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1.47 Viruses and Virus-Like Particles in Biotechnology: Fundamentals and Applications

A Roldão, **AC Silva, and MCM Mellado**, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal and Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal
PM Alves and MJT Carrondo, IBET, Oeiras, Portugal

Glossary

downstream process Technical steps, including all purification steps, of a bioprocess from the product harvest to the purified product.
metabolic engineering The practice of optimizing genetic and regulatory processes within cells to increase the cells' production of a certain substance.
prophylactic A medication or a treatment designed and used to prevent a disease from occurring.
transduction The process of introducing foreign DNA deliberately into cells using viral vectors.

transfection The process of introducing foreign DNA deliberately into cells using nonviral methods.

upstream process Technical steps of a bioprocess from the cell development to product harvest including all cell culture steps.

1.47.1 Introduction

Viruses are microscopic infectious agents and exclusively intracellular organisms that depend on animal, plant, or bacterial cells to multiply. All viruses have one nucleic acid, RNA or DNA, enclosed within a protein coat that protects the viral genes. Many viruses also have a viral envelope covering the nucleocapsids, consisting of host cell membrane lipids and proteins, and viral glycoproteins that protects viruses from degradation outside the cell and helps their attachment to host cell membranes.

Since the initial discovery of tobacco mosaic virus by Martinus Beijerinck in 1898 [1], more than 5000 viruses have been described in detail. Nonetheless, significant progress in virus identification and propagation was possible only in the 1950s upon the establishment of cell culture technology [2, 3]. With this development, animal cell cultures gradually replaced live animals in the preparation of viral antigens for vaccines. More recently, advances in virology and molecular biology allowed the development of platforms for the production of virus-like particles (VLPs) as vaccines against emergent diseases and viral vectors for gene therapy.

VLPs are composed of viral structural proteins that, when expressed in recombinant systems, form multiprotein structures mimicking the organization and conformation of authentic native viruses but lacking the viral genome. Several applications have been proposed for viruses and VLPs: vaccination, gene therapy, drug delivery, nanotechnology, and bioweapons, among others.

This article aims at reviewing the fundamentals, applications, and production strategies of viruses and VLPs, as well as the challenges and perspectives for the future.

1.47.2 Types of Viruses

Viruses are among the simplest biological systems. They behave as intracellular parasites and contain a limited set of genes that encode information for their replication, encapsidation, and cell-to-cell propagation. Two groups of viruses have been under considerable investigation: animal viruses, due to their impact on animal and human health, and bacterial viruses, normally used as a model to study the basic concepts of biology and virology. Plant viruses, despite their agricultural importance, and algal viruses constitute a less-studied class of viruses.

The nature of the viral genome has many implications on the life cycle of the virus. Viral genome consists of one or more fragments of single-stranded (ss) or double-stranded (ds) RNA or DNA. Most DNA and positive (+) ssRNA viruses rely on host cell machinery for initiating the replication and transcription of viral genome, not requiring *de novo* synthesis of other viral gene products. Others such as dsRNA or negative (−) ssRNA viruses have to deliver their own polymerase into the host cell for the synthesis of viral proteins essential for initiating viral replication, as host cells do not possess the machinery to cope with dsRNA or negative (−) ssRNA. In addition, many dsRNA viruses deliver their genome within a protein capsid to avoid contact between the dsRNA and the cell cytosol.

1.47.2.1 Virus with DNA

A DNA virus has DNA as its genetic material and replicates using a DNA-dependent DNA polymerase. The nucleic acid is usually dsDNA; ssDNA is less common, as during replication ssDNA typically expands to dsDNA. This class of viruses belongs to group I or group II of the Baltimore classification system for viruses. Group I virus includes the *Adenoviridae*, *Herpesviridae* (herpes simplex virus – HSV), and *Baculoviridae* (*Autographa californica* multicapsid nucleopolyhedrovirus – see **Figure 1**(**a**)) families, while group II virus includes the *Parvoviridae* and *Circoviridae* families (**Table 1**). Although group VII viruses such as hepatitis B (hepatitis B virus – HBV) contain a DNA genome, they are not considered DNA viruses according to the Baltimore classification, but rather reverse transcribing viruses because they replicate through an RNA intermediate.

1.47.2.2 Virus with RNA

Conversely, an RNA virus has RNA as its genetic material. This nucleic acid is usually ssRNA but may be dsRNA. The International Committee on Taxonomy of Viruses classifies RNA viruses as those that belong to group III, group IV, or group V of the Baltimore classification system for viruses and does not consider viruses with DNA intermediates as RNA viruses. These viruses are included in group VI and possess ssRNA genomes that replicate using reverse transcriptase. Viruses such as the human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), and Rous sarcoma virus (RSV) (members of the *Retroviridae* family – see **Figure 1**(**b**)) are included in this group.

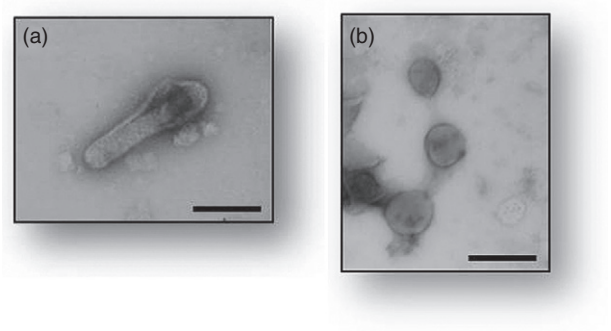

Figure 1 Electron micrographs of negatively stained (a) *Autographa californica* multicapsid nucleopolyhedrovirus and (b) retrovirus. Scale = 100 nm.

Table 1 List of viruses with DNA genomes

Virus family	*Examples (common names)*	*Virion (naked/ enveloped)*	*Capsid symmetry*	*Nucleic acid type*	*Group*
Adenoviridae	Adenovirus, canine adenovírus type 2, human adenovirus type 2 and 5	Naked	Icosahedral	ds	I
Baculoviridae	*Autographa californica* multicapsid nucleopolyhedrovirus	Enveloped	Helical	ds	I
Circoviridae	Transfusion-transmitted virus	Naked	Icosahedral	ss circular	II
Hepadnaviridae	Hepatitis B virus	Enveloped	Icosahedral	ds-RT	VII
Herpesviridae	Herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus, varicella virus	Enveloped	Icosahedral	ds	I
Papillomaviridae	Human papillomavirus, bovine papillomavirus	Naked	Icosahedral	ds circular	I
Parvoviridae	Parvovirus B19, porcine parvovirus, adeno-associated virus	Naked	Icosahedral	ss	II
Polyomaviridae	Polyomavirus, JC polyomavirus, simian virus 40, goose hemorrhagic polyomavirus, murine polyomavirus	Naked	Icosahedral	ds circular	I
Poxviridae	Smallpox virus, vaccinia virus	Complex coats	Complex	ds	I

ds, double stranded; ss, single stranded; RT, reverse transcriptase.
Source: http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/index.htm

1.47.2.2.1 Group III: dsRNA viruses

dsRNA viruses represent a large group of pathogens whose genome can be monopartite or segmented up to 12 fragments. These viruses do not release the free dsRNA genome into infected cells and require that transcription and synthesis of new dsRNA genomes take place in confined environments. Reovirus and rotavirus, members of the *Reoviridae* family, are included in this group (Table 2).

1.47.2.2.2 Group IV: (+) ssRNA viruses

In contrast with the dsRNA or the (−) ssRNA viruses, the genomes of (+) ssRNA viruses are infectious. Icosahedral (+) ssRNA viruses represent a large fraction of all viruses known and include important human pathogens: poliovirus, human rhinovirus, hepatitis A virus, Norwalk virus, astrovirus, alphavirus, and members of the *Flaviviridae* family that carry an RNA-containing nucleocapsid are some examples. Unlike (−) ssRNA viruses, the nucleoproteins responsible for protecting the genome from non-specific cellular RNA binding are not expressed in (+) ssRNA viruses. Thus, the synthesis of progeny viruses requires that the capsid proteins of these viruses specifically package the viral RNA genome while excluding the ubiquitous cellular RNA. Group IV includes the *Flaviviridae* (hepatitis C virus – HCV), *Coronaviridae* (severe acute respiratory syndrome virus – SARS virus), *Picornaviridae*, *Astroviridae*, *Togaviridae*, and *Caliciviridae* families (Table 2).

1.47.2.2.3 Group V: (–) ssRNA viruses

Negative ssRNA viruses are classified into seven families: *Rhabdoviridae*, *Paramyxoviridae*, *Filoviridae*, *Bornaviridae*, *Arenaviridae*, *Bunyaviridae* (Hantaan virus and rift valley fever virus – RVFV), and *Orthomyxoviridae* (influenza viruses). The first four families are characterized by nonsegmented genomes. The remaining three have genomes comprising 2, 3, and 6–8 (−) sense RNA segments, respectively. The large group of (−) sense RNA viruses includes (1) highly prevalent human pathogens such as respiratory syncytial virus, influenza, and human parainfluenza viruses; (2) two of the most deadly human pathogens, namely Ebola and Marburg viruses; and (3) viruses with a major economic impact on the poultry and cattle industries, namely the Newcastle disease virus (NDV) and rinderpest virus (Table 2).

Table 2 List of viruses with RNA genomes

Virus family	*Examples (common names)*	*Virion (naked/ enveloped)*	*Capsid symmetry*	*Nucleic acid type*	*Group*
Arenaviridae	Lymphocytic choriomeningitis virus	Enveloped	Complex	(–) ss	V
Astroviridae	Astrovirus	Naked	Icosahedral	(+) ss	IV
Birnaviridae	Infectious bursal disease virus	Naked	Icosahedral	ds	III
Bornaviridae	Borna disease virus	Enveloped	Helical	(–) ss	V
Bunyaviridae	California encephalitis virus, Hantaan virus, rift valley fever virus	Enveloped	Helical	(–) ss	V
Caliciviridae	Norwalk virus, hepatitis E virus, rabbit hemorrhagic disease virus, norovirus	Naked	Icosahedral	(+) ss	IV
Coronaviridae	Corona virus, severe acute respiratory syndrome virus	Enveloped	Helical	(+) ss	IV
Filoviridae	Ebola virus, Marburg virus	Enveloped	Helical	(–) ss	V
Flaviviridae	Dengue virus, hepatitis C virus, yellow fever virus, Japanese encephalitis virus	Enveloped	Icosahedral	(+) ss	IV
Orthomyxoviridae	Influenza A virus, influenza B virus, influenza C virus, Isavirus, Thogotovirus	Enveloped	Helical	(–) ss	V
Paramyxoviridae	Measles virus, mumps virus, respiratory syncytial virus, human parainfluenza viruses, Newcastle disease virus, rinderpest virus	Enveloped	Helical	(–) ss	V
Picornaviridae	Enterovirus 71, Rhinovirus, Hepatovirus, Cardiovirus, Aphthovirus, Poliovirus, Parechovirus, Erbovirus, Kobuvirus, Teschovirus, Coxsackie, hepatitis A virus	Naked	Icosahedral	(+) ss	IV
Reoviridae	Reovirus, rotavirus, bluetongue virus	Naked	Icosahedral	ds	III
Retroviridae	Human immunodeficiency virus, simian immunodeficiency virus, Rous sarcoma virus, simian–human immunodeficiency virus, lentivirus, Moloney murine leukemia virus	Enveloped	-	ss-RT	VI
Rhabdoviridae	Rabies virus	Enveloped	Helical	(–) ss	V
Tetraviridae	*Nudaurelia capensis* ω virus	Naked	Icosahedral	(+) ss	IV
Togaviridae	Rubella virus, alphavirus	Enveloped	Icosahedral	(+) ss	IV

ds, double stranded; (–) ss, negative single stranded; (+) ss, positive single stranded; RT, reverse transcriptase.
Source: http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/index.htm

1.47.3 Types of VLPs

VLPs are multimeric protein complexes composed of viral structural proteins that assemble spontaneously when expressed in recombinant systems. These structures mimic the organization and conformation of authentic native viruses but lack the viral genome. To date, different types of viruses have been mimicked by VLPs: viruses with single or multiple capsid proteins and with or without lipid envelopes (**Table 3**).

1.47.3.1 VLPs of Structurally Simple Viruses

In most nonenveloped viruses, the nucleocapsids are formed by a single, virally encoded protein. Thus, VLPs of these viruses are relatively easy to generate as the assembly process relies solely on the expression levels of a single protein. Some examples are presented in **Table 3**. One of the most studied VLPs of structurally simple viruses is the human papillomavirus (HPV)-VLP. Although the native virus contains the major and minor capsid proteins of HPV, L1 and L2, respectively [30, 68], the HPV-VLP is formed just by L1 protein organized in 72 pentameric capsomers. Canine parvovirus and porcine parvovirus (PPV)-VLPs are also formed by a single protein, VP2, the major structural protein in both viruses. These VLPs are normally expressed in insect cells and induce high immunogenic responses [56]. In the case of PPV-VLPs, large-scale production is doable [69]. Norwalk virus (NV)-VLPs, VLPs of hepatitis E virus (HEV), and chimeric VLPs from simian virus 40 (SV40) constitute other examples of expression in insect cells. NV-VLPs have been extremely useful as a source of diagnostic antigen to monitor disease outbreaks since the native virus has limited growth in cell culture [68]. These particles have also been shown to be effective at stimulating IgG, IgA, and humoral responses in mice [70]. Preliminary phase I trials in humans have confirmed that they are safe and effectively stimulate IgG and IgA responses [52]. A truncated form of HEV capsid readily assembles into a VLP in insect cells, but this has not yet been tested as a vaccine. In mice immunization studies, these VLPs were able to induce systemic and mucosal immune responses following oral administration [25]. Finally, by genetically manipulating the major capsid protein of SV40 (VP1), it is possible to accommodate foreign peptides on the surface of this protein in such a way that assembled chimeric SV40-VLPs display these peptides on their surface. This confers to chimeric SV40-VLPs the essential features to be used as a controlled, cell type-specific gene delivery system [66].

Table 3 VLPs developed for prophylactic vaccines

Virus	*Family*	*Expression system*	*Recombinant proteins*	*Envelope*	*Structure*	*Product status*	*References*
AAV	*Parvoviridae*	B/IC	VP1, VP2, VP3	No	20–25 nm (sl)	None	[4]
BTV	*Reoviridae*	B/IC	VP2, VP3, VP5, VP7	No	69 nm (sl or dl)	Preclinical	[5]
Ebola virus	*Filoviridae*	Mammalian cells[a, b]	Glycoprotein and VP40	Yes, two proteins	65 nm	Preclinical	[6–8]
Ebola virus	*Filoviridae*	B/IC	Glycoprotein and VP40	Yes, two proteins	70 nm diameter, 800–1500 nm length	Preclinical	[9]
Enterovirus 71	*Picornaviridae*	B/IC	VP0, VP1, VP2	No	25–27 nm	Preclinical	[10, 11]
GPHV	*Polyomaviridae*	B/IC	VP1	No	45 nm	None	[12]
GPHV	*Polyomaviridae*	*Saccharomyces cerevisiae*	VP1, VP2	No	20–45 nm	None	[12]
Hantaan virus	*Bunyaviridae*	B/IC	M and S segments	Yes, two proteins	100–300 nm	None	[13]
Hantaan virus	*Bunyaviridae*	Mammalian cells-VVES	M and S segments	Yes, two proteins	100–300 nm	None	[13]
HBV	*Hepadnaviridae*	*Saccharomyces cerevisiae*	Surface antigen	Yes, three proteins	22 nm (sl)	Licensed (GlaxoSmithKline and Merck & Co.)	[14, 15]
HBV	*Hepadnaviridae*	*Hansenula polymorpha*	Surface antigen	Yes, three proteins	18.1 nm	Licensed (Berna Biotech Korea Corp)	[16, 17]
HBV	*Hepadnaviridae*	Transgenic plants	Surface antigen	Yes, three proteins	17 nm (sl)	Preclinical	[18]
HBV	*Hepadnaviridae*	B/IC	Surface and core antigens	Yes, three proteins	27 nm (sl)	Preclinical	[19]
HBV	*Hepadnaviridae*	CHO cells	PreS1, preS2, and surface antigens	Yes, three proteins	22 nm (sl)	Licensed (BioTechnology General)	[20]
HBV	*Hepadnaviridae*	*Escherichia coli*	Core antigen	Yes, three proteins	35 nm (sl)	None	[21]
HCV	*Flaviviridae*	*Escherichia coli*	Truncated core protein	Yes, two proteins	18–32 nm	None	[22]
HCV	*Flaviviridae*	B/IC	Core, E1, E2	Yes, two proteins	40–60 nm (sl)	Preclinical	[23]
HDV	n.a.	Mammalian cells[c]	Surface antigen	Yes, three proteins	22–50 nm	None	[24]
HEV	*Caliciviridae*	B/IC	Truncated major capsid protein ORF2	No	23.7 nm	Preclinical	[25]
HIV	*Retroviridae*	B/IC	Pr55gag, envelope	Yes, two proteins	100–120 nm (sl)	Preclinical	[26–29]
HIV	*Retroviridae*	Mammalian cells[a,b]	Pr55gag, envelope	Yes, two proteins	100–120 nm	Preclinical	[29, 30]
HIV	*Retroviridae*	Mammalian cells-VVES	Gag, pol	Yes, two proteins	100–150 nm (sl)	None	[31]
HIV	*Retroviridae*	*Saccharomyces cerevisiae*	Gag protein	Yes, two proteins	108–138 nm (sl)	None	[32]
HPV	*Papillomaviridae*	*Saccharomyces cerevisiae*	L1 major capsid protein	No	30–50 nm	Licensed (Merck & Co.)	[33, 34]
HPV	*Papillomaviridae*	*Escherichia coli*	L1 major capsid protein	No	50–60 nm (sl)	Preclinical	[35–37]
HPV	*Papillomaviridae*	B/IC	L1 major capsid protein	No	50 nm	Licensed (GlaxoSmithKline)	[33, 38]
HPV	*Papillomaviridae*	Mammalian cells-VVES	L1 major capsid protein	No	40–50 nm	None	[39]
HSV	*Herpesviridae*	B/IC	VP23, VP5, VP26, VP19c, VP21, VP24, VP22a	Yes	90 nm	None	[40]
IBDV	*Birnaviridae*	Mammalian cells-VVES	VP2, VP3, VP4	No	60–65 nm	None	[41]

(*Continued*)

Table 3 (*Continued*)

Virus	*Family*	*Expression system*	*Recombinant proteins*	*Envelope*	*Structure*	*Product status*	*References*
IBDV	*Birnaviridae*	B/IC	VP2, VP3, VP4	No	60 nm (sI)	None	[42]
Influenza A virus (H1N1)	*Orthomyxoviridae*	B/IC	HA, NA, M1, M2	Yes, three proteins	80–120 nm (sI)	Preclinical	[43]
Influenza A virus (H3N2)	*Orthomyxoviridae*	B/IC	HA, NA, M1, M2	Yes, three proteins	80–120 nm (sI)	Phase IIa (Novavax)	[44]
Influenza A virus (H5N1)	*Orthomyxoviridae*	B/IC	HA, NA, M1	Yes, three proteins	80–120 nm (sI)	Phase I/IIa (Novavax)	[45]
IPCV	n.a.	Transgenic plants	Coat protein	No	85–120 nm	None	[46]
IPCV	n.a.	*Escherichia coli*	Coat protein	No	30 nm	None	[46]
JC polyomavirus	*Polyomaviridae*	B/IC	VP1	No	20 nm	Preclinical	[47]
Marburg virus	*Filoviridae*	B/IC	Glycoprotein and VP40, nucleoprotein	Yes, two proteins	90–100 nm	Preclinical	[48]
Marburg virus	*Filoviridae*	Mammalian cells[a]	Glycoprotein and VP40	Yes, two proteins	50–70 nm	Preclinical	[7, 8]
NDV	*Paramyxoviridae*	B/IC	HN	Yes, two proteins	154–408 nm	None	[49]
No	*Caliciviridae*	*Pichia pastoris*	Capsid protein	No	35 nm	Preclinical	[50]
No	*Caliciviridae*	B/IC	Capsid protein	No	40 nm	Preclinical	[51]
NV	*Caliciviridae*	B/IC	Capsid protein	No	38 nm	Phase I	[52]
NV	*Caliciviridae*	Transgenic plants	Capsid protein	No	23 and 38 nm	Preclinical	[18]
NωV	*Tetraviridae*	B/IC	Coat protein	No	40 nm	None	[53]
Poliovirus	*Picornaviridae*	B/IC	VP0, VP1, VP3	No	27 nm	None	[54]
PPV	*Parvoviridae*	B/IC	VP2	No	22 nm (sI)	Preclinical	[55, 56]
RHDV	*Caliciviridae*	B/IC	VP60	No	40 nm	Preclinical	[57]
Rotavirus	*Reoviridae*	B/IC	VP2, VP6, VP7, and/or VP4	No	70–80 nm	Preclinical	[58–60]
RSV	*Retroviridae*	*Escherichia coli*	Gag protein	Yes, two proteins	70 nm	None	[61]
RVFV	*Bunyaviridae*	B/IC	N, Gn, Gc	Yes, two proteins	56–78 nm	None	[62]
SARS virus	*Coronaviridae*	B/IC	S, E, M	Yes, one protein	100 nm	Preclinical	[63]
SIV	*Retroviridae*	B/IC	Pr56gag, envelope	Yes, two proteins	130 nm	Preclinical	[64]
SV40	*Polyomaviridae*	B/IC	VP1, VP2, VP3	No	45–50 nm	None	[65, 66]
SV40	*Polyomaviridae*	*Escherichia coli*	VP1, VP3	No	50 nm	None	[67]

AAV, adeno-associated virus; B/IC, baculovirus/insect cells; BTV, bluetongue virus; DI, double layer; GPHV, goose hemorrhagic polyomavirus; HA, hemagglutin; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; HN, hemagglutin-neuraminidase; HPV, human papillomavirus; HSV, herpes simplex virus; IBDV, infectious bursal disease virus; IPCV, Indian peanut clump virus; M1, matrix 1; M2, matrix 2; n.a., not assigned; NA, neuraminidase; NDV, Newcastle disease virus; No, norovirus; NV, Norwalk virus; NωV, *Nudaurelia capensis* ω virus; PPV, porcine parvovirus; RHDV, rabbit hemorrhagic disease virus; RSV, Rous sarcoma virus; RVFV, rift valley fever virus; SARS, severe acute respiratory syndrome; SIV, simian immunodeficiency virus; SI, single layer; SV40, simian virus 40; VP, viral protein; VVES, vaccinia vector expression system.

[a]Transient transfection.

[b]Stable cell line.

[c]Baculovirus transduction.

1.47.3.2 VLPs with Lipid Envelope

Many pathogenic viruses are surrounded by an envelope consisting of host cell membrane lipids and proteins, and viral glycoproteins. These proteins are the targets of neutralizing antibodies and essential components of a vaccine. Due to the inherent properties of the lipid envelope, assembly of enveloped VLPs is technically complex. Some examples are presented in **Table 3**. The first VLP vaccine to be produced and characterized consisted of spherical particles with diameter between 17 and 25 nm formed by the surface antigen of the hepatitis B virus (HBsAg) co-assembled with host cellular membranes [71]. Meanwhile, several other enveloped VLPs have been successfully developed. The HCV-VLP, produced in the baculovirus/insect cell system by co-expression of core E1 and E2 proteins, and VLPs of Ebola and Marburg virus, vaccine candidates against these emergent diseases, are two examples. The first have been tested in mice and baboons and shown to be effective at stimulating both cellular and humoral immune responses [23, 72]. The latter have been shown to protect small laboratory animals as well as nonhuman primates against lethal challenge by Ebola and/or Marburg viruses [8]. HSV-VLP [40], NDV-VLP [49], Hantaan-VLP [13], and RVFV-VLP [62] exemplify other enveloped VLPs successfully expressed and assembled in the baculovirus/insect cell system. Substantial efforts were put forward to recreate the envelope of SARS coronavirus [63] and the envelope of viruses from *Retroviridae* family in a form that permits the efficient induction of broadly neutralizing antibodies. Although none of the retrovirus-derived VLPs have gone into clinical trials yet, initial experiments with HIV-VLP [73] and SIV-VLP [64] in animal models look promising. Chimeric VLPs containing the gag capsid protein from SIV and the envelope protein from HIV [74], RSV-VLPs formed by *in vitro* assembly of gag proteins [61], and VLPs for influenza A virus subtypes H3N2 [44] and H5N1 [45] are also part of the group of successfully produced VLPs with lipid envelope.

1.47.3.3 VLPs with Multiple-Protein Layers

VLPs composed of multiple interacting capsid proteins are more challenging to produce than those formed by one or two major capsid proteins, due to the site of protein expression: proteins encoded by multiple discrete mRNAs and not processed from a single polyprotein tend to be differently localized in the cell, significantly affecting the efficiency of the assembly process. Thus, it is essential to guarantee that all interacting capsid proteins are expressed in the vicinity of each other and within the same cell [68]. Assembly of VLPs by expressing more than one structural viral protein has been achieved for various members of *Picornaviridae* (poliovirus [54] and enterovirus 71 [11]) and *Birnaviridae* (infectious bursal disease virus [41, 42]) families. The adeno-associated virus type 2 VLPs are also included in the category of VLPs with multiple-protein layers. These particles are produced by coinfecting insect cells with recombinant baculovirus coding for adenovirus capsid proteins VP1, VP2, and VP3 [4]. VLPs have been efficiently produced for the members of the *Reoviridae* family. These viruses are considerably complex to mimic as they are characterized by multiple concentric layers and different capsid proteins. The bluetongue virus (BTV)-VLP and rotavirus-like particles (RLPs) are two such cases. Intact and biologically active BTV-VLPs are produced in insect cells by simultaneously expressing all four structural proteins of the BTV (VP2, VP3, VP5, and VP7) using a multicistronic recombinant baculovirus [75]; RLPs are also formed in a similar way. The rotavirus itself consists of three concentric protein layers: an inner core of VP2, a middle layer of the polymorphic protein VP6, and an external layer formed by the glycoprotein VP7, with 60 spikes of VP4. When treated with trypsin, VP4 is cleaved into VP5 and VP8, which allows virus binding and entry to cells [76]. Inside the core, small amounts of VP1 and VP3 exist, constituting less than 3% of the total viral protein [77]. Although VP4 is considered to play a key role in RLP stabilization upon assembly, it is consensual that VP2, VP6, and VP7 are sufficient to form a triple-layered particle structurally similar to the native virus and which is biologically functional [59, 60, 78, 79]. RLPs are normally expressed in the baculovirus/insect cell system by co-expression of the three rotavirus structural proteins mentioned above. The diversity of structures normally observed at the end of the culture indicates that the assembly process is highly inefficient (**Figure 2**). These particles are extensively used to study virus structure, role of protein in viral morphogenesis, protein function and biochemical properties, virus interaction with the mammalian host cell, and protein–protein interactions, as reviewed by Palomares and Ramirez [81]. RLPs are also vaccine candidates against rotavirus disease. They are considered safe and induce a robust antibody response and protection in animals if they are engineered to include one or both of the outer capsid proteins VP4 and VP7, properly formulated with a potent adjuvant, and administered intramuscularly [58, 79, 82]. Even intrarectal immunization, inducing a local mucosal response, has been reported as sufficient for protection from rotavirus infection [83]. It is possible that RLPs may provide a viable alternative to the existing live virus vaccines, which has recently raised efficacy concerns in developing countries such as Bangladesh and South Africa [84].

1.47.4 Production Platforms: A Focus on Animal Cell Technology

1.47.4.1 Cell Lines for Virus Production

Production of viruses was initially performed using the natural hosts of the viruses. Upon the establishment of cell culture technology in the 1950s, animal cell cultures gradually replaced live animals in the preparation of viral antigens for vaccines. The observation by Enders and co-workers [2] that non-nervous tissue culture could be used to replicate and produce poliovirus paved the way to large-scale production of vaccines. This discovery led to the development of the first commercial product generated using mammalian cell cultures (primary monkey kidney cells), the poliovirus vaccine. Regrettably, primary monkey kidney cells presented many drawbacks such as the relatively high risk of contamination with adventitious agents

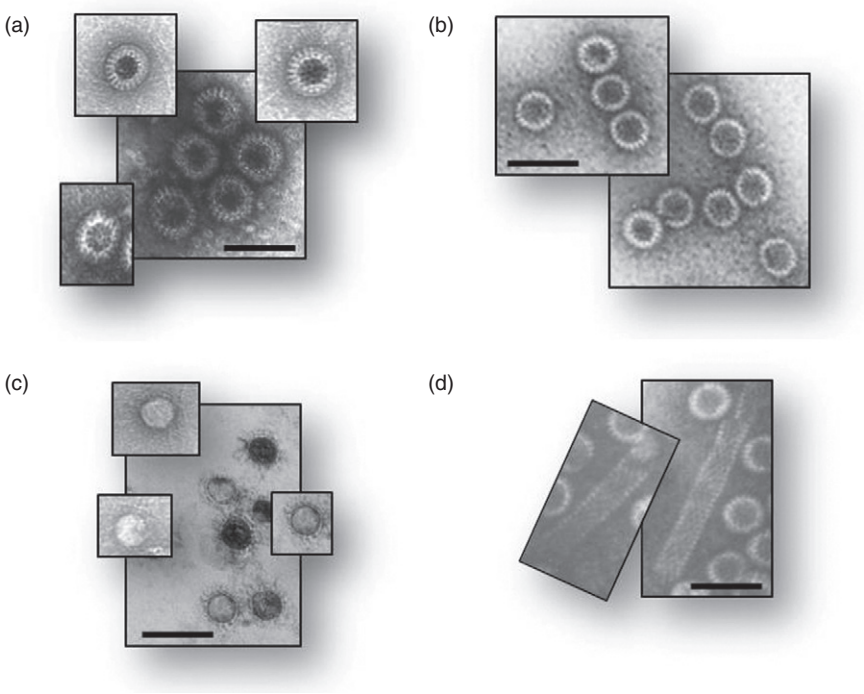

Figure 2 Electron micrographs of negatively stained rotavirus-like particles: (a) triple-layered 2/6/7 particles; (b) double-layered 2/6 particles; (c) single-layered VP2 particles; and (d) VP6 tubes [80]. Scale = 100 nm.

(contamination by various monkey viruses), shortage of donor animals, use of endangered animals as cell source, use of uncharacterized or insufficiently characterized cell substrates for virus production, limited expansion, and obligatorily adherent cell growth [85–88]. In the 1960s, human diploid fibroblast cells, WI-38 [89] and MRC-5 [90], and baby hamster kidney cells (BHK-21 (C13)) were established and used for the production of a vaccine against rabies virus [91] and foot-and-mouth disease [92], respectively. Nowadays, there are several licensed human viral vaccines produced using cell substrates (see **Table 4**) or under clinical trials such as the influenza vaccine produced using Vero [93–95], Madin-Darby canine kidney (MDCK) [96, 97], or PER.C6 [98, 99] cells among others [100]. Despite these significant advances in vaccine manufacturing, there are vaccines that are still produced in eggs such as the recently approved influenza A (H1N1) 2009 monovalent vaccines from CSL, MedImmune LLC, or Novartis Vaccines and Diagnostics. Viruses may also serve purposes other than being vaccine production platforms. In fact, viruses such as adenovirus, retrovirus, lentivirus, and adeno-associated virus are commonly used as gene delivery vectors for gene therapy among other applications (see Section 1.47.5).

1.47.4.1.1 Cell lines used to produce adenovirus

The most common and well-documented packaging cell line for adenovirus production is the human embryonic kidney 293 (HEK 293) cell line, which contains the E1 region of the adenovirus [101]. Homologous recombination between the left terminus of first-generation adenovirus vector or helper virus DNA and partially overlapping E1 sequences in the genome of HEK 293 cells normally leads to the generation of replicative-competent adenoviruses (RCAs) [102]. The presence of RCAs is clearly undesirable as they may replicate in an uncontrolled manner. In recombinant adenovirus batches to be used in human patients, RCA is potentially hazardous, especially in immunocompromised patients, being associated with inflammatory responses [103]. Alternative host cell lines have been developed to overcome this problem either by reducing the overlapping sequences or by eliminating any overlap, as is the case of N52.E6 [104] and PER.C6 [105] cells. The PER.C6 cell line, derived from human embryonic retinal cells, has been established for industrial applications (e.g., full traceability available) due to its reduced propensity to generate RCAs and its capacity to achieve high yields of adenovirus vectors. Another disadvantage of human adenovirus vectors is their limited clinical use; 90% of the population has developed preexisting humoral and cellular immunity to those vectors [106]. Sustainable platforms for the generation of vectors from different human serotypes or of those derived from nonhuman adenovirus at titers similar or greater than those obtained with human adenovirus vectors and free of detectable levels of RCAs are required [107, 108]. One example is the production of canine adenovirus type 2 (CAV-2) vectors in dog kidney cells [106, 109–111]. With these vectors, the risks associated with RCAs are diminished, if not completely eliminated, because CAV-2 vectors do not propagate in human cells; also, CAV-2 vectors transduce human-derived cells at an efficiency similar to that of human adenovirus type 5, and are amenable to *in vivo* use [106]. Novel cell lines, with emphasis on MDCK cells, for the production of CAV-2 vectors are under evaluation [112, 113].

Table 4 Licensed viral vaccines using cell substrates

Vaccine name	*Manufacturer*	*Trade name*	*Cell line for production*	*Virus*	*Virus type*	*Virus family*
Hepatitis A virus vaccine, inactivated	GlaxoSmithKline Biologicals	Havrix®	MRC-5	Hepatitis A virus	(+) ssRNA	*Picornaviridae*
Hepatitis A virus vaccine, inactivated	Merck & Co., Inc.	VAQTA®	MRC-5	Hepatitis A virus	(+) ssRNA	*Picornaviridae*
Japanese encephalitis virus vaccine, inactivated	Intercell Biomedical	IXIARO®	Vero	Japanese encephalitis virus	(+) ssRNA	*Flaviviridae*
Measles virus vaccine, live, attenuated	Merck & Co., Inc.	ATTENUVAX®	Chick embryo cell	Measles virus	(–) ssRNA	*Paramyxoviridae*
Measles, mumps, and rubella virus vaccine, live, attenuated	Merck & Co., Inc.	M-M-R II®	Chick embryo cell	Measles virus	(–) ssRNA	*Paramyxoviridae*
			Chick embryo cell	Mumps virus	(–) ssRNA	*Paramyxoviridae*
			WI-38	Rubella virus	(+) ssRNA	*Togaviridae*
Measles, mumps, rubella, and varicella virus vaccine, live, attenuated	Merck & Co., Inc.	ProQuad®	Chick embryo cell	Measles virus	(–) ssRNA	*Paramyxoviridae*
			Chick embryo cell	Mumps virus	(–) ssRNA	*Paramyxoviridae*
			WI-38	Rubella virus	(+) ssRNA	*Togaviridae*
			MRC-5	Varicella virus	dsDNA	*Herpesviridae*
Poliovirus vaccine, inactivated	Sanofi Pasteur SA	IPOL®	Vero	Poliovirus	(+) ssRNA	*Picornaviridae*
Rabies virus vaccine, inactivated	Sanofi Pasteur SA	Imovax®	MRC-5	Rabies virus	(–) ssRNA	*Rhabdoviridae*
Smallpox (vaccinia) vaccine, live	Acambis Inc.	ACAM2000®	Vero	Vaccinia virus	dsDNA	*Poxviridae*
Varicella virus vaccine, live, attenuated	Merck & Co., Inc.	Varivax®	MRC-5	Varicella virus	dsDNA	*Herpesviridae*
Varicella-zoster virus vaccine, live, attenuated	Merck & Co., Inc.	Zostavax®	MRC-5	Varicella-zoster virus	dsDNA	*Herpesviridae*

ds, double stranded; (–) ss, negative single stranded; (+) ss, positive single stranded.
Source: http://www.fda.gov

1.47.4.1.2 Cell lines for retrovirus and lentivirus production

The NIH 3T3 mouse embryonic fibroblast cell line, a ferret brain cell line, the human cell lines HT1080, TE671, HEK293 (which can grow in suspension), and CEM (which is an obligatory suspension cell line) all have been used for the establishment of retrovirus vector producer cell lines; other detailed examples can be found elsewhere [114]. Recently, highly versatile producer cell lines such as Flp293A and 293 have been developed. Based on HEK 293 cells and equipped with flippase recognition target sites containing a murine leukemia virus (MLV)-green fluorescent protein (GFP) vector, these cells allow the efficient Flp recombinase-mediated cassette exchange of MLV vectors; thus, after cassette exchange, the tagged retrovirus producer cell clone is capable of producing vectors containing the transgene of choice at levels similar to those observed for the mother producer cell line [115, 116].

The major problem in the production of lentiviruses has been the development of a packaging cell line. Stable expression of lentivirus particles has proven to be more difficult than that of oncoviruses [117, 118], partly due to the expression of proteins such as rev and viral proteases which appear to be toxic to cells [119]. Consequently, lentiviral vectors have been produced by transient transfection of high-expressing cell lines such as COS [120] and 293T [121, 122] generally using four different plasmids (gag-pol, env, rev, and lv-vector). Lentiviral vectors can also be produced by transduction of 293T cells with baculoviruses; sustained transgene expression was observed after lentivirus transduction of HeLa cells [123].

1.47.4.1.3 Cell lines for adeno-associated virus production

Recombinant adeno-associated viral (rAAV) vectors can be produced using stable cell lines containing the required genes or by transient transfection. Transient transfection employs the use of 293 or A549 cells co-transfected with two plasmids containing the rAAV vector and the rep and cap genes, followed by an infection with helper virus to induce the replication of rAAV. Other possibility is to co-transfect cells with three plasmids containing the rAAV vector, the rep and cap genes, and the adenovirus helper genes [124, 125]. Stable cell lines, on the other hand, require only the presence of a helper virus to initiate rAAV production since they already contain the rAAV vector and the rep and cap genes of adeno-associated virus. It is possible to use HeLa (the most common), 293, and A549 cells as stable cell lines. This system is better suited for large-scale production of rAAV than transient transfection; nevertheless, generation of such stable cell lines can be tedious and time consuming.

It has been estimated that 10^{12}–10^{14} rAAV particles are required for clinical human use [126]. Independent of the production strategy, maximum rAAV titers are typically around 10^{7} infectious particles (IP) per ml, clearly insufficient to fulfill the needs. In order to overcome this limitation, recent studies have focused on producing rAAV vectors in insect cell cultures, using the recombinant baculovirus system [127]. Production of rAAV particles is achieved by coinfecting *Spodoptera frugiperda* (*Sf*)-9 cells with three baculovirus vectors, BacRep, BacCap, and Bac-rAAV; these encode the respective components of the rAAV production machinery. This system lends itself to large-scale production under serum-free conditions, as *Sf*-9 cells are grown in suspension.

1.47.4.2 Expression Systems for VLP Production

The most popular expression system for the production of VLPs is the yeast system due to its easy protein expression, ability to scale-up, and cost of production. However, appropriate posttranslational modifications (PTMs) such as protein glycosylation and correct protein folding, protein assembly, and codon optimization may dictate alternative production systems. Within those, mammalian cell lines (either transiently or stably transfected or transduced with viral expression vectors), the baculovirus/insect cell system, and various species of bacteria and plant cells have been receiving special attention (**Table 5**).

1.47.4.2.1 Bacteria and yeast cells

VLPs of structurally simple viruses are usually produced in bacteria or yeast. Three licensed VLPs are currently produced in *Saccharomyces cerevisiae*: two HBV-VLPs (Recombivax HB®, Merck & Co. and Engerix-B®, GlaxoSmithKline) and one HPV-VLP (Gardasil®, Merck & Co.). A different species of yeast, *Hansenula polymorpha*, has been used for the production of a licensed HBV-VLP (Hepavax-Gene®, Berna Biotech Korea Corp). Other VLPs produced in yeast, either in *S. cerevisiae* or in *Pichia pastoris*, namely the HIV-VLP [32], polyomavirus-VLP [12, 129], norovirus (No)-VLP [50], and HCV-VLP [130], are under investigation. VLPs produced in bacteria (i.e., *Escherichia coli*) have not yet reached the market. In fact, little or no immunogenic information is available for HBV-VLP [21], HPV-VLP [35, 37], HCV-VLP [22], Ebola-VLP [131], SV40-VLP [67], RSV-VLP [61], infectious hypodermal and hematopoietic necrosis virus-VLP [132], No-VLP [133], and Indian peanut clump virus-VLP [46]. This is due to the inability of the proteins expressed in prokaryotic cells undergo PTM such as protein gycosylation, a key feature in most VLP-derived vaccines. Another common difficulty of these cells is the expression of a soluble and full-length product free of toxins, heat shock proteins (HSPs), and chaperone proteins. Since limited solubility of recombinant viral proteins promotes the formation of inclusion bodies [35, 37], the downstream processing is significantly compromised. Consequently, the process route becomes potentially more expensive than the eukaryotic route. Even in downstream processing of soluble fractions, the VLP precursors often require separation of HSPs and molecular chaperone proteins (e.g., GroEL) that can remain attached to the capsomeres, creating a significant bioseparation problem [67]. Protease degradation and codon bias can also be a source of reduced manufacturing precision, contributing to lower yields and possibly to nonhomogeneity of the final VLP architecture [134].

To overcome these limitations, expression systems such as the baculovirus/insect cell system should be used instead of bacteria or yeast. Another alternative is the disassembly and reassembly of VLPs *in vitro* [128], a method described in detail in the following sections.

Table 5 Hosts used to generate VLPs

Hosts	*Examples of VLPs*	*Comments*
B/IC	Most of the VLPs shown in **Table 3**	A versatile and efficient system, relatively high expression levels with simple post-translational modifications and production of complex VLPs
Bacteria	HBV, HPV, HCV, Ebola, SV40, RSV	High expression levels, with limited protein solubility, unable to undergo post-translational modifications and production limited to structurally simple VLPs
Mammalian cells	IBDV, HBV, HDV, HPV, HIV, Marburg, Ebola, Hantaan	Able to undergo complex post-translational modifications with higher production costs
Transgenic plants	HBV, Norwalk virus	Low expression levels, VLPs degradation in edible vaccines but their production can be easily scaled-up
Yeast	HBV, HPV, HIV	Low-cost VLP producers, able to undergo simple post-translational modifications, and production limited to structurally simple VLPs

B/IC, baculovirus/insect cells; IBDV, infectious bursal disease virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; SV40, simian virus 40; RSV, Rous sarcoma virus.
Source: Several references presented in table 3.

1.47.4.2.2 Baculovirus/insect cell system

VLPs formed by several proteins require simultaneous expression of multiple proteins. A versatile and efficient system for the production of these recombinant proteins is the baculovirus/insect cell system. Insect cells are initially grown to a desired cell concentration after which they are infected with one or several recombinant baculoviruses containing the gene or genes coding for the proteins of interest. The construction of recombinant baculovirus is simple and fast, although multicistronic constructs can pose some problems, providing a high versatility to the expression system. This is very important when producing vaccines for rapidly changing viruses, a fundamental requirement in an efficient approach to contend with potential pandemics in a timely manner. For instance, an influenza vaccine production campaign based on the baculovirus/insect cell system can be completed within 1.5 months after having identified the particular circulating viral strain, whereas an egg-based or other cell culture-based platform would require 7–9 months [135]. A list of VLPs produced using this system is shown in **Tables 3** and **6**.

Table 6 Examples of chimeric VLPs developed for prophylactic and therapeutic vaccines, and drug and gene delivery

VLP platform	*Chimeric antigen and plasmids*	*Expression system*	*Status*	*References*
BPV	CTL epitopes of HPV and HIV, L2 HPV epitopes	B/IC	Preclinical	[136, 137]
HBV (core)	GFP, *Plasmodium falciparum* circumsporozoite protein epitopes (malaria vaccine candidate)*, bacterial and protozoan epitopes, HPV 16 E7 oncoprotein epitopes, B- and T-cell epitopes of HCV	*Escherichia coli*	Preclinical	[21, 138–142]
HBV (surface)	Plant signal peptides, dengue virus envelope, HIV gp41 2F5 epitope, HCV HVR1, HPV 16 E7 oncoprotein epitopes	Mammalian cells, tobacco plants, yeast	Preclinical	[142–147]
HEV	HEV B-cell epitope	B/IC	Preclinical	[148]
HIV, SHIV gag	Various HIV env epitopes	B/IC, mammalian cells	Preclinical	[149, 150]
HPV	SHIV (HIV tat, rev; SIV gag), HPV E6/E7, HBV core protein epitopes	B/IC	Preclinical	[151–154]
JC polyomavirus	DNA fragment coding for EGFP	B/IC	None	[155]
Murine polyomavirus-VLP	Immunodominant H-2Kb-restricted ovalbumin 257-264 epitope	*Escherichia coli*	Preclinical	[156]
Parvovirus B19	Dengue-2 glycoprotein epitopes	B/IC	Preclinical	[157]
Phage Qβ	Nicotine, angiotensin II, IL-1β, A β 1-6 peptide	*Escherichia coli*	Phase II	[158–164]
RHDV	Short peptides	B/IC	Preclinical	[165]
SV40	Plasmid DNA up to 17.7 kb, foreign peptides	B/IC	Preclinical	[66, 166]

B/IC, baculovirus/insect cells; BPV, bovine papillomavirus; CTL, cytotoxic T lymphocyte; HCV, hepatitis C virus; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; HBV, hepatitis B virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; IL, interleukin; RHDV, rabbit hemorrhagic disease virus; SHIV, simian–human immunodeficiency virus; SV40, simian virus 40.
*phase I

1.47.4.2.3 Mammalian cells

Proteins expressed in mammalian cell culture systems can undergo complex PTMs by copying nature, a significant advantage over other systems that are limited to high-mannose glycoprotein modifications (baculovirus/insect cell system and yeast) or incapable of any type of PTM (bacteria). Thus, the assembly of these proteins into VLPs closely resembles the formation of native virus particles. The main disadvantages of this system are its low controllability and high production costs. Different mammalian cells can be used for VLP production; for example, BSC-1 [41], SW480 [31], BHK-21, and Vero E6 cells [13] are able to produce VLPs when infected with recombinant vaccinia vectors carrying the genes coding for viral proteins of different viruses. VLPs have also been produced by stably or transiently transfecting CHO [20] and 293T cells [7, 29]. Wang *et al.* [24] have used recombinant baculovirus to transduce HuH-7, HepG2, HeLa, BHK, and primary rat articular chondrocyte cells.

1.47.4.2.4 Transgenic plants

Recent advances in plant biotechnology have made possible the use of transgenic plants as potentially viable alternatives to cell culture systems for the production of recombinant edible subunit vaccines [167]. HBV-VLP and NV-VLP are the most studied VLPs produced by three different species of transgenic plants: *Solanum tuberosum* (potato) [168, 169], *Lycopersicon esculentum* (tomato) [18], and *Nicotiana benthamiana* (tobacco) [18, 46, 170]. Edible plants offer a palatable oral delivery system that would preclude the costly purification process of injectable vaccines. In theory, the scale-up of production would not require large investments in hardware and culture media. Few resources would be needed such as agricultural practice, a stable transgenic line, and acreage for cultivation. The main disadvantages of using transgenic plants are the low expression levels obtained and antigen degradation taking place during *in vivo* delivery.

1.47.5 Applications: Prevention and Treatment

1.47.5.1 Virus Applications

The main areas of application of animal, bacterial, plant, and algal viruses include vaccine development and gene therapy.

1.47.5.1.1 Vaccine development

Vaccines based on live viruses have been traditionally effective and relatively easy to produce. The elimination of smallpox was accomplished through mass vaccination with the live vaccinia virus, a mildly pathogenic animal virus related to smallpox. Likewise, live attenuated vaccines are well tolerated and highly immunogenic. The live attenuated poliovirus vaccine developed by Dr. Albert Sabin in 1961 eradicated poliomyelitis disease in the Western hemisphere and drastically reduced its incidence rate worldwide. Vaccines against infectious diseases such as yellow fever, typhoid fever, mumps, and shigella are also based on live attenuated viruses. The attenuation of viruses is accomplished through one of the following methods: (1) attenuation of the pathogen by physical means; (2) selection of naturally occurring mutants that lead to infection with abortive replication of the pathogen while retaining its immunogenicity. Inactivated (killed) vaccines can also stimulate a protective immune response. The inactivated poliovirus vaccines (IPOL®, Sanofi Pasteur SA), influenza vaccines (Fluarix®, GlaxoSmithKline), and typhoid fever vaccines (Typhim Vi®, Sanofi Pasteur MSD) constitute some examples. The disease-causing organism is inactivated with chemicals such as formaldehyde; the main drawback of these vaccines is that they require boosting for continuous, efficient immune response. Nowadays, using molecular biology and DNA manipulation methods, it is possible to express protective proteins in adequate live vectors with the purpose of designing live vaccines against various types of pathogens. In addition, the development of reverse genetics systems for the recovery of viruses from cDNA has made it possible to rapidly generate recombinant attenuated derivatives. **Table 4** lists some examples of current licensed viral vaccines.

1.47.5.1.2 Gene therapy

In the late 1970s and the early 1980s, the emergence of techniques for subcloning mammalian genes into prokaryotic plasmids and bacteriophage was correctly foreseen as the stepping-stone to precursors of techniques for human gene therapy. Parallel investigations on the biology of avian and murine onco-retroviruses led to the development of retroviral vectors, which began to be used in the mid-1980s as a tool for gene transfer into mammalian cells. The first human trial of gene transfer was carried out in the late 1980s for the treatment of patients with advanced metastatic cancer. The process consisted in introducing the gene coding for neomycin resistance into human tumor-infiltrating lymphocytes by retroviral-mediated gene transduction, before their infusion into patients, thus, using the new gene as a marker for the infused cells [171]. Since then, there has been a remarkable expansion in the number of vector systems available to express human genes directly associated with disease states for therapeutic purposes.

Gene therapy trials using retroviral vectors to treat X-linked severe combined immunodeficiency (X-SCID) constitute the most successful application of gene therapy to date. SCID is a disease in which the patient has neither cell-mediated immune responses nor the ability to generate antibodies. A high rate of immune system reconstitution was observed in patients treated in the X-SCID gene therapy trials [172], but 5 out of more than 20 patients developed a leukemia-like illness, of which 4 fully recovered after conventional anti-leukemia treatment [173, 174]. Despite these results, gene therapy trials to treat SCID due to deficiency of the adenosine deaminase enzyme continue with relative success in the United States, France, the United Kingdom, Italy, and Japan. In the last decade, the process of retroviral vector production has been under considerable investigation as it presents many difficulties,

mainly due to vector instability and low cell productivities hampering the attainment of high viral titers. Strategies based on the manipulation of sugar carbon sources [175, 176], lipids [177], temperature [178], or osmotic pressure [179] used in bioreaction and on the establishment of pioneering packaging cell lines such as 293 FLEX [180] and Flp293A [116] show potential to increase the yields of infectious retroviral vectors. This will allow the generation of high-quality clinical preparations for gene therapy applications.

Adenovirus vectors are also efficient vehicles for delivering nucleic acids into mammalian cells. The human adenovirus type 2 and 5 are the most used vector backbones for adenovirus-mediated gene transfer. However, due to a number of significant disadvantages such as the need to immunosuppress or tolerize patients to a potentially debilitating virus [106, 111], vectors from different serotypes or those derived from nonhuman adenovirus (bovine, sheep, and birds) were developed [107, 108]. Recently, CAV-2 vectors, produced in dog kidney cells, have been gaining increasing attention due to their emerging potential for the study of the pathophysiology and potential treatment of neurodegenerative diseases like Parkinson's, Alzheimer's, and Huntington's , among others [181]. The first clinical trial using recombinant adenovirus was carried out in 1993 in cystic fibrosis (CF) patients [182]. Two years later, the first recombinant adeno-associated virus trial was initiated in CF patients [183]; trials in hemophilia B patients commenced shortly after [184]. Inevitably, lentivirus and recombinant herpesvirus vectors have also entered into clinical trials [185–187]. All the four viral vector systems mentioned above are highly efficient systems for gene transfer and expression *in vivo* in nondividing cells. In fact, more than 50% of all viral vectors currently undergoing clinical trials are adenovirus (24.1%), retrovirus (21.2%), adeno-associated virus (4.4%), or HSV (3.3%). A detailed description of the cell lines used for the production of these vectors and the respective production strategies are presented in **Table 7**.

Due to their inability to replicate and absence of toxicity in mammalian cells, baculoviral vectors have emerged as gene therapy vehicles for the treatment of a wide range of human diseases. Recently, a genetically modified recombinant baculovirus encoding for a cherry-red fluorescent protein under the control of a strong mammalian cell promoter (cytomegalovirus promoter) proved to be effective in transducing a human liver carcinoma cell line, HepG2 [212]. Other studies indicate that baculoviruses show promising gene expression efficiencies in liver [213], skeletal muscle [214], brain [215], and eye [216]. Importantly, baculoviral vectors present efficiencies similar to those of adenoviral vectors in transducing human smooth muscle cells, human cardiomyocytes, and fibroblasts [217]. The major challenge is the production of high titers of recombinant baculovirus [218]. Although metabolic engineering approaches have shown to improve baculovirus titers at high cell densities [219, 220], platforms for the production of baculoviral vectors to be used in gene therapy clinical trials have not yet been implemented.

1.47.5.2 VLP Applications

VLPs can be used as prophylactic or therapeutic vaccines against a wide variety of diseases. The recent developments in molecular biology and virology renewed the interest in VLPs as versatile systems for gene and drug delivery.

Table 7 Viral vectors used in gene therapy

Viral vector	*Clinical trials (%)*[a]	*Most used type*	*Cell lines used for production*	*Production strategies*	*References*	*Indications in clinical trials*[a]
Adenovirus	23.8	Ad-5	HEK 293, PER.C6, HeLa	Infection	[105, 188–195]	Cancer diseases (64.5%)
		Ad-2	PER.C6	Transfection		Cardiovascular diseases (8.9%)
Retrovirus	21.2	Mo-MLV Lentivirus	Packaging cell lines derived from NIH 3T3, HT1080, TE671, and HEK 293 cells	Stable cell line	[114, 116, 118, 120, 123, 180, 196–199]	Monogenic diseases (8.2%)
			293T, 293 EBNA-1, COS	Transient transfection		Infectious diseases (8%)
			293T	Transduction		Gene marking (3%)
						Healthy volunteers (2.3%)
Adeno-associated virus	4.5	AAV-2	B130, High Five *Sf-9* and HEK 293	Infection	[200–204]	Neurological diseases (1.8%)
			HEK 293F and HeLa	Transfection		Ocular diseases (1.1%)
			BHK and HEK 293	Transfection + infection		Others (2.4%)
			HeLa	Stable cell line		
Herpes simplex virus	3	HSV-1	Vero	Infection	[205–211]	

AAV-2, adeno-associated virus type 2; Ad-5, adenovirus type 5; Ad-2, adenovirus type 2; HSV-1, herpes simplex virus type 1; Mo-MLV, Moloney murine leukemia virus.
[a]Source: http://www.wiley.co.uk/genetherapy/clinical(2010)

1.47.5.2.1 Prophylactic vaccination

VLPs used for vaccination are normally devoid of any DNA of viral or cellular origin. VLPs have been produced from the capsid or envelope proteins of a wide variety of viruses for the purpose of studying viral assembly and for developing vaccines. While HBV-VLPs and HPV-VLPs are successful vaccines, VLP-based vaccines against pathogens that directly affect immune cells and successfully evade the immune system, such as HIV-1 and HCV, are proving to be extremely difficult to develop. Another important and forthcoming application of VLPs is their use in the generation of immune responses against foreign protein epitopes by fusing or by coupling them to VLPs of different origins, resulting in the so-called chimeric VLP (**Table 6**) [221]. The vaccine against HPV infection is an example of a chimeric VLP, in which the L2 protein epitopes are inserted into the L1 protein [137] to confer protection against a broader range of HPV types. In the end, tailoring of VLPs depends on their final application as vaccine (prophylactic or therapeutic) and may require adaptations in their structure (particle size, envelope structure, etc.), target host (dendritic cells, mucosal surfaces, etc), and route of administration (intranasal, intramuscular, etc) to achieve the desired immune response [30].

1.47.5.2.2 Therapeutic vaccination

In cancer immunotherapy, a T-cell response is often more desirable than a B-cell response. Additionally, a T-cell epitope localized inside the VLP avoids interference with VLP uptake by the natural receptors of the native virus. Thus, a VLP vaccine candidate does not necessarily have to display on its surface-specific epitopes in order to be recognized by the immune system. This is the case of HPV 16 VLPs and HBV-VLPs. In the first case, the HPV 16 E6 and/or E7 peptide containing a T-cell epitope was fused to HPV L1 protein and inserted into the VLP [221]. In the second case, HPV 16 E7 epitopes were inserted into the surface or core proteins of HBV-VLPs [142]. The HPV-VLP was shown to induce E7-specific cytotoxic T cells and to protect mice against a challenge with an HPV 16-transformed tumor [154]. Results on purified chimeric HBV-VLPs have also confirmed their high immunogenicity in mice [222]. Most of these VLPs have a common feature: to induce an immune response against a non-self-antigen (the only exception being conjugated VLPs). However, for immune therapy of cancers of nonviral origin, the strategy is to induce an immune response against a self-antigen. In one study, a CD8+ T-cell epitope derived from ovalbumin, a well-studied melanoma tumor antigen, was inserted directly into murine polyomavirus-VLP [156]; VLPs carrying this ovalbumin epitope were then injected into mice in a therapeutic setting, with two injections after the melanoma tumor challenge. Complete protection against tumor development was obtained and induction of T cells specific for the ovalbumin epitope was demonstrated.

1.47.5.2.3 Gene delivery

Many capsid proteins used for VLP formation, such as HPV L1 and polyomavirus VP1, have the ability to bind non-specifically to viral or cellular DNA [223]. In 1983, it was demonstrated that murine polyomavirus-VLP could package viral DNA and transduce it into cells *in vitro*, resulting in expression of the viral gene products [224]. Later on, experiments with plasmid DNA demonstrated the feasibility of using these VLPs for gene transfer [225]. Since then, a number of studies have been performed in order to evaluate and optimize DNA packaging and transduction by VLPs derived from different members of *Polyomaviridae* family [226]. An example is the SV40-VLP produced in the baculovirus/insect cell system. Using an efficient methodology for *in vitro* packaging of plasmids with less than 17.7 kb, SV40-VLPs [166] have proven to be efficient for gene delivery *in vivo* [227]. Another example is the JC polyomavirus-VLP produced in the baculovirus/insect cell system; by VLP disassembly and VP1 pentamers reassembly *in vitro*, it was possible to insert a 1.6 kb DNA fragment coding for enhanced green fluorescent protein (EGFP) inside a JC polyomavirus-VLP [155]. VLP transduction results in TC-620 cells, measured by flow cytometry, showed that 70% of cells were expressing EGFP.

1.47.5.2.4 Drug delivery

Protein epitopes or other small molecules can be attached to the surface of already formed purified VLPs [163, 228]. This presents some advantages over VLP-forming methods that directly insert the epitopes into the VLP proteins. One advantage is that the already formed VLP can be used as a basis for the attachment of a number of different drugs, either proteins or smaller molecules, providing a flexible platform for drug delivery. Second, the attached molecule may provide a stronger and more efficient immune response due to its localization in the VLP surface. One example is the attachment of peptides or small molecules to VLPs consisting of the coat protein from the bacteriophage Qβ or AP205; these VLPs have been developed for the generation of antibody responses against nicotine and angiotensin II [158, 163]. In phase I trials, healthy nonsmokers vaccinated with nicotine-conjugated Qβ-VLPs [163] demonstrated a strong nicotine-specific IgM/IgG response. Noteworthy is that nicotine *per se* does not induce an antibody response. Only when conjugated with a VLP, nicotine potentially helps smokers quit smoking by reducing the satisfaction derived from nicotine intake. In phase II clinical trials, an increase in long-term abstinence was observed in smokers who attained high antibody levels following vaccination [160]. This same platform has been used to couple IL-1β molecules and Aβ1-6 peptide to VLPs for rheumatoid arthritis and Alzheimer's treatment, respectively [159, 161, 164]. Peacey *et al.* [165] genetically modified rabbit hemorrhagic disease virus (RHDV)-VLP with various short peptide sequences that are capable of being presented to and recognized by immune cells. However, this approach does have limitations, including the time involved in engineering each chimeric VLP, size constraints imposed on introduced peptides, increased instability of modified capsids leading to limited yield, and the inaccessibility and altered conformation of introduced residues. To overcome these difficulties, a chemical linker was used to covalently conjugate both small peptides and whole protein to the RHDV-VLP scaffold. This rapid approach enabled surface conjugation of a substantial range of antigens without the constraints imposed by subunit folding and VLP formation. Attachment of antigen to

RHDV-VLP conferred the immuno-stimulatory properties of the underlying viral shell to the conjugated antigen, and so enabled the initiation of both antigen-specific humoral and cell-mediated immune responses [165]. The results demonstrated that RHDV-VLP can be utilized as a versatile molecular scaffold in many applications, from vaccine development to biological nanotechnology. Drug delivery may also be achieved with a VLP carrying the desired drug inside the capsid. This can be accomplished, for example, with steps of *in vitro* disassembly and reassembly. Lee and Tan [21] have successfully encapsulated GFP inside HBV-VLP by disassembling the VLP into monomers with urea followed by reassembly using dialysis with GFP molecule.

1.47.6 Bioengineering Challenges

Viruses and VLPs have been successfully produced *in vivo* or *in vitro* since the beginning of the twentieth century and in the late 1980s, respectively [229–232]. The production of these bioproducts involves several bioengineering issues that must be carefully addressed in order to control upstream and downstream processing, and to maximize performance and reduce production cost.

1.47.6.1 The Production Strategy

Product yields are strongly dependent on the production strategy chosen. Batch, fed-batch, continuous, and perfusion strategies are normally used for the production of viruses and VLPs. In batch production, it is important to control the accumulation of toxic products as well as the depletion of essential nutrients for cellular growth [219, 233]. This can be avoided by selective addition of nutrients (glucose and glutamine) and/or amino acids, and complete medium addition [218, 234–236] using fed-batch strategies. The yields are significantly improved but the scale-up becomes difficult and expensive since culture medium is inefficiently used. The alternative, a continuous system, presents a short throughput time and a small number of production steps. Nonetheless, continuous reactors operated for long periods (>1 month) are prone to generate defective viruses that either directly reduce viral yields or indirectly impact on VLP yields by competing with the host cell for the protein expression machinery [237, 238]. In addition, the complexity of the bioproducts produced in this system is generally low, which constitutes a major drawback compared to other systems. Perfusion strategies enhance cellular growth and product yields [239, 240]. However, it requires the use of large volumes of media, significantly increasing the production cost. In addition, the devices used for separating cells from the medium are difficult to scale-up and normally induce cellular damage, impacting negatively on cellular growth rate and subsequently on viral or VLP production rates.

1.47.6.2 The 'Envirome'

The 'envirome' affects viral replication and VLP production at the level of cellular growth and metabolic state, DNA transcription and replication, mRNA translation, and protein PTMs. Among others, the 'envirome' enfolds the dissolved oxygen concentration, pH, temperature, agitation rate, cell and substrate concentration, inlet gas flow and composition, volume, pressure, fluid dynamics, power input, and osmolarity. Most of these variables differ from process to process and are possible to monitor and control *in situ* by continuously adjusting bioreactor parameters to certain predetermined set points [241, 242]. For example, recombinant protein production in insect cells is maintained constant at osmolarities between 300 and 380 mOsm [243]. If osmolarity falls out of this range by 30 mOsm, productivities are significantly reduced. Depending on the sensitivity of the cell line used, the stress generated while sparging and by bubble entrainment during agitation may impact negatively on viral and VLP productivities [26, 55]. This can be solved using head space aeration, albeit the productivities achieved in these culture systems are remarkably lower; a more valid solution is the use of nonionic copolymers such as the Pluronic F-68, which lowers the culture medium surface tension and impedes the attachment of cells to bubbles, which liberate lethal energy during bursting [194, 244]. Pluronic F-68 also interacts with the cell membrane, increasing its rigidity and making it more resistant to hydrodynamic forces. Oxygen limitation or excess can induce protease synthesis and subsequent degradation of the product of interest [245]. Concomitantly, oxygen-derived free radicals present at high levels of dissolved oxygen tension can cause oxidative stress to cells or oxidative damage to proteins [246]. It is also important to bear in mind that temperature fluctuation induces different oxygen solubility levels: increasing temperatures induce lower oxygen solubility.

1.47.6.3 The Downstream Processing

The absence of contaminant proteins and DNA (host or viral), the absence of incorrectly assembled macrostructures, and endotoxin levels below those specified by regulatory agencies (Food and Drug Administration (FDA) and European Medicines Agency (EMEA)) ensure the quality and efficacy of the downstream process and are critical for the success of the process technology used. This is particularly a challenging task as in most cases produced viruses and VLPs do not differ significantly in size and molecular weight from other protein complexes or defective viruses that need to be removed. The nature of the bioproduct, either extracellular or associated with cellular structures, also influences the purification strategy [59, 212, 247–251]. Products that are secreted into the media are easily processed since they do not require a high number of purification steps. If produced intracellularly, extraction prior to purification is essential. In both the cases, fusion tags can be used to facilitate monitoring and downstream

processing [252, 253]. Nonetheless, care must be taken when placing these fusion tags as they may affect virus maturation and protein expression, indirectly compromising their biological activity [254, 255]. In medium containing serum, the major problem is the high protein content that complicates the downstream processing [256, 257]. The use of serum-free medium is recommended as it does not contain animal-derived supplements, which pose safety concerns, and facilitates downstream operations. The nature of the outer protein of viruses and VLPs confers to these bioproducts specific and unique characteristics that strongly impact on the design of the downstream strategy. In the end, there is a clear trend in downstream processing from classical laboratorial purification methods like sucrose or cesium chloride gradient centrifugations toward more sophisticated techniques like tangential flow filtration, gel permeation chromatography, liquid chromatography, ion exchange chromatography, affinity chromatography, and size-exclusion chromatography, including the use of newer, disposable membrane technology [249, 258, 259].

1.47.6.4 Transfection versus Transduction

Heterologous proteins, the basis of viral particles and VLPs, are normally expressed by transfection in transient or stable systems or by transduction using viral expression vectors. The availability of stable packaging cell lines capable of continuously expressing a specific gene represents a step toward the scaled-up production of viral vector stocks to be used as drug delivery systems or in applications such as gene therapy [260]. Although the productivities of some viral vector producer cell lines remain lower than expected [261], the generation of acceptable viral yields and the expression of secreted and insoluble proteins are normally favored by these stable transfected cell lines. The use of a constitutive active promoter that integrates into the genome and does not require infection for its activation is essential [262]. In applications such as protein characterization and high-throughput screening of gene functions, transient transfection is more appropriate [204, 263]. The use of viral expression vectors for virus and/or protein synthesis is another alternative. Although it requires the additional and always fiddly step of viral infection, viral yields can be as high as 10^{10} IP ml^{-1} as reported for baculovirus or adenovirus production [218, 264] and protein expression levels can be between 1 and 500 mg l^{-1} [60, 265–267]. The strength of the promoter(s) controlling protein(s) expression for either virus encapsulation or VLP assembly, as well as the time at which the promoter(s) become(s) active, drives the productivity levels and thus can be optimized [268, 269]. The promoter strength must be carefully evaluated as it is common that very strong promoters overwhelm the processing capacity of the endoplasmic reticulum, thus reduce the final yields. Indeed, the secretion and complete complex glycosylation of recombinant proteins in the baculovirus/insect cell system improve when genes are under the control of the p10 promoter instead of the stronger polyhedrin promoter [270]. The use of early instead of late or very late promoters is a difficult choice; with very late promoters, the expression of proteins occurs toward the end of the culture when cells are in the death phase and proteases influence the correct protein PTM, negatively impacting final yields. On the other hand, early promoters induce lower protein productivities, as in most cases the enzymes and transcriptional factors necessary for protein expression are not yet fully active at such an early stage. The combination of promoter strength with the correct time for promoter activation is essential to assess high product yields and mathematical modeling of intracellular events can be used to identify best strategies to obtain the optimal stoicheometry and thermodynamic conditions for VLP assembly [271].

1.47.6.5 The Key Process-Related Parameters

Process-related parameters such as the multiplicity of infection (MOI), time of infection (TOI), time of harvest (TOH), and cell concentration at infection (CCI) strongly influence virus and VLP titers. Adequate MOI, defined as the number of virus per cell, is determinant for attaining optimal yields and robust production systems. The MOI to be used depends on the target product, the production process, and the dimension of viral stocks, and normally relies on predictions of the Poisson distribution [267, 272–274]. Low MOIs (0.01–1 virus per cell) have the advantage of requiring low concentrations of viruses. The number of viruses is normally insufficient to infect all the cells; thus, a high percentage of cells remain healthy and grow upon initial infection. A steep increase in infected cell concentration is observed as a second generation of viruses start to infect the uninfected cell population [267]. At the end of bioreaction, the concentration of infected cells is sufficiently high to sustain the production of viruses and/or VLPs to high levels. The main drawback is the action of proteases. Since the overall process (infection plus viral and/or protein synthesis) is slow, the bioproduct is exposed to cellular proteases for long periods of time, which may compromise the quality and quantity of the final product. On the contrary, high MOIs (>1 virus per cell) require large viral stocks and favor the selection of fast-replicative defective virus [275]. In addition, volumetric yields are considerably lower than those achieved at low MOIs as a result of lower concentration of infected cells at the end of bioreaction. In theory, such differences could be compensated by increasing the CCI. However, at high CCI, the change in cell's energetic state upon infection induces a significant drop in cell-specific productivity [219]. Medium replacement at TOI and the use of fed-batch or perfusion cultures are strategies capable of maintaining specific productivities similar to those reported at low CCI; the major inconvenience is that they are neither practical nor necessarily economical at large scale. This creates an opportunity for the development of novel techniques for process optimization based on metabolic engineering and systems biology. Another important parameter is the TOH. Delayed harvest times increase the exposure of viruses and VLPs to intracellular or extracellular proteases [276] and induce a more pronounced cell lysis. Additionally, the release of contaminant proteins (degraded or not), host and viral DNA, cell compartments, and viral or protein macrostructures to the extracellular medium will complicate the downstream processing of the product of interest. Optimal harvest times are normally between 72 and 120 hpi (hours postinfection) (40 and 70% of cell viability) [27, 234, 277]. The interplay between all the above-mentioned parameters is complex and normally requires substantial experimentation. To avoid this, mathematical models can be used. They significantly reduce the amount of work

required and can assist the definition of optimal process-related parameters such as MOI, TOI, TOH, and CCI in complex biological systems for maximization of process performance.

1.47.6.6 Process Monitoring and Product Quality Control

The commercial success of a bioproduct requires a controlled and monitored process and a well-characterized product. Unfortunately, most monitoring systems and characterization techniques are complicated to handle, extremely costly, semiquantitative, and in many cases non-existent, which significantly compromises the robustness and scalability of the process. In viral quantification, there is no accurate method to measure both infectious and total particles. Titration assays such as plaque-forming unit assay, growth cessation and cell size assay, alamar blue assay, tissue culture infectious dose 50, microculture tetrazolium assay, reverse transcriptase activity assay, and electron microscopy directly or indirectly assess viral titers of either infectious or total particles, but not both simultaneously [278–280]. Other methods such as flow cytometry, real-time quantitative polymerase chain reaction (Q-PCR), immunoblotting [281–283], and high-performance liquid chromatography (HPLC) [284, 285] are semiquantitative. In the end, the most appropriate titration method depends on the type of virus and the detection system available. The quantification of VLPs involves a higher degree of complexity. Most methods are based on immunoassays such as Western blot, enzyme-linked immunosorbent assay (ELISA), and bicinchoninic acid (BCA) protein quantification assay [55, 60, 286]. Electronic microscopy and real-time Q-PCR are alternatives. However, all these methods are semiquantitative. They are unable to differentiate proteins that are part of a correctly assembled VLP from others that are in incomplete VLPs, viral particles, or other macromolecular structures. Thus, overestimated or underestimated VLP yields are frequently obtained [266]. Recently developed methods such as gel-permeation HPLC [266], sodium dodecyl sulfate-capillary gel electrophoresis [80], new application of intact cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) methodology [287], and capillary zone electrophoresis [288] may allow a simple, fast, and low-cost quantification of viral proteins and VLPs in purified and bulk samples. In the near future, with the development of novel detection sensors based on acoustic resonance, turbidity, and fluorescence, it will be possible to monitor the kinetics of VLP formation *in situ* and online, as it has already been made possible with a wide variety of culture constituents. One of those techniques is the two-dimensional (2D) fluorometry that, using optical fiber technology, allows the simultaneous monitoring of several compounds present outside (envirome) and inside (metabolome) the cells [289, 290].

1.47.6.7 Thermodynamic Assembly of Viruses and VLPs

The assembly of viral particles, VLPs, and other spherical polymers – closed structures composed of several protein subunits – is poorly understood. There is little experimental information describing intra- and intersubunit binding energies, rates and orders for assembly reactions, and formation of nucleating structures [291]. During the last decade, mathematical models have become a prominent and reliable source of knowledge for understanding what is driving the building of a virus capsid or a polyhedral protein macrostructure. Many mathematical models have been proposed based on theoretical constants of association and dissociation (K_n and $K_{d,app}$, respectively), free energies associated with intersubunit contact (ΔG^0_c) and assembly pathways [42, 292–296]. The way in which these constants and energies relate with specific culture parameters such as pH, calcium concentration, ionic strength, and others needs to be better understood. *In vitro* experiments of assembly and disassembly of VLPs are helpful in assessing these kinds of relationships. A recent study of *in vitro* disassembly of RLPs (triple-layered 2/6/7 particles) into DLPs (double-layered 2/6 particles) and the assembly of DLPs and VP7 monomers into RLPs addresses the effect of physicochemical parameters (pH, ionic strength, and temperature) on the formation and stability of RLPs and DLPs [297]. The results indicate that both particles are stable within a specific pH (3–7) and temperature range (5–25 °C). Outside those thresholds, particle aggregation ($T \in [35–45\,^\circ C]$), disassembly ($T > 65\,^\circ C$), and instability (isoelectric point of RLPs = 3 and DLPs = 3.8) become evident. In addition, the reaction rates of RLP disassembly are correlated with the temperature and ionic strength; low temperatures and low ionic strengths induce low disassembly reaction rates. On the other hand, RLP assembly reaction rates decrease with the increase in pH, ionic strength, temperature, and calcium concentration. These findings clearly demonstrate that process optimization of complex protein macrostructures is feasible by manipulation of physicochemical parameters. Nonetheless, care must be taken when extrapolating *in vitro* results to *in vivo* experiments, as in most cases the best conditions for subunit interaction and particle formation are not the same due to the different environmental culture conditions of the two systems.

1.47.7 Concluding Remarks and Future Trends

Viruses and VLPs are protein-related macrostructures and preferential vehicles for many applications. As complex products, the design of efficient upstream and downstream strategies, the definition of appropriate quality control and monitoring methods, and the attainment of high productivities are as complicated as they are essential. Thus, it is imperative to constrain the 'envirome' effect on the quality and quantity of the product, so that viral particles and VLPs can be further used in their respective areas of application.

Viruses are widely used in gene therapy [298, 299], high-throughput screening of gene functions [204, 263], drug delivery [300], *in vitro* assembly studies to design antiviral drugs [22], recombinant protein production [60, 301], and bioinsecticide

[302] and bioweapon [303, 304] preparation. In material science and engineering, they can be the building blocks for electronics, biosensors, and chemistry [305]. VLPs, on the other hand, are established as a powerful tool for vaccine development [58, 306, 307].

Furthermore, improved knowledge points out toward larger and promising applications of virus and VLP platforms to other areas of research in the near future, such as nanotechnology, where they can be used as tools for biomedical science [308–311].

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