Seong, 2017). Internalized VLPs are processed and presented by major histocompatibility complex (MHC) class II molecules for the activation of T helper cells (Fig. 3).

Due to their highly ordered structure, VLPs resemble pathogen-associated molecular patterns, so they are recognized by specific receptors, triggering an interferon response and inflammation, and increasing MHC class I antigen presentation and, consequently, the priming of cytotoxic T lymphocytes (CTLs) (Fig. 3) (Yan et al., 2015; Shirbaghaee and Bolhassani, 2016).

As VLPs can induce both humoral and cellular responses, they are of great interest for vaccination strategies, with prophylactic and therapeutic objectives. The strong immunogenicity of VLPs, together with their self-adjuvant properties and their high stability, results in stronger immune responses with lower doses applied compared to subunit vaccines, leading to cost reduction (Koudelka et al., 2015; Mohsen et al., 2020; Roldão et al., 2010; Shirbaghaee and Bolhassani, 2016).

Finally, VLPs can be engineered to present two or more different antigens (chimeric VLPs) (Koudelka et al., 2015). This is particularly useful when intending to develop multivalent vaccines or to present an antigen with no self-assembling properties in the immunostimulant structural context of a VLP.

Environmental exposure to prevalent strains of a VLP parent virus, or previous vaccination with a VLP vaccine may generate anti-VLP antibodies, thus having deleterious interference (Jegerlehner et al., 2010). This can be overcome by alternating between different VLP vectors (Zhou et al., 2011), or by masking VLP surface antigens (Jariyapong et al., 2013).

2.1.2. Virosomes

The paradigmatic example of virosomes are influenza virosomes (Crisci et al., 15), which allow influenza antigens to be presented in a non-pyrogenic yet immunogenic platform (Almeida et al., 1975; Metcalfe and Gluck, 2004). The two glycoproteins of the influenza virus membrane, HA and NA, permit virosomes to fuse with the plasma

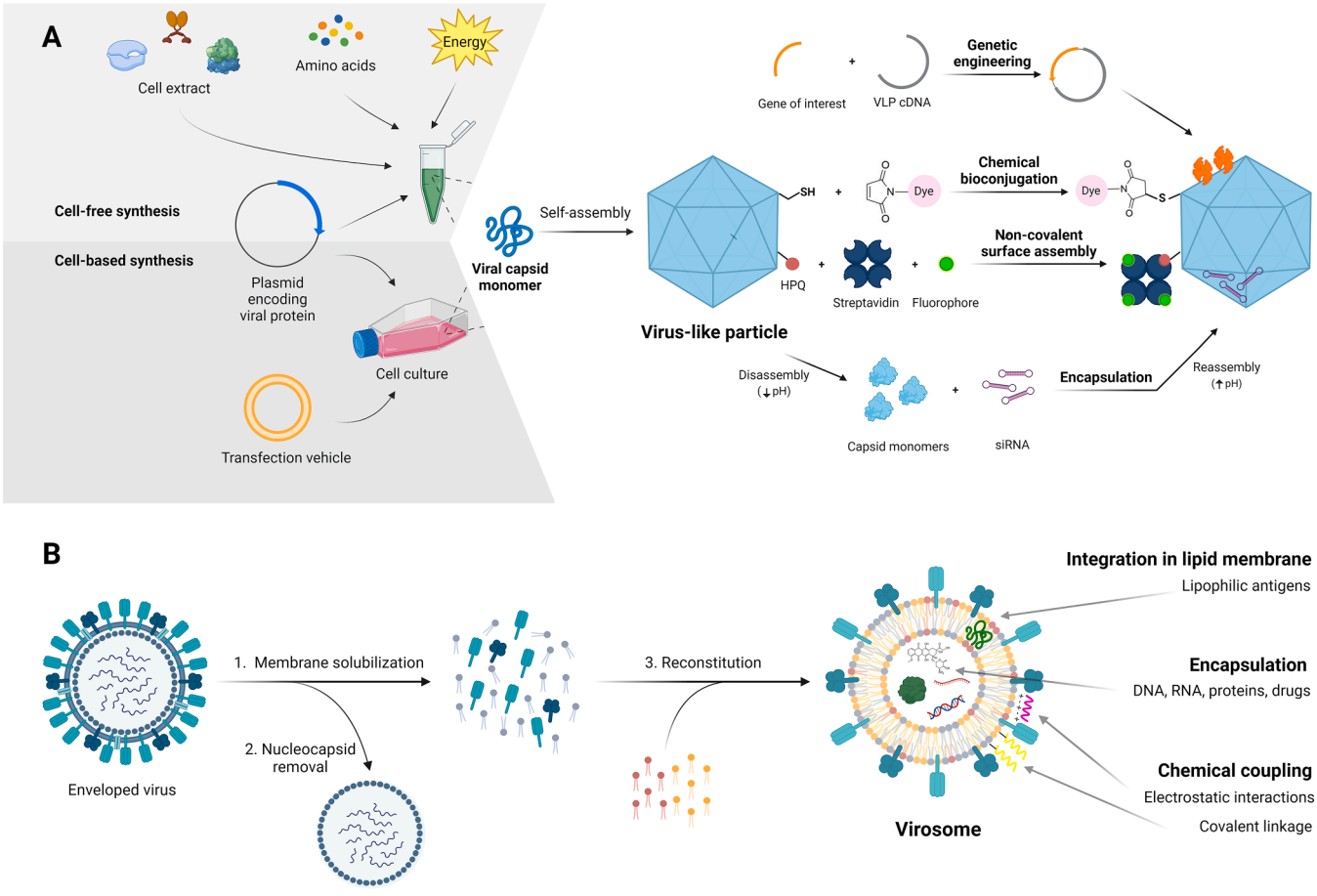

Fig. 1. Synthesis and modification strategies of ViP NPs. (A) Production of VLPs using cell-based or cell-free expression systems, and subsequent modification through genetic engineering, chemical bioconjugation, non-covalent surface assembly, or by cargo encapsulation. HPQ: homophenylalaninylmethane sequence of a biotin-mimicking peptide. (B) Main steps in the production of virosomes from enveloped viruses, and potential modifications, including integration in the lipid membrane, chemical coupling, and encapsulation. Created with BioRender.com.

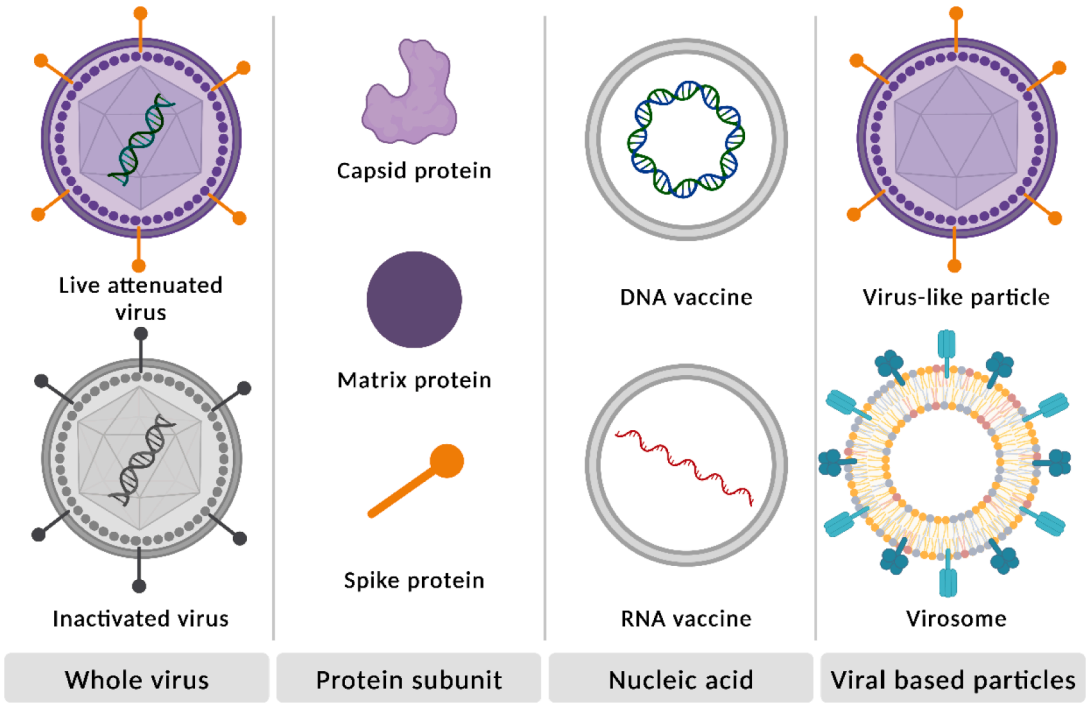

Fig. 2. Types of vaccines. The structural differences among the diverse particles used for vaccination are represented. Created with BioRender.com.

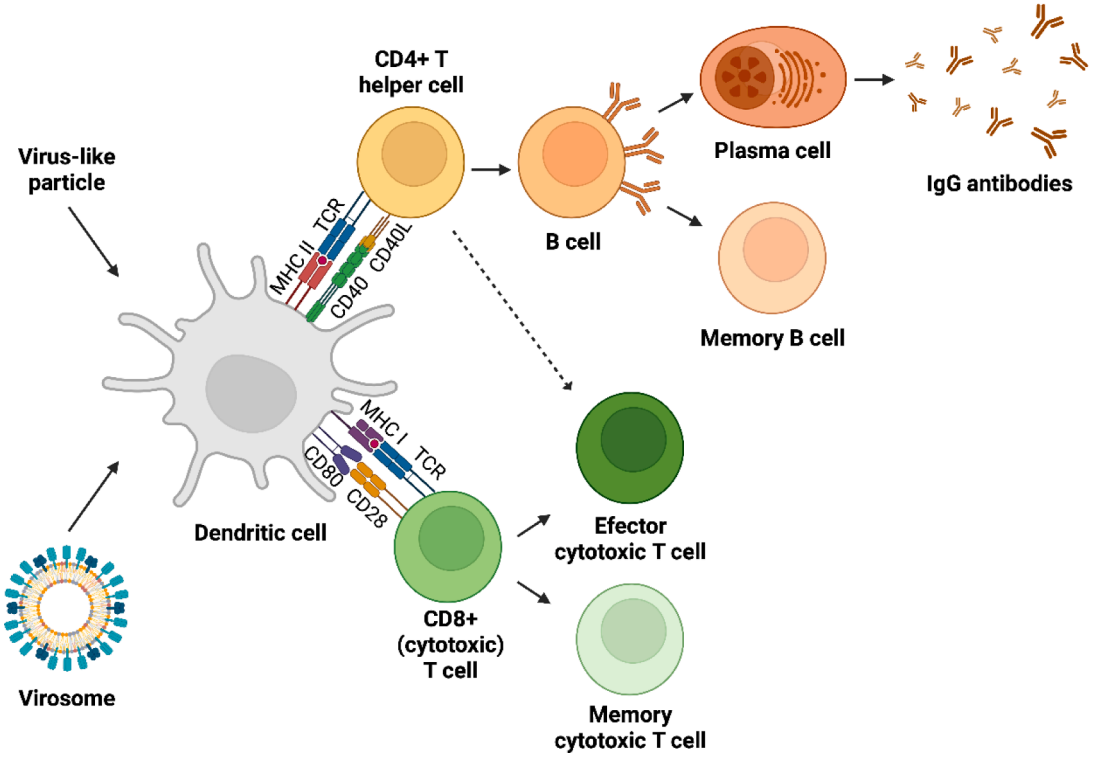

Fig. 3. Immunostimulation by VLPs and virosomes. Both ViP NPs are taken up by APCs, such as dendritic cells, where antigens are presented on class I and II MHC molecules. Therefore, they activate CD4+ and CD8+ T cells that eventually induce humoral and cellular immune responses, respectively. Created with BioRender.com.

membrane of non-phagocytic cells. There, the antigen is processed and presented on MHC class I molecules that induce a specific CTL response. HA also targets virosomes to receptors containing sialic acid on the membranes of APCs, promoting their endocytosis. After virosome degradation in the endosome, the antigen is presented on MHC class II molecules and induces a humoral response (Fig. 3) (Mak and Saunders, 2006).

The capacity of influenza virosomes to induce strong humoral and cellular immune responses makes them useful as influenza vaccines. Moreover, they show intrinsic adjuvant capacity, enhancing the immune

response to unrelated antigens. When a substantial amount of external lipids is added to influenza virosomes, the resulting platforms are termed immunopotentiating reconstituted influenza virosomes (IRIVs). IRIVs are biodegradable and biocompatible, showing an excellent safety profile. They can be administered by most routes, combined with other adjuvants, and used for multiantigen delivery (Asadi and Gholami, 2021). These properties have resulted in the FDA authorization of IRIV-based nanocarriers for human use. Their major limitation is their rapid disintegration in the bloodstream (Asadi and Gholami, 2021; Liu et al., 2015), although it can be overcome by conjugating a targeting ligand that drives virosomes to their target sites within a short time after administration.

The applications of virosomes are not limited to viral infections. For example, they can deliver tumor-associated antigens (TAAs), serving simultaneously as carriers and adjuvants, and promoting the generation of immune responses against cancer cells (Wiedermann et al., 2010).

2.2. ViP NPs in prophylactic vaccination

The immunogenic properties of VLPs and virosomes have lead to the approval of several vaccines for the prevention of infectious diseases. Also, some candidates are undergoing clinical trials and many prototypes are under preclinical investigation. Here, we will focus on VLP-based and virosome-based vaccines that are commercially available or have reached clinical evaluation, which are summarized in Tables 1 and 2, respectively.

2.2.1. Hepatitis B virus

In the 1980s, hepatitis B surface antigen (HBsAg)-VLPs isolated from the blood of infected individuals were commercialized as vaccines under the trade names Heptavax B® and Hevac B® (Mohsen et al., 2017; Gomes et al., 2017; Tornesello et al., 2022). Although they were considered the first VLP-based vaccines, they were irregular lipoprotein complexes, different from the highly ordered protein structures generally described as VLPs (Jennings and Bachmann, 2008). The safety issues related to blood products, the dependence on blood availability and the advances in recombinant DNA technology and biotechnology led to the development of recombinant HBsAg (Roldão et al., 2010; Gomes et al., 2017).

Second-generation vaccines Recombivax HB® (Merck and Co., Inc.) and Engerix B® (GlaxoSmithKline S.A.) were approved by the FDA in 1986 and 1989, respectively. Both vaccines were based on the recombinant expression of HBsAg in *Saccharomyces cerevisiae,* where HBsAg spontaneously assembles into 20 nm lipid-containing particles. They were safer and more immunogenic than first-generation vaccines (Jennings and Bachmann, 2008; Shirbaghaee and Bolhassani, 2016; Diaz et al., 2018; Mohsen et al., 2017; Roldão et al., 2010; Donaldson et al., 2018; Tornesello et al., 2022).

Even though the commercialization of these vaccines meant a great success, their combination with adjuvants could improve responder rates in immunocompromised subjects and increase protection without increasing the number of doses (Jennings and Bachmann, 2008). Fendrix® vaccine, adjuvanted by AS04, received marketing authorization from the EMA in 2005 to be used in patients with renal insufficiency (Machiels et al., 2019).

More recently, the third generation of HBV-VLP-based vaccines was developed to further increase protection levels, particularly in some sectors of the population, such as immunocompromised patients

Table 1
ViP NP-based prophylactic vaccines that have received marketing authorization.

Name	Target agent	Antigen	ViP NP type	Expression system	Manufacturer	First approval
Heptavax B®	Hepatitis B virus	HBsAg	VLP	Blood-derived	Merck Pharmaceuticals	1981 (FDA) Discontinued in 1990
Hevac B®	Hepatitis B virus	HBsAg	VLP	Blood-derived	Institute Pasteur	1981 (France)
Recombivax HB®	Hepatitis B virus	HBsAg	VLP	*Saccharomyces cerevisiae*	Merck and Co., Inc.	1986 (Germany and FDA)
Engerix B®	Hepatitis B virus	HBsAg	VLP	*Saccharomyces cerevisiae*	GlaxoSmithKline S.A.	1986 (Belgium) 1989 (FDA)
Fendrix®	Hepatitis B virus	HBsAg	VLP	*Saccharomyces cerevisiae*	GlaxoSmithKline S.A.	2005 (EMA)
Sci-B-Vac®	Hepatitis B virus	S, Pre-S1, and Pre-S2; M and L proteins	VLP	Chinese hamster ovary cells	VBI Vaccines (Delaware) Inc.	2017 (Israel and East Asia)
GenHevac B®	Hepatitis B virus	S, Pre-S1, and Pre-S2; M protein	VLP	*Hansenula polymorpha*	Pasteur-Mérieux Aventis	1989 (France)
Heplisav B®	Hepatitis B virus	HBsAg	VLP	*Hansenula polymorpha*	Dynavax Technologies Corporation	2017 (FDA)
Gardasil®	Human papillomavirus	L1 protein (HPV 6, 11, 16, and 18)	VLP	*Saccharomyces cerevisiae*	Merck and Co., Inc.	2006 (FDA)
Cervarix®	Human papillomavirus	L1 protein (HPV 16, and 18)	VLP	Trichoplusia ni (High Five) cells	GlaxoSmithLine S.A.	2009 (FDA)
Gardasil® 9	Human papillomavirus	L1 protein (HPV 6, 11, 16, 18, 31, 33, 45, 52, and 58)	VLP	*Saccharomyces cerevisiae*	Merck Sharp & Dohme Corp.	2017 (FDA)
Cecolin®	Human papillomavirus	L1 protein (HPV 16, and 18)	VLP	*E. coli*	Xiamen Innovax Biotech Co., Ltd.	2019 (China)
Hecolin®	Hepatitis E virus	L1 protein (HPV 6, 11, 16, and 18)	VLP	*E. coli*	Xiamen Innovax Biotech Co., Ltd.	2011 (China)
Mosquirix®	*Plasmodium falciparum*	Secreted circumsporozoite protein (CSP)	HBsAg-VLPs	*Saccharomyces cerevisiae*	GlaxoSmithKline S.A.	2022 (WHO prequalification)
Epaxal®	Hepatitis A virus	Formalin-inactivated Hepatitis A antigen (strain RG-SB)	IRIV	HAV virions: MRC-5 cells	Berna Biotech Co., Ltd. Crucell	1994 (Switzerland) 1999 (EMA) Discontinued in 2011
Inflexal® V	Influenza virus	HA and NA from annual strains	IRIV	Fertilised chicken eggs	Berna Biotech Co., Ltd. Crucell	1997 (Switzerland) 2001 (EMA) Discontinued in 2011
Porcilis® PCV	Porcine circovirus type 2	ORF2 capsid protein	VLP	Trichoplusia ni (High Five) cells	Intervet International BV	2009 (EMA)
Ingelvac CircoFLEX®	Porcine circovirus type 2	ORF2 capsid protein	VLP	Spodoptera frugiperda 9 (Sf9) cells	Boehringer Ingelheim Vetmedica GmbH	2008 (EMA)

Table 2
ViP NP-based prophylactic vaccines that have reached clinical trials.

Name	Target agent	Antigen	ViP NP type	Expression system	Phase	Registration number	Status
	Hepatitis B virus	HBsAg	VLP	Transgenic tubers (*Solanum tuberosum)*			
	Hepatitis B virus	HBsAg	VLP	Transgenic lettuce (Lactuca sativa L.)			
R21/Matrix-M	Malaria	CPS from *Plasmodium falciparum*	HBsAg-VLP		III	NCT04704830	Active, not recruiting
					II	NCT05252845	Recruiting
MalariVax (ICC-1132)	Malaria	CPS from *Plasmodium falciparum*	HBcAg-VLP	*E. coli*	I	NCT00587249	Completed
PEV301&302	Malaria	Synthetic peptides derived from apical membrane antigen-1 (PEV301) and from CSP (PEV302) from *Plasmodium falciparum*	Virosome		Ib	NCT00513669	Completed
ACAM FLU-A	Influenza virus	M2 peptides from the influenza A virus	HBcAg-VLP	*E. coli*	I	NCT00819013	Completed
	Influenza virus		VLP		IIa	NCT00903552	Completed
NanoFLu (Quad-NIV)	Influenza virus	Recombinant HA protein	VLP	Sf9 insect cells	III	NCT04120194	Completed
	Norwalk virus	Major capsid protein (VP1)	VLP	Sf9 insect cells			
	Norwalk virus	Major capsid protein (VP1)	VLP	Transgenic potatoes			
	Norwalk virus	VP1	VLP	Baculovirus expression system	I	NCT00806962	Completed
VAI-VP705	Human parvovirus B19	VP1 and VP2 proteins	VLP	baculovirus-insect cell expression system	I/II	NCT00379938	Terminated
VRC—CHKVLP059–00-VP	Chikungunya Virus	E1, E2, and capsid proteins	VLP	VRC293 cells	II	NCT02562482	Completed
HIV p17/p24:Ty-VLP	HIV	p17 or p24	Ty VLP	S. cerevisiae	I II	NCT00001053	Completed
CoVLP	SARS-CoV-2	Mutated S2P (stabilized prefusion form)	SARS-CoV-2 VLP	*Nicotiana benthamiana*	I	NCT0445000	Complete
					I/II	NCT05065619	Terminated (for ethical reasons to prioritize ancentral vaccination)
					II/III	NCT04636697	Active, not recruiting
					III	NCT05040789	Withdrawn (further development terminated)
VBI-2902 VBI-2905	SARS-CoV-2 B.1.351 variant	S protein (prefusion state)	Enveloped MLV-VLP		I	NCT04773665	Completed
VBI-2901	SARS-CoV-2 SARS-CoV MERS	S protein	Enveloped MLV-VLP		I	NCT05548439	Active, not recruiting
ABNCoV2	SARS-CoV-2	RBD from S protein	Acinetobacter phage AP205 capsid-like particle	E. coli (Tag-CLP)	I	NCT04839146	Completed
				Drosophila melanogaster (RBD-Catcher)	II	NCT05077267	Active, not recruiting
					III	NCT05329220	Active, not recruiting
SARS-CoV-2 VLP vaccine	SARS-CoV-2	S, E, M, and N proteins	VLP		I	NCT0481828	Completed
					II	NCT04962893	Completed
RBD SARS-CoV-2 HBsAg VLP Vaccine	SARS-CoV-2	RBD	HBsAg-VLP	Yeast	I/II	ACTRN12620001308987	Stopped early est vaccine no longer developed for commercial reasons)
					I/II	ACTRN12620000817943	Recruiting
LYB001	SARS-CoV-2	RBD	VLP vector		II/III	NCT05602558	Not yet recruiting
					II	NCT05663086	Not yet recruiting
					II/III	NCT05137444	Not yet recruiting

(Shirbaghaee and Bolhassani, 2016; Diaz et al., 2018; Donaldson et al., 2018). Sci-B-Vac®, developed by VBI Vaccines Inc., was first approved in Israel and East Asia in 2017 and is composed of three surface antigens (S, Pre-S1, and Pre-S2), together with M and L proteins, expressed in mammalian Chinese hamster ovary cells (Mohsen et al., 2017; Roldão et al., 2010). It combines glycosylated and non-glycosylated HBsAg, thus eliciting higher immunogenicity than yeast-derived vaccines. The GenHevac B® vaccine contains the same three surface antigens together with the M protein (Roldão et al., Oct), expressed in *Hansenula polymorpha* yeast, and is registered in India (Shivananda et al., 2006). Finally, the vaccine Helipsav B, manufactured by Dynavax Technologies Corporation and approved in the United States, contains HBsAg-VLPs adjuvanted with CpG sequence 1018 (Tornesello et al., 2022).

A main goal in vaccine development is the production of edible vaccines. Current studies take advantage of the assembly of HBV-VLPs in transgenic plants (Jennings and Bachmann, 2008; Fuenmayor et al., 25). Thanavala et al. generated HBsAg transgenic tubers in *Solanum tuberosum*. Subjects who have previously received a vaccine against hepatitis B showed higher titers of specific anti-HBsAg antibodies after eating the transgenic potatoes (Thanavala et al., 2005). Kapusta et al. introduced the HBsAg gene into *Agrobacterium tumefaciens* to obtain transgenic lupin and lettuce. Human volunteers fed with this transgenic lettuce developed a specific humoral response (Kapusta et al., 2001).

2.2.2. Human Papillomavirus

In the 1990s, it was discovered that the L1 major capsid of HPV can self-assemble into VLPs. They were identical to the wild-type virus and induced both humoral and cellular responses (Jennings and Bachmann, 2008; Fuenmayor et al., 25; Roldão et al., 2010; Tornesello et al., 2022; Roy and Noad, 2008). Currently, three HPV VLP-based vaccines are available in Europe and the United States: Gardasil® (Merck and Co., Inc.), Cervarix® (GlaxoSmithKline S.A.), and Gardasil® 9 (Shirbaghaee and Bolhassani, 2016; Diaz et al., 2018; Donaldson et al., 2018).

Gardasil®, approved by the FDA in 2006, is a tetravalent vaccine composed of VLPs of the recombinant L1 protein of HPV types 6, 11, 16, and 18. VLPs of each HPV type are produced in a separate culture of *Saccharomyces cerevisiae,* adsorbed onto aluminum hydroxyphosphate sulfate, combined, and formulated for intramuscular administration (Jennings and Bachmann, 2008; Fuenmayor et al., 25; Mohsen et al., 2017; Roldão et al., 2010; Tornesello et al., 2022).

Cervarix®, licensed in Europe in 2007, is a bivalent vaccine against HPV types 16 and 18. In this case, a recombinant baculovirus containing the L1 protein was used to infect *Trichoplusia ni* insect cells. The purified L1 protein is assembled into VLPs, which are adjuvanted with AS04 (Jennings and Bachmann, 2008; Fuenmayor et al., 2017; Mohsen et al., 2017; Roldão et al., 2010; Tornesello et al., 2022).

Gardasil® 9, licensed in the USA in 2014, is a variation of Gardasil® that covers nine HPV types (6, 11, 16, 18, 31, 33, 45, 52, and 58) (Mohsen et al., 2017; Tornesello et al., 2022). Gardasil® and Cervarix® are still in clinical trials in some countries (Roldão et al., 2010). The results show that they induce not only protection against the HPV serotypes included in the vaccine, but also some response to others not included (Smith et al., 2007; Harari et al., 2016). Another clinical study is comparing the immunogenicity of fractional doses (one-fifth of the standard dose) of Cervarix® and Gardasil® 9 (NCT04235257) (Tornesello et al., 2022).

The main limitation of commercialized vaccines is their high production costs (Tornesello et al., 2022), which most developing countries cannot afford. Xiamen Innovax Biotech Co., Ltd. (Innovax) has recently developed a bivalent VLP vaccine against HPV types 16 and 18 using an *Escherichia coli* expression system (Qian et al., 2020). Clinical trials show excellent safety and similar induction of neutralizing antibodies to the reference vaccines (Gu et al., 2017; Qiao et al., 2020). It has been first marketed in China in 2019 under the name Cecolin®.

Gardasil®, Cervarix®, Gardasil® 9, and Cecolin® have received WHO prequalification, which ensures they meet international quality, safety, and efficacy standards, and accelerates their introduction to the global public vaccine market (Regan and Graham, 2021).

Additional clinical studies would help to expand vaccine use, decrease costs, and address supply needs (Kuter et al., 2021). Some studies are looking for a therapeutic vaccine against cervical cancers related to HPV (Chen et al., 2011; Cai et al., 2022). In addition, the production of HPV VLPs in transgenic plants (potatoes) has been achieved by two groups (Biemelt et al., 2003; Garcea and Gissmann, 2004; Warzecha et al., 2003; Naupu et al., 2020). This would open the possibility of developing edible vaccines, which would be very beneficial for patient comfort.

2.2.3. Hepatitis E virus

Innovax has developed HEV 293, a VLP-based vaccine produced in *E. coli* using a truncated form of the viral capsid protein of the hepatitis E virus (HEV). It showed both high immunogenicity and safety after intramuscular administration in clinical trials (Zhu et al., 2010) and was approved in China in 2011 under the trade name Hecolin® (Fuenmayor et al., 2017; Gomes et al., 2017; Donaldson et al., 2018; Roy and Noad, 2008).

2.2.4. Malaria

GlaxoSmithKline developed the first advanced vaccine against the pre-erythrocytic stage of *Plasmodium falciparum* (Hawkes, 2015). RTS, S/AS01 vaccine (Mosquirix®) is produced by fusing parts of the secreted circumsporozoite protein (CSP) of the parasite to the surface of HBV VLPs. It was produced in yeast and formulated with AS01 adjuvant. Phase III clinical trials showed that three monthly intramuscular administrations of Mosquirix® to children from 6 weeks to 17 months old help to protect them against malaria (RTS SCT Partnership, 2012; RTS SCT Partnership, 2015). Although it is still not approved by FDA and EMA, the latter issued a positive opinion for its use outside the European Union (European Medicines Agency, 2015). Currently, Mosquirix® is administered to populations where malaria is prevalent. At the same time, several clinical trials evaluating Mosquirix® are still ongoing. In September 2022, it has been prequalified by the WHO, being the first case for a malaria vaccine (GSK, 2022).

A VLP-based vaccine, named R21, showed positive results in phase I/IIa clinical trials. In contrast to Mosquirix®, here the CPS antigen is directly fused to HBsAg, so the ratio CSP/VLP is higher. Moreover, it is formulated with potent adjuvants (Matrix-M and Abisto-100) (Plummer and Manchester, 2011). A phase I/IIb clinical trial in children in Burkina Faso (NCT03896724) suggests that vaccination with R21/Matrix-M could have a significant impact in areas of highly seasonal malaria transmission in Africa (Cornuz et al., 2008). This trial has progressed to phase III (NCT04704830). To assess the safety and immunogenicity of R21/Matrix-M in Asian populations, a phase II clinical trial in Thai adults was planned in February 2022 (NCT05252845).

MalariVax (ICC-1132) is a candidate vaccine produced in *E. coli* where CSP is fused to the surface of hepatitis B core antigen (HBcAg)-VLPs (Nauck et al., 2021). The results of a phase I trial demonstrated clinical efficacy against malaria (Torresi et al., 2011).

Finally, there are some virosome formulations for malaria vaccination under clinical evaluation. PEV301 and PEV302 are virosomal formulations containing synthetic peptides derived from apical membrane antigen-1 and CSP from *Plasmodium falciparum*, respectively. Vaccination of healthy semi-immune adults and children with a combination of PEV301 and PEV302 produced a significant decrease in malaria incidence without important adverse effects (Passalacqua et al., 2018).

2.2.5. Hepatitis A virus

Epaxal®, commercialized in 1994 (Herzog, 2006) consists of formalin-inactivated hepatitis A antigen adsorbed to the surface of IRIVs (Bovier, 2008). The substitution of aluminum-based adjuvants by IRIVs resulted in similar immunogenicity but fewer local reactions (Holzer et al., 1996). After a single injection, Epaxal® is highly immunogenic

and well-tolerated (Bovier, 2008).

The formulation for children, called HAVpur® Junior or Epaxal® Junior, has shown great potential for mass vaccination programs (Van Der Wielen et al., 2007). A phase IV clinical trial in India reported high immunogenicity and safety (Jain et al., 2015).

2.2.6. *Influenza virus*

A virosome-based vaccine against influenza was first marketed in Switzerland in 1997 under the name Inflexal® V. Currently, it is approved in many countries, in some of them with a different name: InfectoVac Flu® in Germany, Isiflu® V in Italy, and Viroflu® in the United Kingdom (Herzog et al., 2009). It is a trivalent vaccine that results from the mixture of three monovalent virosome pools, each of them carrying the specific HA and NA from the influenza strains annually recommended by the WHO and the EMA (Mischler and Metcalfe, 2002; Gerdil, 2003). This is the only adjuvanted influenza vaccine that has been licensed for all age groups, showing excellent tolerability and high immunogenicity, not only in healthy adults but also in immunocompromised individuals (Herzog et al., 2009).

Alternative targets to HA and NA, such as the influenza M2 gene with a low mutation rate eliminate the requirement for annual evaluations of influenza vaccines (Kaiser, 2006). Although M2 does not self-assemble into VLPs, it can be displayed on the surface of recombinant VLPs to increase its immunogenicity (Neirynck et al., 1999). Sanofi Pasteur developed a VLP vaccine based on HBcAg-VLPs produced in *E. coli* and decorated with M2 peptides from the influenza A virus on their surface (Roldão et al., 2010). Phase I clinical trials show that this vaccine, ACAM FLU-A, induces seroconversion to M2 ectodomain antigens, especially when adjuvanted with Stimulon QS 21 (Fiers et al., 2009).

The pharmaceutical company Novavax developed a trivalent vaccine based on influenza VLPs, including the following strains: H1N1 A/Brisbane/59/2007, H3N2 A/Brisbane/10/2007, and B/Florida/04/2006. They reported positive results in preclinical and clinical trials (Roldão et al., 2010). Novavax also developed a quadrivalent influenza vaccine, called NanoFLu, produced in the baculovirus-insect cell expression system and adjuvanted with Novavax's patented saponin-based Matrix-M™. Phase 3 clinical trials comparing NanoFLu and Fluzone, an inactivated tetravalent influenza vaccine, demonstrated enhanced humoral and cellular immune responses with NanoFLu and a similar safety profile (Shinde et al., 2022).

2.2.7. *Norwalk virus*

The main capsid protein of the Norwalk virus (VP1) undergoes spontaneous assembly into VLPs when expressed in *Spodoptera frugiperda 9* (*Sf9*) insect cells. NV-VLPs have also been produced in transgenic plants (potatoes, tomatoes) (Jennings and Bachmann, 2008; Fuenmayor et al., 2017). In phase I clinical trials, oral administration of NV-VLPs, either in water or in transgenic potatoes, induced mucosal IgA production (Ball et al., 1999; Tacket et al., 2000; Tacket et al., 2003).

LigoCyte Pharmaceuticals Inc. is developing two formulations of NV-VLPs as vaccine candidates (Roldão et al., 2010). One of them is a dry powder composed of lyophilized VLPs combined with chitosan and GlaxoSmithKline's MPL adjuvant. Phase I clinical studies showed good tolerance and high immunogenicity after intranasal administration (El-Kamary et al., 2010). The other one is a liquid containing VLPs, alum, and MPL for intramuscular administration (WO2013009849A1).

2.2.8. *Human parvovirus*

VLPs composed of VP1 and VP2 proteins of human parvovirus B19 can be produced in a baculovirus-insect cell expression system (Kajigaya et al., 1991). A phase I/II clinical trial in combination with MF59 adjuvant had to be halted because of the development of rashes in some participants (Shira et al., 2011). Due to the possible relation between this adverse effect and the phospholipase A2 activity of the VP1 protein, a parvovirus B19 VLP vaccine lacking phospholipase A2 activity has been produced in human embryonic kidney 293T cells. Results of preclinical trials showed a decrease in inflammatory responses in mice without affecting immunogenicity (Suzuki et al., 2022). Therefore, these mutant B19 VLPs could be good candidates for human vaccination.

2.2.9. *Chikungunya virus*

A phase II clinical study (NCT02562482) evaluated the safety and immunogenicity of a Chikungunya VLP-based vaccine (VRC—CHKVLP059–00-VP) composed of E1, E2, and capsid proteins of Chikungunya virus. The administration of two intramuscular injections separated by a period of 28 days was safe and induced high titers of specific antibodies (Chen et al., 2020).

2.2.10. *Human immunodeficiency virus*

A vaccine candidate was developed by fusing the HIV structural proteins p17 or p24 to Ty VLPs (Adams et al., 1987). A phase I study reported that the vaccine was immunogenic and well tolerated in healthy volunteers (Weber et al., 1995). Despite increasing CD4 counts in asymptomatic seropositive subjects, significant humoral or cellular responses were not achieved in a pilot phase II clinical trial (Peters et al., 1997).

2.2.11. *SARS-CoV-2*

Vaccines against SARS-CoV-2 target mainly the spike (S) protein, and more specifically, its receptor-binding domain (RBD), which is associated with increased transmissibility of the virus (Prates-Syed et al., 2021; Sharifzadeh et al., 2022). At least six VLP-based vaccines developed against SARS-CoV-2 have reached clinical trials.

Medicago developed the CoVLP, displaying the stabilized prefusion form of the S protein, and adjuvanted with AS03 from Glaxo Smith Kline. After the administration of two doses, volunteers showed higher neutralizing antibody titers in serum than convalescent individuals (Lehner et al., 1992). CoVLP has reached phase II/III trials (NCT04636697).

VBI Vaccines used its proprietary technology for the development of enveloped VLPs, and produced two monovalent VLP-based vaccines containing the S protein in its prefusion state: VBI-2902, directed against the ancestral/Wuhan SARS-CoV-2 strain, and VBI-2905, directed to the beta (B.1.351) variant. Both are adjuvanted with aluminum phosphate, and they have completed phase I trials (NCT04773665).

The Radboud University together with AdaptVac developed the ABNCoV2 candidate, consisting of the RBD from the S protein displayed in a bacteriophage scaffold using SpyTag/SpyCatcher technology. Basically, the RBD was genetically fused to the Catcher and expressed in *Drosophila melanogaster* Schneider 2 insect cell system (ExpreS2). On the other hand, the Tag was added to the capsid protein of *Acinetobacter phage AP205* and expressed in *E. coli.* Finally, both proteins were mixed, resulting in a VLP displaying the RBD. Moreover, during its production in *E. coli*, the VLP encapsulates bacterial RNA that may serve as a Th1-type adjuvant via Toll-like receptors (TLRs) 7/8 (Fougeroux et al., 2021). The vaccine is currently undergoing phase II/III clinical trials (NCT05077267, NCT05329220).

SpyBiotech, in collaboration with the Serum Institute of India (SIIPL), developed another vaccine candidate that uses the SpyTag/SpyCatcher technology to display the RBD antigen on HBsAg-VLPs. The construct is produced in a yeast expression system. This candidate is in phase I/II trials in Australia (ACTRN12620000817943). One of them (ACTRN12620001308987), has stopped early since the test vaccine was no longer developed for commercial reasons.

The Scientific and Technological Research Council of Türkiye (TÜBİTAK) has developed a VLP-based vaccine including the four structural proteins of SARS-CoV-2 (S, E, M, and N proteins). This vaccine has completed phase II trials (NCT04962893) (Mandal, 2021).

Yantai Patronus Biotech developed the LYB001 candidate, consisting of the RBD antigen displayed on a VLP vector, which has also reached phase II/III trials (NCT05663086, NCT05602558).

Once past the urgent need for vaccine candidates of the COVID-19

pandemic, efforts have been directed towards the rational design of versatile platforms that enable rapid responses to new variants. Also, the development of broad-spectrum vaccines against different coronaviruses might help to deal with future pandemics. In this sense, VBI Vaccines has developed a trivalent vaccine (VBI-2901) that uses enveloped VLPs to express the S proteins of SARS-CoV-2, SARS-CoV, and MERS-CoV (Sharifzadeh et al., 2022). After successful results in the preclinical phase, it has entered phase I clinical trials (NCT05548439).

Regarding virosome-based vaccines, some candidates contain the RBD antigen from the S protein of SARS-CoV-2 integrated into the lipid membrane of virosomal nanoparticles. One of these candidates is formulated as a thermostable powder vaccine that can be stored at 4 °C or even higher temperatures. It is intended for intranasal administration, aiming to induce mucosal immunity in the upper respiratory tract. Up to now, it has shown safety and both humoral and cellular immune responses in rats (Cremona et al., 2022). However, to our knowledge, no virosome-based candidates have entered the clinical phase up to now.

2.2.12. Veterinary infections

Although most commercialized vaccines are based on conventional platforms, veterinary vaccinology research is increasingly focusing on subunit vaccines and, especially, in VLPs. In veterinary vaccination, the lower doses of antigen required in VLP vaccines in comparison to monomeric ones is a key factor, since the cost of a vaccine needs to be compared to the value of the animal (Crisci et al., 2012).

There are two commercialized VLP-based veterinary vaccines, both against porcine circovirus type 2. The first one, Porcilis PCV, consists of VLPs expressing the ORF2 capsid protein of porcine circovirus type 2 in a baculovirus-insect cell expression system, assembled, and formulated with alfa-tocopherol and a liquid paraffin-based adjuvant (Fan et al., 2007). It was approved for vaccinating pigs at three days of age (Shirbaghaee and Bolhassani, 2016; European Medicines Agency, 2009). The latest one, called Ingelvac CircoFLEX, is based on the same antigen and expression system but adjuvanted with an aqueous polymer (Impran-FLEX). However, it has still not been demonstrated whether this recombinant protein assembles into VLPs (Crisci et al., 2012).

Several VLP-based veterinary vaccines are undergoing clinical trials. Some examples are VLPs from parvovirus (Ju et al., 2011), mink enteritis virus (Wu et al., 2020), chicken anemia virus (Tseng et al., 2019), Newcastle disease virus (Park et al., 2014), influenza virus (El-Husseiny et al., 2021), and Bluetongue virus (Pérez de Diego et al., 2011). In addition, human, plant, or animal VLPs are being studied for the display of animal virus antigens (Crisci et al., 2012).

2.3. ViP NPs in therapeutic vaccination

Our immune system has evolved to avoid reactions against self-antigens. However, many chronic diseases are related to the accumulation of self-proteins or the excessive production of inflammatory cytokines (Gomes et al., 2017). In these cases, overcoming self-tolerance would let our immune system fight the disease. Monoclonal antibodies are a successful strategy, but their high costs of production limit their application. Therapeutic vaccination is a cost-effective alternative approach (Gomes et al., 2017).

ViP NPs are great candidates as vehicles for the presentation of self-antigens (Yan et al., 2015). The ordered and repetitive antigen presentation allows to overcome B-cell tolerance, thus inducing the production of antibodies to self-proteins implicated in the disease (Yan et al., 2015; Jennings and Bachmann, 2008; Neek et al., 2019; Jeong and Seong, 2017). Up to now, there are no ViP NP-based products in the market for therapeutic vaccination, but great strides have been made in the preclinical and clinical settings. In this section, we will review the ViP NP-based vaccines undergoing clinical evaluation for therapeutic purposes, which are summarized in Table 3.

2.3.1. Cancer

Cancer vaccines aim to induce specific CTL responses toward cancer cells. To do so, it is necessary to stimulate the immune system against TAAs (Neek et al., 2019). Therapeutic vaccines may have several advantages over chemotherapy (Koudelka et al., 2015), such as possible outpatient administration, fewer side effects, avoidance of drug resistance, and long-term memory. However, cancer vaccines consisting of

Table 3
ViP NP-based therapeutic vaccines currently in clinical trials.

Name	Application	Antigen	ViP NP type	Phase	Registration number	Status
CYT004-MelQbG10	Malignant melanoma	Melan-A/Mart1	Qß-VLP	II	NCT00651703	Completed
				IIa	NCT00306514	Completed
				IIa	NCT00306566	Completed
				II	NCT00306553	Completed
Vidutolimod (CMP-001)	Pancreatic cancer	No TAA	Qß-VLP	Ib/II	NCT04387071	Terminated (drug no longer available)
	Melanoma			II/III	NCT04695977	Active, not recruiting
				II	NCT04698187	Active, not recruiting
				Ib	NCT03084640	Completed
	Cervical cancer	E7 oncoprotein	L1 HPV-VLP			
PEV7	Recurrent vulvovaginal candidiasis	Aspartyl proteinase-2 from *Candida albicans*	Virosome	I	NCT01067131	Completed
CAD106	Alzheimer's disease	Beta-amyloid(1–6) peptide	Qβ-VLP	IIb	NCT01097096	Completed
				II/III	NCT02565511	Terminated
CYT006-AngQb	Hypertension	Modified angiotensin-II peptide	Qβ-VLP	I/II	NCT00500786	Completed
CYT002-NicQb	Nicotine dependence	Nicotine	Qβ-VLP	II	NCT00369616	Completed
CYT013-IL1bQb	Type II diabetes	Interleukin-1β	Qβ-VLP	I	NCT00924105	Completed
PEV2A PEV2B	Chronic hepatitis C	One CD4 epitope and two CD8 epitopes	Virosome	I	NCT00445419	Completed
CYT003-QbG10	House dust mite allergy	No allergen	CYT003-QßG10 VLP	II	NCT00800332	Completed
	Allergic bronchial asthma			II	NCT00890734	Completed
CYT005-AllQbG10	House dust mite allergy	House dust mite allergen extract	CYT003-QßG10	I/II	NCT00652223	Completed
				II	NCT00574704	Completed
				II	NCT00574223	Completed
CYT005-AllQbG10	Grass pollen allergy	Grass pollen allergen	CYT003-QßG10	I/II	NCT00293904	Completed
Qb-Der p 1		Synthetic sequence of allergen Der p 1	Qβ-VLP	I		
Fel-CuMV (HypoCat™)	Cat allergy sympthoms	Major cat allergen Fel d 1	CMV-VLP	Not applicable	NCT03089788	Completed

tumor antigens are unable to overcome self-tolerance (Neek et al., 2019). To increase their efficacy, several strategies have been proposed, including the use of whole tumor lysates (Reyes et al., 2013), the addition of adjuvants (Dubensky and Reed, 2010), or their formulation in carriers (Park et al., 2013; Krishnamachari et al., 2011).

A VLP-based vaccine against melanoma, called Qß(G10)-Melan-A, was developed by linking the melanoma antigen Melan-A/Mart1 to Qß-VLPs and packaging inside G10 CpG. A phase I/II trial in stage II/IV melanoma patients revealed the generation of Melan-A-specific T-cell responses after subcutaneous or intradermal administration (Speiser et al., 2010). The addition of topical imiquimod enhanced central memory CD8+ T cell responses, allowing a more sustained immune response (Goldinger et al., 2012).

Another candidate, CMP-001, consisting of Qß-VLPs loaded with a TLR-9 agonist (A-type CpGs) but without any TAA, is being evaluated in clinical trials for melanoma and pancreatic cancer. The first results in melanoma patients demonstrated that CMP-001 combined with pembrolizumab can overcome PD-1 blockade resistance for long periods (Ribas et al., 2021). A phase Ib/II clinical trial in patients with stage IV pancreatic cancer (NCT04387071) receiving CMP-001 combined with the monoclonal antibody INCAGN01949, which targets the costimulatory receptor OX40, has been reported to delay tumor growth.

Regarding cervical cancer, a chimeric VLP was developed by conjugating HPV-VLPs made up by the self-assembly of L1 protein together with E7 oncoprotein (Greenstone et al., 1998; Schäfer et al., 1999). The results of the clinical trials show high titers of anti-L1 antibodies but low titers of anti-E7 antibodies. Still, the vaccine shows adequate CTL responses against both proteins and a safe profile (Kaufmann et al., 2007).

VLP-based therapeutic vaccine candidates have been developed against other types of cancers, such as breast cancer or hepatocellular carcinoma. Although several preclinical studies are showing promising results, no candidate has reached clinical trials up to now.

2.3.2. Recurrent vulvovaginal candidiasis

Pevion Biotech developed a virosome-based vaccine candidate against recurrent vulvovaginal candidiasis. PEVION7 (PEV7) vaccine consists of virosomes displaying aspartyl proteinase-2 from *Candida albicans* on their surface (De Bernardis et al., 2015). Two dosage forms were investigated in a phase I clinical trial (NCT01067131): an intravaginal capsule (PEV7C) and a reconstituted lyophilizate for intramuscular application (PEV7B). All the vaccinated women generated specific B-cell memory. Both intravaginal and intramuscular administration showed a rapid and specific response in serum and/or in cervicovaginal secretions. These results encourage the potential of the vaccine as a therapeutic candidate (De Bernardis et al., 2015).

2.3.3. Alzheimer's disease

Novartis Pharmaceuticals has developed a therapeutic vaccine called CAD106, consisting of beta-amyloid(1–6) peptide covalently linked to Qβ-VLPs (Jennings and Bachmann, 2008). Phase II clinical trials in patients with mild Alzheimer's disease showed that CAD106 was well tolerated and induced strong antibody responses against the amyloid beta peptide (Vandenberghe et al., 2017).

A phase III study was initiated to test whether CAD106 or CNP520, a beta-site amyloid precursor protein cleaving enzyme 1 inhibitor, each administered to a different cohort of patients, could delay the onset and progression of clinical symptoms in people at risk of developing Alzheimer's disease (NCT02565511). Unfortunately, the study was terminated due to unexpected changes in cognitive function, and loss of brain volume and body weight. Although these effects were associated with CNP520, the study arm treated with CAD106 was also terminated.

2.3.4. Hypertension

A vaccine targeting components of the renin–angiotensin–aldosterone system could be useful to downregulate blood pressure (Michel, 2004). An anti-angiotensin II VLP vaccine was produced through the conjugation of a modified angiotensin-II peptide to Qβ VLPs (Ambühl et al., 2007). After three subcutaneous injections of the VLP, combined with an aluminum hydroxide wet gel suspension adjuvant, a significant reduction in ambulatory blood pressure was observed (Tissot et al., 2008). This was the first report of a VLP-conjugate vaccine able to overcome B-cell tolerance in humans, inducing antibodies against angiotensin II (Ambühl et al., 2007).

2.3.5. Nicotine dependence

Current strategies to tackle nicotine dependence, based on replacement therapies or drugs such as bupropion and varenicline, have limited efficacy. Vaccination to induce antibodies against nicotine emerges as an alternative therapeutic approach. The antibodies would capture nicotine in the blood forming a large complex that would be unable to cross the blood-brain barrier, thus restricting nicotine effects in the brain (Jennings and Bachmann, 2008).

Nicotine itself is not immunogenic, so it needs to be linked to a larger protein to trigger antibody production (Jennings and Bachmann, 2008). Nicotine has been covalently coupled via a succinate linker to the surface of Qβ-VLPs at a nicotine:VLP ratio of 580 to 1 (Maurer et al., 2005). This multivalent display of nicotine resulted in the induction of neutralizing antibodies in phase I and II clinical trials (Maurer et al., 2005; Cornuz et al., 2008). Novartis Pharmaceuticals possesses a manufacturing and commercial license for this nicotine-VLP vaccine (Jennings and Bachmann, 2008).

2.3.6. Type II diabetes

Type II diabetes prevalence is continuously increasing around the globe. Despite the approval of novel drugs, the number of patients with controlled diabetes is still low, and new strategies are being sought (Nauck et al., 2021). Several investigations are focusing on interleukin-1β, a key cytokine involved in inflammatory illnesses, including type II diabetes. A therapeutic vaccine composed of Qβ-VLPs coupled to interleukin-1β has completed phase I clinical trials (NCT00924105).

2.3.7. Hepatitis C virus

Pevion Biotech developed an innovative virosome-based vaccine against chronic hepatitis C. PEV2A PEV2B is a modular therapeutic vaccine that results from combining two platforms (PeviPRO and PeviTER) using synthetic peptide antigens from the hepatitis C virus. PeviPRO (PEV2A) consists of a virosome-formulated CD4 epitope that provides a helper T cell response. PeviTER (PEV2B) consists of two virosome-formulated CD8 epitopes that induce a specific CTL response. In June 2008, the company completed a phase I clinical trial of this vaccine (NCT00445419) (Torresi et al., 2011).

2.3.8. Allergy

The only available therapeutic strategy against type I allergies is allergen-specific immunotherapy (AIT), which pursues the induction of tolerance through an adaptive allergen-specific immune response. This could be reached by subcutaneous or oral administration of increasing doses of allergen extracts and their efficient uptake by APCs (Passalacqua et al., 2018). The main limitations of AIT are: (i) the risk of anaphylactic reactions; (ii) the long duration of the treatment; (iii) the low quality of allergen extracts; and (iv) the poor efficacy in polysensitized subjects (Klimek et al., 2013). AIT can take advantage of VLPs, either alone or combined with an allergen, to overcome these problems (Anzaghe et al., 2018).

There are two strategies to induce tolerance to an allergen using VLPs alone: (a) to package TLR-9 ligands into the VLPs, or (b) to display cytokines on their surface (Anzaghe et al., 2018). All studies with VLPs for AIT use the CYT003-QßG10 VLP developed by Cytos Biotechnology (Klimek et al., 2011; Beeh et al., 2013). It consists of a Qß-VLP where G10 CpG is encapsulated during assembly. These VLPs have reached phase II clinical trials. The VLPs were well tolerated and reduced allergic

symptoms in patients suffering from house dust mite allergy (NCT00800332) and from persistent allergic asthma (NCT00890734) (Klimek et al., 2011).

VLPs can also be combined with allergens: they can be packaged into the VLPs, displayed on their surface, or simply mixed with them. The objective is to activate allergen-specific CD8+ T cells to downregulate IgE responses (Anzaghe et al., 2018). The immunotherapeutic effect of CYT003-QßG10 was also studied in clinical trials in combination with allergen extracts such as grass pollen allergen (NCT00652223) or house dust mite extract (NCT00293904, NCT00574704, NCT00574223). In patients with house dust mite allergy, that combination showed a significant reduction of rhinitis and allergic asthma symptoms (Senti et al., 2009). In a phase I trial on healthy volunteers, the house dust mite allergen Der p 1, covalently coupled to Qβ-VLPs, was safe and highly immunogenic (Kündig et al., 2006).

An innovative approach consisted of the vaccination of cats with FelCuMV (HypoCat™) to treat cat-induced allergies to their owners. The vaccine consisted of the major cat allergen Fel d 1 displayed on CMV-VLPs. Cats developed neutralizing antibodies, resulting in lower levels of the allergen and, consequently, a reduction of allergic symptoms in the owners (Thoms et al., 2020).

Overall, VLPs seem to be good candidates for AIT. Efficacy seems higher if the allergen is displayed on the surface of the VLP rather than packaged inside. In the future, it is expected that VLPs will co-deliver allergens, immune-stimulating proteins, and adjuvants to a single APC. Through these combinations, they would restore the balance of immune responses towards a tolerogenic state by boosting specific responses without inducing bystander activation of specific Th2 responses (Anzaghe et al., 2018).

3. ViP NPs for gene/drug delivery

An ideal vector for the delivery of cargo to specific cells should remain stable in the bloodstream and, after extravasation, target the intended cells without affecting the rest. In many cases, it is also desirable that the vector can escape the endosome and release the intact cargo in the cytosol (Rohovie et al., 2017). A wide variety of synthetic nanoparticles have been developed to address these challenges, but most of them show problems related to physicochemical heterogeneity, instability in physiological conditions, low functionality, and/or toxicity (Diaz et al., 2018). On the other hand, viruses have been extensively used as vectors for drugs or genes owing to their natural role as nucleic acid vehicles, but their small packaging capacity and safety concerns for both patients and manufacturers limit their use (Thomas et al., 2003). At the interface between viruses and synthetic nanoparticles, ViP NPs can take advantage of both systems and fit better the demands of the ideal vector.

In this section, we will explore the inherent properties of ViP NPs that position them as promising candidates for drug/gene delivery. Additionally, we will provide an in-depth analysis of the latest advancements in the development of ViP NPs for delivering various types of molecules, including small drugs, proteins, and nucleic acids. To provide a comprehensive overview, we have summarized the ViP NP-based delivery systems currently undergoing preclinical evaluation in Table 4.

VLPs self-assemble creating homogeneous populations, are thermodynamically stable, allow flexible functionalization, and are biocompatible and nontoxic (Yan et al., 2015; Ding et al., 2018). Some disadvantages of VLPs as delivery systems, such as phagocyte-mediated clearance and difficult extravasation of icosahedral VLPs from blood vessels, can be addressed by functionalizing VLP surface (Ding et al., 2018).

The advantages of virosomes over viruses and synthetic nanoparticles as drug delivery systems include their biocompatibility and biodegradability, and ease of production, modification, and scale-up (Kalra et al., 2013; Khoshnejad et al., 2007). Virosomes share many properties with liposomes, but the presence of glycoproteins on the virosomal surface endows them with additional properties. The most significant is the potential induction of immune responses, which is beneficial for vaccination but not for drug delivery purposes. This problem can be minimized by surface coating with hydrophilic polymers such as polyethylene glycol (PEG) (Sabu et al., 2018). Besides, while liposomes are rapidly cleared by the reticuloendothelial system, virosomes usually protect the encapsulated agent until they reach their target cells (Liu et al., 2015).

Table 4
ViP NP-based systems under preclinical evaluation for drug/gene delivery.

Application	Cargo	External moieties	Vehicle
Glioblastoma	Doxorubicin	PEG	MS2-VLPs
Glioblastoma	Doxorubicin	PEG	TMV-VLPs
Breast cancer	Doxorubicin	Fab' fragments of anti-rat Neu monoclonal antibody	Virosomes
Prostate cancer	Decitabine	–	EMHVs (Erythro-Magneto-Hemagglutinin Virosomes)
Arterial thrombosis	–	PEG-tPA (tissue plasminogen activator)	TMV-VLPs
Base editing: treatment of genetic diseases	Therapeutic ribonucleoproteins	–	Engineered VLPs (Friend murine leukemia virus/ vesicular stomatitis virus)
Gene therapy	miR146a	HIV-1 Tat_{47-57} peptide	MS2-VLPs
Hepatocellular carcinoma	miR122	HIV-1 Tat_{47-57} peptide	MS2-VLPs
Glioblastoma	siRNA	Cell-penetrating peptide Apolipoprotein E peptide	Green fluorescent Qβ-VLPs
Gene therapy targeted to the liver	Plasmid DNA	–	Sendai virosomes
Vaccination/ Gene therapy	HIV DNA	–	HEV-VLPs
Vaccination	CpG ODNs	p33 peptide from lymphocytic choriomeningitis virus	HBcAg-VLPs Qβ-VLPs

3.1. Targeting and intracellular transport of VIP NPs

Both VLPs and virosomes may retain intrinsic targeting properties inherited from native viruses. For example, HEV has a selective tropism for the liver, so HEV-VLPs can be used as vehicles for specific delivery to the liver (Lee et al., 2019). Another example is the neurotropic JC polyomavirus (JCPyV), which infects glial cells and oligodendrocytes. Thus, JCPyV-VLPs have shown the ability to deliver genetic material to U87-MG cells in orthotopic xenograft models, exhibiting potential for the treatment of metastatic brain tumors (Chao et al., 2018). Moreover, some viruses target receptors that are upregulated in tumor cells. For example, canine parvovirus VLPs have intrinsic ability to target cells expressing transferrin receptors (Singh et al., 2006). The shape, size, and aspect ratio of the VLP also influence its targeting properties. Tubular or filamentous VLPs show enhanced flow and margination toward vessel walls, which eventually leads to reduced clearance and increased tumor accumulation (Fig. 4) (Shukla et al., 2013).

Virosomes also inherit targeting properties from the native virus, provided that these properties are imparted by the glycoproteins incorporated on their surface (Fig. 4). Thus, influenza virosomes and influenza viruses share cell binding and uptake, as well as endosomal escape properties, which are conferred by HA. This poses the challenge

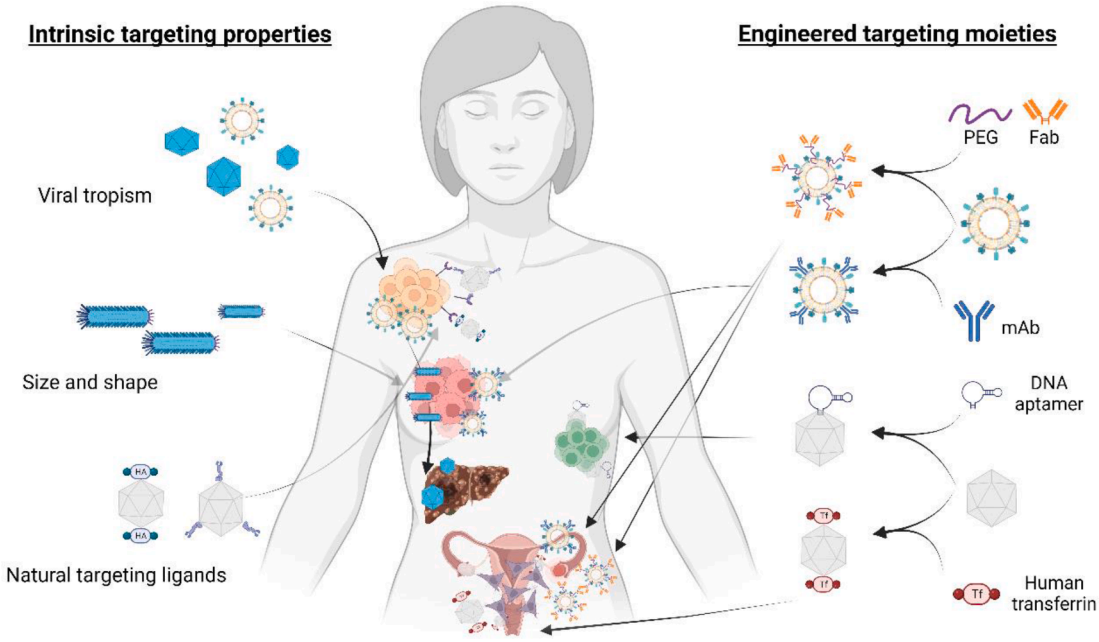

Fig. 4. ViP NPs as vehicles for targeted delivery of therapeutic agents. ViP NPs have intrinsic targeting properties derived from the viral tropism, their size and shape, and the presence of natural targeting ligands, such as hemagglutinin. Furthermore, additional targeting can be provided by modifying ViP NPs with different targeting molecules, including monoclonal antibodies (mAb), antigen-binding fragments (Fab), DNA aptamers, and ligands for overexpressed receptors, such as transferrin. Created with BioRender.com.

of maintaining the targeting properties of influenza virosomes while avoiding immune system recognition when looking for drug delivery applications (Khoshnejad et al., 2007). Another example of natural targeting properties is that of Sendai virosomes, which present specificity for hepatocytes due to the F protein on their surface (Bagai et al., 1993).

Targeting properties can also be artificially imparted to ViP NPs by functionalizing them with targeting ligands (Fig. 4). There are several examples of ligands conjugated to the surface of VLPs that target receptors overexpressed in specific cell types. Those ligands include small molecules such as folic acid, peptides as arginyl-glycyl-aspartic acid (RGD) sequences, proteins like antibodies, and polynucleotides like aptamers (Shirbaghaee and Bolhassani, 2016; Koudelka et al., 2015; Rohovie et al., 2017). For example, lactobionic acid conjugated to the surface of a rotavirus VLP allowed specific targeting of hepatocytes or hepatoma cells bearing asialoglycoprotein receptors (Zhao et al., 2011). MS2-VLPs displaying human transferrin on their surface were selectively internalized into HeLa cells (Galaway and Stockley, 2013). DNA aptamers attached to MS2-VLPs promoted the targeting of Jurkat leukemia T cells (Tong et al., 2009). Targeting ligands can also be genetically displayed on VLPs. This is the case of a chimeric M13-VLP displaying an αv-integrin-binding peptide for vascular targeting (Chen et al., 2004).

Virosomes can be easily modified to incorporate specific targeting ligands, including cytokines, peptides, monoclonal antibodies, and tumor-specific monoclonal antibody fragments (Liu et al., 2015; Kalra et al., 2013). There are successful examples of virosomes tailored to target tumor cells, erythrocytes, hepatocytes, and cells of the respiratory and gastrointestinal systems (Liu et al., 2015). Several proof-of-concept studies demonstrated that it is possible to provide specific targeting to influenza virosomes despite the relatively ubiquitous binding character of the viral HA to sialic acid residues (Khoshnejad et al., 2007). For example, influenza virosomes coated with PEG covalently linked to an antigen binding fragment that recognizes epithelial glycoprotein 2 fused with ovarian carcinoma cells in a pH-dependent manner (Mastrobattista et al., 2001). Monoclonal antibodies that bind specifically to TAAs can help to systemically administer virosomes and reach cancerous tissues. For example, monoclonal antibody fragments conjugated to the surface of doxorubicin-loaded virosomes targeted them to human cancer cells (Waelti et al., 2002). Also, affibody molecules of the human epidermal growth factor receptor 2 displayed on virosomes allow them to target breast and ovarian cancer cells (Nishimura et al., 2013). Additional innovative strategies are also arising such as the use of magnetic force to drive drug-loaded virosomes to tumors. Erythro-magneto-hemagglutinin virosomes (EMHVs) are a novel erythrocyte-based drug delivery system endowed with both super-paramagnetic nanoparticles inside the erythrocytes and HA glycoprotein on their membrane (WO2010/070,620). EMHVs have been used for the delivery of decitabine with promising results (Naldi et al., 2014).

Additionally, the delivery system must release its cargo into the cytosol or the cell nucleus according to the application of interest. This step usually poses important limitations related to inefficient uptake, endosomal entrapment, and cell damage.

Cellular uptake of nanoparticles commonly relies on endocytosis, which eventually leads to the destruction of the nanoparticles after the fusion of endosomes with lysosomes. Several strategies have been developed to promote the release of synthetic nanoparticles from endosomes before lysosomal degradation. Basically, they induce a destabilization of the endosomal membrane, after which the cargo may be released into the cytosol through pore formation, membrane rupture, or membrane fusion. To achieve membrane destabilization, cationic molecules and membrane-destabilizing peptides are frequently incorporated into the nanoparticles. Most strategies are based on viral and bacterial infection pathways since they have evolved to escape from endocytic degradation and deliver their genome into the cytosol within the infected cells (Martens et al., 2014).

As it occurs with synthetic nanoparticles, VLPs that are internalized through the endocytic pathway are exposed to the same risk. When a VLP is intended for the delivery of a small drug, the degradation of the vehicle in the lysosome may not affect the functionality of the drug. However, when more complex cargo is to be delivered, such as nucleic acids or proteins, lysosomal degradation more compromises their stability and functionality (Rohovie et al., 2017).

Researchers have exploited the ability of retroviruses to efficiently

deliver cargo intracellularly by synthesizing derived VLPs with similar transport characteristics (Kaczmarczyk et al., 2011). Another strategy widely studied is the display of cell-penetrating peptides, such as HIV-1 transactivator of transcription (TAT) peptide, on the VLP surface, which aids both in the initial cell uptake and in endosome escape steps (Anand et al., 2015). For example, MS2-VLPs (Pan et al., 2012; Wang et al., 2016) have been functionalized with the HIV-TAT peptide, and an arginine-rich R5 peptide has been displayed on CPMV-VLPs (Wu et al., 2012). Due to the cationic charge of these peptides, electrostatic interactions may be established with the negatively charged phospholipids of the endosomal membrane, resulting in its destabilization and, eventually, in endosomal lysis (Martens et al., 2014; Blanco et al., 2015).

Also, peptides containing protonatable secondary and/or tertiary amines, such as histidine-rich H5WYG peptide, have been displayed on MS2-VLPs (Ashley et al., 2011). In this case, the endosomal escape is produced through the "proton sponge effect". Basically, when the pH in the endolysosomal compartment decreases, the peptide acts as a buffering agent by absorbing protons. The electrochemical gradient created across the endolysosomal membrane results in an associated influx of water and counter-ions. As a result, there is a swelling that leads to the rupture of the endosome (Fig. 5) (Martens et al., 2014; Blanco et al., 2015).

Another kind of peptides that have been displayed on VLPs to promote endosomal escape are membrane fusion peptides. Such peptides are based on the HA2 peptide from the influenza virus. When pH decreases, the protonation of the peptide results in a conformational change to a helical structure that promotes the fusion of the virus with the endosomal membrane, thus releasing its content into the cytosol (Lear and DeGrado, 1987). Synthetic peptides have been engineered that mimic this behavior, such as the GALA peptide. It has been displayed, for example, on the surface of HBsAg-VLPs, conferring them the ability of endosomal escape (Nishimura et al., 2014).

In the case of virosomes, the fusion activity of glycoproteins is a key piece for the intracellular delivery of the cargo. Depending on the native virus, virosomes can fuse with the plasma membrane at a neutral pH or with the endosomal membrane at an acidic pH. For example, Sendai virosomes fuse at neutral pH, while influenza virosomes do it at a pH of 5.5–5.8 (Daemen et al., 2005).

HA plays a key role in receptor-binding and membrane-fusion of influenza virosomes. HA promotes receptor-mediated endocytosis of virosomes, and the acidic environment of the endosome triggers HA-mediated membrane fusion, allowing the therapeutic agent to reach the cytosol of the target cell. Based on this mechanism, virosomes displaying HA can significantly enhance the cytosolic delivery of their cargo (Fig. 5) (Kalra et al., 2013; Huckriede et al., 2005). Moreover, because virosomes can exit the endosomal compartment before lysosomes are formed, these systems can protect therapeutic agents from proteolytic and acid degradation in this compartment (Kalra et al., 2013). Therefore, virosomes are particularly useful for the delivery of nucleic acids and other macromolecules to the cell cytosol.

The fusogenic properties of virosomes can be compromised when these systems are PEGylated to avoid their immune recognition. However, these limitations can be solved by strategies that use a pH-sensitive PEG that is eliminated in the acidic conditions of endosomes. With this technology HA is exposed again in endo-lysosomes, restoring the endosomal escape properties of the system (Khoshnejad et al., 2007).

3.2. Delivery of small drugs

Most of the efforts on developing VLPs and virosomes as delivery vehicles of small drugs are destined to cancer treatment, and most use doxorubicin (DOX) as cargo. For example, DOX has been both encapsulated and attached to the surface of red clover necrotic mosaic virus-VLPs, promoting a bimodal release of the drug: a rapid release of surface DOX followed by a slow release of the encapsulated drug (Cao et al., 2014).

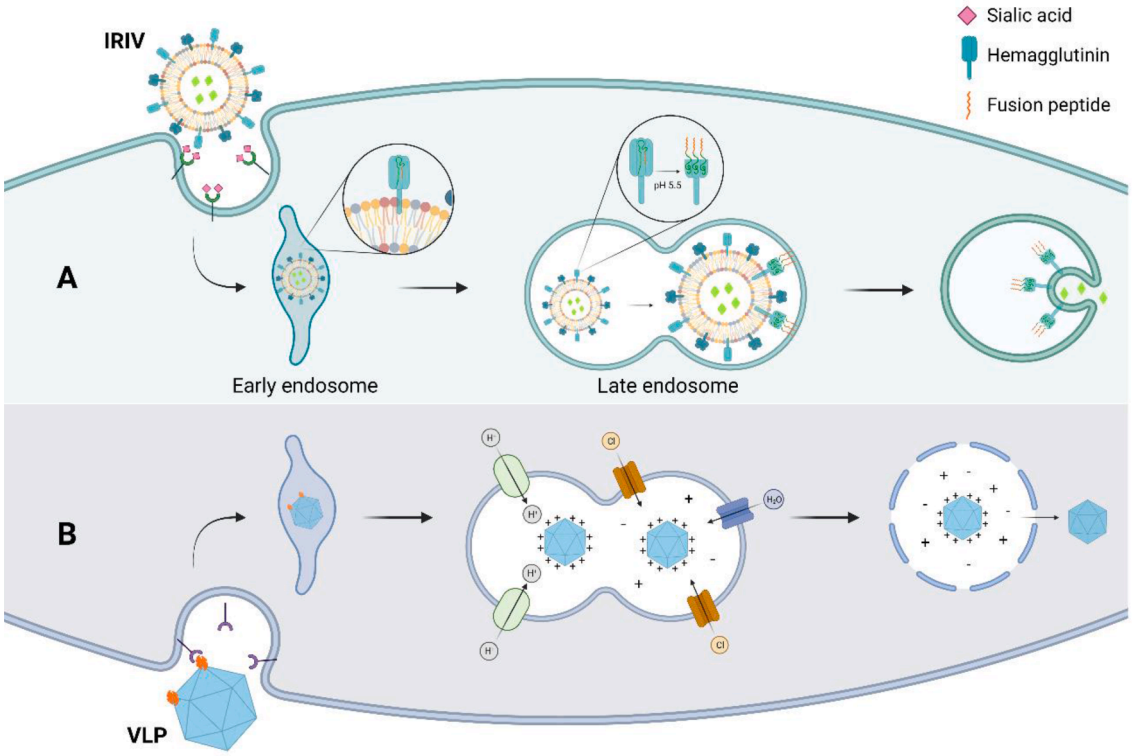

Fig. 5. Endosomal escape of ViP NPs. (A) Due to the fusion activity of the HA2 peptide, that suffers a conformational change when the pH inside endosomes decreases, IRIVs can deliver their cargo into de cytosol. (B) Model VLP displaying protonable secondary/tertiary amines that promote endosomal escape through the "proton sponge effect". Created with Biorender.com.

Furthermore, DOX-loaded VLPs capable of releasing their cargo in response to different stimuli have been designed. Qβ-VLPs displaying PEG and photocleavable DOX complexes on their surface showed controllable release and cytotoxic effects only after photoactivation (Chen et al., 2016). Truncated HBcAg-VLPs displaying folic acid and encapsulating DOX and polyacrylic acid delivered the antitumoral only upon reaching extracellular tumor tissues or intracellular endosomes with a pH of approximately 5–5.5. At pH 7.4 the interaction between DOX and polyacrylic acid prevented drug release (Biabanikhankahdani et al., 2016). Also, thermoresponsive VLPs were developed based on Macrobrachium rosenbergii nodavirus-VLPs displaying folic acid and loaded with DOX. DOX was released in a sustained manner, but the release rate increased at 43 °C compared to 37 °C. This was possibly due to weaker protein-protein and protein-RNA interactions that cause a flux of water molecules towards the VLP cavity, followed by swelling and bursting (Thong et al., 2019).

Convection-enhanced delivery (CED) of chemotherapeutics could also benefit from VLP vehicles as they allow higher penetration of the drug in the tumor tissue. DOX delivered by either bacteriophage MS2-VLPs or tobacco mosaic virus (TMV)-VLPs significantly increased the survival times of small tumor-bearing mice, with TMV-DOX treatment having the highest efficacy (Finbloom et al., 2018).

Other anticancer drugs have also been loaded into VLPs. For example, TMV-VLPs displaying mannose and lactose moieties and loaded with cisplatin showed enhanced cytotoxicity in MCF-7 and Hep G2 cancer cell lines (Franke et al., 2018).

As with VLPs, chemotherapeutic drugs are the most frequently encapsulated in virosomes. Waelti et al. were able to inhibit tumor progression in a mouse model with a virosome formulation encapsulating DOX and displaying phospholipid-PEG anchored antibodies for targeting (Waelti et al., 2002). Also, EMHVs have been used to encapsulate decitabine. This system resulted in improved drug pharmacokinetics and efficacy, and a further reduction of tumor mass in a xenograft model of prostate cancer as compared to the free drug (Naldi et al., 2014).

3.3. Delivery of peptides/proteins

Most VLPs displaying foreign peptides or proteins are for vaccination purposes and have been described before (Section 2). However, there is also research trying to take advantage of VLPs for the delivery of other therapeutic proteins.

One objective is the delivery of enzymes that catalyze the transformation of pro-drugs into their active form, with the final goal of increasing their efficacy/toxicity ratio. This is achieved by activating the drugs with the enzymes only at the target tissue, which reduces side effects and the required doses. A bacterial CYP variant encapsulated inside cowpea chlorotic mottle virus (CCMV)-VLPs was able to transform tamoxifen and resveratrol into active chemotherapeutics (Sánchez-Sánchez et al., 2014). P22-VLPs encapsulating a cytochrome P450 with enhanced peroxygenase activity, CYPBM3, was used to deliver this enzyme to human cervix carcinoma cells. These results supported the potential of biocatalytic VLPs for these promising therapeutic applications (Sánchez-Sánchez et al., 2015).

Cancer treatment is also being pursued by delivering cytotoxic peptides. P22-VLPs encapsulating two different peptides with synergistic cytotoxic effects were able to provide cell-specific release of this cargo. Concretely, this controllable release was triggered by cathepsin B, a protease overexpressed in many tumors (Wang et al., 2018).

The conjugation of peptides to the surface of VLPs can also be advantageous. Preclinical studies in mouse models of arterial thrombosis showed similar therapeutic efficacy of free tissue plasminogen activator (tPA) and tPA conjugated to TMV-VLPs. However, the safety profile was improved with the tPA-TMV formulation (Pitek et al., 2018).

An innovative approach that can benefit from VLPs is the therapy based on gene editing agents. The delivery of ribonucleoproteins instead of DNA would reduce off-target editing because of the short lifetime of ribonucleoproteins in cells. By delivering those proteins in VLPs, the possibility of viral vector integration into the genome of transduced cells is avoided. Banskota et al. developed an engineered VLP platform for packaging and delivering therapeutic ribonucleoproteins, with successful results both in vitro and in vivo (Banskota et al., 2022).

In the case of virosomes, the delivery of peptides and proteins has been restricted to antigens, a topic covered in detail in Section 3.

3.4. Delivery of nucleic acids

The internal surface of viral capsids contains basic polypeptide domains that neutralize the negative charge of nucleic acids, creating stable and nonspecific ionic interactions with these molecules (Garcea and Gissmann, 2004). This natural structure designed for the accommodation of polynucleotides makes VLPs promising candidates as vehicles for gene medicine.

Many examples of VLPs encapsulating nucleic acids are aimed at vaccination, for example through DNA vaccines. This is the case of an HIV DNA vaccine encapsulated in HEV-VLPs for oral administration (Takamura et al., 2004). In other vaccination modalities, polynucleotides can be introduced as adjuvants. For instance, the packaging of CpG ODNs into VLPs enhances their immunostimulatory properties while reducing their toxicity (Storni et al., 2004). Biddlecome et al. developed a vaccine/gene delivery platform encapsulating self-amplifying mRNA into CCMV-VLPs, and preliminary in vitro studies showed an increased effect compared to naked mRNA or VLPs alone (Biddlecome et al., 2019).

Besides vaccination, other applications of VLPs in gene delivery have been investigated. Systemic delivery of small interference RNA (siRNA) is limited by its poor stability and low cell penetration. To circumvent these problems, Shao et al. loaded siRNA into adeno-associated virus type 2-VLPs coated with polyethyleneimine (Shao et al., 2012). Similarly, the cellular delivery of microRNAs (miRNAs) is a major obstacle that might be addressed by using VLPs. An example of this is a carrier based on MS2-VLPs loaded with miR146a and displaying HIV-1 cell-penetrating peptide TAT on their surface. This carrier was able to induce miRNA expression both *in vitro* and *in vivo*, delaying the progression of systemic lupus erythematosus in mice, and repressing osteoclast differentiation (Pan et al., 2012). When MS2-VLPs were loaded with miR122, and TAT was genetically incorporated instead of chemically crosslinked, results were significantly better both *in vitro* and *in vivo* (Wang et al., 2016).

VLPs can also help to cross the blood-brain barrier and deliver their cargo into the brain. As a proof-of-concept, fluorescent VLP/siRNA nanocomplexes functionalized with a cell-penetrating peptide and apolipoprotein E demonstrated an adjuvant cytotoxic effect on U87 orthotopic brain tumors in mice (Pang et al., 2019).

Virosomes are particularly useful for delivering nucleic acids since they combine the capacity of liposomes to entrap DNA with the cell-binding and fusion properties of viruses (Daemen et al., 2005). Virosomes containing cationic lipids such as DOTAP, DODAC, or DOSPER may be preferred as they form stable complexes with the negatively charged groups of nucleic acids. Through this ionic complexation, the carriers might protect them and enhance their cellular uptake (Schoen et al., 1999; De Jonge et al., 2007).

Most studies of the virosomal delivery of nucleic acids use influenza or Sendai virosomes (Khoshnejad et al., 2007). Ramani et al. were the first to report the delivery of exogenous DNA by virosomes. They prepared virosomes derived from purified Sendai virus F-protein in the presence of plasmid DNA. Gene expression was detected both in vitro in HepG2 cells (Ramani et al., 1997) and *in vivo*, where transfection was restricted primarily to the liver (Ramani et al., 1998). Zakaria et al. took advantage of the hepatocyte-specificity of Sendai virosomes to deliver short hairpin RNA against oncogene c-Myc in hepatocarcinoma cells (Zakaria et al., 2014). The encapsulation of plasmid DNA in

reconstituted influenza virosomes protected DNA from degradation and increased transfection efficiency up to 90% of the available cells (De Jonge et al., 2007).

Apart from being encapsulated, DNA can also be transported on the virosome surface. Influenza virosomes containing 30% DODAC for DNA complexation showed high *in vitro* uptake and gene expression (Schoen et al., 1999). The same cationic lipid was used to increase the loading of siRNA into virosomes used for intraperitoneal injection. This system was able to deliver siRNA efficiently to cells at the injection cavity (de Jonge et al., 2006).

4. ViP NPs for diagnosis, biosensing, and disease monitoring

In vivo imaging using non-invasive techniques has great potential for early diagnosis and treatment of disease, leading to lower costs and higher quality of life (Lanza et al., 2002; Rudin and Weissleder, 2003; Boles et al., 2010; Van Oosten et al., 2013; Bakos et al., 2018; Nicolson et al., 2019; Baumann et al., 2021). Conventional methods are based on generalized imaging techniques that require the use of nonspecific dyes or image contrast agents (Rudin and Weissleder, 2003). By contrast, new techniques rely on targeting tissues of interest by identifying specific ligands.

Several strategies have been developed for *in vivo* imaging using specific targeting. The most used are computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and ultrasonography (Rudin and Weissleder, 2003; Key and Leary, 2014; McCarthy et al., 2020). More recently, optical strategies emerged as promising approaches. For example, near-infrared (NIR) fluorescent probes proved useful to image fluorescently-labeled nanoparticles *in vivo* (He et al., 2010; Lee et al., 2012).

4.1. Nanoparticles for in vivo imaging

Nanotechnology appears as a promising tool for *in vivo* imaging. It allows targeting specific ligands, displaying imaging moieties, and transporting therapeutic molecules. Therefore, diagnosis, therapy, and monitoring can be achieved simultaneously (Thoms et al., 2020; Mir et al., 2017). Several types of nanoparticles have been used for this "theragnostic" approach (Manchester and Singh, 2006), including virus-based nanoparticles (Sun et al., 2016; Low et al., 2016). Indeed, ViP NPs show many advantages as tools for in vivo imaging (Fig. 6). Targeting and imaging moieties can be displayed on the external surface of ViP NPs or encapsulated in their inner cavity, either by genetic fusion, chemical conjugation (Koudelka et al., 2015; Ding et al., 2018; Manchester and Singh, 2006), or infusion (Yildiz et al., 2013). Many molecules can be incorporated per particle, thus facilitating flow cytometry, confocal microscopy, and *in vivo* imaging (Sen et al., 2005; Robertson and Liu, 2012). For example, the genetic fusion of green fluorescent protein or mCherry to the coat protein of the potato virus X enabled the *in vivo* imaging of human tumor xenografts in mice (Shukla et al., 2014). Furthermore, it is possible to control the spacing and orientation of the displayed molecule. Thus, the sensitivity of detection can be modulated, and the signal-to-noise ratio can be adjusted (Koudelka et al., 2015). Finally, in contrast to synthetic methods, the self-assembly of ViP NPs ensures a monodisperse population (Manchester and Singh, 2006).

Overall, these properties promoted the investigation of ViP NP-based systems for *in vivo* imaging. Those that have reached preclinical studies are summarized in Table 5.

4.1.1. ViP NPs in optical imaging

In vivo optical imaging relies on the penetration of light into tissues. This goal can be achieved through NIR imaging, which can take advantage of ViP NPs loaded with NIR dyes. For example, bacteriophage MS2-VLPs displaying fibrin-targeting ligands on their surface and loaded with NIR dyes allows the detection of blood clots (Koudelka et al., 2015). Sun et al. produced Simian virus 40 (SV40)-VLPs encapsulating NIR quantum dots and an anticoagulant drug, and decorated these VLPs with atherosclerosis-targeting peptides. This system appears as a promising multifunctional tool for *in vivo* theragnosis in atherosclerosis (Sun et al., 2016).

Second NIR (NIR2) imaging can penetrate more deeply into tissues than NIR (Koudelka et al., 2015). SV40-VLPs were also used to encapsulate two kinds of organic NIR2 fluorescent molecules (CH1 and CH2). They exhibited a highly uniform size, strong fluorescence, high photostability, and good biocompatibility. The CH1-SV40 prototype enabled blood vessel imaging and image-guided surgery with an excellent signal-to-background ratio (Min et al., 2021).

4.1.2. ViP NPs in MRI

MRI is a non-invasive technique commonly used to image tissues. The sensitivity and resolution of MRI can be increased by using specific contrast agents. ViP NPs can be useful to reach this goal because they concentrate contrast agents, thus optimizing the signal-to-noise ratio while protecting tissues from the toxicity of the encapsulated molecules (Koudelka et al., 2015).

TMV nanoparticles loaded with Gd-DOTA proved as useful imaging tools for the delineation of atherosclerotic plaques at sub-micromolar doses. Their filamentous shape conferred optimal flow properties and promoted their movement toward vessel walls (Bruckman et al., 2013;

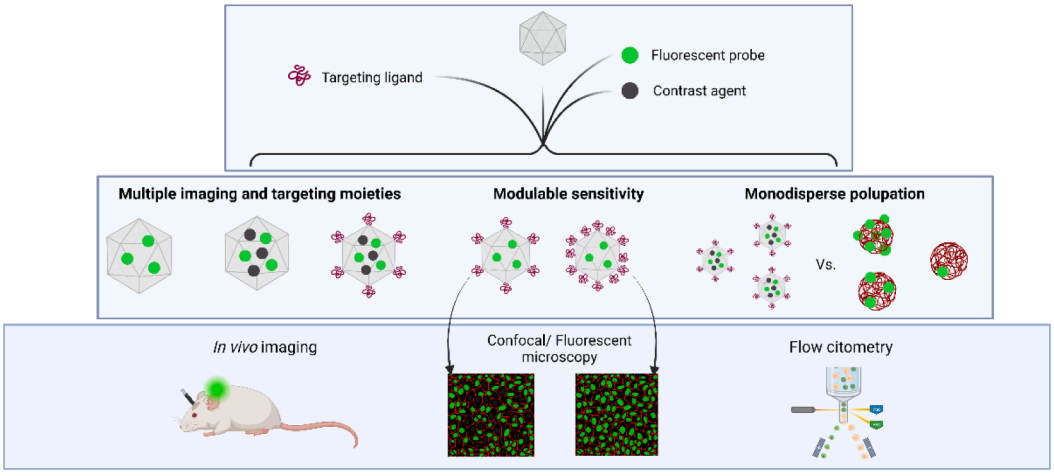

Fig. 6. Advantages of ViP NPs for in vivo imaging. The possibility of displaying imaging and targeting molecules simultaneously, controlling the spacing and orientation of such molecules, and the monodispersity of their populations enable sensible and specific imaging. Created with BioRender.com.

Table 5
ViP NP-based systems under preclinical evaluation for in vivo imaging.

Application	Imaging technique	Cargo	External moieties	Vehicle
Targeted delivery of anticoagulant drugs to atherosclerotic plaques	NIR	NIR quantum dots Hirulog (drug)	Targeting peptides for VCAM-1, macrophages, and fibrine	SV40-VLPs
In vivo imaging (vasculature, tumor resection…)	NIR	CH1/CH2 fluorophores	–	SV40-VLPs
In vivo imaging of atherosclerotic plaques	Dual optical and MR imaging	Sulfo-Cy5-azide dye Gd-DOTA	VCAM-1 targeting ligands PEG	TMV nanoparticles
Targeted *in vivo* imaging of prostate cancer	MRI	–	SPARC binding peptide Iron oxide nanoparticles	Bacteriophage M13-VLP
In vivo biodistribution	NIR fluorescence MRI	–	IRDye800CW	EMHVs
In vivo biodistribution	PET-CT	^{64}Cu-DOTA	PEG	MS2-VLPs
Glioblastoma treatment	PET-fluorescence dual-imaging	Epirubicin	^{68}Ga-DOTA Cell-penetrating peptide	Green fluorescent Qβ-VLPs

Bruckman et al., 2014). Another example are CCMV-VLPs, which can bind gadolinium (3+), resulting in paramagnetic nanoparticles useful for MRI (Allen et al., 2005).

The two basic types of MRI images are T1-weighted and T2-weighted images. T1 shortening agents create a bright signal, while T2 agents originate a dark one (Koudelka et al., 2015). Iron oxide nanoparticles (T2 agents) have been displayed on bacteriophage M13 VLPs for *in vivo* imaging of prostate cancer (Ghosh et al., 2012). They have also been encapsulated into VLPs derived from the brome mosaic virus for imaging *Nicotiana benthamiana* plants (Huang et al., 2011). On the other hand, P22 capsids loaded with paramagnetic manganese (III) protoporphyrin IX complexes demonstrated potential as a T1-enhancing contrast agent (Qazi et al., 2014).

MRI and NIR proved useful for real-time imaging of the biodistribution of innovative virosome-based systems. Precisely, EMHVs were used for drug delivery to the lungs, achieving effective targeting after intravenous injection thanks to an external magnetic field. Real-time imaging, together with magnetic-guided targeting, renders this system a fascinating example of a theragnostic tool (Vizzoca et al., 2022).

New MRI contrast approaches, such as chemical exchange saturation transfer (CEST) and hyper-CEST imaging can also benefit from ViP NPs (Koudelka et al., 2015). The combination of icosahedral bacteriophage MS2 capsid and filamentous bacteriophage fd with xenon-based sensors are representative examples (Meldrum et al., 2010; Palaniappan et al., 2013).

4.1.3. ViP NPs in PET

MS2-VLPs with DOTA groups covalently linked to their inner surface were used to chelate ^{64}Cu radioisotopes for PET. This system allowed imaging in mice for prolonged times, since the radioisotope remained for 24 hours in the bloodstream (Farkas et al., 2013). Pang et al. reported how PET can be used to monitor the delivery of drugs to brain tumors by CED. They fused green fluorescent protein to Qβ coat protein and produced fluorescently labeled VLPs in *E. coli*. These VLPs were loaded with epirubicin, modified with a cell-penetrating peptide, and labeled with ^{68}Ga-DOTA. CED infusion of the described system in animal models results in a synergistic therapeutic effect without systemic toxicity. PET/CT allowed to examine the distribution of multifunctional VLPs in the brain and their *in vivo* biodistribution in tumor-bearing mice (Pang et al., 2019).

5. Conclusions

The discovery of VLPs and virosomes opened a wide spectrum of possibilities in the pharmaceutical field. ViP NPs take advantage of their viral nature but avoid safety problems related to the manipulation of viruses. Throughout this review, we made an overview of the progress made on ViP NPs.

Up to now, prophylactic vaccination against viral infections benefited the most from ViP NPs. Additional ongoing research pursues innovative vaccination strategies which aim to simplify vaccine production and administration, thereby reducing costs and reaching developing countries. In addition, therapeutic vaccination with VLPs and virosomes showed promising results in clinical trials to treat cancer and other chronic diseases.

Current research is also investigating the possibilities that ViP NPs offer for drug/gene delivery and for diagnostics, biosensing, and disease monitoring. The data suggest that ViP NPs have intriguing cell targeting and intracellular transport capabilities that merit further study.

In summary, ViP NPs are already important tools in the pharmaceutical arsenal. It is envisaged that as new advances in biotechnology, synthetic biology, and nanotechnology are implemented in the following years, these fascinating platforms will extend to new therapeutic areas and reach new milestones in clinical use.

CRediT authorship contribution statement

Rocio Mellid-Carballal: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft. **Sara Gutierrez-Gutierrez:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft. **Carmen Rivas:** Writing – review & editing, Supervision, Project administration. **Marcos Garcia-Fuentes:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgments

This publication is part of the following grants: PID2021–124986OB-I00 (Project "IMMMA") funded by MCIN/AEI/ 10.13039/501100011033 and by "ERDF A way of making Europe"; AC21_2/00046 (Project "RAIN") funded by Health Institute Carlos III/MCIN/AEI/ 10.13039/501100011033 and by "European Union NextGenerationEU/

EURONANOMED 3; PID2021–126510NB-I00 funded by MCIN/AEI/10.13039/501100011033 and "ERDF A way of making Europe"; and ED431G 2019/02 grant funded by Xunta de Galicia. RMC is a recipient of a predoctoral grant from the Ministerio de Educación y Formación Profesional (FPU20/01525).

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