



Viral protein nanoparticles (Part 1): Pharmaceutical characteristics

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ABSTRACT

Viral protein nanoparticles fill the gap between viruses and synthetic nanoparticles. Combining advantageous properties of both systems, they have revolutionized pharmaceutical research. Virus-like particles are characterized by a structure identical to viruses but lacking genetic material. Another type of viral protein nanoparticles, virosomes, are similar to liposomes but include viral spike proteins. Both systems are effective and safe vaccine candidates capable of overcoming the disadvantages of both traditional and subunit vaccines. Besides, their particulate structure, biocompatibility, and biodegradability make them good candidates as vectors for drug and gene delivery, and for diagnostic applications. In this review, we analyze viral protein nanoparticles from a pharmaceutical perspective and examine current research focused on their development process, from production to administration. Advances in synthesis, modification and formulation of viral protein nanoparticles are critical so that large-scale production of viral protein nanoparticle products becomes viable and affordable, which ultimately will increase their market penetration in the future. We will discuss their expression systems, modification strategies, formulation, biopharmaceutical properties, and biocompatibility.

1. Introduction

Viruses have always engaged the imagination of the biomedical field, serving as continuous inspiration. They present inherent properties for biodistribution, tissue homing, cell recognition, and intracellular trafficking that have become a model for the whole nanomedicine field (Wegst et al., 2015). These properties have resulted in the widespread use of viruses, often inactivated or genetically modified, first as vaccines and later as gene therapy carriers (bin Umair et al., 2022). Currently, viruses are also explored for other pharmaceutical applications, a

testament to their flexibility and potential. Still, viruses present clear safety concerns, already at the point of manufacturing and when administered to the patient. For instance, during production, manufacturers not only need to observe strict sterility conditions, but they require special safety barriers that prevent the accidental spreading of the product (Thomas et al., 2003). From the patient perspective, viruses can cause severe immune reactions, and some entail risks associated with mutagenesis or reversion to an infective strain. For all these reasons, tools to recapitulate viral morphology, structure, and functionality while minimizing risks are of obvious medical interest, and this has

Abbreviations: AAV, Adeno-associated virus; AHA, Azidohomoalanine; APC, Antigen-presenting cell; BPV, Bovine papillomavirus; CCMV, Cowpea chlorotic mottle virus; CHO cells, Chinese hamster ovary cells; CpG ODNs, Cytosine-phosphate-guanosine oligodeoxynucleotides; CPMV, Cowpea mosaic virus; CTL, Cytotoxic T lymphocyte; dsRNA, Double-stranded RNA; EV71, Enterovirus 71; eVLP, Enveloped virus-like particle; HA, Hemagglutinin; HBcAg, Hepatitis B core antigen; HBsAg, Hepatitis B surface antigen; HCV, Hepatitis C virus; HEK293T cells, Human embryonic kidney 293T cells; HEV, Hepatitis E virus; HIV, Human immunodeficiency virus; HPV, Human papillomavirus; IRIV, Immunopotentiating reconstituted influenza virosome; M2e, Matrix protein 2 ectodomain of influenza A virus; MPL, 3-O-desacyl-4'-monophosphoryl lipid A; NA, Neuraminidase; neVLP, Non-enveloped virus-like particle; pAF, p-amino-phenylalanine; PD-1, Programmed cell death protein 1; PEG, Polyethylene glycol; PPV, Porcine parvovirus; PTM, Post-translational modification; PVX, Potato virus X; SHIV, Simian-human immunodeficiency virus; SIV, Simian immunodeficiency virus; SMPH, Succinimidyl 6-(beta-maleimidopropionamido)hexanoate; SFV, Semliki Forest virus; RSV, Respiratory syncytial virus; PhMV, Physalis mottle virus; ssRNA, Single-stranded RNA; Th1/2, T helper 1/2 lymphocyte; TLR, Toll-like receptor; UNAA, Unnatural amino acid; ViP NP, Viral protein nanoparticle; VLP, Virus-like particle.

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spurred research in the incorporation of viral proteins in natural and synthetic nanoparticles.

Different types of nanoparticles showing viral proteins have been investigated, from polymers conjugated to small viral peptides to complete or attenuated viruses. For instance, Tat sequences from the human immunodeficiency virus (HIV) have been incorporated in a variety of polymeric nanoparticles and liposomes as a cell-penetrating domain (Rao et al., 2008; Zhang et al., 2020). Other viral integrin-binding sequences have been conjugated to a variety of platforms, with the aim of improving tissue targeting properties.

Synthetic carriers displaying small viral domains have been reviewed elsewhere (Zhang et al., 2020) and will not be described here. Instead, this manuscript studies nanoparticles depleted of any viral genome where proteins are core components of the structure. These structures are globally termed herein viral protein nanoparticles (ViP NPs). Concretely, we focus on ViP NPs with the highest therapeutic potential: virus-like particles and virosomes. In the following lines, we include a description of their structural characteristics, formulation, biopharmaceutical properties, and biocompatibility. In a separate review paper (Mellid-Carballal et al., 2023), the therapeutic applications of ViP NPs are described.

2. Structure of viral protein nanoparticles

2.1. Virus-like particles

The concept of virus-like particles (VLPs) was first employed to refer to diverse biological structures similar in morphology to viruses, or empty protein aggregates of viral origin that lack genetic material (Zeltins, 2013). The first recorded observation of these systems was from the 1960s, when some viral particles depleted of nucleic acids were identified as the capsids of the hepatitis B virus (Blumberg et al., 1969). Further studies found that these particles were able to trigger immune responses comparable to those of the complete hepatitis virus (Bayer et al., 1968). These pioneer studies, together with key advances in biotechnology, triggered many investigations resulting in our current understanding of these virus-based structures.

Nowadays, VLPs are defined as virus-derived structures made up of copies of one or several viral structural proteins that self-assemble into capsids, mimicking the overall structure of the native virions but lacking the viral genome (MJ Rohovie et al., 2017; Chroboczek et al., 2014). Consequently, they are neither infective nor replicative, but retain features from the native viruses. For instance, VLPs present affinity for cellular receptors, engage in host cell entry mechanisms, and are recognized by the immune system (Naskalska and Pyrc, 2015). These properties make them attractive tools for a broad spectrum of therapeutic applications.

2.1.1. VLP structure: enveloped and non-enveloped

In vivo assembly of VLPs results from the aggregation of viral capsid monomers after their translation in the cells (Naskalska and Pyrc, 2015). These minor structural subunits self-assemble to form the capsid (Perlmutter and Hagan, 2015). Capsid growth is driven by a thermodynamic equilibrium based on van der Waals, hydrogen bonding, hydrophobic, and electrostatic interactions. The number, arrangement, nature, and interactions of capsid monomers will determine the morphology and properties of the resulting VLPs (Cuervo et al., 2013; Mejía-Méndez et al., 2022). VLPs may appear helical, icosahedral, spherical, or have complex shapes (Le and Müller, 2021). Some VLPs can also be surrounded by a lipid envelope acquired from the cell membrane (Naskalska and Pyrc, 2015).

Enveloped viruses interact and enclose themselves within the cell membrane, from where they bud to the extracellular environment. Some VLPs (enveloped VLPs; eVLPs) share this release mechanism that helps them to cloak within host cell components (Naskalska and Pyrc, 2015). Self-assembly of eVLPs includes two main steps: first, capsid monomers

self-aggregate forming the scaffold protein; then, the particles acquire the membrane during exocytosis. This mechanism has been observed, for instance, for retroviruses and filoviruses (Sukenfik et al., 2021). The VLP envelope usually consists of lipids, proteins, and glycoproteins. The envelope composition impacts VLP structure, stability, and activity. For instance, cellular glycoproteins could act as surface antigens in eVLPs, improving immunogenicity and granting tissue targeting.

Other VLPs lack any cellular component in their structure, and those are called non-enveloped VLPs (neVLPs). neVLPs are composed of one or more viral proteins that can be modified post-translationally (Sukenfik et al., 2021). These capsid monomers self-assemble in the cytoplasm to form neVLPs, but in this case, the release mechanism does not require the active participation of the cell membrane or other cell structures. The release of neVLPs from the cell typically entails cell membrane disruption and even apoptosis (Glasgow and Tullman-Ercek, 2014).

As potential vaccines, eVLPs might benefit from additional immune stimulation from their shell glycoproteins, but they are structurally more complex than neVLPs and more difficult to produce. Because of these differences, most of the developed VLP-based therapeutic systems are based on neVLPs (Buonaguro et al., 2011).

2.1.2. VLP production

The VLP synthesis method is not only important from a process optimization perspective but also for the resulting VLP characteristics. VLPs can be generated in cells or by cell-free synthesis. The type of organism or cell extract used for VLP production is critical to their final composition and structure. It affects post-translational modifications (PTMs), intracellular trafficking and assembly of capsid monomers, and envelope composition.

Cell-based synthesis is the classical VLP synthesis method and relies on the natural process of virus replication. The expression of VLP proteins can be achieved both by infecting a host cell with a complete viral genome and collecting fully assembled virions, or by just inducing the expression of the desired protein after inserting its gene sequence in a vector (e.g. a plasmid) (Rabindran and Dawson, 2001). The first method mimics natural virus replication and ensures VLP morphology and properties. However, genome removal is a critical step that requires either chemical degradation or disassembly and separation. These processes could disturb capsid structure, and there is always some risk of keeping some residual viral genome. The transfection of cells with a plasmid encoding the protein of interest is an effective way of producing large amounts of VLPs, but some aspects of virus replication might not be perfectly recapitulated (Tissot et al., 2010).

There are different expression systems for VLP cell-based production, including bacteria, yeast, insect, mammalian, and plant cells (Mett et al., 2008; Kim and Kim, 2016; Roy and Noad, 2009; Diminsky et al., 1997; Warfield et al., 2003). A comparison of the main characteristics of these different systems is shown in Table 1.

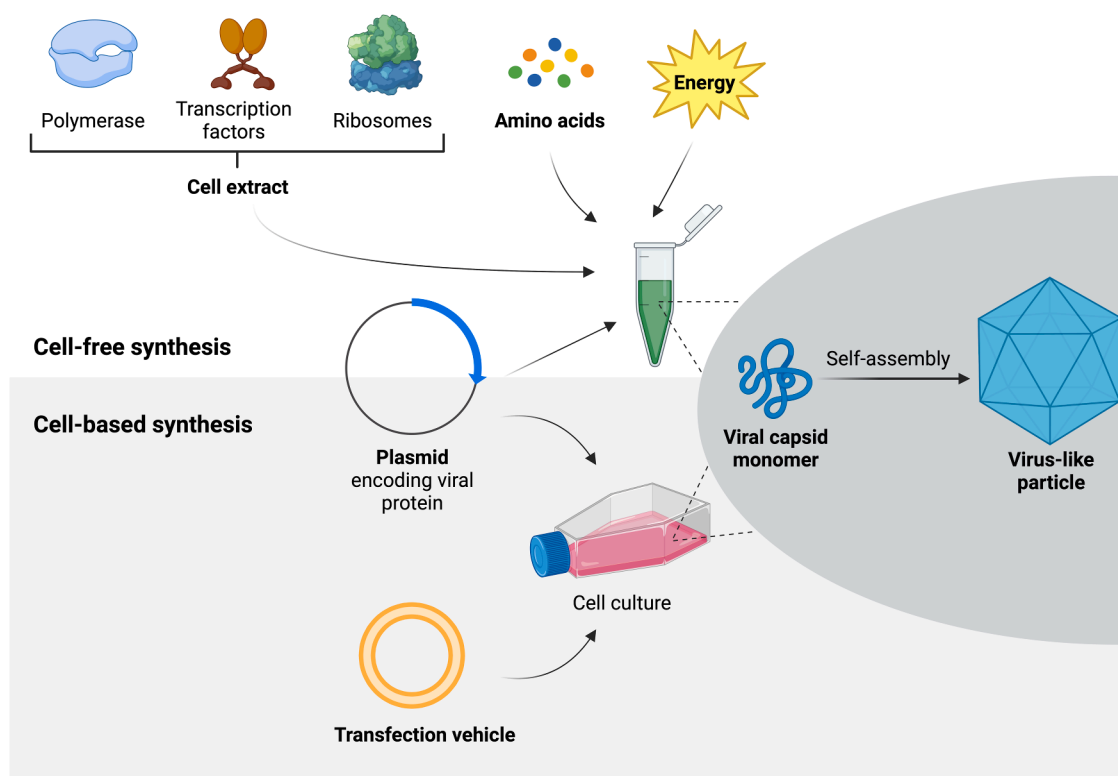
Cell-free protein synthesis technology uses cell extracts to produce in vitro coupled transcription-translation processes (Fig. 1). One of the main advantages of this system is its open nature; protein synthesis occurs without the constraints of cellular membrane barriers and the genome of the host cell (Shadiac et al., 2013). There is no need to maintain the cells alive, and all the available resources can be focused on the expression of the proteins of interest. Furthermore, synthesis conditions can be manipulated at any point during the process, granting additional flexibility (Gregorio et al., 2019). However, this method has some disadvantages as it is difficult to know whether the full range of PTMs are recapitulated. Additionally, capsid monomer assembly occurs in an environment different from the cytoplasm, and no cell membrane is available for VLP envelopment. All this might result in structural and functional differences (Dondapati et al., 2020).

There are many different cell-free synthesis methods depending on the cell line from which the production extract is derived. The selection of the optimal system should be done according to the sequence, conformation, and PTMs of the protein of interest (Shadiac et al., 2013;

Table 1

Properties of the different cell-based expression systems for VLP production.

EXPRESSION SYSTEM	PTMs	VLP TYPE	YIELD	COST	SAFETY	SCALE-UP
Bacteria • <i>E. coli</i>	Limited	neVLPs	High	Low	Potential endotoxin contamination	Easy
Yeast • <i>Saccharomyces cerevisiae</i> • <i>Pichia pastoris</i>	Dissimilar to mammalian ones	neVLPs	High, but lower than bacteria	Low	High	Yield loss
Insect cells • <i>Spodoptera frugiperda</i> (Sf9/Sf21) • <i>Trichoplusia ni</i> (Tn5)	Similar to mammalian ones	neVLPs eVLPs	High	High	High	Feasible, but requires optimization
Mammalian cells • CHO cells • HEK293T cells • Fibroblast-like cells (COS-1)	Original	neVLPs eVLPs	Low	High	Risk of opportunistic pathogens	Time-consuming High financial investment
Plant cells • <i>Nicotiana benthamiana</i> • Edible plants (potato, lettuce...)	Plant specific	neVLPs eVLPs	Variable	Low	High	Easy

**Fig. 1.** Schematic representation of VLP production using cell-free or cell-based synthesis.

Gregorio et al., 2019). Although cell-free systems can be prepared in-house (Shadiac et al., 2013), commercial kits are already available for most platforms. Cell-free production is fast and easy to introduce for direct VLP preparation but can become costly for producing large amounts of protein (Gregorio et al., 2019).

2.1.3. VLP modification

Virus-like particles can serve as platforms to present heterologous antigens, to deliver drugs, or to display targeting moieties. The generation of such platforms requires the modification of the VLP, which can be achieved through different strategies: genetic engineering, chemical bioconjugation, and other approaches involving weak forces. In this section we will describe each of these approaches (Fig. 2).

2.1.3.1. Genetic engineering. Genetic engineering allows the insertion, elimination, and substitution of amino acid residues on the surface of VLPs. In this way, it is possible to introduce purification/immunodetection tags, epitope sequences, targeting ligands, etc. (Koudelka et al., 2015).

There are two main approaches for the genetic modification of VLPs. The simplest one is the direct insertion of peptides at the N- or C-terminal regions of the VLP structural proteins, or within loop regions of the capsid subunits (Shirbaghaee and Bolhassani, 2016; D Diaz et al., 2018). Another approach consists of the manipulation of genes that encode VLP structural proteins and their translation (Mohsen et al., 2017), which allows to change or remove amino acid residues, or to introduce unnatural amino acids (UNAAs) (D Diaz et al., 2018).

The advantages of genetic modification strategies include: (i) the

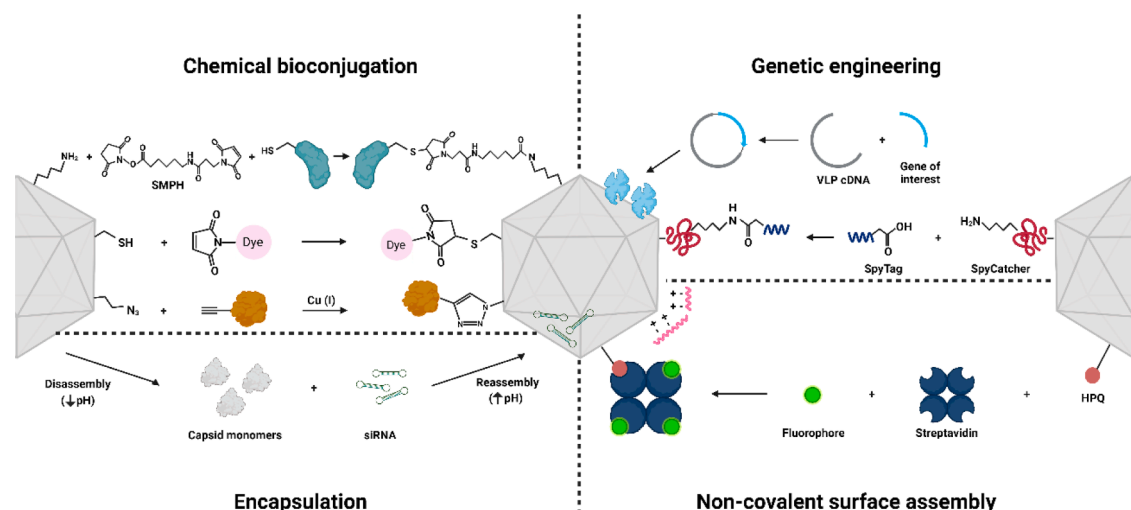


Fig. 2. Summary of the main modification strategies used for virus-like particles. Chemical bioconjugation: (i) lysine-based modification through a N-hydroxy succinimide ester reaction using succinimidyl-6-(beta-maleimidopropionamido)hexanoate (SMPH), (ii) cysteine-based modification using a maleimide compound, and (iii) copper-catalyzed azide-alkyne cycloaddition. Genetic engineering: (i) insertion of a gene of interest into the VLP coding sequence, and subsequent synthesis of a fusion protein that maintains self-assembly ability; and (ii) spontaneous isopeptide bond formation between SpyTag (fused to a model peptide) and SpyCatcher (fused to the VLP). Encapsulation: in vitro loading by pH-driven disassembly/reassembly of the VLP in the presence of siRNA as a model cargo. Non-covalent surface assembly: (i) electrostatic interactions between the positively charged VLP surface and a negatively charged polymer, and (ii) affinity interaction between streptavidin, carrying a model fluorophore, and the HPQ (homophenylalaninylmethane) sequence of a biotin-mimicking peptide attached to the VLP surface. Created with BioRender.com.

formation of stable bonds between the VLP and the ligand (Shirbaghaee and Bolhassani, 2016), (ii) the possible insertion of hundreds of peptides per VLP (MJ Rohovie et al., 2017), and (iii) the feasibility of incorporating different antigens by identifying distinct fusion positions (Shirbaghaee and Bolhassani, 2016).

Genetic modification has also limitations: (i) non-peptide antigens cannot be incorporated (Jennings and Bachmann, 2008 May 1); (ii) only small peptides (below 30 amino acids) can be presented without interfering with VLP assembly (Shirbaghaee and Bolhassani, 2016); (iii) even with small peptides, their effect on self-assembly is unpredictable and may impair proper VLP and antigen functionality (Shirbaghaee and Bolhassani, 2016; MJ Rohovie et al., 2017; Jennings and Bachmann, 2008 May 1); and (iv) expression and purification yields are often reduced (Jennings and Bachmann, 2008 May 1).

To avoid some of these limitations, new genetic modification strategies have been developed. One example is SplitCore, based on the splitting of major immunodominant regions of the hepatitis B core antigen (HBcAg) into two parts, followed by the addition of heterologous sequences to the N-terminus (CoreN) or C-terminus (CoreC). This method provides higher flexibility for introducing proteins larger than 300 amino acids on the surface of VLPs, without affecting their assembly (Baltabekova et al., 2015; Walker et al., 2011). A similar method, called Tandem core, relies on the stabilization of HBcAg-VLPs by fusing two monomers of the major immunodominant regions and displaying larger proteins in every second region (Peyret et al., 2015).

Finally, the SpyTag/SpyCatcher technology, based on the rational engineering of an adhesin from *Streptococcus pyogenes* (Zakeri et al., 2012), has proved very useful. SpyCatcher oligomers are fused to the VLP while SpyTag is fused to the antigen of interest. Then, SpyCatcher and SpyTag motifs are spontaneously linked through an isopeptide bond, generating the chimeric VLP (Brune et al., 2016). Apart from a significant number of publications using this technology to display antigens on the surface of VLPs for vaccination purposes (Thrane et al., 2016), there are promising proof-of-concept studies in other areas, such as the development of hydrogels for tissue engineering (Reddington and Howarth, 2015). In contrast to SplitCore and Tandem core, which are limited to HBcAg-VLPs, SpyTag/SpyCatcher technology can be applied to any VLP. Moreover, different variants of these peptides are

commercially available, thus facilitating the application of the technology in different research areas.

2.1.3.2. Chemical bioconjugation. In this approach, antigens and VLPs are generated individually and then coupled together by covalent interactions. Covalent conjugation requires exposed functional groups on the internal or external surface of the VLPs, such as amino, carboxylic, sulfhydryl, or hydroxyl groups (Ding et al., 2018; MO Mohsen et al., 2017). According to the modified amino acid, modifications can be divided as follows (D Diaz et al., 2018; MJ Rohovie et al., 2017):

- A. Lysine-based modifications are the most popular. They are based on N-hydroxy succinimide ester reactions, which can occur at physiological pH (D Diaz et al., 2018; MJ Rohovie et al., 2017). This method was used to couple gp41 peptides to the surface of bacteriophage AP205-VLPs, aiming to develop a new vaccination approach against HIV (Pastori et al., 2012).
- B. Cysteine-based modifications are often based on the spontaneous formation of disulfide bonds between thiol groups (MJ Rohovie et al., 2017). Cysteine modifications have been used to display cell-penetrating peptides, fluorescent probes, and heterologous peptides on different VLPs (Anand et al., 2015; Kang et al., 2008; Pomwised et al., 2016).
- C. Aspartate- or glutamate-based modifications employ carbodiimides as conjugation agents, but these reactions are less used since they require multiple steps (MJ Rohovie et al., 2017). One example is their use to display fluorescent probes on cowpea chlorotic mottle virus (CCMV)-VLPs (Gillitzer et al., 2002).
- D. Another strategy consists of introducing UNAAs like azidohomoalanine (AHA) and p-amino-phenylalanine (pAF) to establish specific bonds to the ligand of interest. AHA, displaying an azide, can participate in click reactions (Patel and Swartz, 2011), while pAF can be conjugated to ligands displaying phenylene diamines and amino phenols (Behrens et al., 2011; Stephanopoulos et al., 2010; Tong et al., 2009).

Covalent coupling of ligands to VLPs has several advantages over genetic engineering: (i) it can incorporate antigens of various sizes and

types (Shirbaghaee and Bolhassani, 2016), including proteins, polypeptides, capsular polysaccharides, and small organic molecules (Jennings and Bachmann, 2008 May 1); (ii) the native VLP and the target antigen are synthesized in parallel, so there is a better chance to combine full-length, correctly folded peptides (Jennings and Bachmann, 2008 May 1); (iii) the coupling location can be optimized to achieve the best exposure of the conjugated antigen (Jennings and Bachmann, 2008 May 1); and (iv) covalent linkage generates stable bonding between the conjugated antigen and the VLP (D Diaz et al., 2018).

The limitations of covalent conjugation are: (i) the reaction must be efficient in an aqueous solvent at physiological pH, use low concentrations of nontoxic reagents, and preserve the tertiary and quaternary structure of both antigen and VLP (Mohsen et al., 2020); (ii) chemical techniques do not always result in full modification of all the capsid monomers building up the VLPs, particularly if large antigens are to be displayed (Mohsen et al., 2017); and (iii) poor selectivity and low reproducibility of some reactions may result in altered conformations and random orientations (D Diaz et al., 2018).

Some limitations can be overcome by using click chemistry, which has proved to be fast, simple, specific, and efficient. The most common click reaction for the modification of VLPs is copper-catalyzed azide-alkyne cycloaddition, but other possibilities are available (D Diaz et al., 2018; Vaidya and Solomon, 2022).

2.1.3.3. Non-covalent surface assembly. Non-covalent attachment of ligands and other materials to VLPs takes advantage of several approaches, such as streptavidin-biotin interactions (Koudelka et al., 2015; Jennings and Bachmann, 2008 May 1), electrostatic interactions (MJ Rohovie et al., 2017), and other affinity-based binding motifs. For example, a system generated by fusing ligands to the “Dec” protein of the bacteriophage L enables the surface display of peptides without modifying the coat proteins. This method was used to display the CD40 ligand and the CD47 “self-peptide” on the surface of P22-VLPs to avoid phagocyte engulfment (Schwarz et al., 2015).

Non-covalent bioconjugation does not require complex chemical reactions but can lead to moiety/VLP disassociation in complex fluids such as blood (D Diaz et al., 2018). In a study using CCMV for the development of electrostatic virus-polymer complexes, the influence of polymer size and ionic strength was examined: large polymers (>20 positive charges) showed affinity towards the external surface of CCMV even at high salt concentrations and could be used to coat these systems; mid-sized polymers (8–9 positive charges) lost their affinity under such conditions, but could coat capsids at low salt concentrations. Small polymers (<4 positive charges) could not form stable complexes under any conditions (Kostiainen et al., 2011). This behavior was also demonstrated for CCMV-VLPs. A different study showed that cationic dendrimers could only interact with the negative surface of P22-VLPs at low ionic strength. Therefore, ionic strength is a key factor when VLP modification is based on electrostatic interactions (Kraj et al., 2021).

2.1.3.4. Encapsulation. Apart from being subject to surface modification, VLPs can encapsulate molecules within their cavities. The following methods have been studied for that purpose:

A. In vitro loading consists of the disassembly of VLPs and subsequent reassembly in the presence of the desired cargo molecules (D Diaz et al., 2018). These structural changes can be controlled by environmental factors such as pH (Cassidy-Amstutz et al., 2016), metals (Lee et al., 2019), reducing agents (Shen et al., 2015), and ionic strength (Sánchez-Sánchez et al., 2014). For example, Physalis mottle virus (PhMV)-VLPs have been engineered to disassemble under physiological conditions so that cargo release occurs at the targeted site (H Hu and Steinmetz, 2020; H Hu and Steinmetz, 2020).

B. In vivo loading relies on the interaction between the cargo molecules and the inner surface of the VLPs during their self-assembly (D Diaz et al., 2018; Patterson et al., 2012).

C. Infusion strategies are based on the introduction of cargo molecules through pores between capsid subunits (D Diaz et al., 2018). For example, Toll-like receptor 9 (TLR9) agonists have been encapsulated in PhMV-VLPs by infusion (Hu and Steinmetz, 2021).

An efficient encapsulation implies that the cargo is retained in the cavity of the VLP and released only at the target site. For that, the cargo can be covalently attached to the inner surface or it can be retained by electrostatic interactions with nucleic acids or internal amino acid side chains (MJ Rohovie et al., 2017; Ding et al., 2018). A variety of molecules can be encapsulated, from small drugs to nucleic acids or even nanoparticles.

While there are multiple examples of VLPs encapsulating small molecules through covalent bonds, e.g. doxorubicin (Bruckman et al., 2016) or fluorescein (Ochoa et al., 2006), encapsulation through non-covalent interactions was only shown with polymerized cargo or by the association of the cargo within nucleic acids. The development of new non-porous VLPs would allow efficient loading and the retention of small molecules inside the VLPs (MJ Rohovie et al., 2017).

Encapsulation of nucleic acids is easier than for small molecules as viral capsids have naturally evolved to carry genetic cargo (MJ Rohovie et al., 2017). The disassembly/reassembly procedure (Gomes et al., 2019) or an enzymatic digestion method can be used (Mohsen et al., 2019; Keller et al., 2010; Storni et al., 2004). As nucleic acids are negatively charged, they interact with the positively charged inner surface of the capsids (D Diaz et al., 2018; Jeevanandam et al., 2019). One interesting possibility is to encapsulate nucleic acid-based adjuvants such as cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODNs). This strategy enables the co-delivery of antigens and adjuvants to the same antigen-presenting cell (APC) (Mohsen et al., 2020).

Regarding nanoparticles, it was shown that loading colloidal contrast agents inside VLPs can improve image resolution in diagnostic applications. Shen et al. reported the efficient encapsulation of magnetic nanoparticles inside HBcAg-VLPs. This approach resulted in higher cellular uptake and r_2 values compared to the nanoparticles alone, which makes them good candidates as T2 contrast agents for magnetic resonance imaging (Shen et al., 2015).

2.2. Virosomes

In 1975, Almeida et al. reported the successful inclusion of the viral spike proteins hemagglutinin (HA) and neuraminidase (NA) in a liposome. Due to their similar aspect to native influenza virions when observed by electron microscopy, they were called virosomes (Almeida et al., 1975). Today, virosomes are defined as a special type of enveloped VLPs lacking the protein capsid (B Donaldson et al., 2018). Structurally similar to unilamellar liposomes carrying viral envelope proteins, virosomes show a spherical shape and a diameter between 60 and 200 nanometers (van der Velden et al., 2022).

The preparation of virosomes can be divided into three main steps, as shown in Fig. 3 (Huckriede et al., 2003; Asadi and Gholami, 2021; Mak and Saunders, 2006): solubilization of the viral membrane in a detergent, removal of the nucleocapsid (usually by ultracentrifugation), and reconstitution of the viral envelope proteins into a liposome (e.g. by removal of the detergent). In this last step, additional lipids, other viral components (nucleic acids, proteins), and antiviral drugs can be added (Asadi and Gholami, 2021).

Since their discovery, virosomes have been developed using proteins from several viruses, such as Sendai virus (Bagai and Sarkar, 1993), Epstein-Barr virus (Grimaldi et al., 1995), and vesicular stomatitis virus (Shoji et al., 2004). However, influenza virosomes are the most widely used due to their immunogenic and delivery properties (van der Velden et al., 2022). For this reason, they are frequently called

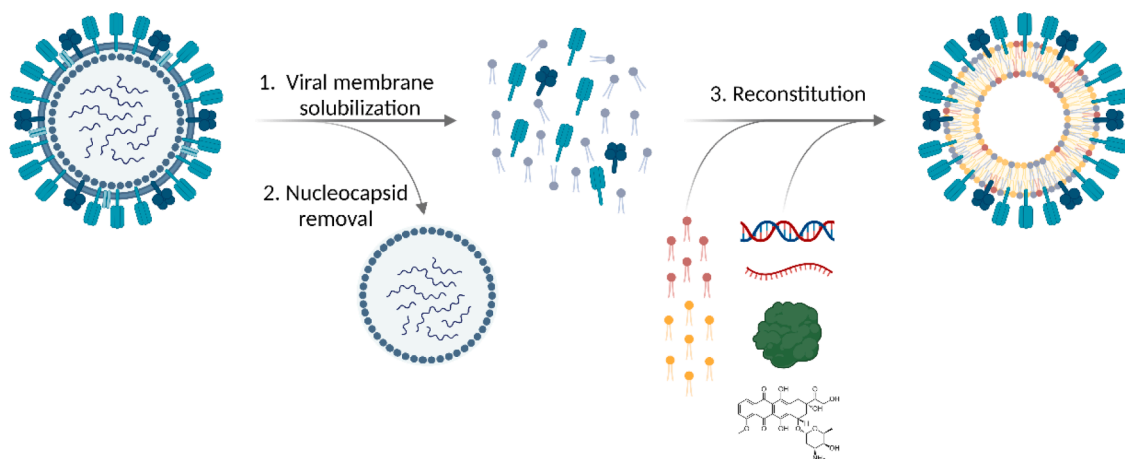


Fig. 3. Main steps in virosome preparation. Created with BioRender.com.

immunopotentiating reconstituted influenza virosomes (IRIVs). Nevertheless, other viruses can be used for virosome production to target different cells (Raska and Turanek, 2015).

2.2.1. Modification strategies

The versatility of virosomes enabled the development of several strategies to incorporate drugs or biomolecules into them (Fig. 4) (van der Velden et al., 2022; Metcalfe and Gluck, 2004):

- A. Integration in the lipid membrane. Antigens or drugs with intrinsic hydrophobic domains, or antigens modified to increase their hydrophobicity, can be incorporated between the phospholipids of the lipid bilayer of virosomes (Metcalfe and Gluck, 2004).
- B. Chemical coupling. Some antigens are covalently linked to the virosome surface (e.g. by click chemistry reactions), while others are adsorbed non-covalently to their surface. The nature of the elicited immune response will depend on whether the antigen is on the

surface of the virosome or inside the virosome. As an example, Pevion Biotech possesses two virosome-based platforms for vaccine development. They differ in whether the epitopes of the antigen are located on the surface of the virosome (PeviPRO™) or inside the virosome (PeviTER™). This structural change results in different immune responses: PeviPRO induces mainly humoral responses whereas PeviTER induces cellular responses (Asadi and Gholami, 2021). Furthermore, the virosome surface can be modified to increase their circulation time (e.g. with polyethylene glycol (PEG) or polyvinylpyrrolidone), and to target the virosomes to specific cells (Asadi and Gholami, 2021; Mastrobattista et al., 2001).

- C. Encapsulation. The transport of antigens inside virosomes is essential to induce strong cellular immune responses and is required for therapeutic vaccination. Traditional methods consisting of passive loading of antigens inside liposomes are inefficient and compromise protein conformation and virosome fusion. Therefore, new methods to encapsulate peptides and small molecules are of great interest

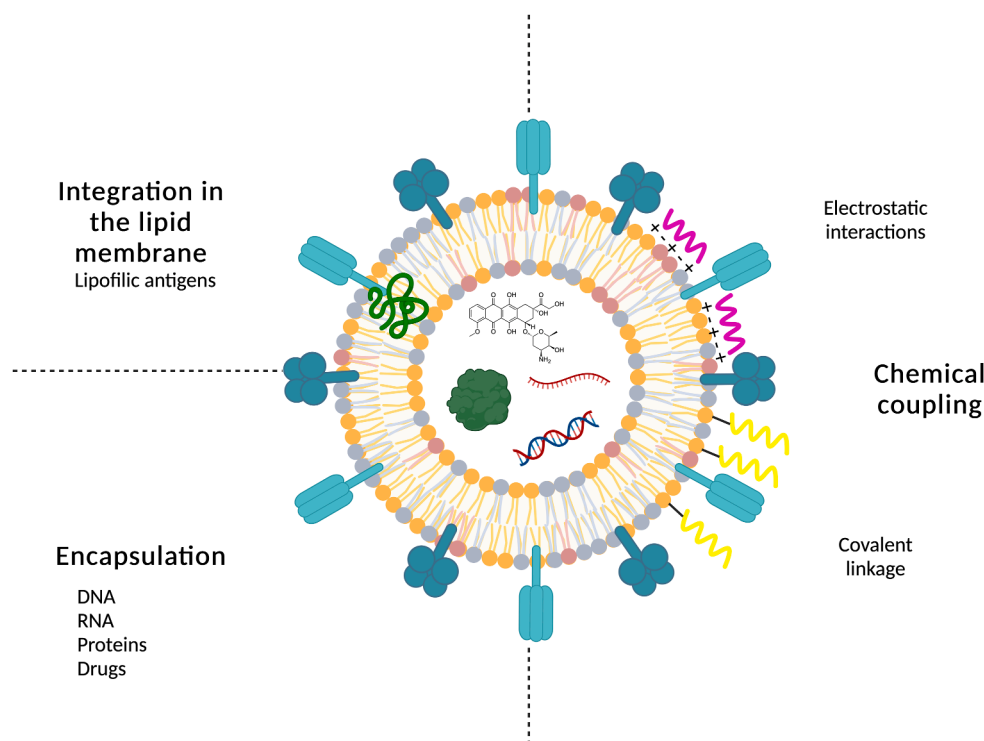


Fig. 4. Summary of possible modification strategies of virosomes. Created with BioRender.com.

(Metcalfe and Gluck, 2004). One example is the loading of influenza virosomes with ammonium sulfate so that a transmembrane proton gradient is created that promotes the active loading of weak bases such as doxorubicin (Waelti et al., 2002). Importantly, when the antigen and the IRIV are physically associated, the immunopotential properties are higher than when they are simply mixed (R Zurbriggen et al., 2000).

3. Formulation, stability, and storage

3.1. Formulation of ViP NPs

Because all ViP NPs that are currently approved are vaccines, we will focus on the formulation strategies of this type of products. Formulation of conventional vaccines, because of their complexity, relies on empirical processes (Rueckert and Guzmán, 2012). Due to their inherent instability, an optimal formulation is required to maintain their potency from production to administration (Jain et al., 2015). Vaccines based on ViP NPs can benefit from many of the advances in the formulation of recombinant proteins. However, when focusing on their immunogenicity, there is an important difference regarding their need for adjuvants. Subunit vaccines are generally poorly immunogenic despite the use of adjuvants. By contrast, the ordered structure and particulate nature of ViP NPs endow them with self-adjuvanting properties (Jain et al., 2015). In any case, the stability of antigens, excipients, and whole products (Braun et al., 2009) must be considered for all vaccine formulations, and during their complete life cycle (Dumpa et al., 2019).

3.1.1. Excipients in ViP NP formulation

Excipients in ViP NP formulations comprise salts and other compounds used to maintain the integrity and stability of ViP NPs in solution. A proper formulation requires the optimization of pH, ionic strength, and other physicochemical properties (B Donaldson et al., 2018).

Most VLP-based vaccines in the market or clinical trials are liquid suspensions. This importantly limits their storage and distribution (B Donaldson et al., 2018). Some common excipients used to preserve their stability in such conditions are: (i) surfactants such as polysorbate 80, to prevent VLP aggregation (Shi et al., 2005); (ii) buffering agents such as L-histidine, sodium borate, and sodium phosphate (Rustandi, 2022; Lua et al., 2014); and (iii) preservatives such as 2-phenoxyethanol (Lua et al., 2014) (Table 2).

VLPs must be protected from pH and temperature fluctuations by selecting an appropriate buffer and by adding thermoprotective and lyoprotective agents, respectively. For example, it was demonstrated that sodium phosphate-based buffers were superior to citrate or Tris buffers for the maintenance of enterovirus 71 (EV71)-VLP stability (Lin et al., 2014). For VLPs that are not stable at neutral pH, the addition of polyanions has proved useful (Kramer et al., 2013). A hepatitis B vaccine was rendered stable to both freezing and heating by the addition of polyols such as propylene glycol (Braun et al., 2009; Clapp et al., 2011). The addition of carbohydrates, such as trehalose, sucrose, and glycerol, has been widely used to increase VLP stability against temperature changes (Kissman et al., 2008; Peixoto et al., 2007).

Gardasil® provides an illustrative example of the importance of excipients in formulation stability. Early formulations of Gardasil® consisted of human papillomavirus (HPV)-VLPs purified from yeast and containing 0.15 M sodium chloride. This early formulation lost 80% of its potency after 12 months at 4 °C. By increasing sodium chloride concentration to 0.32 M and adding 0.01% polysorbate 80 and 10 mM L-histidine (pH 6.2), the new vaccine formulation preserved its potency under the same conditions. However, the preparation was still unstable at room temperature. After the addition of 0.1% polyanions, the vaccine could preserve its potency for up to 12 months at room temperature (Shi et al., 2005; Shi et al., 2007).

Another strategy to improve the structure and stability of HPV-VLPs

Table 2

Adjuvants and excipients in commercialized ViP NP-based vaccines.

Vaccine	Adjuvants	Excipients
Recombivax HB®	• Amorphous aluminum hydroxyphosphate sulfate	• Sodium chloride • Sodium borate • Water for injections
Engerix B®	• Aluminum hydroxide	• Sodium chloride • Disodium phosphate dihydrate • Sodium dihydrogen phosphate dihydrate
Fendrix®	• AS04 (MPL + aluminum phosphate)	• Sodium chloride • Water for injections
Sci-B-Vac®	• Aluminum hydroxide	• Sodium chloride • Potassium chloride • Disodium phosphate dodecahydrate • Potassium dihydrogen phosphate • Sodium hydroxide (for pH adjustment) • Hydrochloric acid (for pH adjustment) • Water for injections
GenHevac B®	• Aluminum hydroxide	• Formaldehyde • Sodium chloride • Polysorbate 80 • Monopotassium phosphate • Disodium phosphate dihydrate • Water for injections
Heplisav B®	• CpG 1018	• Sodium chloride • Disodium phosphate dodecahydrate • Sodium dihydrogen phosphate dihydrate • Polysorbate 80 • Water for injections
Gardasil®	• Amorphous aluminum hydroxyphosphate sulfate	• Sodium chloride • Histidine • Polysorbate 80 • Borax • Water for injections
Cervarix®	• AS04 (MPL + aluminum phosphate)	• Sodium chloride • Sodium dihydrogen phosphate dihydrate • Water for injections
Gardasil® 9	• Amorphous aluminum hydroxyphosphate sulfate	• Sodium chloride • Histidine • Polysorbate 80 • Borax • Water for injections
Cecolin®	• Aluminum hydroxide	• Sodium chloride • Sodium dihydrogen phosphate dihydrate • Disodium hydrogen phosphate dihydrate • Polysorbate 80 • Water for injections
Hecolin®	• Aluminum hydroxide	• Sodium chloride • Disodium hydrogen phosphate • Potassium dihydrogen phosphate • Thiomersal • Water for injections
Mosquirix®	• AS01 (QS-21 + MPL)	• Powder: sucrose, polysorbate 80, disodium phosphate dihydrate, sodium dihydrogen phosphate dihydrate • Suspension: dioleoyl phosphatidylcholine (DOPC), cholesterol, sodium chloride, disodium phosphate anhydrous, potassium dihydrogen phosphate, water for injections
Epaxal®	• IRIVs	• Sodium chloride • Water for injections
Inflexal® V	• IRIVs	• Sodium chloride • Disodium phosphate dihydrate • Potassium dihydrogen phosphate

(continued on next page)

Table 2 (continued)

Vaccine	Adjuvants	Excipients
Porcilis® PCV	• DL-α-tocopheryl acetate • Light liquid paraffin	• Lecithin • Water for injections • Polysorbate 80 • Simethicone • Sodium chloride • Potassium chloride • Disodium phosphate dihydrate • Potassium dihydrogen phosphate • Water for injections
Ingelvac CircofLEX®	• Carbomer	• Sodium chloride • Water for injections

is the incorporation of a disassembly/reassembly step after their purification from the yeast culture. These reassembled VLPs seem to be not only more stable but also more immunogenic (Mach et al., 2006).

Currently, there is ongoing research on VLP formulations aiming to reduce dosage, improve immunogenicity, enhance stability during long-term storage, and avoid the cold chain. An innovative strategy is the use of GelSite®, a mucoadhesive polysaccharide derived from *Aloe vera* L., for the intranasal administration of VLPs in a dry powder vaccine. The in situ gelation of this vehicle delays mucociliary clearance, resulting in higher systemic and mucosal antibody production than liquid formulations (Shira et al., 2011).

In the case of virosomal formulations, the essential excipients are the inactivated influenza virus, egg-derived or synthetic phospholipids, and buffering agents (Moser et al., 2013). Optional excipients include cationic lipids, such as cholesterol derivatives, and carbohydrates such as sucrose, mannitol, or inulin (Moser et al., 2013; de Jonge et al., 2007; De Jonge et al., 2007).

The approved virosome-based vaccines Epaxal® and Inflexal® V are both liquid suspensions. Their excipients are mainly buffering agents such as sodium chloride, disodium phosphate dihydrate, or potassium dihydrogen phosphate (Health Products Regulatory Authority, 2012; Health Products Regulatory Authority 2014). A different case is PEV7, a vaccine candidate against recurrent vulvovaginal candidiasis, which has been studied in clinical trials in two dosage forms (NCT01067131): as a reconstituted lyophilizate for intramuscular injection and as a capsule for intravaginal administration (De Bernardis et al., 2015). To produce the capsules, the excipients used were spray-dried lactose, crospovidone, hydrophilic fumed silica, sodium phosphate dibasic dihydrate, potassium phosphate, and hydroxypropyl methylcellulose capsules (Vacher et al., 2012).

Despite these advances, the formulation of ViP NP-based products is mainly empirical. Each product requires the screening of many conditions, consuming much effort (Lua et al., 2014). The development of a rational preformulating strategy for the early phases of vaccine manufacturing would accelerate the development of ViP NP-based vaccines (Volkin and Middaugh, 2010; Cox and Onraedt, 2012; Hem et al., 2010). Advances in biophysical and mass spectrometry technologies are thought to improve our understanding of the nature of ViP NPs and their interactions with excipients and adjuvants. This information might provide new avenues for effective preformulating processes (Hem et al., 2010; Kamerzell et al., 2011; Morefield, 2011).

One example of such advances is the development of a high-throughput analytical tool called empirical phase diagram. In this technique, an antigen is characterized by different biophysical techniques under multiple conditions (pH, temperature, ionic strength, etc.). The obtained information is submitted to a mathematical model and represented in an empirical phase diagram. Color variations represent changes in the structure of the macromolecule. This allows to select the optimum solution conditions, which would be the starting point for the design of a high throughput assay to screen excipients (Maddux et al., 2011; Ausar et al., 2006; Hu et al., 2011). To expedite this process, accelerated stress conditions are commonly used. However, real-time

studies may be necessary to validate the results (Singh, 2013).

Recently, a miniaturized system for high-throughput screening of formulation conditions was developed. It is based on a dynamic light scattering microplate reader that uses two milligrams of sample per formulation. The most promising ones are then analyzed through asymmetric flow field-flow fractionation – multiangle light scattering (Mohr et al., 2013). Finally, different dosage forms are evaluated based on these initial tests.

3.1.2. Adjuvants in ViP NP-based vaccines

Adjuvants in approved vaccines for human use include aluminum salts, oil-in-water emulsions such as MF59, saponin-derivatives such as QS-21 (*Quillaja Saponaria*: fraction 21), and Toll-like receptor (TLR) agonists such as 3-O-desacyl-4'-monophosphoryl lipid A (MPL). Virosomes and VLPs play also a role as adjuvants in some licensed vaccines (Montomoli et al., 2011).

Aluminum salts are, by far, the most used adjuvants. In fact, almost all the licensed VLP-based vaccines, despite their self-adjuncting properties, are formulated with aluminum salts (Table 2) (Lua et al., 2014). Formulation with novel adjuvants such as oil-in-water emulsions, lipid A, and CpG ODNs is being explored in the search for more efficacious vaccines against the hepatitis B virus (Ambrosch et al., 2000; Wang et al., 2016). Among them, a group of adjuvants that has gained much attention in the last decades are nucleic acids.

Nucleic acid adjuvants include double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and non-methylated CpG ODNs. All of them are ligands of TLRs located in the endosomes of dendritic cells. dsRNA, as well as its synthetic equivalent polyinosinic:polycytidylic acid, are recognized by TLR3 and trigger type-I interferon production, enhancing cytotoxic T lymphocyte (CTL) responses (Alexopoulou et al., 2001). ssRNA is recognized by TLR7/8, which trigger T helper 1 (Th1)-polarized responses and cytokine production (Dowling, 2018). Non-methylated CpG ODNs, including different synthetic DNA oligonucleotides, are recognized by TLR9 and induce strong Th1 and CTL responses (Schwarz et al., 2003; Hemmi et al., 2000). In VLP-based vaccines, nucleic acid adjuvants are commonly encapsulated into the VLPs. The packaging of nucleic acid adjuvants inside VLPs has three main advantages over their co-administration in free form: it ensures they reach the endosomal TLRs in dendritic cells where VLPs are degraded (MO Mohsen et al., 2017); it prevents their degradation by nucleases; and it avoids systemic side effects (e.g. the splenomegaly induced by CpG ODNs in mice) (Storni et al., 2004; Klimek et al., 2018). A successful example is Heplisav B®, adjuvanted with CpG 1018 (European Medicines Agency, 2022).

The Adjuvant System is an interesting strategy developed in the 1990s by GlaxoSmithKline to design vaccines against difficult pathogens such as HIV and malaria parasites. This approach consists of a combination of novel and classic adjuvants, aiming for an additive effect. Currently, five Adjuvant System families have been investigated in clinical trials: AS01 (QS-21 and MPL combined with liposomes), AS02 (QS-21 and MPL combined with an oil-in-water emulsion), AS03 (oil-in-water emulsion with alpha-tocopherol), AS04 (MPL adsorbed onto aluminum hydroxide or aluminum phosphate), and AS15 (CpG 7909, QS-21 and MPL combined with liposomes) (Garçon and Di Pasquale, 2017). As for VLP vaccines, AS04 is used in the formulation of Cervarix® and Fendrix® and demonstrated to enhance the activation of antigen-specific T cells (Didierlaurent et al., 2009).

The current consensus is that adjuvants and antigens should be in close proximity in order to be delivered to the same APC (Cho et al., 2000; Eckl-Dorna and Batista, 2009; Jennings and Bachmann, 2007). However, it was demonstrated that adjuvants unbound to VLPs can also reach the same APC. This can be of great value when formulating VLPs that cannot package adjuvants (Gomes et al., 2017; MO Mohsen et al., 2017). Besides this, recent research on vaccine formulation increasingly considers what type of immune response (Th1 or Th2 biased) is required when selecting the optimal adjuvants (Leroux-Roels, 2010; Cimica and

Galarza, 2017; Visciano et al., 2012; Cribbs et al., 2003).

Virosome-based vaccines are a special case because, in general, virosomes themselves are the adjuvants of those vaccines. The biodegradable nature of virosomes explains the reduced side effects observed with virosome-based vaccines compared to non-virosomal adjuvanted vaccines, such as those adjuvanted with aluminum (Bovier, 2008; Herzog et al., 2009).

Epaxal® consists of inactivated hepatitis A virions, acting as antigens, adsorbed to the surface of IRIVs, which play the adjuvant role. The other excipients of the vaccine are sodium chloride and water for injections. Since the year 2000, no thiomersal or other preservatives are included (Health Products Regulatory Authority, 2012; Bovier, 2008). In Inflexal® V, virosomes function both as antigens and adjuvants. As excipients, the vaccine contains sodium chloride, disodium phosphate dihydrate, potassium dihydrogen phosphate, lecithin, and water for injections (Health Products Regulatory Authority 2014).

3.2. Stability and storage

The production of VLPs generally results in highly uniform populations, with low variability between lots (Ding et al., 2018; MJ Rohovie et al., 2017). They are generally more stable than subunit vaccines (Dai et al., 2018) but less than polymeric nanoparticles (MJ Rohovie et al., 2017). The absence of the viral genome makes VLPs more sensitive to changes in external conditions (temperature, pH, ionic strength), particularly during purification and other downstream processing steps (Fig. 5) (Ding et al., 2018; Dai et al., 2018).

One widely used method to increase VLP stability is the introduction of cysteines in the VLP structure to form disulfide bonds between different subunits (Chen et al., 2001; Verardi et al., 2020). However, efforts are focusing on developing new methods that allow to screen the self-assembly and stabilization factors that affect VLPs (Ding et al., 2018).

In general, the self-assembly process of complex VLPs is less efficient than for simple VLPs, and it can be affected when trying to incorporate large antigens (Ding et al., 2018). Also, enveloped VLPs are more sensitive to the environment than non-enveloped ones. In fact, changes in

temperature, shear force, and chemical treatment can affect their integrity. Thermostability can be increased by introducing stabilizing mutations (Dai et al., 2018).

Since most VLP vaccines in the market are liquid suspensions, their storage and distribution require the maintenance of a cold chain at 2–8 °C. Even so, their long-term stability can be limited (B Donaldson et al., 2018). Stabilizing compounds such as glycerol, chitosan, glutamate, and glycine can avoid the aggregation of VLPs in suspension (Shi et al., 2005; Lynch et al., 2012; Correia et al., 2021). To prolong their shelf life, they can also be stored at –20 °C or less by adding a cryopreservative such as glycerol or trehalose to the formulation (Lynch et al., 2012; Correia et al., 2021). However, those storage conditions complicate the delivery of vaccines to developing countries.

To avoid the requirement of a cold chain, alternative methods have been developed. One common technique is freeze-drying, but this process can negatively affect VLP stability (Lang et al., 2009). The addition of cryoprotectants or lyoprotectants can be useful to improve stability (Lan et al., 2018). Research is focusing on the stabilization of VLPs against freeze-drying using polysorbate 20 or trehalose (Lang et al., 2009).

An alternative strategy is spray-drying. In this case, the potential mechanical damage of the freezing process is avoided but the high temperatures required can affect thermolabile VLPs (Kramer et al., 2013; Peabody et al., 2017). Finally, an innovative approach was studied for inactivated influenza virus and influenza VLPs. They were suspended in a carboxymethylcellulose solution, and that suspension was used to coat microneedles using air or vacuum drying methods. Their immunogenicity was retained and their shelf life was prolonged (Koutsouanos et al., 2009; Pearton et al., 2010).

Virosomes do not need such an arduous purification process as VLPs, since they are not derived from host cells. First-generation virosomes, after their formation and incorporation of antigens, are simply diluted to the desired concentration, sterilized, and packaged. This is the case of Epaxal® and Inflexal® V (Moser et al., 2013). However, influenza virosomes have relatively poor stability in aqueous suspensions (Wilschut et al., 2007).

The stability of Inflexal® V has been studied under different storage

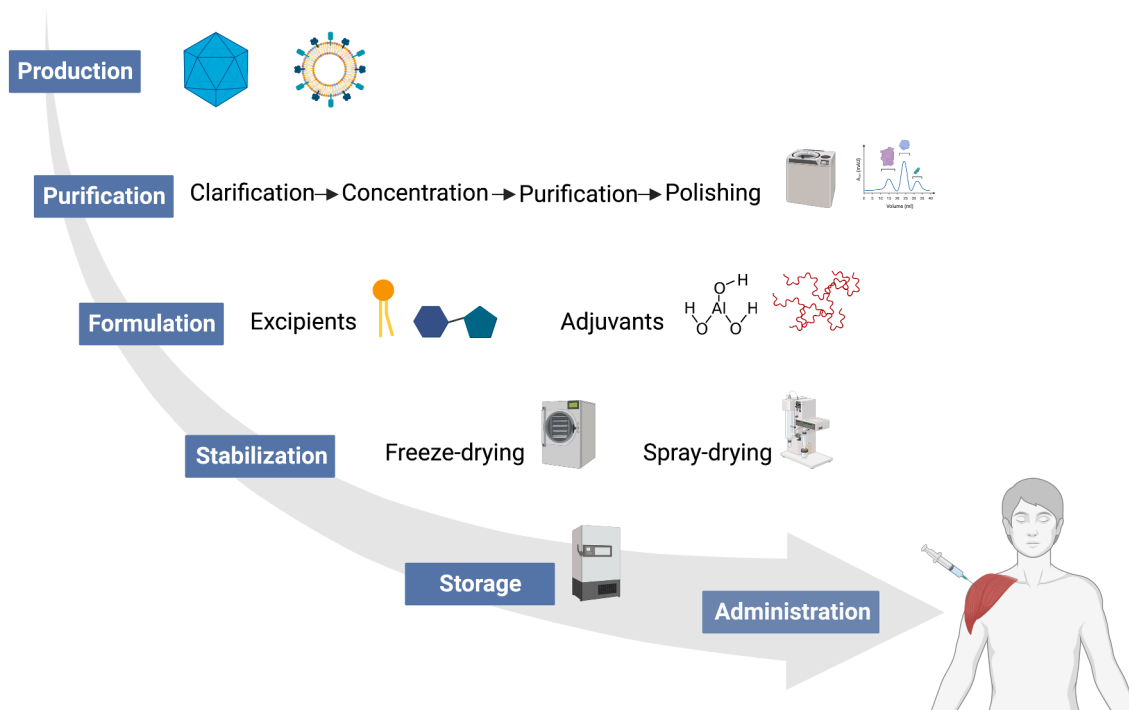


Fig. 5. Main steps of the downstream processing from the production of ViP NPs to the administration of ViP NP-based pharmaceutical products.

conditions. The three virosome pools maintain high levels of HA for 24 months. The stability of the final bulk vaccine is based on endotoxin levels, pH, phospholipid content, potency, sterility, and virosome size. Inflexal® V was stable for at least 18 months at 5 °C, 3 months at 25 °C, and 28 days at 37 °C (Mischler and Metcalfe, 2002).

By contrast, second-generation virosomes, such as malaria, hepatitis C virus, and recurrent vulvovaginal candidiasis candidates, can be freeze-dried (Kammer et al., 2007; Cech et al., 2011). Lyophilization increases their stability and allows the development of alternative administration forms, such as capsules for vaginal mucosal immunization (Vacher et al., 2012).

Although freeze-drying has been shown to increase virosome stability, it is necessary to incorporate a lyoprotectant to preserve virosome structure and function during lyophilization and storage (Brown et al., 2013). Inulin has been evaluated as a lyoprotectant for influenza virosomes. When freeze-dried without lyoprotectants, virosomes lose their structure and fusogenic properties; when inulin was added both characteristics were conserved, as well as their immunogenicity. Moreover, HA potency was retained for at least 12 weeks at 20 °C for the dry powder formulation (Wilschut et al., 2007).

The incorporation of cationic lipids and/or sugars into virosome formulations facilitates freeze-drying as well as the association of negatively charged molecules such as nucleic acids (de Jonge et al., 2007; De Jonge et al., 2007; Kammer et al., 2007; Jamali et al., 2012). Lyophilization has been shown to enhance immune cell responses, while it does not affect antibody responses, compared to non-lyophilized virosomes (Moser et al., 2013).

For example, Pevion Biotech AG developed two malaria vaccine candidates consisting of two antigens (UK39 or AMA49-CPE) formulated in virosomes in a liquid form (Thompson et al., 2008) (PEV3A, NCT00408668) or in a lyophilized form (Cech et al., 2011) (PEV3B, NCT00513669). They were stored at 5 °C for 600 days and at 37 °C for 120 days. Only the lyophilized form maintained the antigen content after both storage conditions (Moser et al., 2013). Furthermore, Pevion Biotech AG holds a patent for an IRIV comprising cationic cholesterol in the lipid membrane, thus allowing appropriate lyophilization and reconstitution of virosomes (WO2006/069,719) (Moser et al., 2013).

In another study, HIV-1 gp41 virosomes were incorporated in different dosage forms for mucosal vaccination: heat spray-dried powders for nasal or oral delivery, and freeze-dried tablets for sublingual administration. The antigenicity of the original liquid formulation was preserved in these solid forms, even after exposure to high temperatures (40 °C), with minimal changes in antigen or adjuvant content in three months. Reconstitution of virosomes from the powder forms did not affect their size, uptake by APCs, or transport to lymph nodes. When exposed to temperatures below −15 °C, mimicking accidental freezing excursions, virosome integrity was preserved. These solid forms appear as thermostable and needle-free candidates for mucosal vaccination against HIV-1, which could simplify their distribution, storage, and administration (Amacker et al., 2020).

4. Biopharmaceutical properties and biocompatibility of ViP NPs

4.1. Administration

4.1.1. Parenteral administration

Traditionally, vaccines have been administered by subcutaneous injection. However, the aluminum adjuvants used in vaccines produced important local reactions such as abscesses and granulomas (Zuckerman, 2000; Garcia-Patos and Pujol, 1995). For this reason, vaccines started to be administered via intramuscular injections, which yield milder local reactions (Mohsen et al., 2020). Although the intramuscular route is characterized by a faster absorption rate than the subcutaneous one (Mohsen et al., 2020), both administration routes result in similar antibody titers (Kündig et al., 2006; Li et al., 2010).

Today, most vaccines are administered via subcutaneous or intramuscular injections (Jain et al., 2015). All marketed VLP vaccines are intramuscularly administered (Mohsen et al., 2020), and so are most in clinical trials (B Donaldson et al., 2018). Similarly, most virosomes in the market (Epaxal®, Epaxal® Junior, and Inflexal® V) and in clinical trials are administered by intramuscular injections (Bovier, 2008; Herzog et al., 2009; Künzi et al., 2009). However, in preclinical studies, the subcutaneous route is the most common due to the ease of injection, the possibility to administer larger volumes, and the flexibility to choose the injection site (Mohsen et al., 2020).

Despite the extended use of subcutaneous and intramuscular vaccination, alternative routes are being investigated to improve efficacy, safety, and administration (Crisuolo et al., 2019). For instance, intradermal administration is being investigated for several VLP-based vaccines, such as simian-human immunodeficiency virus (SHIV)-VLPs (Cubas et al., 2009) or hepatitis C virus (HCV)-VLPs (Christiansen et al., 2019). One innovative approach for this administration route is the use of microneedle patches (Koutsonanos et al., 2009; Zhu et al., 2009; Quan et al., 2013), which enabled painless administration and dose sparing for influenza VLPs (FS Quan et al., 2010; F-S Quan et al., 2010). Intradermal delivery has also been tested for virosomes (Epaxal® and Inflexal® V) with successful results (WO2008/152,052) (Moser et al., 2013; Frösner et al., 2009).

In the field of therapeutic cancer vaccines, VLPs are also injected intratumorally. For example, bacteriophage QB-VLPs packaging A-type CpG ODNs were administered intratumorally in combination with systemic anti-PD-1 checkpoint inhibitors for cancer treatment. Preliminary results show no serious toxicity and suggest the possibility to overcome the resistance to anti-PD-1, resulting in a clinically therapeutic effect (NCT02680184/NCT03084640). Another study showed the efficacy of intratumoral injections of cowpea mosaic virus (CPMV)-VLPs against dermal B16F10 melanomas (P. et al., 2016).

4.1.2. Mucosal delivery

Mucosal immunity relies on the induction of immunoglobulin A (IgA) and immunoglobulin G (IgG) at mucosal sites and has an important role in the prevention of pathogen entry (Boisgérault et al., 2002). A key factor when looking for mucosal responses is the administration route (Boisgérault et al., 2002). Although systemic routes can provide some mucosal protection, antibody titers are usually low. Mucosal delivery needs higher doses of ViP NPs than parenteral injections (B Donaldson et al., 2018), but has several advantages (Jain et al., 2015): (i) it induces both mucosal and systemic immunity; (ii) it does not require needles; and (iii) it avoids infections related to inadequate parenteral administration. The use of VLPs or virosomes rather than subunit vaccines for mucosal vaccination prevents stability problems derived from their exposure to the oral or nasal environment (Vacher et al., 2013; Neutra and Kozłowski, 2006; Holmgren and Czerkinsky, 2005). In addition, the use of TLR7/8 or TLR9 ligands as adjuvants increases IgA production (Mohsen et al., 2020).

Oral administration is a major goal in vaccine research, but the enzymatic digestion of orally administered proteins represents a significant obstacle. One attractive approach for oral delivery of VLP vaccines is their production in edible plants and subsequent ingestion of the plant itself without the need for VLP purification. For example, HBV-VLPs and Norwalk virus-VLPs have been successfully expressed in potato plants (Huang et al., 2005; Mason et al., 1996; Berardi et al., 2017).

Still, most VLPs are administered in liquid oral dosage forms. For instance, the delivery of hepatitis E virus (HEV)-VLPs at mucosal surfaces was studied in several animal models. Oral administration to mice produced an increase of anti-HEV antibodies in their serum (Li et al., 2001). In cynomolgus monkeys, IgM, IgG, and IgA were detected in serum after oral immunization (Li et al., 2004). Chimeric HEV-VLPs have also been evaluated for oral immunization (Niikura et al., 2002; Jariyapong et al., 2013). When incorporating a B cell epitope tag to HEV-VLPs, significant levels of IgG and IgA against the inserted epitope

and the HEV-VLP were detected in intestinal secretions (Niikura et al., 2002).

Another administration route of great interest for ViP NPs is nasal delivery. Intranasal administration of porcine parvovirus (PPV)-VLPs elicited both mucosal and serum antibodies without the need for adjuvants (Sedik et al., 1999). Similar results were obtained with chimeric bovine papillomavirus (BPV)-VLPs containing an epitope from the E7 protein of HPV16 (Liu et al., 1998). Another example is the influenza M2e5x VLP vaccine, based on the matrix protein 2 ectodomain (M2e) of influenza A virus, which induced cross-protection after intranasal administration (Lee et al., 2018). An intranasal influenza vaccine based on virosomes is also under development and protected with a patent (WO2007/107,585) (Moser et al., 2013).

There are other innovative forms of mucosal and dermal delivery of interest (Sanyal and Shi, 2009). An innovative strategy is epidermal powder immunization, a needle-free technology that uses the motive force of helium gas to propel the vaccine powder into the epidermis, which has been successfully applied to hepatitis B surface antigen (HBsAg)-VLPs (Maa et al., 2007). The administration of recombinant simian immunodeficiency virus (SIV) gag p27:Ty-VLPs by vaginal plus oral, or oral plus rectal routes induced strong mucosal and systemic immune responses in primates (Lehner et al., 1992; Lehner et al., 1993). In another study, CPMV-VLPs promoted the regression of B16F10 lung melanoma when administered by inhalation (P. et al., 2016).

4.1.3. Studies comparing administration routes for ViP NPs

Different studies have compared the effect of the administration route on the efficacy of VLP-based vaccines. Intranasal and oral administration of HPV-VLPs have been studied. Baud et al. constructed recombinant *Salmonella* organisms that produce HPV16-VLPs. Those VLPs induced high titers of neutralizing antibodies in mice after a single nasal or oral immunization with live bacteria (Baud et al., 2004). Balmelli et al. compared oral, nasal, and parenteral administration of HPV16-VLPs combined with the cholera toxin as a mucosal adjuvant. Parenteral administration induced specific IgG in serum and genital secretions, but no IgA. Oral immunization was unsuccessful. Nasal administration induced high titers of both IgG and IgA in serum, saliva, and genital secretions (Balmelli et al., 1998).

For SHIV-VLPs, four administration routes were compared: subcutaneous, intramuscular, intraperitoneal, and intradermal. Intradermal administration scored the highest production of antibodies and CTL responses, and involved a higher number of lymph nodes (Cubas et al., 2009). Also, a phase I/II study compared the administration of MelanA-VLPs to patients with stage II-IV melanoma by subcutaneous, intradermal, and intranodal injections. The results, in this case, showed no differences in the generated immune responses (Speiser et al., 2010).

For virosomes, a comparison of the intravaginal and intramuscular routes of vaccination against recurrent vulvovaginal candidiasis with the virosomal vaccine PEV7 has also been carried out (NCT01067131). Both routes showed a rapid and specific response, either in serum and/or in cervicovaginal secretion (De Bernardis et al., 2015).

4.2. Biodistribution

Biodistribution of ViP NPs has not been extensively studied. There are some biodistribution studies of CPMV particles in mice. After intravenous administration, CPMV particles were detected for several days in different organs, such as the liver, kidney, lung, spleen, and lymph nodes. Although they maintained their integrity, they were not infectious, probably due to complement inactivation. When CPMV particles were administered orally, they were found both in the blood and in several tissues. Altogether, the results of these studies show that CPMV particles are bioavailable after oral administration (Rae et al., 2005).

In general, plant viruses are rapidly cleared from plasma and accumulate in several tissues (Rae et al., 2005; Kaiser et al., 2007; Singh

et al., 2007). However, there are some differences in biodistribution among plant viruses. For example, while CPMV accumulates mainly in the liver and only at low levels in the spleen, potato virus X (PVX) shows lower accumulation in the liver and higher in the spleen. When they are PEGylated, the levels in both the liver and spleen are reduced (Lewis et al., 2006).

VLPs of the MS2 bacteriophage (MS2-VLPs) labeled with ^{64}Cu or ^{18}F and injected intravenously in mice also accumulated in the liver and spleen (Hooker et al., 2008; Farkas et al., 2013). Coating of the MS2-VLPs with PEG significantly reduced their retention in the spleen (Farkas et al., 2013). A similar experiment with Q β -VLPs, labeled with gadolinium, showed accumulation in the liver, but not in the spleen. Moreover, changes in the surface charge resulting from the chemical modification of exposed amine groups affected the circulation time of the particles (Prasuhn et al., 2008).

Finally, there are biodistribution studies in mice bearing tumor xenografts. Different levels of MS2-VLPs, CPMV, and PVX were detected in the tumors 24 hours after intravenous injection (Farkas et al., 2013; Shukla et al., 2013). The higher tumor accumulation of PVX compared to CPMV was first attributed to its filamentous shape, which would enhance its extravasation from blood capillaries. However, Shukla et al. observed that PVX levels in tumors were higher after 24 hours than after 5 hours from administration, while it has a plasma half-life of 12.5 minutes, which suggests that the tumor homing mechanism of PVX is not entirely due to an enhanced permeability and retention effect (Shukla et al., 2014). In the case of CPMV, it also accumulates in the tumor after oral administration and intratumoral injection, where it can be detected for several days (Affonso De Oliveira et al., 2022).

Although most biodistribution studies use plant viruses, there are some on the biodistribution of viral vectors derived from animal viruses. Among them, adeno-associated viral (AAV) vectors are the most studied. The biodistribution of AAV vectors depends on the virus-specific serotype tropism and the administration route. Some common patterns can be inferred from the available data: after intramuscular or intravenous administration, AAVs are widely distributed, primarily in well-vascularized organs such as liver, lungs, spleen, and kidneys (Salmon et al., 2014). Ballon et al. studied the biodistribution of iodine-124-labeled AAV vector in nonhuman primates for up to 72 hours after administration. After intravenous administration, the AAV vector was primarily distributed to the liver and heart, but also to the nose, parotid glands, submandibular glands, spleen, long bones, and vertebrae. Pre-existing immunity resulted in a 10-fold increased biodistribution to the spleen. After intracisternal administration, the AAV vector spread through the brain parenchyma, but a significant amount left the cerebrospinal fluid and was distributed throughout the body (Ballon et al., 2020). Also, the biodistribution of the Semliki Forest virus (SFV) vector has been evaluated in mice. High levels of SFV were detected in the heart and lungs after intravenous administration, but not after intraperitoneal injection. In contrast to AAV vectors, SFV vectors do not show a preference for the liver. Of notice, despite the tropism of alphaviruses for the central nervous system, levels of SFV vectors in the brain were low (Rodriguez-Madoz et al., 2007).

An important determinant of the biodistribution of VLPs is viral tropism. Some VLPs maintain the cell tropism of the wild-type virus. For example, HBV-VLPs and rotavirus-derived VLPs show affinity for the liver and the intestine, respectively (Mejía-Méndez et al., 2022; Nooraei et al., 2021). Nevertheless, although knowledge of wild-type viruses can be applied to their derived VLPs, studies of VLP biodistribution would provide a better understanding of VLP behavior after administration.

Virosome biodistribution has not been studied in detail. Zurbriggen et al. used streptavidin as a model antigen to study its biodistribution when bound to IRIVs. After intramuscular administration to mice, high amounts of antigen were detected at the injection site at least after 28 days. Small amounts were detected in the kidney, liver, spleen, and stomach. However, when the antigen was administered alone, it was mostly detected in the kidney. This study shows how the use of the IRIV

carrier prevents the rapid elimination of the antigen through the kidneys and produces a depot effect that may have some implications for the adjuvant effect of IRIVs in vaccines (R Zurbriggen et al., 2000).

A key factor when studying the biodistribution of nanoparticles is the potential formation of a protein corona. Since the term “protein corona” was proposed in 2007 (Cedervall et al., 2007), it has been thoroughly studied for synthetic nanoparticles but not as much in biological particles. However, it is known that a protein corona also forms on viruses and influences viral infectivity (Hills and Howarth, 2022). For example, lipoproteins such as apolipoprotein E are essential for HCV infection (Chang et al., 2007) and soluble heparin sulfonated proteoglycans enhance the infectivity of HPV (Surviladze et al., 2012; Pitek et al., 2016).

Ezzat et al. were pioneers in characterizing the protein corona of an infectious animal virus (the respiratory syncytial virus (RSV)) in different biological fluids (human plasma, bronchoalveolar lavage fluid, fetal bovine serum, and rhesus macaque plasma), and studying its effect on viral pathogenicity. The corona profile was dependent on the nature of the biological fluid, but rather than reflecting its most abundant proteins, it was specifically enriched in certain components. The coronae from the different fluids enhanced RSV infectivity, except in human plasma, where the corona completely neutralized the virus, probably because it was enriched in antibodies and complement factors. Authors speculated that the viral corona factors could be part of the pathogen-associated molecular patterns recognized by the innate immune system (Ezzat et al., 2019).

Indeed, the protein corona does not always hinder VLP-cell interactions. Zackova et al. demonstrated that the protein corona of mouse polyomavirus-VLPs did not prevent the interaction between transferrin molecules conjugated to the VLP surface and their receptors, overexpressed in cancer cells (Zackova Suchanova et al., 2020). Chen et al. went further and took advantage of the protein corona phenomenon to prepare synthetic VLPs by incubating gold nanoparticles in a solution containing an avian coronavirus spike protein (Chen et al., 2016).

The formation of protein coronae has not been directly studied in virosomes. However, it is well-known that liposomes acquire a protein corona in the bloodstream, so we hypothesize something similar would occur to virosomes. Different factors such as liposome composition, surface charge, topology, curvature, etc. influence the characteristics of the protein corona and, therefore, the biodistribution of liposomes. Researchers are trying to exploit this knowledge to develop liposomes that, once in the bloodstream, acquire a protein corona of a desired composition that helps liposomes to achieve their goal (Onishchenko et al., 2021; Caracciolo, 2015). It would be interesting to apply this knowledge to get the best performance of virosome formulations.

In any case, the formation of a protein corona should always be considered when designing pharmaceutical products based on ViP NPs and analyzing their performance.

4.3. Elimination

The clearance of ViP NPs from the blood is similar to that of synthetic particles. However, their elimination from tissues differs significantly. While synthetic particles can persist in the body for weeks (Semmler-Behnke et al., 2008; Oh and Park, 2014; Tarn et al., 2013), ViP NPs disappear within a few days because of proteolytic degradation (Koudelka et al., 2015).

A study of PVX biodistribution and clearance in healthy mice and tumor xenograft mice models revealed that those VLPs were cleared from the blood and accumulated in the reticuloendothelial system within 2–6 hours. This observation agrees with its short plasma circulation half-life (12.5 minutes). In addition, PVX was detected in the kidney and stool, indicative of its elimination through urine and bile. Detection in reticuloendothelial tissues after 72 hours indicates that the clearance from tissues may take several days (Shukla et al., 2014).

CCMV injected intravenously is rapidly dispersed throughout the

body without important tissue preferences except for low levels in the brain. At 1 hour post-injection, the highest levels are found in the bladder, thyroid, liver, spleen, and salivary gland. Twenty-four hours after injection, more than 50% of the particles are excreted. Besides, low levels of CCMV particles are detected in the stool. Overall, CCMV particles are eliminated in 24 hours and do not persist in tissues for long periods (Kaiser et al., 2007).

The elimination of VLPs from animal viruses has not been extensively studied. However, due to their similar characteristics to their parental virus, both in terms of structure and in terms of immunogenicity, it could be expected they are eliminated in a similar way as the complete virion. In fact, it is known that when using VLPs as carriers for other molecules, tolerance may be developed due to VLP neutralization and its subsequent clearance through complement or other immune mechanisms (Jegerlehner et al., 2010).

Regarding virosome elimination, it is expected their constituent phospholipids are degraded by phospholipases and the degradation products are eliminated mainly through the kidney and the liver (Nooraei et al., 2021). However, there are also not comprehensive studies on virosome elimination from the body.

4.4. Biocompatibility

The in vivo toxicity of most viruses and ViP NPs developed for pharmaceutical applications has not been evaluated in detail (Manchester and Singh, 2006; Singh et al., 2006). Some viral vehicles used for gene delivery, such as adenoviruses, induce proinflammatory cytokines that can lead to severe toxicity (Schiedner et al., 2003; Harvey et al., 2002; Christ et al., 2000; Muruve, 2004; Cotter and Muruve, 2005; Reid et al., 2002; Higginbotham et al., 2002; Engler et al., 2004). However, most viruses used in bionanotechnology are not human pathogens, so they do not interact with receptors present in human cells. Therefore, it is unlikely that they activate intracellular pathways that lead to toxic effects, such as the acute induction of proinflammatory cytokines promoted by adenoviruses (Manchester and Singh, 2006). For example, studies in mice comparing the toxicity of CPMV and adenoviral particles revealed that even five times higher doses of CPMV were not toxic as compared to the adenoviral particles. For CPMV, only a lymphoblastic response in the spleen was reported, suggesting a minor immune reaction (Manchester and Singh, 2006).

Although the primary desired outcome of a ViP NP-based vaccine is to induce a potent immune response, this could be a problem when ViP NPs are used as delivery vectors for drugs or heterologous genes. For example, the administration of chimeric HPV-VLPs carrying HPV16-E7 protein to mice with pre-existing immunity did not protect them from an HPV16 E7-positive TC-1 tumor challenge, as compared to mice lacking pre-existing immunity (Da Silva et al., 2001).

Several strategies have been studied to reduce these unwanted immune responses to ViP NPs. The most studied one relies on coating the ViP NP with stealth polymers (Singh et al., 2006). For example, adenoviruses coated with multivalent polymers were less toxic and immunogenic (Green et al., 2004). PVX and CPMV coated with PEG had longer retention time in the circulation and were less immunogenic (Lee et al., 2015). Also, influenza virosomes have been coated with PEG to prevent HA fusion activity (Asadi and Gholami, 2021). However, the repetitive administration of PEGylated nanoparticles can induce anti-PEG antibodies (Qi Yang, 2015), and thus, other approaches are being investigated (Neek et al., 2019). One example is the surface display of the “self-marker” membrane protein CD47 ectodomain and its self-peptide. When displayed on VLPs, it decreases their phagocytosis in vitro (Schwarz et al., 2015).

5. Conclusions

The discovery of VLPs and the development of virosomes endowed researchers with new versatile tools of great pharmaceutical potential.

Modification strategies such as genetic engineering, chemical bio-conjugation, and encapsulation open the scope of possible applications, from their use as vaccines and immunotherapies to gene and drug delivery, and to diagnostics.

Most ViP NPs are formulated as liquid suspensions, and thus, considerations regarding stability are paramount for their formulation. When ViP NPs are to be used as vaccines or immunotherapies, adjuvants are also frequently added. For the moment, alum salts continue to be the most used ones, but research on better alternatives is active. Nucleic acids such as CpG ODNs offer some of the most intriguing results in their combination with ViP NPs.

Pharmaceutical research is also focusing on the generation of ViP NP dry forms, by using freeze-drying and spray-drying. This will not only help to generate formulations with better stability but might enable the development of other types of dosage forms. In this sense, several studies show the therapeutic possibilities offered by the integration of ViP NPs in alternative dosage forms, of particular interest for transdermal or mucosal administration.

All the commercialized ViP NP-based vaccines are administered intramuscularly, but other administration routes are being researched to reduce treatment invasiveness and enhance mucosal immune effects. In general, once absorbed, ViP NPs are rapidly cleared from the blood through their accumulation in organs of the reticuloendothelial system. In tumor-bearing animals, some of the ViP NPs accumulate also at the tumor site. Biodistribution and circulation patterns can be modulated by particle coating. Elimination occurs mainly by proteolysis and urine elimination, but some ViP NPs might be excreted in feces, probably through biliary excretion.

ViP NPs are less toxic than many synthetic particles and, due to their lack of genetic material, safer than viruses. However, some side effects can be derived from their immunogenicity, particularly when used as carriers for drug/gene delivery. Again, polymer coating and decoration with self-proteins can be used to prevent these problems.

In summary, ViP NPs' flexible structure, favorable biopharmaceutical profile, and intrinsic immune characteristics support their undisputed interest for pharmaceutical use. In a second part of this review (Mellid-Carballal et al., 2023), we will cover their applications in drug/gene delivery, diagnostics, and vaccination.

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.

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