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Virus-like particles (VLPs): A promising platform for combating against Newcastle disease virus

Mohammad Sadegh Taghizadeh [a], Ali Niazi [a,*], Alireza Afsharifar [b]

[a] Institute of Biotechnology, Shiraz University, Shiraz, Iran
[b] Plant Virus Research Center, School of Agriculture, Shiraz University, Shiraz, Iran

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ABSTRACT

The global poultry industry plays a pivotal role in providing eggs and meat for human consumption. However, outbreaks of viral disease, especially Newcastle virus disease (NDV), within poultry farms have detrimental effects on various zootechnical parameters, such as body weight gain, feed intake, feed conversion ratio, as well as the quality of egg and meat production. Cases of vaccine failure have been reported in regions where highly pathogenic strains of NDV are prevalent. To tackle this challenge, virus-like particles (VLPs) have emerged as a potential solution. VLPs closely resemble natural viruses, offering biocompatibility and immune-stimulating properties that make them highly promising for therapeutic applications against NDV. Hence, this review emphasizes the significance of NDV and the need for effective treatments. The manuscript will contain several key aspects, starting with an exploration of the structure and properties of NDV. Subsequently, the paper will delve into the characteristics and benefits of VLPs compared to conventional drug delivery systems. A comprehensive analysis of VLPs as potential vaccine candidates targeting NDV will be presented, along with a discussion on strategies for loading cargo into these NDV-targeting VLPs. The review will also examine various expression systems utilized in the production of NDV-targeting VLPs. Additionally, the manuscript will address future prospects and challenges in the field, concluding with recommendations for further research.

1. Introduction

Newcastle disease virus (NDV) is a highly contagious poultry disease that affects the respiratory, nervous, and digestive systems, causing significant losses to the poultry industry [1]. The disease is well-documented, with 18 genotypes and over 236 susceptible avian species reported worldwide [1–3]. The disease is characterized by respiratory, neurological, and gastrointestinal symptoms, leading to high morbidity and mortality rates in infected birds [4]. The ongoing challenge in the development of NDV vaccines lies in the ever-changing nature of genetically varied genotypes that are widely dispersed across different regions [5]. Vaccines, including live attenuated and inactivated formulations, are commonly used to protect poultry from NDV [6]. However, the administration of live vaccines can lead to respiratory symptoms, and the occurrence of frequent vaccine failures can be attributed to pre-existing conditions and the interference caused by maternal antibodies [7]. To address these challenges, recombinant viral vector vaccines have been developed, utilizing various vectors such as Fowlpox virus (FPV) and Herdwicks visna maedi virus (HVT) expressing NDV proteins [5]. These vaccines offer advantages in terms of stability, in vivo replication, and the ability to co-express multiple heterologous proteins [5]. NDV itself is used as a viral vector for bivalent vaccines, demonstrating its potential for creating vaccines against other avian pathogens [8,9]. Additionally, a linear immunodominant epitope in the NDV protein has been identified, showing promise for epitope-based vaccine development [10]. However, viral vector vaccines entail a more intricate manufacturing process and carry the risk of genomic integration. Moreover, the immune response to these vaccines can be dampened by pre-existing immunity against the vector [11].

In recent years, virus-like particles (VLPs) can be emerged as a promising tool for the cargo delivery or vaccine against NDV. VLPs are intricate formations composed of viral structural proteins that closely resemble both the physical structure and antigenic properties of actual viruses. However, they do not contain the viral genome [12]. This unique characteristic makes VLPs non-infectious and safe for therapeutic applications. Moreover, the utilization of VLPs as cargo delivery systems presents several advantages compared to traditional nanoparticles. Firstly, VLPs can be engineered to display specific viral surface

* Corresponding author at: Isfahan-Shiraz Hwy., Bajgah District, Institute of Biotechnology, Shiraz University, Shiraz, Iran.
E-mail address: niazi@shirazu.ac.ir (A. Niazi).

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proteins, enabling targeted delivery of antiviral drugs to the infected cells. By exploiting the natural tropism of NDV, VLPs can be designed to selectively bind to the viral receptors expressed on the surface of the infected cells, thereby enhancing drug uptake and reducing off-target effects [13]. Secondly, VLPs provide a stable and protective environment for encapsulating antiviral drugs, shielding them from degradation and improving their stability during circulation [14]. This encapsulation not only enhances drug solubility but also enables controlled release at the site of infection, ensuring sustained therapeutic concentrations of the drug [14]. Third, VLPs can be easily modified and functionalized to enhance their drug delivery capabilities. Surface modifications, such as the addition of targeting ligands or PEGylation, can improve the pharmacokinetics, biodistribution, and cellular uptake of VLPs [15], further enhancing their therapeutic potential against Newcastle virus. Furthermore, VLPs have inherent immunomodulatory properties due to their structural similarity to native viruses [16]. They can stimulate the immune system, leading to enhanced immune responses and potential synergistic effects with antiviral drugs [16]. Finally, VLPs are generally considered biocompatible and well-tolerated by the body [17]. They have a low risk of inducing adverse immune reactions or toxicity, making them suitable for therapeutic applications [17]. Therefore, the unique characteristics and advantages of VLPs make them promising carriers for antiviral drugs against NDV.

In this comprehensive review, the objective is to provide a detailed overview of the utilization of virus-like particles (VLPs) as vaccines against Newcastle disease virus (NDV). The paper will cover various aspects including the structure and properties of NDV, the characteristics and advantages of VLPs as compared to conventional drug delivery systems. A thorough examination of NDV-targeting VLPs as potential vaccine candidates will be presented, followed by a discussion on strategies for cargo loading into NDV-targeting VLPs. The review will also explore different expression systems employed for the production of NDV-targeting VLPs. Furthermore, future prospects and challenges in the field will be addressed, along with recommendations for further research. By exploring the potential of VLPs as an innovative cargo delivery platform for antiviral therapy against NDV [18], this review aims to contribute towards the development of more effective and targeted treatments for NDV, thereby minimizing its impact on both the poultry industry and public health.

2. Structure and properties of Newcastle virus

Newcastle disease virus (NDV) belongs to the genus *Avulavirus* within the *Paramyxoviridae* family. The virus possesses an enveloped structure and harbors a non-segmented, negative-sense RNA genome spanning approximately 15,192 bp [19]. The viral particle consists of several structural components that contribute to its infectivity and pathogenicity [19,20].

The body of the virus consists of envelope proteins, matrix protein, and ribonucleoprotein complex [21]. The envelope of NDV is derived from the host cell membrane during the process of viral budding. The envelope proteins contain viral glycoproteins, including the hemagglutinin-neuraminidase (HN) and fusion (F) proteins, which play key roles in viral entry, attachment, and fusion with host cells [22]. The HN protein is a multifunctional glycoprotein present on the surface of NDV and mediates the attachment of the virus to sialic acid-containing receptors on host cells. Additionally, the HN protein facilitates the release of newly generated virions from infected cells due to its neuraminidase activity [22]. Upon attachment, the F protein takes on the role of merging the viral envelope with the host cell membrane [23]. It undergoes conformational alterations upon binding to receptors, initiating the fusion of viral and cellular membranes, and enabling the viral genome to enter the host cell. The matrix protein (M) lies beneath the viral envelope and provides structural integrity to the virus [24]. It is involved in viral assembly, budding, and regulation of viral RNA synthesis. The Ribonucleoprotein Complex (RNP) comprises the viral RNA genome associated with nucleoprotein (NP), phosphoprotein (P), and large polymerase protein (L) [22]. Once inside the host cell, the viral RNA is released, and the viral RNP complex is uncoated. The viral RNA serves as a template for both genome replication and synthesis of viral mRNAs by using the P and L proteins [25]. Subsequently, the viral mRNAs are translated by host cell ribosomes to produce viral proteins, including non-structural and structural proteins [22]. The structural proteins, such as NP, P, and L, are involved in viral particle assembly [25]. The newly synthesized viral components are transported to the plasma membrane, where viral budding occurs, resulting in the release of mature viral particles [20].

NDV is a viral pathogen capable of infecting over 200 species of wild birds and domestic poultry [26]. It spreads when infected birds release the virus through their mouth and rear opening, and other birds can catch it by breathing it in or eating it [27]. The presentation of NDV symptoms can vary based on the specific virus strain and the species of bird affected. These symptoms range from reduced food intake and decreased egg production in layer hens to an exceptionally high mortality rate, reaching up to 100 % in unvaccinated birds [26]. Scientists use four categories to describe how harmful the different types of NDV are when they experimentally infect chickens that don't have any other diseases. These categories are determined by how strong the virus is in infecting chickens, i.e. velogenic with high morbidity and mortality, mesogenic with high morbidity and low mortality, lentogenic with low morbidity and mortality, and asymptomatic enteric [26]. Although all NDV are part of the same group, they have different genes and proteins and keep changing over time [27,28]. The symptoms of NDV usually appear within two to fifteen days after a bird gets infected, but sometimes it can take up to four weeks [26]. Scientists have found that NDV can be divided into two groups based on their full genome sequence: one group is mainly not very harmful, and the other has 20 subgroups with different levels of harmfulness [27,28]. These subgroups have preferences for infecting certain bird species and are found in different places around the world [27,28]. Because the number of NDV cases is increasing, and the viruses are changing a lot over time [3,26], it's crucial to have effective ways to control and prevent the disease. Understanding the structure and properties of NDV is crucial for developing effective antiviral strategies. This knowledge provides a foundation for exploring the potential of VLPs for cargo delivery against NDV, which will be further discussed in subsequent sections.

3. Understanding virus-like particles (VLPs)

Since VLPs are comprised of structural proteins from viruses that self-assemble into well-organized structures, they possess the morphology and antigenic properties similar to those present in natural viruses [29–32]. The resulting VLPs resemble intact viruses in terms of size, shape, and surface antigens, making them ideal candidates for various applications, including drug delivery and vaccine [33–36].

VLPs possess several important characteristics that contribute to their suitability as drug carriers [37–39]: (i) lack the viral genome necessary for replication and are therefore non-infectious, resulting in enhanced their safety profile, minimizing the risk of causing disease in recipients. (ii) possess inherent self-assembly properties, meaning they spontaneously form from the viral structural proteins without the need for additional components, which attribute simplifies their production and enhances their stability during storage and circulation. (iii) can elicit robust immune responses due to their structural similarity to native viruses and activate both innate and adaptive immune responses, making them valuable tools for vaccine development and immunotherapy.

VLPs are classified into three main groups based on their structural diversity: non-enveloped, enveloped, and chimeric (Fig. 1). Enveloped VLPs, complex structures with a host-cell-derived membrane and glycoproteins, have been utilized for drug delivery and vaccine development [40]. In a specific case, Rous sarcoma virus (RSV) enveloped VLPs

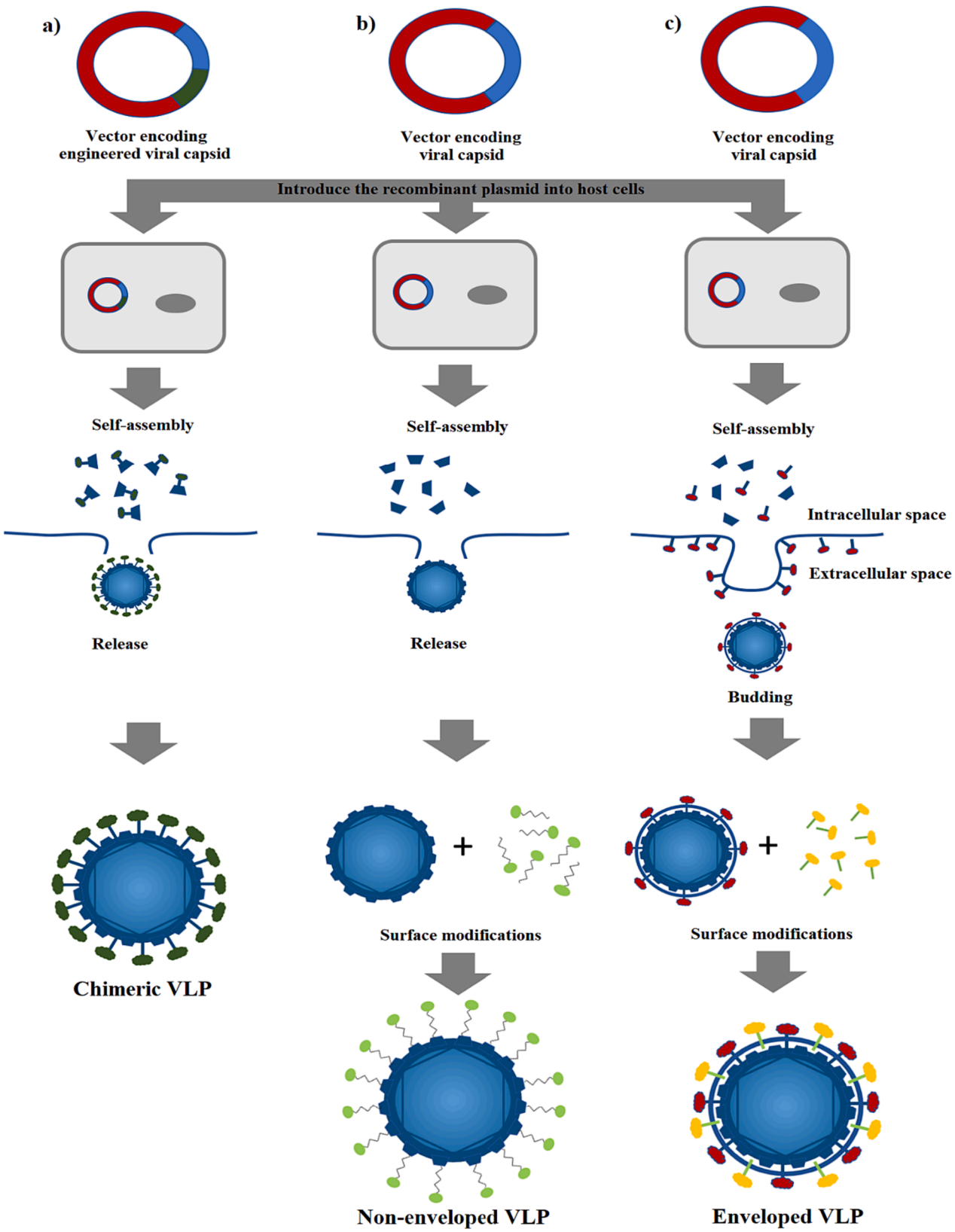

Fig. 1. Schematic diagram of the recombinant production process and variety structures of enveloped, non-enveloped, and chimeric VLPs. Image was . adapted from [41]

were modified to display a humanized CC49 antibody fragment (hCC49) on silkworm larvae. This modification allowed the targeted delivery of doxorubicin to human colon carcinoma cells. The drug was loaded into the hCC49-modified RSV VLPs using electroporation (Fig. 2) [40]. Second group, non-enveloped VLPs, are nano-constructs comprised of single or multiple capsid proteins, lacking cell membranes [41]. They offer the advantage of surface manipulation through chemical and genetic methods, allowing them to expose peptides or epitopes on their surfaces. This manipulation enables them to elicit broader immunological responses [41]. For example, non-enveloped VLPs obtained from the coxsackievirus B3 antigen have been observed to elicit improved humoral immune responses and confer protection against myocarditis in murine models [42]. Finally, chimeric VLPs are nano-constructs that combine structural proteins from different viral serotypes [43], allowing for the modification of the VLP core with antigens and the encapsulation of multiple therapeutic or diagnostic molecules [44]. They offer a range of benefits such as presenting foreign epitopes, encapsulating diagnostic molecules or multiple therapeutic, and specifically targeting cells, tissues, or organs [45]. In a particular study, chimeric VLPs were synthesized using two capsid proteins, gag and M1, obtained from the influenza virus A/swine flu/Iowa/15/30/H1N1, within silkworms [46]. These chimeric VLPs were modified to contain a glycosylphosphatidylinositol-anchored single-chain variable fragment region, enabling specific targeting of colon carcinoma cells [20]. Additionally, these VLPs were employed as carriers for the delivery of doxorubicin at a concentration of 13.7 nM [46]. However, the production of chimeric VLPs is influenced by factors such as glycosylation patterns, steric effects, protein conjugation, antigen length, and cell type [39].

Since NDV is an enveloped virus, the VLPs produced from it possess surface glycoproteins that exhibit correct folding and insertion into membranes, displaying repeating patterns characteristic of enveloped viruses [25]. Studies have indicated that paramyxovirus VLPs can be generated through the expression of either the M protein alone or in combination with different glycoproteins and NP [47–50]. In fact, cells that express the NDV HN, F, NP, and M proteins are capable of releasing particles that exhibit both structural and functional similarities to authentic virus particles [51,52]. The distinctive characteristic of ND VLPs, setting them apart from other paramyxovirus VLPs and numerous other types of VLPs, is their remarkable efficiency of release [52]. In previous studies, the efficiency of release for various paramyxovirus VLPs, as determined by the release of M protein, has been reported to range between 10 % and 50 % [49,50], while ND VLPs exhibit an impressive release efficiency of 84 % [51]. Consequently, it is relatively straightforward to generate substantial quantities of these particles, even from cells that have undergone transient transfection [51,53]. Hence, the M protein is positioned on the inner surface of the NDV envelope and plays a crucial role in the production of ND VLPs [52]. The M protein alone is capable of facilitating VLP assembly. Nevertheless, for efficient VLP budding, it is necessary to co-express the N protein along with either the F or HN glycoprotein in conjunction with the M protein [54]. Therefore, the presence of biologically active glycoproteins within

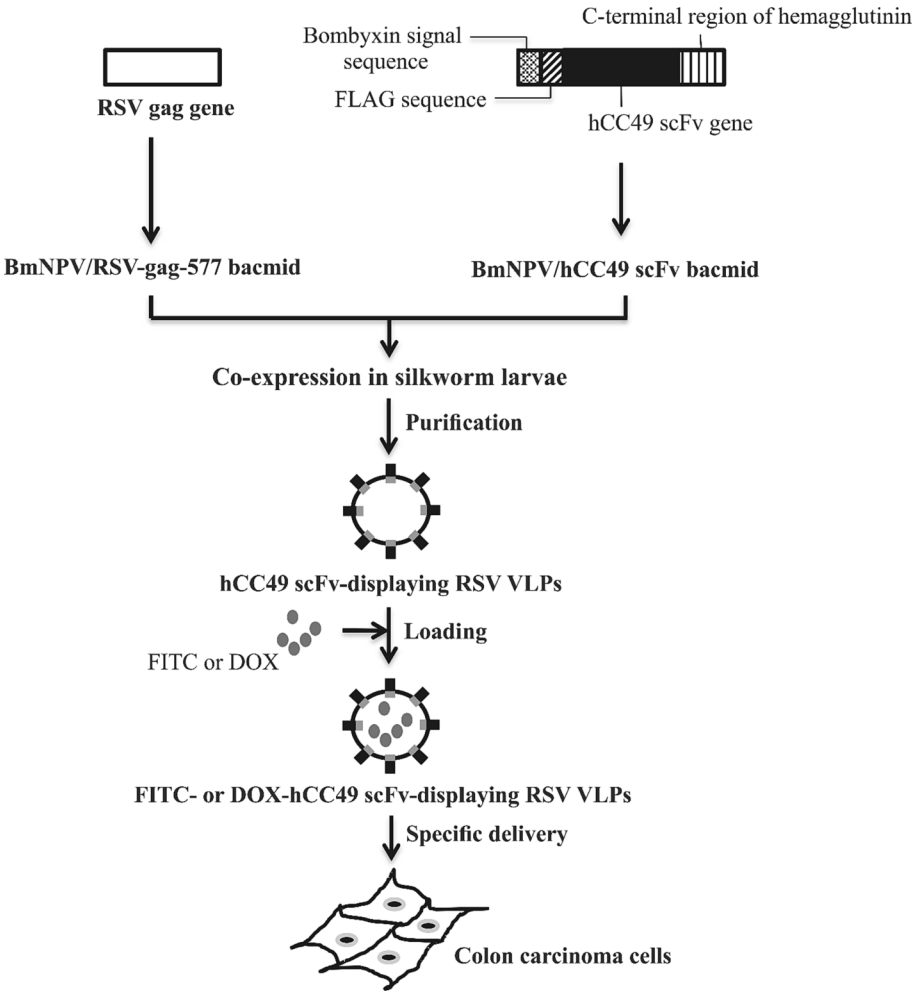

Fig. 2. Schematic presentation of the application of an enveloped VLP in drug delivery. Image was . adapted from [40]

ND VLPs suggests that these proteins have properly folded into an authentic conformation during the process of VLP assembly. Much like the HN protein associated with virions, the HN protein associated with VLPs plays a role in cell binding and exhibits neuraminidase activity [51]. Similar to the F protein associated with virions, the F protein associated with VLPs is capable of facilitating the fusion between the VLP membrane and red blood cell membranes [51]. Furthermore, the assembly of NDV glycoproteins (HN and F proteins) into VLPs is attributed to the specific interactions between the cytoplasmic (CT) and transmembrane (TM) domains of the glycoproteins and the core proteins of NDV. In contrast, the ectodomain of the glycoproteins has minimal influence on the assembly process [55]. In a study conducted by Gravel, McGinnes [56], it was demonstrated that the precise sequence of the transmembrane (TM) domain in the NDV F protein plays a crucial role in determining the conformation of the ectodomain in its preactivation form. This sequence also influences the interactions between the F protein and the HN protein, as well as the fusion activity of the protein.

4. The possibility of VLPs as anti-NDV compounds carriers

The preferred approach to prevent the NDV involves the utilization of killed or live attenuated vaccines. Instances of vaccination failure have been documented in areas where the highly pathogenic strains of NDV are prevalent [3,57,58]. However, the continuous emergence of new variants and their ability to cause outbreaks, even in vaccinated chickens [59], highlights the high mutation rate of NDV. Addressing this issue, an alternative solution would be the administration of antiviral drugs targeting NDV using VLPs, although currently, such drugs are not accessible on the market.

While numerous antiviral compounds have the ability to freely permeate cells and diffuse randomly, there is a need to achieve a more targeted concentration of the drug in specific cells. This targeted delivery aims to enhance drug efficacy and minimize potential side effects [60]. Based on the aforementioned characteristics, VLPs emerge as a prime candidate for this purpose. Promising anti-NDV agents from diverse sources are highlighted in Table 1, demonstrating effective efficacy against NDV. Despite the natural tropism of VLPs, their efficacy and ability to target specific cells are commonly enhanced through chemical modifications of their surface to present anti-NDV agents and signal molecules, respectively [61]. Current antiviral candidates against NDV face limitations such as drug resistance, potential side effects, and restricted efficacy against diverse viral strains [62]. However, utilizing VLP formulation may show potential in addressing these challenges. To date, there have been no documented instances of incorporating

Table 1
Potential candidate anti-NDV agents derived from various sources.

Anti-NDV compound	Source	Efficacy	Characterizations	Ref.
Nitazoxanide	An approved medication	IC_{50} = 60 μM	Mode of action against NDV and other viruses: (i) impeding influenza virus replication by hindering the maturation of the hemagglutinin protein, (ii) causing misfolding of the Sendai virus F protein followed by preventing its transport to the plasma membrane, (iii) reducing the size of viroplasm in rotavirus and resulting in a decrease in dsRNA formation, (iv) the activation of the protein kinase R (PKR) of Hepatitis C virus, subsequently triggering the phosphorylation of eukaryotic initiation factor 2α (eIF2-α), and (v) disrupting the replication of bovine viral diarrhea virus by triggering endoplasmic reticulum stress.	[110–115]
n-Docosanol	An FDA-approved saturated fatty alcohol	Survival rate from 37.4 % to 53.2 %	Reducing the NDV load in digestive tissues (26.2 % to 33.9 %), respiratory tissues (38.3 % to 63 %), nervous tissues (26.7 % to 51.1 %), and lymphatic tissues (16.4 % to 29.1 %); decreasing virus shedding in oropharyngeal discharge and feces, effectively restricting the spread of NDV.	[116]
Propolis flavone	Derived from plants, or collected and processed by bees	–	A valuable adjunct and antiviral agent, particularly in chickens administered inactivated or activated vaccines; exhibits the capacity to enhance both humoral and cellular immune responses, thereby bolstering immune activity; successfully developed live vaccines against NDV using chitosan nanoparticles.	[117,118]
Fucoidan	A sulfated polysaccharide found in the cell wall of brown algae	IS_{50} > 2000	Revealed a 48 % reduction in viral infection and decreased expression of the NDV HN protein.	[119]
C4- and C5-substituted 2,3-unsaturated sialic acid derivatives	Synthesized compounds	IC_{50} values ranging from 0.03 to 13 μM	Decrease in the infection of NDV within Vero cells through inhibitory activity against neuraminidase; exhibited minimal toxicity.	[120]
9-butyl-harmol	β-carboline derivatives	–	Targeting the GSK-3β and HSP90β, thereby suppressing the infection of NDV.	[121]
Maackiain (SR-1) and echinoisoflavanone (SR-2)	Flavonoids extracted from *Sophora interrupta* Bedd. plant	–	Inhibit viral entry, replication, and transcription against NDV.	[122]
Telomycin	Derived from *Streptomyces coeruleorubidus*	–	Inhibit the hemagglutination activity of NDV strain (MN635617) with $log10^6$ infectivity titers (EID_{50}/mL).	[123]
Diethyl phthalate	derived from *Streptomyces misakimycin*	–	Inhibition of the hemagglutination (HI) activity of NDV strain (MN635617) at $log10^7$ infectivity titers (EID_{50}/mL).	[124]
Limonin	Derived from citrus fruits	–	Reduces NDV replication in various cell lines and down-regulates the expression of NDV HN and matrix genes.	[125]
Solomonseal Polysaccharide (PS) and Sulfated Polysaccharide (sPS)	Derived from *Codonopsis pilosula*	–	Killing the virus and suppressing the expression of viral antigen during *in vitro* tests; Lowest mortality and morbidity rates, with the highest cure rate during in vivo tests.	[126]
Ulvan	A sulfated polysaccharide derived from *Ulva clathrate*	IC_{50} value of 0.1 μg/mL	Inhibiting cell–cell spread through direct interaction with the F0 protein.	[127]
Lithium Chloride	A chemical compound	–	Effectively inhibited NDV replication and reduced the levels of a stress-inducible protein called GRP78	[128]
Emetine	A natural product derived from *Hedera helix*, *Alangium longiflorum*, and other organisms	–	Inhibit the replication of NDV viral RNA at noncytotoxic concentrations; impede viral entry into host cells; decrease in the synthesis of viral polymerase.	[129]
Degraded β-chitosan	Derived from crustaceans	–	Effectively reduces the hemagglutination titer to zero, indicating its strong antiviral properties	[130]

antiviral compounds specific to NDV into VLPs. However, this concept holds promise for future investigations, as the combination of VLPs and antiviral candidates can achieve synergistic effects. Hence, the immunomodulatory properties of VLPs can enhance the antiviral activity of candidates by promoting immune activation and reducing viral replication. This combined approach holds promise for improving therapeutic outcomes and overcoming drug resistance. The use of VLPs as drug carriers presents an innovative strategy to enhance drug delivery, improve efficacy, and potentially minimize side effects associated with conventional drug formulations [34]. This approach holds promise for advancing antiviral therapies against NDV.

5. Application of VLPs for combating against NDV

Vaccination plays a critical role in combating viral infections. However, vaccines developed for NDV have primarily relied on lentogenic genotype II NDV strains, such as clone 30, B1, and LaSota, provided many years ago [63]. The genetic distances between these vaccine strains and the currently prevalent genotype VII strains range from 18.3 % to 26.6 % [27]. While these vaccines effectively prevent clinical symptoms, they do not completely suppress virus shedding in individuals infected with virulent NDV strains [3]. VLPs not only possess immunogenic properties but also have the potential for cargo delivery and gene therapy by carrying and encapsulating various molecules, enabling targeted delivery to specific cells, tissues, or organs [64]. Cells employ receptor-mediated endocytosis to uptake VLPs, which are then transported through endosomes and lysosomes for degradation [64]. However, this limits drug delivery efficacy. To overcome this, VLPs are utilized as nanocarriers due to their capacity to evade lysosomal degradation, enhancing cargo delivery potential [64]. To address the limitations of current inactivated commercial vaccines for NDV, researchers developed dendritic cell (DC)-targeting strategies to enhance adaptive and mucosal immune responses against respiratory pathogens [65]. During a study, chimeric VLPs decorated with a DC-binding peptide (DCpep) were created, incorporating haemagglutinin–neuraminidase (HN) derived from NDV and haemagglutinin (HA) derived from avian influenza virus (AIV) [65]. The DCpep-decorated chimeric VLPs effectively stimulated DCs *in vitro* and elicited robust immune responses in chickens, including increased secretion of secretory immunoglobulin A (sIgA) and differentiation of splenic T-cells [65]. Administration of 40 μg of chimeric VLPs provided complete protection against both heterologous and homologous NDV strains, as well as AIV H9N2 [65]. Moreover, intranasal administration of DCpep-decorated chimeric VLPs resulted in superior immune responses compared to intramuscular administration, as evidenced by enhanced secretion of sIgA and reduced shedding period of virus [65]. Overall, these chimeric VLPs hold promise as vaccine candidates for controlling AIV H9N2 and NDV, offering a versatile platform with multivalent antigens. Importantly, in cases of infection with a homologous NDV strain, the use of H9/F-DCpep-cVLPsIN led to a 100 % protection rate, ensuring survival without any viral shedding [66]. In contrast, the inactivated NDV vaccine exhibited an approximate 90 % protection rate [66]. In another investigation, the immunostimulatory effects of VLPs incorporating the M and HN proteins of genotype VII NDV were examined on DCs *in vitro* [67]. Subsequently, the immunogenicity of these VLPs was evaluated in mice. The findings revealed that when stimulated by VLPs, immature bone marrow-derived dendritic cells (BMDCs) exhibited an up-regulation in the expressions of MHC II, CD86, CD80, and CD40 molecules [67]. Additionally, VLPs induced increased secretion of IFN-γ, TNF-α, IL-12p70, and IL-6 cytokines by BMDCs [67]. Moreover, VLPs elicited the immunostimulatory potential of DCs, resulting in the proliferation of autologous T-cells [67]. Furthermore, VLPs demonstrated the ability to elicit strong cellular and humoral immune responses [67]. The administration of VLPs In C57BL/6 mice resulted in the recruitment of mature DCs to the spleen. This was supported by a notable enhancement in the proliferation of CD11c + CD86 + cells, indicating their double-positive phenotype [67]. In an independent study, VLPs were constructed by assembling the three key structural proteins of velogenic NDV, which include HN, F, and M glycoproteins [68]. Mice receiving three doses of NDV VLPs with a 10-day interval between each vaccination exhibited significantly elevated levels of IFN-γ, TNF-α, IL10, and IL2 ($p \leq 0.05$) in their splenocyte suspension cells as assessed by ELISA, while mice receiving two doses of NDV VLPs with the same interval and mice receiving a B1 live vaccine booster displayed comparatively lower cytokine levels [68]. The group of chickens that received only NDV VLPs had higher levels of CD8 + cells, and all of them survived when exposed to NDV sub-genotype VII, whereas two out of six chickens that received NDV VLPs followed by a B1 live vaccine did not survive the infection. In conclusion, these findings strongly suggest that the T-cell immune response is more crucial than the B-cell response in NDV infection [68]. A study was undertaken to develop a bivalent vaccine capable of providing a single immunization against both AIV and NDV [69]. The researchers constructed a chimeric VLP by combining the M1 protein and HA protein of AIV with a chimeric protein containing the cytoplasmic and transmembrane domains of AIV neuraminidase protein (NA) and the ectodomain of NDV HN protein (NA/HN) [69]. When chickens were immunized with this chimeric VLP vaccine, they produced antibodies specific to both AIV H5 and NDV. The hemagglutination inhibition (HI) titers and specific antibodies elicited by the chimeric VLPs were comparable, in a statistically significant manner, to those induced by the respective commercial monovalent vaccines [69]. Furthermore, chickens that received the chimeric VLP vaccine and were subsequently challenged with the NDV F48E9 displayed complete protection [69]. In another study, an approach was employed the infectious bronchitis virus (IBV) S1 protein and the ectodomain of NDV F protein were separately fused with the transmembrane and carboxy-terminal domain of IBV S protein (STMCT) [70]. This resulted in the creation of two distinct components, rS and rF. These components were then combined with the IBV M protein to construct a chimeric VLPs (IBV-NDV VLPs). The findings revealed that immunization with the chimeric IBV-NDV VLPs effectively stimulated both humoral and cellular immune responses [70]. Furthermore, in a challenge study, administration of 100 μg VLP of the chimeric IBV-NDV VLPs provided complete protection (100 %) against virulent challenges of both IBV and NDV, resulting in a significant reduction in viral RNA levels in tissues and swabs [70]. Overall, these findings highlight the highly immunogenic nature of the chimeric IBV-NDV VLPs and their ability to completely protect against virulent challenges of IBV and NDV [70]. In another study, a bac-to-bac expression system was utilized to create VLPs based on a genotype VII strain of NDV [71]. The NDV VLPs were constructed using the NDV M protein as a structural framework, with protective antigen NH and F glycoproteins exposed on the surface [71]. The immunization assay conducted in the study demonstrated that NDV VLPs provided a longer duration of protection, reduced virus presence in tissues, and a shorter period of virus shedding compared to the commercially available LaSota-based vaccine when challenged with a genotype VII NDV strain [71]. These findings suggest that NDV VLPs have the potential to serve as an alternative to current live vaccines that may not match the circulating genotypes, offering improved control and elimination of NDV in avian populations. In a separate investigation, a bivalent VLPs vaccine was generated, consisting of the M1 and HA glycoproteins derived from the AIV H5N1, alongside a hybrid protein integrating the ectodomain of the F protein derived from NDV with the transmembrane and cytoplasmic domains of the HA protein derived from AIV [72]. Chickens immunized with this chimeric VLP vaccine demonstrated high levels of antibodies against H5N1 AIV and NDV, as well as protection against subsequent lethal infections caused by both viruses [72]. These results suggest that the chimeric VLP vaccine holds promise as a strategy for simultaneously controlling AIV and NDV in poultry. In a different research, researchers produced NDV VLPs composing the NDV F and AIV M1 proteins using the baculovirus/insect cell expression platform [73]. The healthy chickens were then vaccinated with NDV VLP vaccines

formulated with oil emulsion, containing different doses of VLPs [73]. The vaccines induced the production of antibodies against NDV and offered protection against a lethal challenge in a dose-dependent manner. A single vaccination with NDV VLP vaccine at concentration of 10 or 50 μg resulted in complete protection of chickens from a lethal challenge and significantly reduced the amount of virus shed during the challenge [73]. Through a separate study, researchers examined VLPs produced by NDV proteins strain AV, including NP, M, HN, and F [51]. The VLPs were produced in specific quantities and given to BALB/c mice as an immunization agent without any adjuvant [51]. The resulting immune responses were similar to those obtained with equal quantities of inactivated NDV vaccine. Additionally, the researchers successfully incorporated F and HN glycoproteins derived from NDV B1 strain into these VLPs [51]. Moreover, the successful integration of foreign peptides into the VLPs was achieved by fusing them with the HN or NP protein [51]. Significantly, the ectodomain of the Nipah virus G protein was effectively integrated into NDV VLPs by combining it with the transmembrane and cytoplasmic domains of the NDV HN protein [51]. According to described methodologies, the application of VLPs for combating against NDV holds significant promise. Further research and development efforts are needed to optimize VLP design, improve targeting strategies, evaluate long-term safety, and translate these approaches into practical clinical applications.

6. Strategies for loading cargo within NDV-targeting VLPs

Efficiently loading cargo VLPs is a critical step in the development of VLP-based delivery systems. This process can be accomplished through a range of approaches, including chemical, biological, and physical strategies. For a comprehensive understanding of these strategies, a detailed review is available elsewhere [60], providing thorough insights into their implementation and effectiveness. These strategies contain a range of techniques, including fusion of foreign proteins with VLPs [25,53,55,74], de novo packaging with nucleic acids [75], osmotic shock [76], polymer-mediated adsorption [77–80], disassembly and reassembly [81–83], chemical linking [84–86], and physical interaction between VLPs and cargo [87–89]. However, among these techniques, the current method firstly employed for loading cargos into VLPs designed for NDV entails the fusion of a foreign protein to the VLPs [90]. To enable the delivery of cargo proteins, they are either fused to or inserted into the component proteins of VLPs using a linker [90]. Once incorporated, these cargo proteins are transported to the target cells or cell nuclei and subsequently released, aided by viral protease or other mechanisms [90]. In a specific study, the sequences of F glycoprotein derived from the NDV NA-1 strain and HA glycoprotein derived from the AIV H9N2 strain were applied for the purpose of cargo loading into VLPs [65]. A fusion sequence, referred to as H9/F, was created, which encoded the ectodomain of HA and the transmembrane domain of F [65]. In order to improve cargo loading efficiency, the DCpep sequence (FYPSYHSTPQRP) and the melittin signal peptide derived from the baculovirus expression vector pFastBac1-HM were added at the 5′ end of H9/F and then inserted into the pFastBac1 vector [65]. The Bac-to-Bac baculovirus/insect expression platform was utilized to generate rBV-H9/F and rBV-H9/FDCpep. Co-transfection of rBV-H9/F, rBV-HN, and rBV-M resulted in the production of H9/F-cVLPs, whereas co-transfection of rBV-H9/F-DCpep, rBV-HN, and rBV-M led to the production of H9/F-DCpep-cVLPs (Fig. 3) [65]. This approach has been employed not only for antigen presentation but also for loading and delivering proteins with various characteristics and functions, including antibodies, transcription factors, and enzymes [60]. Initially, the packaging and delivery of relatively small proteins within VLPs were constrained by the limited space and size of the VLPs. However, in recent years, the field has witnessed significant advancements in gene editing technology for therapeutic applications. This progress has sparked growing interest among researchers in exploring the protein delivery or protein fusion with larger molecular weights, such as dCas9-base editors and Cas9 proteins [91–94]. However, it remains unclear whether the function or dynamics of the cargo is affected by the scaffold protein. Other techniques for cargo delivery using NDV-targeting VLPs have not been fully explored yet, but hold potential for future research.

7. Expression systems for producing NDV-targeting VLPs

Choosing the proper expression system is of utmost importance in

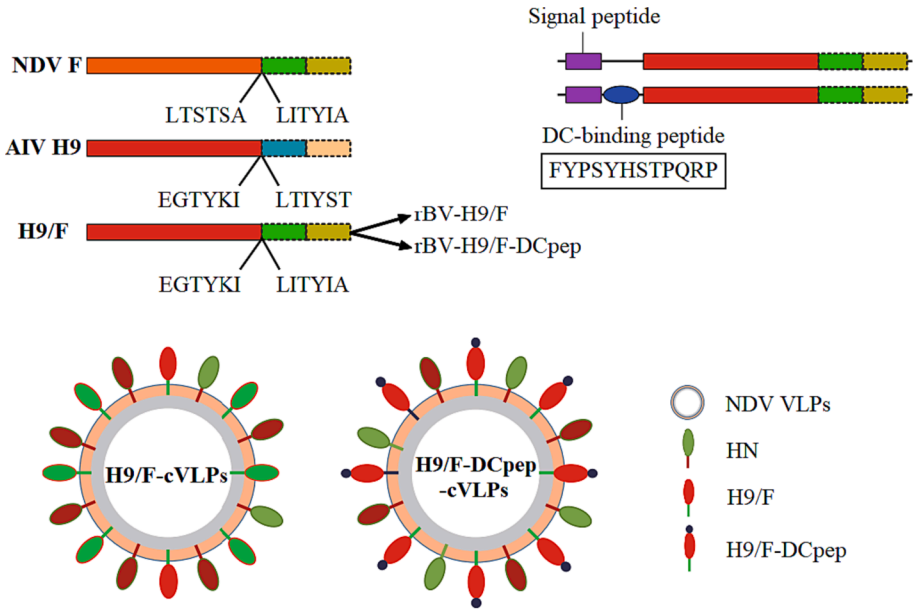

Fig. 3. A visual representation of the strategy involving the fusion of a foreign protein with VLPs for targeted delivery against NDV. The ectodomain of the NDV F protein was substituted with the ectodomain of the AIV H9 protein, resulting in the formation of H9/F. To targeted cargo delivery, the DCpep sequence was fused to the amino terminus of the H9/F protein using a GS-linker. The H9/F protein, along with the NDV HN protein, was co-expressed on the surface of NDV VLPs. Image was .
adapted from [65]

VLP production to ensure correct protein folding and post-translational modifications. These modifications, including glycosylation and phosphorylation, can significantly affect the quaternary structure of VLP proteins, ultimately impacting the immunogenicity of the vaccine [95]. A wide range of expression platforms have been employed for this purpose, including bacteria, yeast, baculovirus/insect cells, plant cells, avian and mammalian cells, and cell-free systems [34]. Among them, baculovirus/insect, plant, and avian expression platforms have been utilized for the production of NDV-targeting VLPs so far.

The baculovirus/insect expression system is widely employed for the production of VLPs [96]. The presence of baculovirus in this expression system offers convenience and rapidity, making it proper for producing viral vaccines that frequently alter their surface antigens between outbreaks [97]. Furthermore, insect cell expression systems provide numerous advantages for VLP production, including the ability to form multi-protein VLPs, high protein yield comparable to bacterial or yeast systems, and the ability to make post-translational modifications [95]. The insect cell lines derived from *Trichoplusia ni* (Tn5) and *Spodoptera frugiperda* (Sf9/Sf21) are commonly used for this purpose [96]. Research investigations focusing on the production of NDV-targeting VLPs consistently indicate that the baculovirus/insect expression system was the prevailing choice among expression systems for this purpose. In a study, genes encoding M and HN proteins were cloned into the baculovirus pFastBac Dual transfer vectors and recombinant baculovirus was produced using baculovirus/sf9 insect expression system, resulting in generated 3.5 mg/mL of the purified NDV VLPs [67]. In a separate study, the pFast/GFP/F/M/HN construct was introduced into DH10 Bac competent cells to generate recombinant Bacmid, which was subsequently expressed in sf9 insect cell lines. This expression process yielded a substantial production of 635 μg/mL of NDV VLPs [68]. Using a similar expression system, Shen, Xue [69] successfully generated recombinant baculovirus, which led to the production of an impressive 1.56 mg of chimeric VLP in a 1 L sf9 cell culture. Similar to previous studies, Noh, Park [72] employed the sf9 insect expression system to generate recombinant baculovirus. However, they utilized the pHAM1F/HA vector as their cloning construction in this study. While the baculovirus/insect cell platform offers numerous benefits for VLP production, it does have a potential drawback. In comparison to mammalian cells, the N-glycosylation pattern of expressed glycoproteins in insect cells is relatively simpler, which may limit the production of some VLPs [98]. In addition, studies indicate a lack of efficient assembly of ND VLPs in insect cells [99]. However, a promising approach has been developed to address this issue. By enhancing the N-glycosylation machinery of insect cells, particularly in specific strains like Ea4, the production of therapeutic human glycoproteins can be simplified [100]. This strategy holds potential for overcoming the limitations of insect cell-based glycosylation and improving the applicability of the baculovirus/insect cell platform for diverse VLP applications.

Plants serve as an attractive option for VLP vaccine manufacturing due to their ability to produce abundant recombinant proteins at a low cost. They possess the necessary machinery for post-translational modifications, ensuring proper protein folding and assembly. Plant-based expression systems carry a lower risk of introducing human pathogens compared to other systems. Moreover, plants can perform N-glycosylation, unlike bacteria-based expression systems [101]. Nevertheless, in the current study, a plant expression platform was established to produce NDV-targeting VLPs transiently in *Nicotiana bethamiana* plants [102]. Through the expression of the HN and/or F glycoproteins derived from a genotype VII.2 strain, NDV VLPs were effectively generated within the plant expression system [102]. In this study, genes encoding HN, F, and M glycoproteins of NDV were cloned into the pEAQ-HT vector and transferred into plant cells using *Agrobacterium tumefaciens* strain *AGL*-1 [102]. The authors indicated that based on their findings, a conservative estimate suggests that 1 Kg of infiltrated leaf material would be enough to conduct vaccination for 10,000 chickens. Each dose would contain 1024 hemagglutination (HA) units (equivalent to 10 log2) [102]. In addition, other studies have successfully demonstrated that plant expression systems, specifically in potato (0.3–0.6 μg of HN protein per mg of total leaf protein) [103], tobacco (3 μg of HN protein per mg of total leaf protein) [104], maize (max. 1.66 μg/mL of F protein and max. 2.4 μg/mL of HN protein) [105], and rice [106], exhibit robustness in producing structural proteins of NDV, including F and HN proteins. Hence, these plant expression systems present a viable alternative for producing plant-based NDV VLPs in future studies. It should be noted that plants faced limitations in VLP production due to lower yields and unique N-glycosylation patterns, compared to mammalian expression systems [107]. Nevertheless, recent advancements in plant expression systems and plant glycoengineering have overcome these challenges [101].

Expression systems based on animal cells continue to be highly valuable platforms for the production of VLPs [96,97]. These animal cell expression platforms are known for their efficiency in generating recombinant proteins, primarily due to their capability to perform intricate and precise post-translational modifications necessary for correct protein folding [108]. CHO, ELL-0, CAP-T, HEK293, Vero 9, and BHK-21 cell lines are widely employed for recombinant VLP production [97]. Among them, CHO stands out as the most commonly utilized cell line due to its non-human origin, thereby reducing the risk of contamination with human viruses [97]. In a specific study, a chimeric VLP was constructed against NDV [51]. Accordingly, genes encoding NDV NP, M, F, and HN proteins were introduced into the pCAGGS expression vector [51]. In addition, Nipah virus G protein cDNA was linked into HN gene, generating a chimeric construction (HN/NiVG). Subsequently, the chimeric construction transfected into the ELL-0 cell lines using Lipofectamine to produce chimeric VLPs. The expressed VLP had a yield of 509 μg/mL [51]. Another study has been investigated the essentiality and adequacy of the M protein in the production and budding of NDV VLPs [52]. For this purpose, authors cloned genes encoding uncleaved F (F-K115Q), M, HN, and NP proteins of NDV into pCAGGS vector and transferred into the ELL-0 cell lines using Lipofectamine for evaluating the production of NDV VLPs. The findings exhibited that the efficiency of VLP release is 83.8 % ± 1.1 % [52]. Nonetheless, the utilization of mammalian cell expression systems for clinical material production is accompanied by significant potential drawbacks, including prolonged expression time, high production costs, low protein yield, and the risk of cell lines being contaminated with mammalian pathogens [96]. It should be noted that the efficient production of ND VLPs can be accomplished in avian cells, eliminating the issues with production in mammalian cells [12,69,109].

8. Challenges and future perspectives

Scaling up the production of VLPs for clinical applications presents various challenges. The optimization of VLP production systems, including cell culture and recombinant protein expression platforms, becomes essential to meet the high demand for large-scale manufacturing. Addressing regulatory requirements, cost-effectiveness, and preserving the integrity and quality of VLPs during scale-up processes are crucial considerations. VLPs can be susceptible to environmental factors, such as temperature, pH, and freeze–thaw cycles, making it vital to ensure their stability during storage, transportation, and administration, as this directly affects their structural integrity and effectiveness in delivering cargo. Strategies to stabilize VLPs and establish appropriate storage conditions must be explored. Precisely targeting specific cells or tissues infected with Newcastle virus using VLPs remains a challenge. Identifying and validating specific receptors or biomarkers that can be targeted by VLPs becomes necessary for efficient drug delivery. Further research is required to enhance targeting specificity and minimize off-target effects. Additionally, VLPs can trigger immune responses, which can influence their safety and therapeutic efficacy. Understanding and controlling the immunogenicity of VLPs is crucial to minimize adverse reactions and enable repeated

dosing if needed. Strategies such as surface engineering or incorporating immunomodulatory agents should be investigated to modulate immune responses effectively.

Looking ahead, the future of VLPs in cargo delivery against NDV appears promising, driven by ongoing advancements in genetic engineering, chemical conjugation, and hybrid VLP technologies. These advancements will enable the creation of more sophisticated and customizable VLP-based cargo delivery systems. By integrating additional functionalities such as stimuli-responsive release, multi-stage targeting, or combination therapies, the therapeutic outcomes can be further enhanced. Combining VLPs with other antiviral agents like small-molecule drugs or nucleic acid-based therapeutics can yield synergistic effects and effectively target various aspects of NDV infection, including viral replication, immune evasion, and host cell interactions. However, to bring these advancements to fruition, extensive preclinical and clinical studies are required to evaluate the safety, efficacy, and pharmacokinetics of VLP-based cargo delivery against NDV. Collaboration among researchers, clinicians, and regulatory authorities is crucial to expedite the translation of VLP technologies into clinical practice. Furthermore, adherence to regulatory guidelines and careful consideration of manufacturing, quality control, and product characterization are essential. VLP-based cargo delivery systems hold great potential in combating NDV infections, and by overcoming current challenges and capitalizing on future opportunities, the development of safe, effective, and personalized therapies for this viral disease can be realized.

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CRediT authorship contribution statement

Mohammad Sadegh Taghizadeh: Conceptualization, Writing – original draft, Writing – review & editing. **Ali Niazi:** Funding acquisition, Writing – review & editing. **Alireza Afsharifar:** Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ali niazi reports was provided by Shiraz University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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