VIRUSES AND VLPS IN NANOMEDICINE:

IMPORTANCE OF SEPARATION AND QUANTIFICATION

APPLICATION NOTE

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CONTENTS

1 / INTRODUCTION

Pathogenic viruses are a huge threat to public health; not only several hundred different types can cause infectious diseases directly in humans, but they also threaten crops and livestock. The ongoing COVID-19 pandemic is an acute reminder of the devastation that viruses can cause, with an immense death toll and financial crisis being experienced worldwide.

Viruses are nanoparticles with typical diameters of less than 300 nanometres, comprised off a similar overall structural composition: one type of a protein-coated nucleic acid (DNA or RNA) inside a capsid made of protein subunits. Depending on the virus' life cycle, the capsid may be enveloped by a host cell-derived lipid layer. An infecting virus strictly depends on its cellular host for energy and machinery for macromolecule synthesis, and ultimately, its own replication[1].

The use of viruses has been critical to one of the greatest successes in public health: vaccine development. Due to their versatile, bioengineer-able characteristics, viruses have enabled the prevention and control of many viral diseases and non-viral infectious diseases[2]. Vaccine development for viral diseases utilises different approaches; where antigens contained in the administered vaccine may consist of a whole virus particle, or different parts of the wildtype virus. Throughout the years, these approaches have led to a wide range of vaccines, including live-attenuated, inactivated, protein-based, and DNA or RNA vaccines – in addition to viral vectors.

In recent years, virus-like particles (VLPs) have emerged as promising candidates for vaccine components. VLPs are synthetic, non-infective whole virus particles that do not contain any genetic material and can be effective stimulants of innate and adaptive immune responses. VLPs attractiveness as vaccines resides on the fact, they can be bioengineered to present heterologous antigens and scalable manufacturing processes.

Despite major advances in viral research, challenges remain. Many of these hurdles are shared by other fields of nanoparticle research or therapeutics, such as the need for precise and reliable virus purification and quantification methods. Here, we present challenges and considerations in virus and VLP purification and quantification, and discuss how different research sectors are impacted.

2 / ISOLATION

Robust purification procedures are needed for many aspects of research and development. For example, viruses must be isolated to enable viral load measurements during infection and pathogenesis studies, or to investigate the effects of potential therapeutics. Virus purification is also required for bioprocess optimisation, such as the manufacture of virus/VLP formulations for vaccines or therapeutics.

Viruses

As briefly mentioned before, there are two types of viruses regarding their external structure: non-enveloped viruses with only protein capsid and enveloped viruses with lipid layer covering the capsid. The primary mechanism by which **non-enveloped** viruses exit host cells is via lysis of the host cell membrane[3]. As a result, non-enveloped viruses can be isolated in vitro by culturing and artificially lysing virus-harbouring cells (adherent or suspension cells), then purifying the virus from the cell lysate supernatant[4]. Cell lysis methods are optimised to maintain the integrity of viruses, typically through the use of lysis buffers, sonication or freeze-thaw cycles[4,5]. In turn, **enveloped** viruses acquire their envelope as they bud off from the host cell membrane, therefore isolation of viruses 'naturally' released into extracellular space is the most used approach for these types of viruses, by collecting supernatant from infected cultured cells without the need of cell lysis[6].

As nanoparticles in suspension of complex cell-derived fluids, viruses are subject to traditional techniques for their purification from non-virus structures/molecules; for instance, ultracentrifugation (UC), density gradient centrifugation (DGC) (or sucrose cushion ultracentrifugation), precipitation, and size exclusion chromatography (SEC)[7]. Certainly, all isolation methods have their own strengths and limitations related to input volumes, yield and the purity of virus preparations. For example, UC and DGC require expensive specialised equipment and present clear scalability issues. SEC, on the other hand, offers a quick, cost-effective, low-effort and standardisable approach. As a result, SEC is widely used in nanoparticle research, including for the isolation of viral particles. An important consideration to have when choosing a virus isolation protocol, is that viruses have substantial similarities to other relevant biological particles, like Extracellular Vesicles (EVs). Thus, EVs and viruses sharing biophysical parameters (e.g. size, density), cell-derived membrane in enveloped viruses and abundant cell-originated release to the extracellular space has resulted in often parallel isolation protocols, but also leading to virus purification challenges when it comes to separating viruses from EVs. Ultimately, virus isolation protocol is based on the specific experimental needs but also greatly depends on size and density of virus of interest and any overlapping of these parameters with EVs, with non-enveloped viruses tending to be smaller (approximately 20-100 nm size) than enveloped viruses (approximately 100-1000 nm size). Therefore, choosing the right method is critical, as it may lead to successful or unsuccessful separation of viruses from other non-virus particles, specially EVs, which have concentrations far higher than viruses in plasma (during peak infection) or in supernatants of viruses that are propagated in vitro in cell cultures[7].

SEC qEV columns have demonstrated enabling fast, non-destructive, and reproducible isolation of virions from cell culture or from biofluids[8–10], whilst also achieving clear separation from bulk total proteins. The two types of qEV isolation columns, qEV/35nm and qEV/70nm, are adequately optimised for the isolation of particles between 35–350 nm and 70–1000 nm, respectively, covering the majority of virus sizes. Molecules or particles smaller than the isolation range (35nm or 70nm) are slowed because they enter the pores of the stationary phase resin within the column, while larger particles – which cannot enter the pores – flow around the resin and are eluted from the column more quickly. Therefore, for very small viruses, such as Enteroviruses with an average size of ~25 nm, virions will be separated by qEV columns from the main population of quick-eluting EVs and elute in later fractions in slower-eluting fractions[5]. Filtering the sample prior to separation with the qEV column may improve purification, by separating cell products that are very different in size and enhancing the effectiveness of the SEC column. The inclusion of a filtering step and choice of filter size (0.22 or 0.45 µm mostly used) will depend on the size of the viral particle of interest.

To illustrate the complexity of virus isolation, there is the case of Ebola virus, an enveloped ssRNA virus. On average, Ebola virions are 1-2 µm long, therefore can be separated from the majority of small EVs generated in cell culture using a 0.22 µm filter. Additionally, improved separation from 'large' (larger than 0.22 µm) cell-derived products can be performed using qEV isolation columns (SEC), where Ebola virions can be purified in early qEV fractions in this particular experiment set up, separate from consecutive but also early EV-enriched qEV fractions (**Figure 1**)[8]. In this case, recovery of virions occurs thanks to the pore size of the qEV column excludes both the virus and EVs, leading to quick but separate elution fractions. Another example can be seen in the isolation of Lentivirus, an enveloped 80-100 nm virus, with size range very similar to small EVs. By using qEV column, authors showed that one fraction contained most, or highest enrichment of lentiviral particles as confirmed by purity assessment by several measurement methods such as protein quantification, particle count, reverse transcriptase activity assay and lentiviral capsid immunoblotting (**Figure 2**)[10].

Nevertheless, attention must be paid to antibody-based virus separations or immunoblot-based purity assessments, since for example, the Ebola virus capsid protein VP40 can be associated with EVs, particularly with exosomes (size of small EVs), as it has been shown that VP40 proteins interact with known exosome biogenesis ESCRT pathway[8]. Therefore, separating Ebola virions by the criteria of absence/presence of capsid protein antigens, in this case, may lead to virion preparations of high yield but low purity (mixture of virions and EVs).

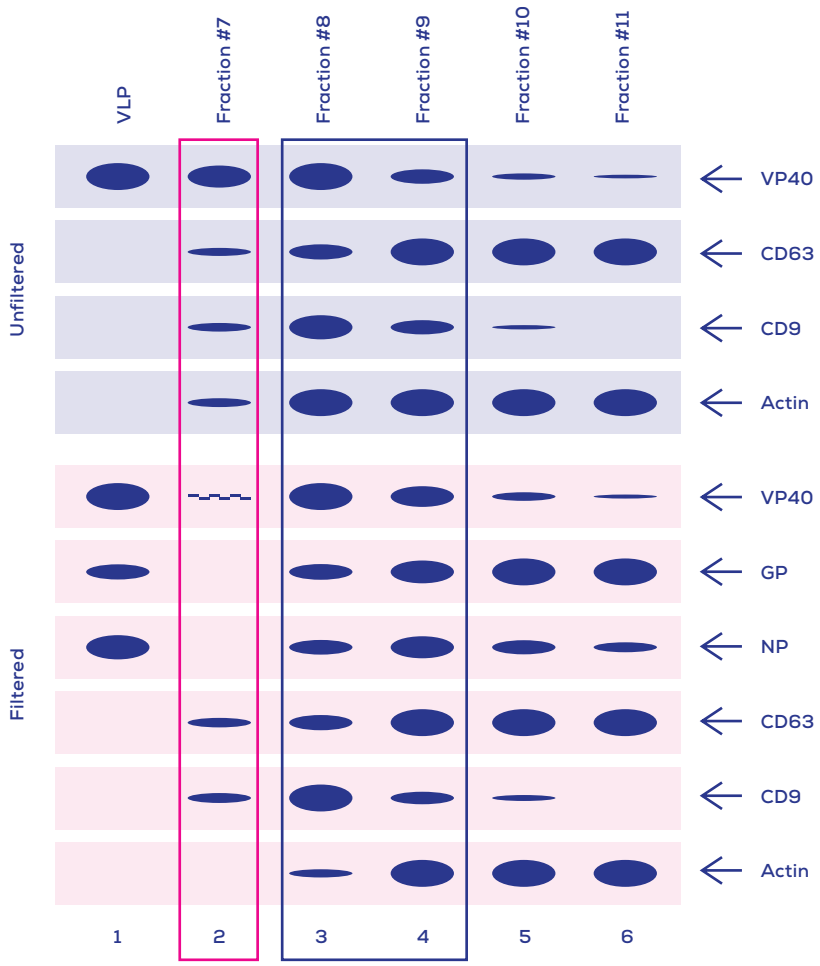

Figure 1. Separation of enveloped Ebola virus VLPs from EVs by size with qEV columns (IZON). Positive control VLP marker (VP40, nucleoprotein (NP) and GP) profiles are shown in lane 1, whereas a typical profile of qEV collection volumes of unfiltered (top panel) or filtered through 0.22 m (bottom panel) supernatants from VLP- expressing cells are shown in lane 2-6. Markers for EVs are CD63, CD9, Actin. qEV collection volumes where VLPs are known to elute are indicated by pink box and fractions containing only EVs and no viral particles are indicated by blue box. Adapted from [8].

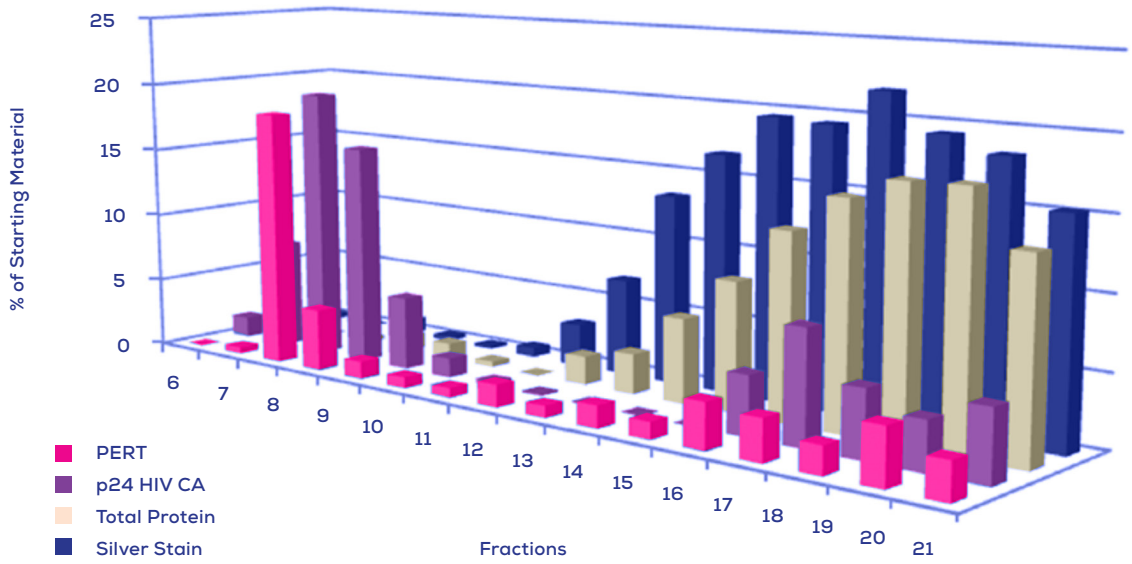

Figure 2. Biochemical characterisation of enveloped lentiviral virions purified by qEV columns (IZON). Aliquots from each qEV fraction were analysed by PERT (Product-enhanced reverse transcriptase) assay (pink columns), immunoblot for p24 HIV CA protein (purple columns), total protein (beige columns) and silver staining (blue columns). This shows virus-specific markers (PERT and p24) are most prominent in early fractions (7–10) yet clustered around fraction 8. Adapted from [10].

Thus, efficient qEV-based separation of virions from EVs will be mostly influenced by the size of the virus particle under study. Izon recommends carrying out preliminary qEV isolation runs to identify the virus and EV-containing fractions and evaluate the purity before determining the final protocol. For any therapeutic applications, virus purification techniques within the manufacturing processes must ensure impurities or contaminations are removed to acceptable limits, acknowledging that depending on the nature of the virus preparation, it may still hold bioburden associated with it. Impurities such as host-cell derived DNA (hc-DNA) are well reported in virus preparations, either as separate large genomic DNA molecules or associated with virions [6,11,12]. It has been shown that nuclease treatment of concentrated virus samples before loading into SEC columns effectively removes contaminating hcDNA, yielding highly pure virus preparations [11,12]. Although anion-exchange chromatography following SEC has been shown to reduce hcDNA by 500-fold in Human influenza virus (H1N1), remaining DNA in virion preparations was still higher than allowed for use as attenuated or inactivated virus vaccines[6].

Virus-like Particles

VLPs are virions without genetic material. There are multiple capsid structural variations from which VLPs may be comprised of, for example one to four structural proteins, one to two layers of capsid proteins, with or without envelop, unmodified or modified capsids, with or without heterologous nucleic acid/protein packaging [13]. Also, numerous cellular expression systems can be used to generate VLPs: bacteria [14], yeasts [15], insect cells [16] or mammalian cells [17]. The method used to generate the VLP depends on the desired VLP structure. In some cases, one plasmid-coded capsid protein expressed in a mammalian host cell is able to self-assemble and be 'naturally' released from the cell, such as enveloped HIV-1 Gag VLPs [18]. In other cases, more complex genetic vectors (coding for multiple recombinant elements) are needed to efficiently express capsids within the cell expression system. This was shown for non-enveloped Foot-And-Mouth Disease VLPs, where toxicity levels of assembly-required proteases were minimised and VLP stability was improved by mutating one of the four capsid proteins [16]. Moreover, VLP assembly can occur outside the cell or in vitro, as demonstrated for dialysis-based assembly of wild-type or recombinant purified capsid units (capsomeres) of non-enveloped Murine Polyomavirus (MuPyV) generated in bacteria Escherichia coli [14]. The utility of the latter approach lies on being low-cost and scalable to very large outputs. Thus, depending on the approach used for production, VLPs from cell cultures can be fairly pure (free of most cell derived non-VLP particles) and may be straightforward to continue with downstream analyses.

As is the case for viruses, non-enveloped and enveloped assembled VLPs are initially retrieved from cell culture systems in cell lysates [15,16] or cell supernatants [17,18], respectively. Again, the chosen cell lysis method (if needed) might be critical for isolating VLPs as some might be more sensitive to disassembly due to stress shear during lysis[19]. Enveloped VLPs isolated from cell culture are also subject to purification challenges, such as abundant presence of EVs or hcDNA, which may be reduced with protocol optimisations like SEC and nuclease treatments, respectively [20]. SEC (coupled with binding selection) has been used for the isolation of VLPs, providing recovery rates of 75-95% by particle counts or 94% by capsid protein immunoblotting [20], yet these column's resolution can be low and isolation procedure can be time-consuming, requiring buffer optimisation, long column equilibration, and calibration steps.

As VLPs have same morphology and size as their virion counterparts, VLPs can be efficiently purified by SEC qEV columns, as these do not require harsh or combination of buffers for binding or eluting of VLPs. Thus, VLP integrity is not altered, and their antigen/epitope exposure is not modified in any way, especially important when VLPs' intended use is to produce protective immune response. Prior to purification by qEV columns, an initial clarification step is recommended; this includes bulk separation from cells, cell debris, and other nanoparticles (by filtration or ultra-filtration)[19].

If the VLPs under study are very similar in size to EVs, a second purification step involving heparin-binding may help in separation. This was shown with HIV-1 gag VLPs, normally with a size of 100 nm, where passing SEC-purified sample through Heparin affinity chromatography column assisted further in separation of EVs and VLPs. However no pure populations were achieved as both EVs [21] and enveloped VLPs have cell membrane derived Heparan Sulfate Proteoglycans which bind to heparin [20].

3 / QUANTIFICATION OF VIRUS AND VLPs

The rapid assessment of virus/VLP load in a buffer suspension has traditionally been performed by measuring the peak UV absorbance at ~214, ~260, or ~280 nm [4,11,14,20], which indicates the quantity of peptides, DNA and proteins, respectively [22,23]. However, as an indirect and approximation method, such optic approaches have their limitations; for instance, individual particles are not counted. As virion/VLP preparations are derived from heterogenous biofluids containing varying proportions of nucleic acids and proteins, this approach does not provide an accurate insight.

Depending on characteristics and the intended purpose of the virus/VLP preparation, few other quantification methods are far more accurate and suitable. For example, virus preparations made for live-attenuated virus vaccines are expected to be able to replicate inside the recipient and induce an immune response similar to a natural infection. Live-attenuated vaccines are usually generated by serial passage in cultured cells, where they lose virulence for the natural host [1]. Nevertheless, during the production some virions within this preparation may be defective particles or not infectious, containing partial nucleic acid content in a single capsid, or empty capsid with no nucleic acid at all. Under this scenario, to complement absolute particle quantification, activity assays are mandatory for correlation of infectiveness of virions. In this way, virion concentrations are correlated to hemagglutination activity assays for Influenza virus [6] or to reverse transcriptase enzyme activity assays for retroviruses such as Lentivirus or HIV [10].

Virus preparations made for inactivated vaccines undergo through physical or chemical inactivation to eliminate their infectivity or ability to replicate, causing a higher requirement of antigens or virus particles to reach desirable immune response. Due to this vaccine approach limitation, accurate virus quantification becomes more critical to meet dose concentrations standards. VLPs preparations made for vaccines are regarded as inactivated virus ones in terms of quantification, as they require similar high doses of viral particles for immune response, however their surface antigens are not modified or damaged as it may happen during inactivation process of virus vaccines.

Thus, in vaccine development and production, accurate quantification of the particles of interest is crucial as reliable measurement of yields is essential for quality control, and accurate concentration of the product must be known to ensure appropriate dosing. Alternative methods or surrogates to virus particle quantification methods have been used and may involve classic plaque forming assay, antibody-based techniques such as enzyme-linked immunosorbent assays (ELISA), bioactivity-based assays, PCR, flow cytometry of labelled particles, among others [24]. However, these methods are often laborious and time-consuming, and are not always sufficiently sensitive or compatible with downstream analyses. Electron microscopy (EM)-based imaging techniques including Transmission EM, CryoTEM and Scanning EM have been fundamental in virology history, enabling precise size measurements and ultrastructure analysis of viruses/VLPs that can be as small as ~20 nm, such as Hepatitis B virus/VLPs [25]. Nevertheless, the resolution of EM-based methods for physical characterisation of particles is hampered by the clear lack of high throughput sample processing capacity, as sample preparation and analysis require not only lengthy protocols but also expensive and specialised equipment and user's skillsets.

There is currently no single, universally accepted method of virion/VLP quantification, and there is an urgent need for simple, fast, and reliable methods which can be streamlined into clinical settings. Single particle analysis methods enable particle-by-particle measurements allowing assessment of key parameter distributions within a heterogeneous population of nanoparticles, as expected for production in cell culture systems. However, virus/VLP quantification not only can be challenging due to the similar size of EVs and some virus particles but also due to the presence of viral proteins and nucleic acids in virus-hosting cell derived EVs, leading to inaccurate quantification of virions based on those markers.

Recent technological advances in electrical sensing of nanoparticles, as opposed to optical quantification, has led to this approach having utility in many research fields, such as virology. In general, Tunable Resistive Pulse Sensing (TRPS) uses the temporal resistance increase or pulse by the passage of viruses/VLPs across a nanopore for quantification purposes [24]. The characteristics of the current pulses are used to determine virus/VLP concentration, size, and effective charge (zeta potential) by measuring the number of pulses, pulse magnitudes and pulse durations, respectively. TRPS is a single-particle method which enables high-throughput measurements, not requiring pre-analytical processing, fluorescent labelling, or highly skilled protocols, highlighting the shift in the field toward direct measurement techniques [24].

TRPS has been extensively and successfully applied to the accurate measurement of virions [4,10,26–30] and VLPs [31,32]. The insights that can be obtained from TRPS can be used to create a better understanding of manufacturing processes; for example, batch-to-batch variation can be assessed by calculating the ratio of total isolated viral particles to infectious particles (**Table 1**) and purification optimisation can be accelerated when manufacturers are informed of accurate size distribution of virus particles separated for example, by qEV isolation columns (**Figure 3A**).

Apart from the fact that the small size of some viruses/VLPs is a challenge for other quantification techniques, TRPS provides an accurate picture of the true particle size distribution and heterogeneity of a sample. Unlike some optical-based techniques which provide an average particle size, TRPS provides single-particle measurements and can precisely resolve multimodal or polydisperse samples, such as those derived of cell culture systems [33]. Furthermore, TRPS has shown the capacity to measure a broader particle concentration range with less variation than the 'gold standard' EM-based quantification approach [28].

VLPs have revolutionised the field of vaccinology, not only exhibiting enormous recombinant antigen-presenting possibilities but also opportunities to improve their efficacy through smart synthetic biology design [13,34]. Antigens in the capsids of non-enveloped VLPs are usually modified through genetic engineering or chemical conjugation, while antigens on the lipid layer of enveloped VLPs are modified by a process called pseudotyping, which includes the modification of epitopes presented in the membrane of the host cell system [32,35]. Whilst morphological characterisation (by high resolution imaging) is critical to corroborate functionality and correct assembly of protein units, reliable and high throughput quantification methods are still required for comprehensive analysis of VLP preparations and dose optimisation studies.

Of note, TRPS has been used to obtain precise measurements for formulation stability studies, including Ebola virus VLPs intended for vaccines. In this study, manufacturing process was improved by disruption (sonication) of large filamentous VLPs (**Figure 3B**) and thermal stability was increased by lyophilization of VLPs, while retaining the protective immune response in tested animals [32]. Thus, TRPS ensures continuous measurements and ability to obtain reliable, consistent measurements at various time points allowing sample stability and aggregation to be assessed over both short and long time periods – essential analyses for vaccine development.

Table 2. Summary of total/infectious particle ratio in a VSV (vesicular stomatitis virus) preparation measured by TRPS (qNano, IZON) and plaque assay, respectively. This shows consistency of total particles counts obtained between biological replicates and a low proportion of infectious particles. Adapted from [28].

VSV population replicates	Virus titer per mL		Total-to-infectious particle ratio
	Particle counts by TRPS	Infectious units by plaque assay	
1	9.4×10^9	9.5×10^9	0.99
2	2.9×10^{10}	8.3×10^9	3.53
3	2.3×10^{10}	5.0×10^9	4.64
4	1.5×10^{10}	6.0×10^9	2.58
5	1.5×10^{10}	1.2×10^{10}	1.29
6	2.5×10^{10}	5.8×10^9	4.42
Average	$1.9 \times 10^{10} \pm 6 \times 10^9$	$7.7 \times 10^9 \pm 2.4 \times 10^9$	2.91 ± 1.42

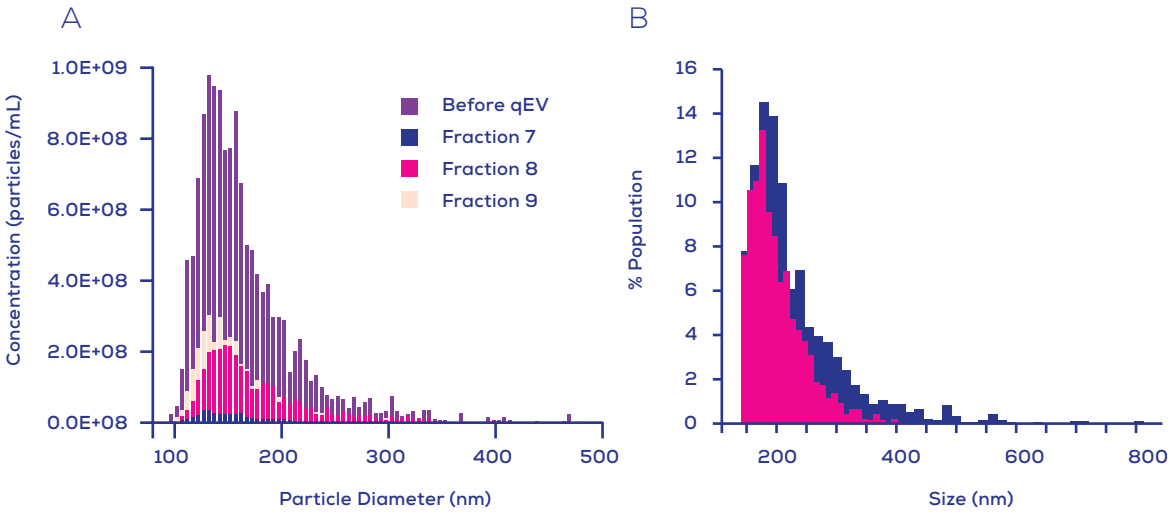

Figure 3. TRPS quantification in virus/VLP preparations. A, Particle size distribution assessed by qNano (IZON) from qEV (IZON) particle-rich fractions (7, 8 and 9) of a STAR-A-HV virus preparation. This shows no clear changes in size distribution from each of the particle-rich fractions. Adapted from [10]. B, Particle size distribution assessed by qNano (IZON) of Ebola virus VLP preparation after sonication and passage through a 0.45 µm (blue) or 0.8/0.2 µm (pink) filter. This shows that double filtration retains smaller particles that are more amenable for manipulation of vaccine formulation. Adapted from [32].

Many relevant biological interactions between nanoparticles depend on their charge or zeta potential. The ability of TRPS technology to measure particle-by-particle zeta potential offers a unique opportunity to perform more comprehensive characterisation of virion/VLP preparations (**Figure 4**). Zeta potential is a measure of effective charge of a nanoparticle in a certain medium and it represents the colloidal stability of particle-particle and particle-medium interactions. Therefore, the tendency of virus/VLP preparations to aggregate or remain in suspension depends on the nanoparticle zeta potential and may be subject to different formulation optimisation approaches. Additionally, it has been shown that the charge of nanoparticles can influence the cellular uptake efficiencies and cytotoxicity effects in recipient host cells [36]. To highlight the importance of charge in virus uptake, studies have demonstrated that single amino acid replacement of recombinant epitope in Tobacco Mosaic tobamovirus (TMV) coat protein (CP) is able to change their predicted isoelectric point (representative of protein charge) and radically change the infectivity in their natural plant host cells [37].

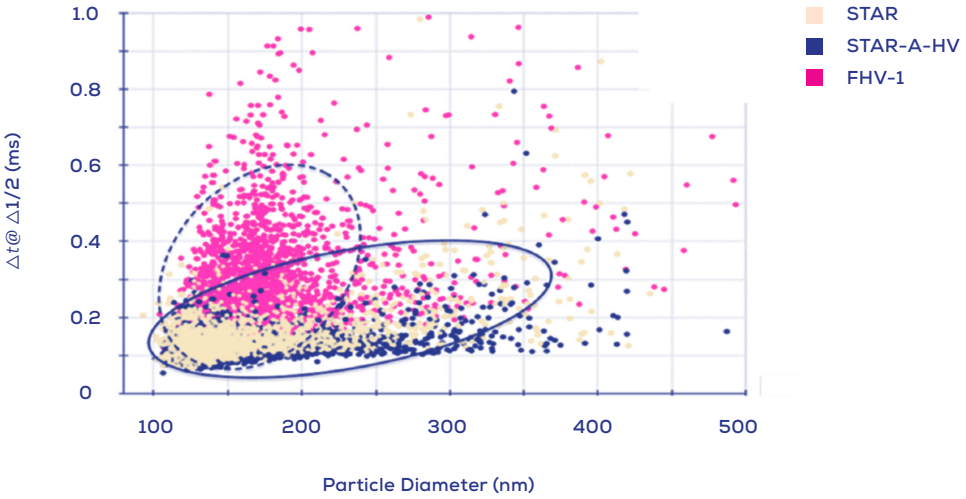

Figure 4. Analysis of zeta potential of different virion preparations by TRPS (qNano, IZON). Plot shows virion particle diameter versus zeta potential of three virus preparations: STAR and STAR-A-HV derived particles as well as FHV-1 particles. This shows no difference in zeta potential between two Lentivirus preparations from STAR and STAR-A-HV cells (full ellipsoid), however the Herpesvirus FHV-1 shows a different pattern, shifted to more negatively charged (broken ellipsoid). Adapted from [10].

4 / VIRUSES IN RESEARCH

Determination of viral load (also referred to as viral titre or burden) is an essential aspect of assessing the severity of infection or responses to treatment. Viral load refers to the absolute number of viral molecules in a given fluid (usually plasma, saliva, nasal secretions, etc). In *in vivo* models, isolation of virions from biofluids presents additional challenges due to the complexity of the fluids as well as the presence of contaminating particles such as protein aggregates and lipoproteins. Firstly, the separation of viral particles from EVs is particularly important as EVs are likely to have specific roles in the pathogenesis of viral infection, meaning that a lack of separation could lead to inaccurate and misleading results. qEVs have shown reliable and efficient separation from bulk proteins and cellular debris normally contaminating cell system derived virus/VLP preparations. When there are no time constraints or necessity for high-throughput quantification, more labour-intensive method can be utilised, such as the plaque assays, which remains as one of the best methods to determine actual infective virus load, however it may take up to a week to get results, with several steps in the method that are subject to variability and optimisation.

5 / VIRUSES IN DIAGNOSTICS

The diagnosis of viral infections relies on accurate methods for detecting and identifying viruses in biofluids. Modern virus diagnostics in humans, heavily depend on quick results, high through-put analysis and close collaboration with clinicians. In this context, virus isolation, virus quantification and/or their culture in cell systems is becoming largely outdated in diagnostic applications. In general, the detection and quantification of virus particles for diagnostic purposes involves the detection and quantification of viral genomes. Genomic indirect quantification methods can be utilised by known correlations to the whole virion, with thorough and prior research providing knowledge of the virus structure and composition. For example, qPCR-based techniques allow rapid virus detection/identification by specific amplification of sequences unique to one type of virus, and virus quantification by the correlation of absolute amount of nucleic acid to nucleic acid per virus particle. As these provide indirect measurements of the number of viral particles, they are associated with limitations; the accuracy of measurement relies on the stability of the extracted nucleic acids (especially critical in RNA extraction), appropriate calibration and efficiency of the PCR reaction, and cross-reactivity or comparison to a reference standard (and, therefore, the availability of appropriate standards). Furthermore, these techniques require pre-analytical preparation including nucleic acid extraction and specialised users and equipment.

6 / VIRUSES AS OTHER THERAPEUTICS

The nature of viruses has positioned them as resourceful stages to therapeutics, since these biological entities are specifically recognised, taken up and processed by host cells in an exploitable manner. Viruses have demonstrated to be promising gene therapy tools, i.e., vehicles for delivering genetic material (i.e. DNA or RNA) to target cells and causing a genetic modification that confers some benefit in a disease context [38]. Virus-based gene therapies have been used successfully to treat several different types of disorders, such as cancers, muscular, metabolic, cardiovascular, hematologic, neuronal, among others [39]. The best gene therapy candidates are analysed based on their manufacturing efficiency and scalability, nucleic acid-packing capability, tropism to and ability to transduce (enter) target cells. Within these parameters, the most studied and used virus vectors are lentivirus, adenovirus and adeno-associated virus (AAV) [38,39]. One of the main challenges related to the production of virus vectors for gene therapy can be traced back to the high particle counts necessary for administration to humans (~1000-10,000 billion virions/kg body weight). In this context, there are many opportunities to optimise cell culture procedures, virus separation and purification techniques. Often, a combination of methods is required to achieve high yields and high purity.

7 / CONCLUDING REMARKS

Although the attention of the general public has been drawn to viruses as nasty infectious disease etiological agents, the reality is that viruses are invaluable contributors to medicine and public health. Viruses' unique life cycle and diversity has converted them as practical platforms for numerous applications in the biomedical field and treatment of many diseases which are urgently needing on alternative approaches. As nanoparticles, viruses/VLPs share challenges with other important biological and synthetic nanoparticles, in terms of mass production, purification and quantification. The advancement of technologies aiding to scaling and accelerating virus/VLPs manufacturing will continue, as will their meaningful contributions to medicine.

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