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Review

Purification of Bionanoparticles

The recent demand for nanoparticulate products such as viruses, plasmids, protein nanoparticles, and drug delivery systems have resulted in the requirement for predictable and controllable production processes. Protein nanoparticles are an attractive candidate for gene and molecular therapy due to their relatively easy production and manipulation. These particles combine the advantages of both viral and non-viral vectors while minimizing the disadvantages. However, their successful application depends on the availability of selective and scalable methodologies for product recovery and purification. Downstream processing of nanoparticles depends on the production process, producer system, culture media and on the structural nature of the assembled nanoparticle, i.e., mainly size, shape and architecture. In this paper, the most common processes currently used for the purification of nanoparticles, are reviewed.

Keywords: Bionanoparticles, Downstream processing, Gene therapy

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1 Introduction

The entire DNA sequencing of the human genome [1, 2] and the identification and characterization of 50,000–100,000 human genes will lead to the understanding of several human diseases in which normal and aberrant genes play important roles [3]. These developments provide several opportunities to interfere in disease processes by delivering proteins, pharmacological agents or genetic material to the target cells affected by disease. This approach to targeting disease, designated molecular therapy, is likely to play an increasingly important role in medicine throughout this century [3].

Gene therapy is a particular approach of molecular therapy in which nucleic acids are delivered to control the genetic flow. The first human gene therapy phase I trial was carried out in 1989 for the correction of ADA (adenosine deaminase) enzyme in SCID (Severe Combined Immunodeficiency Disease) patients [4]. Since then, significant advances have been experienced in the field of human gene therapy, driven by the increased knowledge and understanding of the molecular mechanisms of diseases, as well as by the advances in vector design and technology to produce more effective, efficient and safer delivery vectors [5]. Since 1989, more than one thousand gene therapy clinical trials have been conducted worldwide for the treatment of several diseases (www.wiley.co.uk/genetherapy/clinical). Cancer is the main target with about 67 % of the total approved clinical trials. Further targets of gene therapy clinical trials include cardiovascular pathologies (9 %), monogenic diseases (8 %) and infectious diseases (6.6 %), among others.

Molecular therapy requires the intracellular delivery of biologically active compounds [6]. Due to their rapid elimination from the circulation and widespread delivery to non-targeted organs and tissues, these biologicals need to be administrated in large quantities. This is often economically unfeasible and may lead to several complications owing to product toxicity [7]. In vivo delivery is also a complex process that involves the passage through different biological barriers, which include the cell membrane with their lipophilic nature that restricts the direct intracellular delivery of these potential therapeutics [6]. These bottlenecks have driven research to the development of novel molecular delivery vectors and associated production technologies, mainly aimed at improving safety and efficacy.

The first generation of molecular therapy vectors explored the ability of viral systems to deliver mostly genetic information into eukaryotic cells in order to regulate cellular functions or to express therapeutic proteins [8, 9]. Even though viral vectors are still the most popular delivery systems used in laboratory studies and clinical trials, there are several disadvantages associated with such vectors, including possible adverse immune responses and random insertion into the genome. The advantages and disadvantages of the main delivery systems used in molecular therapy are summarized in Tab. 1. Non-viral delivery systems have several advantages over viral vectors. Such systems, which include liposomes, DNA-protein and polymeric complexes, can be constructed to be less immunogenic in order to enable repeated administrations, have no the-

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Table 1. Description of the main delivery systems used in molecular therapy: major advantages and disadvantages.

Delivery system	Characteristics/Advantages	Disadvantages
Non-viral vectors		
Liposomes	• Improve vector association with specific cells as well as DNA expression • Increase specificity for gene delivery (targeting of cell types containing specific receptors or recognition of certain molecules over other cells) • Long survival times in the circulation system and effective target recognition in vivo • Less hazardous in terms of antigen-specific immune responses	• Specific targeting • Low transfection efficiency • Only transient expression
Cationic polymers	• Provide protection to DNA from nuclease degradation during gene delivery • Facilitate DNA release into the cell (acting as a proton sponge that destabilizes the endosomal compartment allowing DNA release into the cytoplasm) • Less toxic delivery agents • Reduced toxicity (biodegradable nature of the molecules)	• Specific targeting • Low transfection efficiency • Only transient expression
Polymer nanoparticles	• Compartmentalization of therapeutic pDNA into a nano-container suitable for blood circulation • Release of therapeutic genes in response to external stimuli (acting as environmentally responsive polyplexes) • Less hazardous in terms of antigen-specific immune responses	• Low levels of gene expression • Enhanced cytotoxicity by the presence of an excess of positive charges (formation of positively charged DNA condensates/aggregates)
Dendrimers	• Large number of controllable peripheral functionalities • Surface, interior and core can be tailored to different sorts of applications • Safe and non-immunogenic	• Low efficiency of gene transfection • Cytotoxicity and side effects (interaction between the positively charged dendrimer and the negatively charged cellular structure, especially glycosaminoglycans)
Viral vectors		
Oncovirus	• Broad cell tropism • Stable gene expression due to viral genome integration into cell chromosomes • Only infect dividing cells • Can accommodate large gene inserts	• Random insertion of viral genome, which may possibly result in mutagenesis • Retrovirus vector particles are rapidly degraded by complement • Possible recombination with human endogenous retrovirus • Only infect dividing cells • Relatively low titers (10^6–10^7 pfu/mL)
Lentivirus	• Infect non-diving cells • Can be pseudotyped with different envelopes, which enhance their cell tropism and allow for easy purification, as they become more stable and resistant • Stable gene expression due to viral genome integration into cell chromosomes • Can accommodate large gene inserts	• Serum conversion to HIV-1 (in the case of HIV-1-based vector) • Possible recombination with human endogenous retrovirus • Biosafety problems with the production of large quantities of the vector (in the case of HIV-1-based vector) • Relatively low titers (10^6–10^7 pfu/mL)

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Delivery system	Characteristics/Advantages	Disadvantages
Adenovirus	• Very high titers (10^{12} pfu/mL) • Transiently high levels of gene expression • Low pathogenicity for humans • Efficient nuclear entry mechanism • Infect non-diving cells	• Strong immune responses • Restricted tropism • Not suitable for long term expression due to the lack of integration into the host
Adeno-associated virus	• Long-term and efficient transgene expression • Broad cell tropism (including non-dividing cells and hematopoietic stem cells) • High titers (10^{10} pfu/mL) • Non-pathogenic and non-toxic	• Lack of specific integration of recombinant AAV vectors, which may cause cell mutagenesis • AAV requires a helper adeno- or a herpes virus • Relatively restricted packaging capacity • Difficult to obtain high titer stocks of pure virus (helper virus free)
Retrovirus (Herpes simplex virus)	• Ability to transduce non-dividing cells • Lifelong latent infection • Easy production of large quantities of pure vector stocks • Large or multiple transgenes can be readily accommodated within the vector	• Host immune response, inflammatory and toxic reactions in patients

oretical limit to the size of the expression cassette, can be used as a drug delivery system, and some can be produced from chemically defined components [5, 10]. However, a number of obstacles, e.g., lack of specific targeting, low transfection efficiency and transient expression, have limited the application of non-viral based vectors in molecular therapy approaches [11], Tab. 1.

The ideal delivery vector is the one that combines the advantages of both viral and non-viral vectors while minimizing all of their disadvantages. Virus-like particles (VLPs) are bionanoparticles mainly composed by structural proteins of a virus, but usually lacking the correspondent genetic material. They are produced by the recombinant expression of the viral structural proteins, which self-assemble into nanostructures identical to the native viruses. As such, while being non-infectious, VLPs have a similar structure and tropism to the natural virus from which they are derived and demonstrate comparable cellular uptake and intracellular trafficking [12]. VLPs are an attractive candidate for prophylactic vaccination, genetic and molecular therapies, since they are relatively easy to produce and manipulate. However, their successful application depends on the availability of selective and scalable methods for product recovery and purification that integrate effectively with upstream production steps. This paper reviews the most common downstream processing methods suitable for the purification of VLPs.

One of the most striking features of VLPs is their extreme diversification in terms of structure, architecture, and production system. To date, VLPs have been produced for more than thirty different viruses [13], in different types of engineering production systems, including yeast, e.g., *Saccharomyces cerevisiae* and *Pichia pastoris* [14–24], bacteria, e.g., *Escherichia coli* and *Staphylococcus aureus* [21, 25–32], insect cells, e.g., *Drosophila* Schneider-2 cells and *Spodoptera frugiperda*, High Five [33–47], mammalian cells, e.g., HEK 293, Vero, HeLa, human TE FLY A7, MA-104, MDCK, and BHK [48–61], insect larvae [62], plants, e.g., banana (*Musa* spp.) and pHB117 binary vector [63, 64], and crustaceous, e.g., crayfish and shrimp [65–67]. Despite the different structures, architectures and production systems, the methodologies used in the purification of VLPs only vary around a few operations, as outlined in Fig. 1.

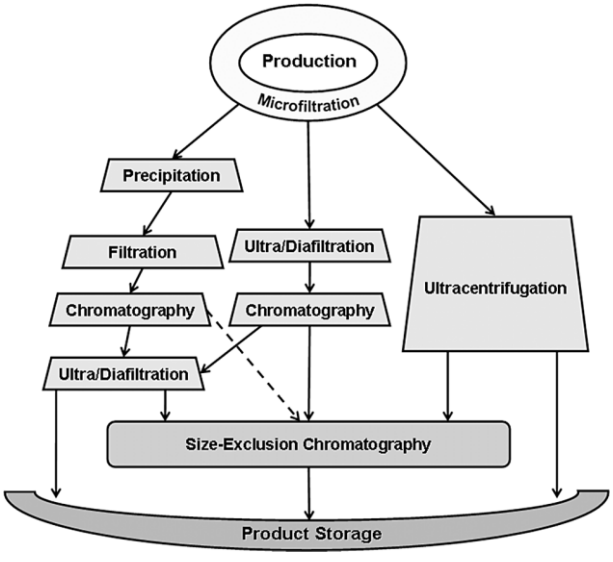

Figure 1. Common methodologies used in downstream processing to purify bionanoparticles based on centrifugation/precipitation processes, membrane operations and chromatography purifications.

2 Protein Nanoparticle Purification Processes

Downstream processing of VLPs depends on their production process, and most importantly on the producer system, culture media and VLP architecture. Similar to other biologicals for molecular therapy [68], the purification of VLPs has specific concerns that are related to the structural nature of the assembled nanoparticles, mainly size, shape and architecture. Producer cell-line derived impurities, as well as some producing media additives such as serum, need to be removed to meet the quality approval standards of the regulatory agencies.

The general rules of thumb for the establishment of purification processes of recombinant proteins may be applied in the downstream processing of VLPs. In particular, the initial operation, aimed at the removal of the most abundant impurities or contaminants, should be selected from those having higher volumetric capacity and throughput, while the final purification steps, aimed at the removal of structurally similar molecules or assemblies, should be selected from the high selective unit operations with lower volumetric capacity and throughput, Fig. 2. Typically, the initial steps are aimed at the separation of culture media from producing cells, product concentration and conditioning, Fig. 1. Microfiltration and diafiltration are the operations generally selected for this stage. The purification stage of the process (see Figs. 1 and 2) involves highly selective operations, traditionally ultracentrifugation and more recently chromatography, in order to remove all impurities and purify the assembled VLPs to high purity standards. The final stage of the process (polishing stage) is aimed at the polishing and further concentration of the product. At this stage, the operations must be selected to enable an additional tight control over misassembled particles and particles with similar sizes although with different final architectures.

As for all biomolecules, the selection of downstream processing operations to purify nanoparticles is highly dependent on the properties and nature of the nanoparticles themselves, their stability, and their production process. For instance, depending on the type of the native virus, e.g., adenovirus, retrovirus, etc., VLPs can be released to the culture media of the producing cell culture or remain soluble or compartmentalized inside the producer cell lines. While the released particles can be easily separated from the producer cells by a simple low-speed centrifugation, the recovery of non-released particles is preceded by a cell lysis process.

While similar choices are usually made when comparing the recovery of excreted versus non-excreted proteins, the release of nanoparticles by the producer cell lines confers additional concerns regarding product properties and stability. Regarding the overall properties, released nanoparticles are involved in a lipid bilayer that renders the outermost surface of the particles negatively charged. Since it is the surface that is presented, and thus targeted, in most of the purification operations, it is very difficult to distinguish such nanoparticles from impurities containing similar outer surfaces, of which cellular vesicles are the most relevant example. The lipidic bilayer of the released nanoparticles is also very labile and overall, the particles become very sensible to shear, osmotic pressure, temperature, pH and ionic strength variations. The degradation of nanoparticles may be inherent to the majority of the purification operations, resulting in the generation of impurities with similar characteristics as the product, and thus, is another critical issue to consider when designing nanoparticles purification processes.

Despite the different methods available to recover crude samples with the desired nanoparticles, and the different production systems, the methodologies used in the purification of VLPs only vary around a few operations, mostly based on centrifugation/precipitation processes, membrane operations and chromatography purifications, Fig. 2. The criteria used for the selection of the appropriate methods for viral concentration and purification include capability for processing large volumes of viral preparations with high yield, preservation of stability of the particle produced, ease of process scale-up, low cost operations, and the final quality standards [68, 69].

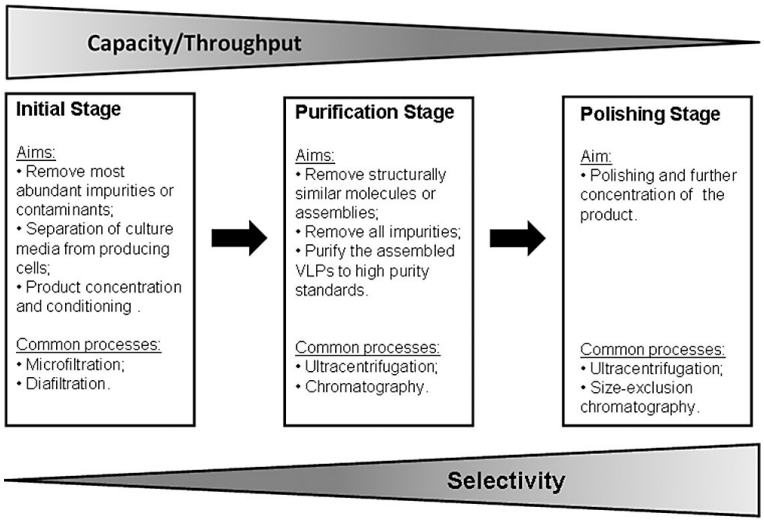

Figure 2. Rules of thumb and objectives of the different stages of the bionanoparticle purification processes. The sequence of unit operations should follow decreasing capacity and throughput and increasing selectivity. Unit operation with higher volumetric capacity and throughput should be selected for operation closer to the bioreactors at the initial steps of the purification process, while at the final purification steps, the criteria should involve the selection of a highly selective unit operation with lower volumetric capacity and throughput.

2.1 Precipitation-Based Methods

As in protein purification, precipitation is an efficient and simple method to purify nanoparticles, Tab. 2. The precipitation mechanism can be interpreted, as for protein solutions at least on a qualitative basis, on the basis of the theory of Derjaguin, Landau, Verweey and Overbeek (DLVO) [70], which defines the stability of dispersions. This DLVO theory views the stability of a dispersion of particles as being determined by the simple alge-

Table 2. Precipitation methods and respective precipitation agent concentrations used in the purification of virus-like particles.

Precipitation Method	Concentration of Precipitation Agent	Bionanoparticle Produced	Reference
Acid precipitation (phosphoric acid)	–	• Dengue virus type 2 envelope protein as a fusion with hepatitis B surface antigen	15
Ammonium sulfate precipitation	• 2.8 M	• IBDV – infectious bursal disease virus – VP2-His6	62
	• 2.8 M	• Turkey coronavirus	71
	• 1.15–1.61 %	• Viral coat protein VP1-Glu	30
	• 2.3 M	• HBc-His6 (Hepatitis B virus core protein)	21
PEG precipitation	• 4 % (w/v) PEG 6000–7000	• Extra chromosomal inheritance of the killer, neutral, and sensitive phenotypes of strains of *S. cerevisiae*	20
PEG and sodium chloride precipitation	• 8 % (w/v) PEG 8000 • 0.02 M NaCl	• Cowpea chlorotic mottle virus (CCMMV)	72
	• 10 % (w/v) PEG 6000 • 0.6 M NaCl	• Mycovirus OMIV	73
	• 10 % (w/v) PEG 8000 • 2.3 % (w/v) NaCl	• Rotaviruses	54
PEG precipitation (6000–9000)	–	• Hepatitis B virus surface antigen – HBsAg	45

braic sum of the potential energies leading to repulsion, which are electrostatic in nature and associated with the diffuse double layer that surrounds charged particles or surfaces in ionic solutions, and the potential energies leading to attraction arising from van der Waals forces (hydrophobic interactions). According to the DLVO theory, nanoparticles are maintained in solution while the total repulsive potential is higher than the total attractive potential. In this DLVO theory, the net balance of attractive and repulsive potential energies is dependent on the surface potentials, the dielectric constant and ionic strength of the medium, and the strength of the van der Waals forces. The dispersion stability is normally associated with the presence of a surface layer of adsorbed ions that can be destabilized by the addition of neutral salts, by changing the dielectric constant, by modifying the surface potential of the particles, and/or by changing the balance of charged versus hydrophobic regions. Whilst the detailed quantitative application of DLVO theory has been criticized, presenting several limitations regarding its application to protein solutions, the theory offers a simple descriptive approach to the understanding of particle-particle interactions, and their effect on dispersion stability.

Both salts and polymers, Tab. 2, can be used as precipitating agents to promote the precipitation of the nanoparticles while keeping most of the impurities solubilized in solution. Salting-out type precipitation of nanoparticles is achieved by adding ammonium sulfate at concentrations of 1.5–4 M. While the mechanism is similar to the salting-out of proteins, the precipitation of nanoparticles explores the higher hydrophobic character of the proteins assembled into nanoparticles as compared with the individualized protein molecules. Therefore, nanoparticle precipitation is achieved with lower concentrations of ammonium sulfate, typically below 2.5 M, Tab. 2.

Precipitation through volume exclusion effects using PEG and other polymers has also been used in the purification of protein nanoparticles, Tab. 2. While in the case of protein precipitation, the increasing effectiveness of PEG as the size of the polymer increases has been documented, systematic studies for protein nanoparticle precipitation are scarce, with no clear tendency being observed, Tab. 2. In addition, and similar to the addition of salts, protein nanoparticles precipitate at lower polymer concentration compared with non-assembled protein molecules, typically in the range of 4–10 % (w/v).

While purification factors can be maximized through optimizing the precipitation conditions, mostly regarding precipitating agent concentration, temperature, and reaction time [49], purification through precipitation is not a selective operation. The co-precipitation of impurities or polymers along with the protein nanoparticles, and loss of their native activity (possibly due to changes in osmotic pressure) limits the use of these methods [74].

2.2 Centrifugation-Based Methods

The use of centrifugation based methods for protein nanoparticle purification has its origin in virology studies, where native viruses and viral particles were purified on the basis of their size and density. These protocols, based on ultracentrifugation and density gradient methods, were the first to be adapted for integration in protein nanoparticle purification for therapeutic use. In these processes, the separation is achieved based on the

specific buoyancy density difference of each component present in the mixture to be purified. Caesium chloride [20, 24, 37–41, 43, 45, 47, 57, 72, 73, 75–78] or sulfate [71], saccharose [36, 42, 45, 59, 61, 63, 71, 72], and potassium or sodium bromide [45, 67], are the agents most commonly used to generate the density gradients, even though other media such as colloidal silica Percoll [54], Nycodenz [64], and iodixanol [79], have also been successfully used to purify viral particles.

Although they are widely used, ultracentrifugation methods are time-consuming, difficult to scale up, and recoveries are very small, mainly due to particle degradation occurring upon pressure forces or osmotic shock [52]. In addition, co-purification of contaminants derived from the culture media and packing cell line, such as membrane cell vesicles released to the culture medium, may also occur [52, 56]. Moreover, the preparation of density gradients requires technical expertise, is time-consuming, and has several practical disadvantages regarding the manipulation of the density gradient agents. In most cases, the recovery yields are very small, at about 1.6–4.4 % [21], mostly due to the high sensitivity of protein nanoparticle assemblies to osmotic pressure. In fact, the viscous and hyperosmotic nature of the commonly used density gradient generating agents, e.g., saccharose and caesium chloride, together with the high shear forces generated in the centrifugation force field, contribute to the disruption of integrity and functionality of assembled protein nanoparticles. Even though iso-osmotic media, such as colloidal silica Percoll [54], Nycodenz [64], and iodoxanol [79] have been successfully used to address these disadvantages, with evidence of preservation of the integrity and functionality of the viral particle, the use of ultracentrifugation-based methods for the large-scale purification of protein nanoparticles is still limited. Nevertheless, ultracentrifugation is a very effective analytical tool to characterize assembled nanoparticles enabling the estimation of nanoparticle size, architecture, mass, density [61] and/or diffusivity.

2.3 Membrane Separation Methods

Membrane separation processes are frequently used in the biotechnology industry to separate the components of a fluid stream on the basis of the hydrodynamic radius difference. Membranes with a wide range of pore diameters are commercially available making membrane separations very versatile operations that can be used for media clarification, product concentration, buffer exchange, and sterile filtration. Membrane operations are particularly suitable for protein nanoparticles purification. The number of published nanoparticle purification processes using membrane operations is increasing, with membranes being used for different objectives. Tab. 3 summarizes some examples of viral nanoparticle purification processes using membrane operations.

Membranes with nominal pore sizes of 0.02–10 μm are used in microfiltration operations, typically to clarify the product stream by removing insoluble particulate materials. Typically producing cells and cell fragments are retained in the membrane while the product goes across the membrane being recovered in the permeate stream [23, 51, 79, 81, 82]. In contrast to centrifugation, microfiltration generates a particle-free harvest solution that requires no additional clarification before subsequent purification [81].

On the other hand, the use of membranes with low nominal pore sizes, typically on the nanometer scale, enables product concentration. Such ultrafiltration membranes are usually rated by the nominal molecular weight cut-off, which is the molecular weight of the globular protein that is 90 % retained by the membrane. Ultrafiltration membranes are selected to ensure rejection of the product of interest while permeating the impurities. Therefore, the product is concentrated inside the membrane and recovered in the retentate stream. Ultrafiltration membranes can also be used for buffer exchange by adding buffer to the membrane feeding reservoir (diafiltration).

Even though suitable for protein nanoparticles recovery, the major disadvantage of membrane separations concerns membrane fowling. According to the Hagen-Poiseuille equation, at the same transmembrane pressure difference, the permeate flux depends mostly on the membrane nominal pore size and viscosity of the streams, with higher fluxes being achieved for larger pores and lower viscosities. As such, larger concentration factors are achieved with membranes of smaller pore sizes. However, higher concentration factors are associated with higher viscosities resulting in lower permeate fluxes. In addition, the concentration polarization on the membrane also increases with increasing retentate concentration, also leading to lower fluxes.

The selection of membrane nominal pore size is also dependent on the nature and composition of the feed streams. Impurities and contaminates with hydrodynamic radii similar to the product are co-purified and co-concentrated with it, mostly due to the non-selectivity of the membranes. In addition and particularly regarding viral nanoparticles as recently shown by Grzenia et al. [83], membrane operation performance is affected by the media composition. In that work [83], the authors observed a decrease of the permeate fluxes upon switching cell growth media to serum-free media. Even though no clear explanation was advanced for this observation, it is advised to develop cell culture and membrane purification in parallel [83].

Upon selection of membranes with nominal pore sizes similar to those of the product, some degree of fractionation can be achieved and purification of viral particles correctly assembled from misassembled particles, protein aggregates, disrupted particles, membrane protein aggregation, and cell vesicles [58, 83].

Despite the disadvantages, some of which were pointed out above, the use of membrane processes is gaining increasing importance in viral nanoparticle production processes. Membranes have been used in microfiltration strategies for media clarification, for particle concentration in ultrafiltration strategies, with possible fractionation, and for buffer exchange in diafiltration strategies. Membrane processes can be easily scaled-up and used in cGMP manufacturing processes [84]. An additional advantage of membrane processes is their ability for process integration. As shown by Subramanian et al. [84], a closed membrane system was successfully scaled-up for the purification of adenoviral particles. In this system, which was effective in recovering, purifying, and concentrating both in-

Table 3. Different pore sizes and membrane types employed in membrane processes used to purify virus-like particles.

Bionanoparticle Produced	Observations	Reference
Adenovirus type 5	• 300 kDa membrane Biomax/Millipore Used in the concentration of the clarified supernatant of the producer cells	77
Adenovirus type 5 particles	• 0.2 μm polyethersulfone membrane/Millipore • 100 kDa MWCO Ultra-15 filter unit/Millipore Used to remove contaminant cellular proteins and to concentrate samples before ultracentrifugation, and after ultracentrifugation to remove CsCl	57
Chimeric infectious bursal disease virus-like particles	• 300 kDa flow cell ultrafiltration unit/Amicon Used as a initial purification step before chromatography to remove low weight contaminants	37
Double-layered rotavirus-like particles	• 300 kDa MWCO Nanosep/Pall Life Sciences Used after a CsCl gradient ultracentrifugation to remove salts before characterization by HPLC	43
HBc – Hepatitis B virus core protein	• Miniultrasette 300 kDa cut-off membrane/Pall Filtron Used to concentrate samples before chromatography	21
HBsAg – Hepatitis B virus surface antigen	• XM-300/Amicon Used in the concentration of supernatants as initial step	45
HIV-1 retrovirus-like particles	• 300-kDa tangential flow ultrafiltration membrane Used for sample concentration and buffer exchange before affinity chromatography	51
Huma Influenza A virus A/PR/8/34 (H1N1)	• 750 kDa MidGee/Xampler/GE Healthcare Used as a concentration step for influenza virus before further purification by chromatography	74
MoMLV-VSV-G pseudotyped	• HT TuffrynR polysulfone membrane (0.45/0.2 μm) capsule filtration/Pall Gelman Sciences • 300 kDa MWCO Omega polyethersulfone membrane disc filter/Pall Gelman Sciences Sequential microfiltration and ultra/diafiltration processes are used for clarification, concentration, and partial purification of retroviral particles from crude supernatants	79

tracellular and extracellular viruses by integrating cell lysis in the membrane module, 80 % particle recovery was achieved with 15- to 20-fold concentration factors in a processing time of less than 2 h [84].

2.4 Chromatographic Methods

Chromatography has become a very popular methodology in downstream processing since it facilitates high recovery rates and high purity products. Moreover is easy to scale up and offers a good platform for large scale production [82]. This methodology has also been applied in the purification of most nanoparticulate material for gene therapy vectors, Tab. 3.

The chromatographic systems used in the purification of VLPs and other nanoparticulate materials must take into account their large diameters, i.e., usually from 80–120 nm. Special resins have to be chosen to overcome the low binding efficiencies and capacities of traditional resins, mostly due to surface adsorption and pore exclusion effects [69, 91, 92]. In fact, most of the currently available chromatographic matrices, having pores within 30–80 nm diameters, were designed to maximize the adsorption of protein macromolecules rather than viruses [69]. As such, the adsorption of nanoparticulate materials will be restricted to the bead surface area while most contaminating proteins have access to the area inside the pores, leading to poor selectivities, low resolution, and very low yields of the adsorbents [21, 23, 69, 91].

In order to circumvent all the problems found with conventional chromatography supports, novel chromatography approaches, aimed at optimizing adsorptive conditions to maximize the binding capacity and product recovery, are been developed.

Membranes and monoliths are gaining particular relevance as alternatives to porous supports. Monoliths are continuous beds consisting of a single piece of highly porous material, characterized by an interconnecting network of channels of up to 4 μm diameter, which can be prepared in a variety of shapes and dimensions using relatively straightforward polymerization chemistry, and that can be derivatized with traditional chromatography ligands [93, 94]. As a consequence of the macroporous structure of these materials, mass transport is mainly based on convection, which overcomes pore diffusional issues encountered in classical porous beads. The large porosity of the support leads to higher nanoparticles that have access to the ligands located inside the monolith channels [69]. The

Table 4. Different resins used in interaction and size-exclusion chromatography for purification of virus-like particles and respective % recovery rates.

Process	Resin	Bionanoparticle Produced	Recovery	Reference
Anion Chromatography	CL6B resin (GE Healthcare)	• Inactivated HIV-1 particles	> 90 %	85
	DEAE Sepharose FF (GE Healthcare)	• Recombinant hepatitis B surface antigen (HBsAg)	80 %	86
		• Retroviral particles	50 %	52
	Mustang Q anion-exchange coin (Pall Corporation)	• rAAV8 – Adeno-associated virus type 8	90 %	56
	POROS 50HQ (Applied Biosystems)	• Chimeric cowpea mosaic virus (CPMV) particles	79 %	87
		• Viral coat protein VP1-Glu	–	30
	Q Sepharose XL (GE Healthcare)	• AAV and AAV2 particles	–	53
		• Adenovirus type 5	50–74 %	88
	Source 15Q (GE Healthcare)	• Adenovirus type 5	–	77
	Streamline Q XL (GE Healthcare)	• Replication-defective adenovirus derived from adenovirus type 5	32 %	76
Cation Chromatography	Fractogel EMD SO3 M (Merck)	• Hepatitis C virus core protein (HCcAg)	50–90 %	18
	Mustang S cation-exchange coin (Pall Corporation)	• rAAV8 – Adeno-associated virus type 8	25–58 %	56
	P11 cationic phosphocellulose (Whatman)	• Human papillomavirus (HPV) HPV16 L1 protein	–	22
	POROS 50HS (Applied Biosystems)	• AAV and AAV2 particles	74 %	53
		• Human Papillomavirus (HPV) major capsid protein L1	10 %	23
Metal Affinity	NTA resin (Qiagen)	• Infectious Bursal Disease Virus capsid protein, rVP2	–	42
	Ni^{2+} immobilized resin (Invitrogen)	• Chimeric infectious bursal disease virus-like particles	–	37
	Ni-NTA resin (Qiagen)	• HBc – Hepatitis B virus core protein	5.6 %	21
		• IBDV – infectious bursal disease virus (rVP2H)	6.73 %	29
		• IBDV – infectious bursal disease virus (rVP2H)	40–55 %	62

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Process	Resin	Bionanoparticle Produced	Recovery	Reference
Size-Exclusion Chromatography	Sephacryl S 1000 (GE Healthcare)	• Triple layered rotavirus like particles	–	89
		• HPV16 L1 protein – Human papillomavirus (HPV)	–	22
		• Turkey coronavirus	–	71
	Sephacryl S 200 (GE Healthcare)	• Full-length RNA-free hepatitis B core particles	–	90
	Sephacryl S 500 media (GE Healthcare)	• Replication-defective adenovirus derived from adenovirus type 5	–	76
	Sephacryl S 55 column (GE Healthcare)	• HIV-1 retrovirus-like particles	–	51
	Sephadex G-25 (GE Healthcare)	• Adenoviral particles	–	88
	Sepharose 4 FF absorbent (GE Healthcare)	• Recombinant hepatitis B virus surface antigen (r-HBsAg)	–	86
	Sepharose CL-4B gel (GE Healthcare)	• Dengue virus type 2 envelope protein as a fusion with hepatitis B surface antigen	–	15
		• MoMLV-VSV-G pseudotyped		79,82
	Sepharose CL-6B column (GE Healthcare)	• Hepatitis C virus core protein (HCcAg)	–	18
	Superdex 200 Hi-Load (GE Healthcare)	• Recombinant rhesus rotavirus (RRV) VP7 – rotavirus outer capsid glycoprotein	–	44
	Superdex 200 prep grade (GE Healthcare)	• Viral coat protein VP1-Glu	–	30
	Superdex 200 (GE Healthcare)	• HIV-1 and virus-like particles produced in serum free medium	–	36
	Ultrahydrogel 2000 (Waters)	• Double-layered rotavirus-like particles	–	43

low pressure drop that characterizes monolith operation is an additional advantage, which enables the use of high flow rates, and thus, leads to higher throughputs as compared with traditional bead matrixes.

Membrane absorbers are also being developed for nanoparticulate material purification [69]. Similar to monolithic columns, adsorptive membranes reflect technological advances on liquid-chromatography based on favorable hydrodynamics. The interaction between the target molecules and the active sites on the membrane occurs in convective flow-through pores, and thus, membrane units also maintain high efficiencies at high flow rates as well as when they are used with large molecules with low diffusivities.

Tentacle supports offer the possibility of increased virus binding capacities. The advantage of using these supports is that they have sterically accessible ligands available for virus capture due to the presence of an inert and flexible spacer arm that separates the ligand from the resin surface. Therefore, large particles can attain access to otherwise sterically inaccessible binding sites. In addition, since they are no longer exclusively based on the surface of the chromatographic bead, larger amounts of ligands are available for binding [82, 95].

Even with the disadvantages of traditional supports, chromatography has been widely used for nanoparticle purification. As summarized in Tab. 4, most interaction chromatography modes, e.g., ion-exchange chromatography, immobilized metal affinity chromatography and hydrophobic interaction chromatography, as well as size exclusion chromatography are suitable for the purification of VLPs.

3 Conclusions

Several literature studies have outlined the practical strategies for the production and purification of bionanoparticles. The small size of viral genomes, the ease with which they can be

manipulated, and the simplicity of the purification process make these protein cages an attractive alternative as transgenic systems for the displaying of antigenic proteins.

The wide clinical application of these vectors for gene therapy will depend on the availability of efficient large-scale manufacturing procedures. Significant advances in the downstream processing of viral vectors have been made in the recent years. Various selective chromatography matrices have been identified and new chromatography technologies, better suited for virus purification purposes, are being developed with very promising results.

There is not a unique and perfect purification method that covers all of the broad range of nanoparticulate products with biotechnological and/or biomedical interest. Current researchers, laboratories and industries have to deal with the mechanism of choosing the best approach to purification of the bionanoparticles of interest. The most suitable downstream process has to take into account the product type, size and production source, as well as the final recovery yield.

Further advances in alternative downstream processing technologies are likely to be based on the development of new materials, e.g., smarter polymers and new ligands, product engineering and new approaches to process integration aimed at tighter coupling of upstream and downstream processing.

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