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Research review paper

Clarification of vaccines: An overview of filter based technology trends and best practices

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ABSTRACT

Vaccines are derived from a variety of sources including tissue extracts, bacterial cells, virus particles, recombinant mammalian, yeast and insect cell produced proteins and nucleic acids. The most common method of vaccine production is based on an initial fermentation process followed by purification. Production of vaccines is a complex process involving many different steps and processes. Selection of the appropriate purification method is critical to achieving desired purity of the final product. Clarification of vaccines is a critical step that strongly impacts product recovery and subsequent downstream purification. There are several technologies that can be applied for vaccine clarification. Selection of a harvesting method and equipment depends on the type of cells, product being harvested, and properties of the process fluids. These techniques include membrane filtration (microfiltration, tangential-flow filtration), centrifugation, and depth filtration (normal flow filtration). Historically vaccine harvest clarification was usually achieved by centrifugation followed by depth filtration. Recently membrane based technologies have gained prominence in vaccine clarification. The increasing use of single-use technologies in upstream processes necessitated a shift in harvest strategies. This review offers a comprehensive view on different membrane based technologies and their application in vaccine clarification, outlines the challenges involved and presents the current state of best practices in the clarification of vaccines.

Contents

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1. Introduction

Vaccines are a key part of our protection against infectious diseases, which still are an alarming cause of mortality. Thanks to immunization, two to three million lives are saved annually from diphtheria, tetanus, pertussis (whooping cough), and measles (WHO, 2014a). Vaccines cover a wide range of products, from small recombinant proteins to entire virus particles and whole bacteria. They can be produced by different systems: eggs, mammalian cells, bacteria, etc. Due to vaccine complexity and diversity, no dominant purification scheme or template exists today, despite the growing interest for a vaccine platform (Ball et al., 2009). Usually, a vaccine process can be split into three sections: upstream (production and clarification), downstream processing (purification involving ultrafiltration, chromatography and chemical treatments), and formulation (finish-fill operation). Independent of the production system, clarification (initial removal of undesirable materials) plays a critical role in defining a robust purification process (Hughes et al., 2007). A suitable clarification step primarily removes whole cells, cell debris, colloids and large aggregates to reduce burden on the downstream processing. In certain cases, clarification also reduces insoluble impurities, host cell proteins (HCPs) and host cell nucleic acids. Like any other purification step, the clarification step needs to be optimized to achieve maximal product yield and purity while accommodating for vaccine specificities and manufacturing constraints.

Table 1
Methods typically used for clarification of vaccines.

Technology	Advantages	Disadvantages
Centrifugation	– Good recovery – Good DNA and HCP removal	– High capital investment – Difficult to scale-up – No scale-down model available
Sedimentation	– Cheap – Simple to operate – Suitable for microcarrier based process	– Unreliable – Time consuming – Product loss
Tubular pressure filters (Fundabac)	– Simple design and operation – Reusable – totally enclosed system for high-containment tasks	– Difficult to scale-up
Tangential flow filtration (plate & frame)	– Very robust – True linear scalability – Low flow rate (Low energy consumption, small piping, compact system size, etc.) – Short flow path (High flux, resolution) – Multiple flow channel configuration (screens)	– Concern for shear – No real open channel
Tangential flow filtration (hollow fiber)	– Multi cycle steamable – Open channel (typically 0.5 to 1.1 mm) – High membrane packing density – Moderate cost – High solid loading	– Robustness – High flow rate (high energy consumption, large piping, large system size, etc.) – Low flux – Non linear scalability
Depth filtration	– Easy to scale-up – Cheap – High capacity	– Issues of virus adsorption – Concern for extractable and leachables – Inherent variability in product
Membrane filtration	– Easy to scale-up – Cheap – Good product recovery – Availability of pre-sterile formats	– Need for extensive optimization

Diverse technologies are used for clarification due to heterogeneity of vaccine types, including centrifugation or filtration technologies (Table 1). Several series of operations are often required to achieve desired clarification. The first operation is aimed at removing larger particles (primary clarification) and a second one for removing colloids and other sub-micron particles (secondary clarification). Low-speed centrifugation as a choice for primary clarification enables cells and cell debris removal by sedimentation. Centrifugation can handle high solid load and has been extensively used in batch or continuous modes with disk-stack centrifuges. It requires high capital investment and maintenance costs and presents challenges related to scale-up due to lack of reliable scale-down model (Yavorsky et al., 2003; Russell et al., 2007). Nevertheless, several commercial vaccine manufacturers employ the use of a centrifuge for large scale manufacturing involving high processing volume and higher number of production campaigns. Development of vaccines targeting niche population or smaller target group demanding less finished dosage, the rise in upstream processing technology and higher titer processes has reduced the size of bioreactors and amount of volumes processed per batch (Genzel et al., 2014a). Because of this, filtration technologies have gained interest for vaccine clarification. Clarifying filtration can be performed either by normal flow filtration (NFF, also known as dead-end filtration) or by tangential flow filtration (TFF, also known as cross-flow filtration). There are also certain filter formats (depth filters) that contain positively charged material and filter aid that enhance retention of cell debris, colloids and negatively charged unwanted components. Membrane filters retain particles by size exclusion and are not of high dirt holding capacity, therefore suitable for a secondary clarification step. Both depth filters and membrane filters are easily scalable and implementable (simple system design). Unlike NFF, TFF is mostly used for primary clarification (microfiltration). Membranes with cut-offs in the range of 0.1–0.65 μm (preferably with an open channel) have been successfully used to retain cells, cell debris and other large contaminants. Most TFF devices are linearly scalable and reusable after cleaning, which significantly reduces consumable cost of the step (Pattnaik et al., 2009; Raghunath et al., 2012).

The clarification step, being at the interface between the upstream and downstream processes, is sometimes neglected during vaccine process development, as time and resources are preferentially allocated to other purification steps such as chromatography or density gradient centrifugation. The limited literature available reflects the lack of systematic efforts on this topic. Even vaccine manufacturing process patents often omit clarification steps. However, clarification efficiency

directly influences the performances of the downstream process. A poorly optimized clarification step can negatively impact the capacity of sterilizing-grade filters or may shorten a chromatography resin lifetime. Today, stricter regulatory expectations tend to make vaccine manufacturers produce purer, well characterized yet affordable vaccines. In such circumstances, each step needs to be given due importance. Current trends indicate that the upstream processes are evolving towards "cleaner" expression systems (i.e. cells grown in serum-free media replacing eggs) and enhanced productivity and higher cell densities. Downstream purification processes are being simplified (less steps) and streamlined, leading to increased purity of the final product (Vicente et al., 2011a; Nestola et al., 2015). To address such changes, the clarification step must not be a bottleneck. In this context, filtration technologies are living up to the new clarification challenges addressing increased process flexibility, possibility of single-use and reduced investment costs.

This article reviews technology trends and best practices for development and optimization of vaccine clarification using filtration through examples, case studies and comparative evaluation of technology and tools.

2. Clarification of viral vaccines

Several types of clarification methods, either in isolation or in combination have been used successfully to clarify vaccine feed stream harvest. A few of them are mentioned in Table 2.

2.1. Considerations for viral vaccine clarification

A wide range of vaccines contains whole or part of virus particles to develop immunity against a viral infection. They are typically split into four main categories (Plotkin et al., 2013):

- *Live attenuated virus (LAV) vaccines*, which are based on a virus strain that has been weakened in order to decrease its virulence. Attenuated viruses can replicate in the body, but are not pathogenic.
- *Inactivated virus (IV) vaccines*, which contain viruses chemically or UV inactivated to eliminate infectivity. Virus particles can be entire, split or purified (antigenic protein only).
- *Viral vector (VV) vaccines*, which are active non-pathogenic viruses presenting antigens of a pathogenic virus. These recently developed constructs are also used for gene therapy applications.
- *Virus-like particle (VLP) vaccines*, which are a specific class of viral subunit vaccines that mimics the overall structure of virus particles without the infectious genetic material.

In most cases virus particles are kept fully integral during the clarification step, even in split virus vaccine processes (splitting is usually performed downstream, in a more purified environment). The main challenge of virus clarification is to recover high yield of viral particles while efficiently removing cell debris, large aggregates and insoluble contaminants. As described in the following sections, several elements can lead to virus degradation or loss. Furthermore, viral yield may be

Table 2
Combination of technologies used for clarification of vaccines.

Vaccine	Production system	Vaccine type	Product location	Scale[a]	Primary clarification	Secondary clarification	References
Meningococcal vaccine	Bacteria fermentation	Conjugated polysaccharide	Extracellular	Pilot	0.2 μm hollow fiber cartridges (TFF)	300 kDa cassettes (TFF)	Robinson et al. (2011)
Hepatitis C	Insect cell culture	Virus like particle	Extracellular	Pilot	Polypropylene filter, Polygard® CN 5.0 μm (NFF)	Polypropylene filter, Polygard® CN 0.3 μm (NFF)	Xenopoulus (2015)
Yellow fever vaccine	Vero cell culture	Inactivated virus	Extracellular	Pilot	Sartopure® PP2 (8.0 μm), NFF	Sartoclean® CA (3.0 μm + 0.8 μm), NFF	Pato et al. (2014)
Rotavirus vaccine	Insect cell culture	Virus like particle	Intracellular	Pilot	Centrifugation at 1000 ×*g* for 10 min at 4 °C	Ultracentrifugation of the supernatant at 100,000 ×*g* for 1 h at 4 °C	Peixoto et al. (2007)
Canine adenovirus vector	MDCK-E1 cell culture	Live viral vector	Intracellular	Pilot	Settling of cells with microcarrier	Sartobrand® P 0.45 μm filter (NFF)	P. Fernandes et al. (2013)
Influenza virus	MDCK cell culture	Inactivated virus	Extracellular	Pilot	0.65 μm polypropylene depth filter (NFF)	NA	Kalbfuss et al. (2007)
Acellular pertussis	Bacterial fermentation	Sub-unit vaccine	Extracellular	Pilot	0.45 μm Prostak™ device (TFF)	NA	Kumar et al. (2009)
Typhoid vaccine	Bacterial fermentation	Conjugated polysaccharide	Extracellular	Pilot	0.45 μm Hydrosart® cassette (TFF)	NA	Kothari et al. (2010) and Kothari et al. (2013)
Pneumococcal vaccine	Bacterial fermentation	Conjugated polysaccharide	Extracellular	Pilot	300 kDa Pellicon® 2 cassettes (TFF)	NA	Macha et al. (2014)
Enterovirus 71	Vero cell culture	Killed viral vaccine	Extracellular		0.65 μm filter (NFF)	NA	Liu et al. (2011)
Rotavirus vaccine	Vero cell culture	Live viral vaccine	Extracellular	Pilot	Centrifugation at 2831 ×*g* for 30 min and 4424 ×*g* for 10 min at 4 °C	0.45 μm hollow fiber (TFF)	Zhang et al. (2011)
pDNA vaccine	Bacterial fermentation	Pladmid DNA vaccine	Intracellular	Pilot	Flocculation	Depth filtration (NFF)	Palmieri et al. (2010)
Diphtheria toxin	Bacterial fermentation	Toxoid	Extracellular	Production	0.45 μm Prostak™ device (TFF)	NA	Sundaran et al. (2002)
Tetanus toxin	Bacterial fermentation	Toxoid	Extracellular	Production	0.22 μm Prostak™ device (TFF)	NA	Muniandi et al. (2013)
Polio virus vaccine	Vero cell culture	Inactivated virus	Extracellular	Production	Diatomaceous earth deposit on a stainless steel mesh filter with 75 μm pore size, or Millistak + ® C0HC (Depth filter)	0.45 and 0.22 μm filtration (NFF)	Thomassen et al. (2013a, b)

[a] Pilot scale: 1–20 L, production scale: more than 20 L.

Table 3
Characterization of virus vaccines produced in continuous cell lines.

Vaccine	Rotavirus vaccine	Foot and mouth disease vaccine	Adenovirus vectors	Inactivated polio vaccine	Japanese encephalitis vaccine	Influenza vaccine
Vaccine type	Live attenuated	Live attenuated	Viral vector	Inactivated	Inactivated	Inactivated
Cell line	Vero	BHK21	PER. C6® HEK293	Vero	Vero	MDCK Vero EB66® PER. C6®
Support for adherent cells	Microcarriers	–	–	Microcarriers	Microcarriers	MDCK, Vero: microcarriers
Cell lysis step	Yes	Yes	Yes	No	No	No
pI[a]	8	3–7	2–5	5–8	5	5–7
Virus size	70 nm	25–30 nm	70–90 nm	30 nm	40–50 nm	80–120 nm
References	Dennehy (2007) and Wainwright (2006)	Grubman and Baxt (2004) and Lombard and Füssel (2007)	Pattnaik and Adams (2013)	Dietrich et al. (2014)	Yang et al. (2004) and Toriniwa and Komiya (2007)	Milián and Kamen (2015) and Palese and Shaw (2007)

[a] Michen and Graule (2010).

difficult to rely on because of the high variability of virus quantification assays at this stage of the process, especially for LAV and VV vaccines. For these vaccines, viral yield is typically assessed using infectivity assays (such as plaque assays or $TCID_{50}$ assays). Indeed, some harvest compounds may interfere with the ability of the virus to infect the indicator cells, which enhances the variability of such quantitation method (Darling, 2002).

2.2. Strategy for viral vaccine clarification

Viral vaccines are extremely diverse in terms of size, structure, shapes and expression system (Table 3). As a result, there is no template for their downstream process in general and for the clarification step in particular. In theory, all available technologies (low-speed centrifugation, microfiltration TFF, NFF) can be selected and potentially combined together to clarify viruses. In fact, the success of a clarification method is influenced by the expression system and the physicochemical properties of the virus of interest.

Recently high cell density process is being explored for viral vaccine process (Genzel et al., 2013, 2014b). High cell density processes pose increased challenges for clarification. Many address this through pretreatment i.e., polymers induced flocculation, precipitation, alternate TFF, etc. As an example, Tomic et al. (2015) described a clarification method for high cell density harvests (>10^7 cells/mL) using a cationic polymer which allows a 4-time reduction of the depth filtration area compared to traditional methods. This technology could permit large fermenter volumes to be harvested without the use of a centrifuge. Similarly, Riske et al. (2007) also show that Chitosan treatment (0.02%) of cell culture harvest (40 L) containing 1.4–2.6% solids can result in a seven-fold increase in capacity on the absolute filter post-depth filtration. They also mentioned that Chitosan appears to improve the clarification efficiency by flocculating the submicron particulates which normally escape sedimentation in the centrifuge. Pretreatment and flocculation methods will most likely continue to be part of the future of vaccine filter clarification (Goerke et al., 2005). Osmolytes, including sugars, sugar alcohols and amino acids, preferentially flocculate viruses (Gencoglu et al., 2014). Virus flocculation using osmolytes, followed by 0.2 μ microfiltration could be used as an integrated process for virus purification (Gencoglu and Heldt, 2015). Osmolytes are able to flocculate hydrophobic non-enveloped and enveloped virus particles by the reduction of the hydration layer around the particles, which stimulates virus aggregation (Saksule and Heldt, 2015). Though it has been demonstrated that osmolytes flocculate viruses, the method has the potential to be a future platform of vaccine purification, the work reported by Gencoglu and Heldt (2015) is at small research scale and its feasibility at pilot or large scale vaccine purification is yet not reported. Flocculation pretreatment methods that will most likely continue to be part of the future of vaccine filter clarification, as reported by Goerke et al. (2005) were done in a 2 L fermenter. To potentially be used in large scale manufacturing operation such methods need to be validated at pilot and production scales.

2.2.1. Impact of expression system

The clarification approach mainly depends on the upstream process and the expression system type, which determines the type and level of contaminants to be removed. Viral vaccines are generally produced in embryonated chicken eggs by mammalian or avian continuous cell lines or by baculovirus/insect cells which is a more complex expression system. Some types of VLPs may also be generated by other heterologous expression systems (bacteria, yeast, plant cells).

2.2.1.1. Viruses produced in embryonated chicken eggs. Vaccines have been produced in eggs for decades. The work dates back to 1931 when Woodruff and Goodpasture successfully used the chorioallantoic membrane of fertile hen's egg as a substrate for growing viruses (Woodruff and Goodpasture, 1931). Today, many human and veterinary vaccines are still made using this age-old process. The most famous is probably the seasonal influenza vaccine. The principle is to inoculate embryonated chicken eggs with the virus of interest, which then replicates in the chorioallantoic membrane. After propagation, the allantoic fluid, rich in virus particles, is collected and purified.

The allantoic fluid is a challenging feed for clarification. Its high mineral and protein (including ovalbumin) content gives it a highly viscous consistency. Allantoic fluid also contains rudimentary tissue compounds from the chicken embryo such as feathers, beaks, blood vessels or blood cells. Because of this high solid content, low-speed centrifugation is the preferred option for primary clarification, typically leading to a recovery yield of around 70% (Hendriks et al., 2011; Eichhorn, 2008). However, implementing filtration techniques for primary clarification is also possible. For NFF, polypropylene and cellulose-based depth filters exhibit fair capacities with allantoic fluid harvests. A surface filter made out of polypropylene or a cellulose-based depth filter can result in capacity between 150 and 210 L/m^2 and reduce the feed stream turbidity up to 3 times (Goyal et al., 1980; Williams and Hughes, 2004). Open feed channel TFF devices are also suitable for allantoic fluid clarification, as the device is better suited to minimal pressure loss across the feed channel resulting in reduced plugging of the channel.

The secondary clarification step can be easily accomplished by NFF. Combinations of polypropylene, cellulose and glass fiber materials generally show good efficiency (Lampson and Machlowitz, 1970). An alternative option for the secondary clarification step is the use of TFF with a 0.65 μm or 0.45 μm microfiltration membrane device operated with permeate flux control. Open (suspended-screen) channel TFF

devices with hydrophilic PVDF or hydrophilic PES membranes can be used at this step (Raghunath et al., 2012).

It is important to note that virus particles may be associated with the insoluble debris material, which can significantly reduce the viral yield during clarification. Hughes et al. (2007) demonstrated that the use of a salt solution could reduce such association between influenza virus and solid debris, resulting in a yield increase of approximately two-fold and without compromising viral particle integrity.

2.2.1.2. Viruses produced in mammalian and avian continuous cell lines. Recently several viral vaccines have moved away from an egg based process and have adopted a cell culture based process (specifically for influenza vaccine). The primary reason for this shift is to prevent vaccine production issues associated with embryonated eggs (i.e., possible shortage of egg supply in the event of any avian disease outbreak) (FDA, 2005; Milián and Kamen, 2015). Consequently, many types of vaccines and viral vectors are currently developed using mammalian or avian continuous cell lines, either conventional (such as Vero, MDCK or HEK293 cell lines) or proprietary cell lines such as PER.C6® and EB66® (Josefsberg and Buckland, 2012). Depending on the expression system, the virus may remain intracellular, necessitating cell lysis step, or extracellular (lytic or budding).

Compared to allantoic fluid, cell culture harvests are considerably cleaner in terms of solid load and soluble content. Therefore, NFF technology is more easily implemented and capacities are significantly higher (Vicente et al., 2011b). However, filter capacity highly depends on the cell culture conditions such as cell density or cell viability at harvest (Iammarino et al., 2007). These parameters influence the amount of cell debris and large aggregates that can plug depth filters and membranes, leading to reduced capacities.

A good example of clarification by NFF was reported by Thomassen et al. (2013a) for the inactivated polio virus (IPV) production process. Vero cells grown on microcarriers were used for virus propagation at a cell density TOI of 0.78×10^6 cells/mL. Pre-clarification was done using a 75 μm stainless steel sieve to remove microcarriers from the harvest. A double layered graded density depth filter (0.2–2.0 μm) was used for clarification, followed by a sterilizing-grade filter. The selected scalable disposable unit was successfully implemented for the preparation of Sabin IPV clinical trial material at a 350 L scale (Thomassen et al., 2013b). Depending on virus serotype, 86% to 96% virus recovery was obtained.

2.2.1.3. Virus like particles produced in baculovirus/insect cell system. The consideration of baculovirus/insect cell system for large-scale production of vaccines is relatively new, but it is gaining interest particularly in the fields of viral vectors and VLPs (Vicente et al., 2011a). The key benefits of the system are that it involves a transient (no need to establish a cell line) and is safe (no complete viral DNA) production (Cox, 2012). There are also drawbacks like the need for baculovirus removal and product stability (Hermens and Smith, 2013; Peixoto et al., 2015). Cervarix®, a VLP vaccine against human papillomavirus infections from GlaxoSmithKline plc. has been the first human vaccine produced commercially in insect cells. Many vaccines based on VLPs and rAAV vectors produced in baculovirus-infected insect cells are currently in development (Krammer and Grabherr, 2010). Insect cell lines derived from the fall armyworm *Spodoptera frugiperda* (Sf9 and Sf21) and from the cabbage looper *Trichoplusia ni* (BTI-TN5B1-4 cells) are most commonly used due to their ability to grow in suspension, which simplifies scale-up of upstream process. After being grown to a desired viable cell density, these cells are infected by recombinant baculoviruses for protein expression during the exponential cell growth phase (Vicente et al., 2011a). Baculovirus' large genome allows expressing up to five or more different proteins, which matches VLPs and viral vector complexity (F. Fernandes et al., 2013).

Downstream processing of insect cell cultures is well described by Bernard et al. (1996). This process is highly variable, reflecting the variety of proteins which have been produced with this technology. The first steps of the purification train are heavily influenced by the characteristics of the bioreactor bulk, namely cell density and viability, or by the nature of the product release, either secreted by budding or cell lysis (Nestola et al., 2015). Baculovirus infection of insect cells causes cell lysis within 3–5 days after infection. Cell disruption may lead to increased proteolytic activity and other environmental factors that can result in degradation of recombinant protein. There have been attempts to develop baculovirus with reduced capability for initiating cell lysis. Such baculovirus shows less than 10% lysis of infected insects (Ho et al., 2004).

Cell lysis can be performed using different methods, such as freeze–thaw, detergents, homogenizer, or sonication (Bernard et al., 1996). Insect cells don't have a cell wall and therefore lyse rapidly. Though sonication has been reported in many bench scale processes, it is rarely used in pilot or commercial scale. The most commonly used method is disrupting the cells using a homogenizer in the presence of a low concentration of detergent (0.1% Triton X-100 or NP-40) (O'Shaughnessy and Doyle, 2011). The use of detergents and homogenization by microfluidization or osmotic shock has been successfully adopted in many large scale manufacturing processes (Cervarix, 2007; ISPE Guide, 2013). Typically insect cells are suspended in lysis buffer (50 mM TRIS pH 7.7, 300 mM NaCl, 5% glycerol, 0.2 mM PMSF and protease inhibitor cocktail) and the lysis is performed by adding Triton-X 100 to a final concentration of 0.1% followed by mild sonication or microfluidization (Vlak et al., 1996). The mix is then centrifuged to remove insoluble particles. At this stage the lysate may look very cloudy and it is difficult to filter using 0.45 μm filters. Sometimes losses as high as 30% can be observed at the lysate clarification step (Wolf and Reichl, 2011). The addition of Benzonase at the lysis step helps solve the filtration problem (Gousseinov et al., 2014). Washing the cells with phosphate buffered saline after the harvest and a quick "freeze–thaw" in high salt (500 mM NaCl) containing lysis buffer help to remove the aggregates.

Clarification of VLPs and viral vectors produced by insect cells happens after cell lysis (either by chemical or mechanical treatment) which releases not only viral particles, but also concomitantly host cell nucleic acid in large quantities. Insect cells are able to grow at high cell densities, from 1 to 9 x 10^6 cells/mL (Ikonomou et al., 2003). Consequently, the clarification step should handle high cell density, high nucleic acid content and if possible, remove baculovirus particles. To make it even more complex, VLPs or viral vectors and baculovirus can have a similar size (baculovirus has a width of 60–80 nm and a length of 300–400 nm). Because of high cell densities, centrifugation has been the preferred technology for primary clarification for decades. However, membrane processes appear as a very attractive alternative as scalability is easily defined. Depth filters have been used efficiently for triple layered rotavirus like particle downstream processes (Peixoto et al., 2007). At laboratory scale, CsCl density gradient ultracentrifugation methods are commonly used for the purification of these complex particles. Peixoto et al. (2007) evaluated not only the clarification step by depth filter but also the whole downstream process (lysis with Triton X-100 and depth filtration followed by ultrafiltration and size exclusion). As a result they achieved higher yield of 37% on the other hand yield of CsCl density gradient ultracentrifugation methods reported the order of 10% (Cheng et al., 2001). As another example, 0.45 μm hollow fiber followed by 500 kDa hollow fiber was used for the recovery and concentration of HIV virus − like particles produced in insect cells (Negrete et al., 2014). In this study, the shear force of hollow fiber was optimized based on cell integrity. As a result they establish low shear force process to replace the bench scale sucrose gradient ultracentrifugation. This process potentially is applicable to large scale production. Depth filters also have been used successfully for recombinant adeno-associated virus production (Cecchini et al., 2011). Cell lysis was conducted with a dual-piston mechanical cell disrupter followed by nuclease treatment. 1.2 μm glass fiber depth filter followed

Table 4
Comparative evaluation of nominally rated pleated polypropylene filter combination for clarification of Hepatitis C virus like particle (VLP) vaccine produced in insect cell culture.

Feed	Filter/filter train	% recovery
Direct harvest	10 μm → 5 μm → 0.6 μm	68%
	5 μm → 0.6 μm	74%
	10 μm → 0.6 μm	68%
	5 μm → 0.3 μm	100%
Centrate[a]	0.6 μm	82%
	0.3 μm	52%

[a] Lab centrifuge was used (300 ×*g* for 10 min) and had a product yield of 90%.

by two-layered 0.8 and 0.2 μm hydrophilic PES were used at the clarification step. Proportionally sized filters were used for process batch from smaller scale to 200 L scale. Depth filters have been used successfully (Cecchini et al., 2011) and TFF with flat sheet membranes or hollow fibers of 0.2 μm or 0.45 μm nominal rating has been reported as very efficient as well (Negrete et al., 2014).

Recent studies at the Instituto de Biologia Experimental e Tecnológica (IBET) in Oeiras, Portugal, clarification of hepatitis C VLP expressed in baculovirus was carried out using NFF without centrifugation (Xenopoulos, 2015). Nominally rated polypropylene filters (10, 5, 0.6 and 0.3 μm) filters were used for the clarification of VLP harvest. The same filter of 0.6 μm and 0.3 μm pore rating were examined for the filtration of a VLP harvest centrate. The filtrates from all studies were tested for HCV-VLP recovery and compared to assess the recommended filtration method. These results are shown in Table 4.

The results showed that a filter train of 5 μm ➔ 0.3 μm filters yielded the highest product recovery (100%) results for the direct harvest hepatitis C VLP feed. Polypropylene 0.6 μm filters yielded the highest product recovery (82%) for the centrate feed and ~70% removal of host cell DNA clearance.

2.2.1.4. Virus like particles produced in bacterial or yeast based systems. The type of clarification method for VLP vaccines expressed in bacterial or yeast based systems depends on the release of VLPs to the extracellular medium. If the VLPs are not efficiently secreted, a cell lysis or other extraction step might be required before the actual clarification step (Vicente et al., 2011a). Although the gold standard in the industry for clarification of proteins expressed in bacteria or yeast based systems has been centrifugation (either continuous or batch), more recently, membrane processes appear as a very attractive alternative due to easy scalability and compatibility to single use processing (Vicente et al., 2011a).

Richter and Topell (2013) explained the use of centrifugation, TFF or combination of both in preparative clarification of VLPs produced in *Escherichia coli*. In their work, *E. coli* homogenate obtained by homogenization was diluted and clarified by employing 0.45 μm TFF membrane at a temperature of 5 °C. They also stated that membrane based TFF is suitable for the processing of high viscosity harvest, preferably using a TFF cassette with an open channel configuration. Clarification by centrifugation was also evaluated as an alternative to TFF. In this case, the homogenate produced was not diluted and subjected to centrifugation at 4 °C for 105 min at 10,000 ×*g*. The supernatant was decanted from the pellet without transferring the soft overlay and re-centrifuged at 4 °C for 60 min at 10,000 ×*g*. The supernatant was then decanted from any pellet present and diluted 1:2 with EB buffer (43.89 mM Tris HCl, 6.11 mM Tris Base, 5.0 mM EDTA, 10% (v/v) Triton X-100), filtered over a 0.22 μm sterilizing grade filtering unit and processed further. A scaled up process of this VLP vaccine produced in *E. coli* at an 800 L scale is also reported to have been clarified by a combination of centrifugation and TFF (Bachman, 2009).

A recombinant hepatitis E vaccine (Hecolin®), HEV 239, has been licensed in China for immunizing adults of 16 year old and above (Wu et al., 2012; Li et al., 2015). The vaccine antigen (VLP) is expressed in *E. coli* and its scale-up of the antigen production process has been demonstrated at 50 L scale (Zhang et al., 2014). The product is extracted by lysis of *E. coli* and inclusion bodies were separated from cellular debris by extensive washing with buffer containing 2% Triton X-100, and then dissolved by homogenization with 4 M urea (Li et al., 2009).

Human papillomavirus VLP produced in *Saccharomyces cerevisiae* (15 L scale) was reported to be clarified by TFF (Cook et al., 1999). The nuclease treated *c*ell lysate was clarified by cross-flow microfiltration in a diafiltration mode using 0.65 μm hollow-fiber filter cartridge. The same has been adopted in the vaccine manufacturing process (Bryan, 2009). Though only a few VLP based vaccines have made it to commercial scale, several VLP based vaccines are in the pipeline (Kushnir et al., 2012), and most of them employ either centrifugation or membrane based technology for clarification.

2.2.2. Impact of physicochemical virus properties

After considering the production system and the way to remove associated contaminants in clarification steps, it is important to take into account virus characteristics and focus on maximizing viral yield.

2.2.2.1. Viruses prone to adsorption. Positively charged materials and filter aids (such as diatomaceous earth) have been developed to improve depth filter efficacy. While positive charges increase nucleic acids and HCP capture, diatomaceous earth is known to bind cell debris and colloids (Yigzaw et al., 2006). However, these materials may also retain viruses by adsorption mechanism. Electrostatic interaction with positively charged filters may occur, since viruses are often negatively charged in the solution. Viruses may also bind by hydrophobic or non-specific interactions with some filter material (diatomaceous earth or glass fibers for example) (Venkiteshwaran et al., 2015). Enveloped viruses, because of their lipidic envelope, are more prone to such adsorption. If virus adsorption occurs on a filter via electrostatic interaction, it is possible to partially recover it by flushing the filter with a high conductivity buffer as virus particles desorb due to salt competition (Roldão et al., 2014). However, this may also elute contaminants such as HCP or nucleic acids. The use of an alternative filter material, such as polypropylene which is more inert, is consequently preferred.

Adenoviruses can be prone to adsorption, but divergent results have been reported. One study by Namatovu et al. (2006) reported good recovery of adenovirus by using positively charged and diatomaceous earth containing depth filters. The findings are summarized in Table 5. Good recovery with borosilicate glass fiber filters were reported as well. On the other hand, a patent deposed by Weggeman et al. (2006) relates 20–40% adenovirus loss when clarifying PER.C6® cell culture with the same positively charged diatomaceous earth containing depth filters. In this case, a nominally rated polypropylene filter showed high virus recovery (>90%).

Influenza virus is also known to be prone to adsorption loss during clarification (Hughes et al., 2007). Hence the use of non-charged filters, i.e., polypropylene based filter is suitable for clarification of influenza harvest. Thompson et al. (2012) reported the use of nominally rated 1.2 μm polypropylene filter followed by 0.45 μm PVDF membrane for clarification of cell based influenza produced by MDCK cells. A total of

Table 5
Adenovirus recovery obtained from clarification of harvest using different grades of Millistak® depth filters.

Sample description	Virus titer (PU/mL)	Virus recovery (%)
Before lysis	2.36e10	100
Post lysis	2.67e10	113
Current process filtrate	2.17e10	92
B1HC filtrate	6.75e9	29
C0HC filtrate	1.98e10	84
30CE filtrate	2.28e10	97
30DE filtrate	2.07e10	88

9 purification runs were performed at the 20 L scale, applying a loading of 111 L/m^2 for the 1.2 μm polypropylene filter and 105 L/m^2 for the 0.45 μm PVDF filter. Results show a good virus recovery for most of the runs (78–154%). They also reported up to 58% hcDNA removal, but no significant HCP removal.

2.2.2.2. Shear sensitive viruses. Some viruses (enveloped or non-enveloped) exhibit low mechanical resistance and may be damaged by shear exposure during centrifugation and membrane filtration steps. Shear forces generated during purification steps involving filtration or chromatography may cause shedding of the viral envelope, thus affecting infectivity (Walther and Stein, 2000). Depending on capsid size, thickness and geometry, the viral capsid may either be brittle or on the contrary resilient to high pressure (Mateu, 2012). Some enveloped viruses such as influenza are resilient to mechanical stress and withstand large deformations. On the other hand, shear forces may cause shedding of the envelope of less resistant viruses, such as retroviruses, compromising virus infectivity (Walther and Stein, 2000). Extracellularly-produced enveloped VLPs are also known to be particularly fragile.

During centrifugation, a high shear rate is generated, mainly at the inlet and outlet parts (where air–liquid interface generates higher shear rate). The transduction ability of some retroviruses is significantly lessened when the viruses are purified by gradient centrifugation (Saha et al., 1994). Relative lability of virus particles to shear forces must be considered while designing centrifugal separations. The centrifugal force is not the only source of shear impact, but more importantly the equipment design, particularly at the inlet and outlet also has significant shear impact (Roush and Lu, 2008). Differences of design across scales may lead to variable yield and recovery of shear-sensitive virus at various scales.

Clarification by TFF should be designed cautiously with shear-sensitive viruses, because both shear stress magnitude and exposure time to the stress (due to recirculation) can be high. Selecting an open-channel device (hollow fibers or open-channel flat sheet devices) is preferred with shear-sensitive virus, to reduce turbulence and shear force inside the feed channels. Operational parameters should also be selected to minimize virus particle damages: low cross flow, moderate transmembrane pressure (TMP) and short processing time (Cruz et al., 2000). Membrane fouling at high pressures can result in loss of viral infectivity, possibly due to shear forces that may act on the viral envelope (Morenweiser, 2005). Membrane based separations are size based and accumulation of large molecular weight viral inhibitors with the viral particles can result in a reduction in infectivity of viral vectors (Andreadis et al., 1999).

Degradation of shear-sensitive viruses during depth filtration is not widely documented in literature. Virus loss in depth filtration is most often attributed to product entrapment, adsorption or time- and temperature-dependent virus degradation (Reeves and Cornetta, 2000). Indeed, even if mechanical stress may occur in a NFF system, the exposure time to shear is extremely short compared to other technologies as products in NFF undergoes a rapid single pass.

2.2.2.3. Retention due to size. Viruses above 100 nm can be retained by mycoplasma removal or sterilizing-grade membranes (0.22 μm and below) (Langfield et al., 2004; Vicente et al., 2011a). In such situations, special attention should be given to filter selection. For a microfiltration TFF step, 0.45 μm or 0.65 μm membranes are preferred to obtain good product passage (Vicente et al., 2014). For NFF multiple-step filtration, the tightest layer should be ≥0.45 μm; and care should be employed while selecting depth filters as some depth filter devices may include a layer of membrane that could result in retention driven product loss (Singh et al., 2013).

Virus aggregation can have a negative impact on virus yield, enhancing retention caused by size. According to a patent by Andre and Champluvier (2010), homogenization can prevent or limit filter plugging by decreasing the size of aggregates, providing a higher yield. Homogenization also improves the filterability of the harvest (filtration capacity increased by 2.4–3 times).

Excess impurities could interfere in virus recovery. Impurities tend to block the filter and plugged membrane pores may result in lower virus passage. In a patent by de Vocht and Veenstra (2013) it was mentioned that direct clarification of high cell density Per.C6® harvest by TFF (0.65 or 0.2 μm membrane) led to null adenovirus virus recovery. Removal of host cell DNA by selective precipitation prior the 0.65 μm TFF step allowed to recover >70% of adenovirus.

2.3. Case study: optimization of viral vaccine clarification

Comprehensive viral vaccine clarification studies are rarely found in literature. At the 2011 BioProcess International™ conference, Sanofi Pasteur presented a rational approach of filter selection for the development of a new clarification sequence of a viral vaccine candidate (Fabre et al., 2011). The study aimed at overcoming issues faced when cell and viral culture processes were optimized. The upstream process modifications led to a 20% yield loss and premature filter fouling during the clarification step, which as a result could not be scaled-up. Entire re-development of the filtration sequence was required, in order to build a robust and scalable clarification step, with a virus recovery higher than 85%.

Based on in-house experience and scientific publications, the team selected 27 filters for a primary screening study. Various filter media (polypropylene, nylon, cellulose esters, glass fibers, charged adsorptive filters) and structure (pleated or depth filters) were individually tested for virus adsorption in small-scale format. Virus yield was assessed by ELISA and clarification efficiencies of the pre-selected filters were also compared by checking turbidity reduction. The preliminary screening study showed that nylon and charged filters were retaining virus particles, with a virus recovery <10%. Polypropylene and polyethersulfone filters exhibited >80% virus recovery. Cellulose esters and glass fiber filter recovery varied depending on the filter evaluated (20% or 90%).

As a second step, Sanofi Pasteur evaluated several combinations (either 2 stage or 3 stage sequence) of seven filters pre-selected in the screening study. Constant flow-rate sizing experiments were performed using small-scale filters. Additionally, this experiment used higher harvest volumes than the screening study. Based on virus recovery and filter capacity results, the team selected the two best combinations for further investigation.

- *Sequence 1* (2 stages): 30 μm nominally rated pleated polypropylene pre-filter followed by a multilayer filter combining cellulose esters and glass fibers (1/0.5 μm porosities)

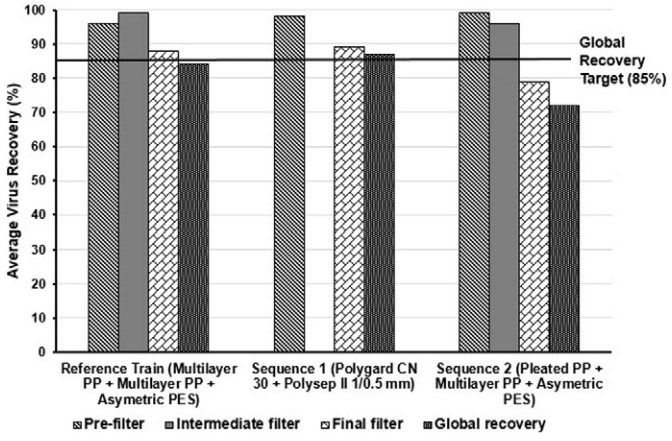

Fig. 1. Average virus recovery obtained at each filtration step. Robustness study with 3 filtration sequences was evaluated, where only sequence 1 using 30 μm nominally rated pleated polypropylene and 1.0/0.5 μm cellulose esters and glass fiber filters reached the global recovery target.

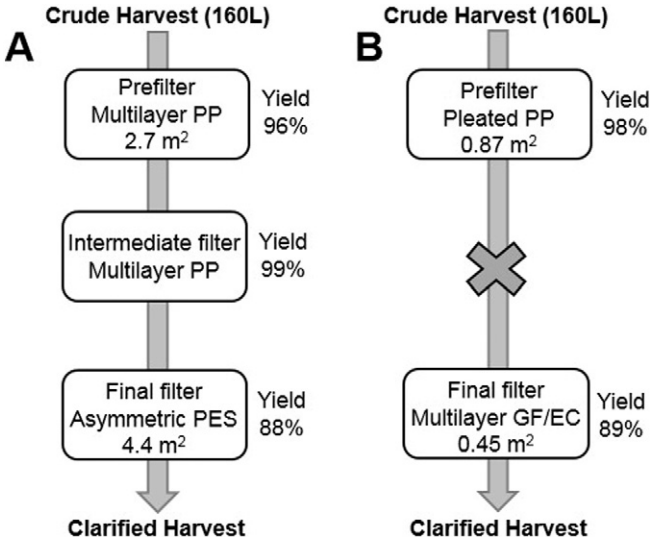

Fig. 2. Graphical representation of filter combination and step yield of clarification trains. Train A is the traditional process and Train B is the optimized process. The optimized train B resulted in 3-time reduction of pre-filter area, removal of intermediate filtration step and 10-time reduction of final filter area that resulted in 3% increase in global virus recovery.

- *Sequence 2* (3 stages): Same pre-filter (30 μm nominally rated polypropylene filter) followed by an intermediate multilayer polypropylene filter and a final asymmetric polyethersulfone membrane.

Robustness of these two clarification sequences was challenged by repeating constant flow-rate sizing experiments with different harvest batches. While both potential sequences confirmed enhanced capacities compared to the reference train, only sequence 1 achieved the virus recovery target (>85%), as shown in Fig. 1.

In parallel, centrifugation technology was also evaluated as the primary clarification step, followed by a 0.45 μm final filtration. Several speed/duration couples were tested. Even though 0.45 μm filterability was 2 fold increased, the final yield was below the 85% target. As a consequence, centrifugation was not further investigated.

Finally, performances of the polypropylene and glass fiber-based filtration sequence were assessed at larger scale (160 L bioreactor size). The filtration train is shown in Fig. 2. Several harvest batches were successfully clarified, with no indication of filter plugging, a process time compatible with manufacturing constraints and a virus yield >85%. There was no impact of the clarification step optimization on the downstream steps and on the critical quality attributes of the vaccine. Consequently, the selected clarification sequence was implemented in the vaccine manufacturing process (1000 L size bioreactor), and performances were successfully confirmed.

3. Clarification of bacterial vaccines

3.1. Considerations for bacterial vaccine clarification

According to the National Library of Medicine MeSH descriptor data (MeSH, 2002), a bacterial vaccine is defined as a suspension of bacteria, attenuated or killed, or their antigenic derivatives administered to induce an immune response for the prevention or treatment of bacterial disease. More generally, bacterial vaccines can be divided into four sub-categories depending on the type of active antigen. This agent can be:

- Killed or attenuated live whole bacteria. i.e., BCG vaccine.
- Purified antigenic determinant (subunit vaccines). i.e., Anthrax or acellular pertussis vaccine.
- Bacterial toxin (toxoid). i.e., diphtheria and tetanus toxoid.
- Plasmid (pDNA).

Because of the broad heterogeneity of products within this family, the challenges in terms of upstream and downstream processes are very dependent on the vaccine type to produce. Thus, the initial fermentation step may or may not be followed by purification, and consequently by a clarification step.

3.2. Strategies for bacterial vaccine clarification

3.2.1. Whole cell bacteria vaccines

Manufacturing processes of live bacterial vaccines (LBV) remained virtually unchanged for decades. The purification process for these vaccines consists of few purification steps which rarely include any clarification steps. For example, cell harvest in the manufacturing process of BCG vaccine is traditionally done with a press filter that allows for drying the bacteria before filling into ampoules and lyophilized. Alternately, cells can be harvested by centrifugation before being mixed with stabilizing agents and lyophilized. They are then re-suspended into WFI prior to injection. But this particular process has significant lot-to-lot variability as *Mycobacterium bovis* tends to aggregate when cultured, which has a direct impact on the potency of the final preparation. Rao et al. (1992) evaluated TFF technology on whole cell pertussis process. Pertussis cells were successfully concentrated 10 times using an open channel device with 0.45 μm PVDF membrane run at low TMP (<0.2 bar). A higher yield than centrifugation was reported (81% vs 70%), and the subsequent vaccine met potency and toxicity requirements.

3.2.2. Bacterial subunit vaccines

Bacterial subunit vaccines are comprised of specific antigen(s) — each vaccine is generally made up of 1 to 20 antigens. Subunit vaccines have been successfully produced for HPV, Hepatitis B, etc. (Plotkin et al., 2013). The recombinant bacterial subunit vaccine is manufactured by placing the antigen's gene in a yeast or bacterial vector. This vector is then modified to express the antigen, which is then taken out of the vector through additional processing using recombinant DNA technology. Because each antigen is different and is expressed in different locations of the bacterial cell, the upstream culture and fermentation conditions also differ, which then impacts the harvest clarification process (Gentschev et al., 2001).

For pertussis vaccine, pertussis toxin, PT (105 kDa) is the major extracellular toxin and detoxified during the process of manufacturing. Filamentous hemagglutinin, FHA (220 kDa) is secreted out of the cell, forms filaments and aggregates and some adhere to cells. Fimbriae 2, Fim2 (22.5 kDa); Fimbriae 3, Fim3 (22 kDa); and Pertactin, PRN (69 kDa) are located on the cell surface. The primary purpose of harvest clarification is to remove the cells (and further purify and isolate FHA, PRN and FIM2/3 through additional processing) and remove PT and FHA from the supernatant. The solid level is relatively low in the harvest. Centrifugation followed by secondary clarification (using depth or membrane filtration) or microfiltration (using TFF) is often used. FHA is shear sensitive so open-channel microfiltration devices using permeate-controlled operation with low TMP and ΔP are often employed. The clarified supernatant is processed through a bioburden reduction (0.45 μm) or sterilizing grade (0.22 μm) membrane-based filter prior to further purification. Open channel TFF device (0.45 μm PVDF membrane) has been successfully used for clarification of acellular pertussis vaccine demonstrating more than 98% recovery of PT and FHA antigen (Kumar et al., 2009).

3.2.3. Toxoids

The two most common toxoids manufactured for vaccine purpose are diphtheria and tetanus, which are produced respectively by *Corynebacterium diphtheriae* and *Clostridium tetani*. The production of

those two vaccines is bound by strict regulatory requirements. The WHO Technical report N°800 and later annexes (N°980) specify recommendations to assure the quality, safety and efficacy of tetanus and diphtheria vaccines (WHO, 1990;2014b). General Good Manufacturing Practices apply to the production of those two vaccines, and employees must be appropriately trained and receive booster immunization against the diseases. GMPs precise that both purity and quality of the final product must be demonstrated. The potency of tetanus final vaccine has to be determined, according to WHO and EP, by comparing it in vivo – or with any other validated method – with an appropriate reference material calibrated in international units (IU) against the International Standard for tetanus toxoid. In 2011 were released the latest requirements in terms of potency that may vary depending on the assessment method. Vaccine safety (absence of toxin and reversion to toxicity) must also be demonstrated for each batch. At last, the stability of the vaccine, and especially the real-time stability, must be addressed.

3.2.3.1. Tetanus vaccine. Tetanus vaccine is prepared by treating the tetanus toxin with formaldehyde to render it nontoxic without losing its immunogenic potency. In a recent technical report, WHO recommends filtration of the crude harvest (WHO, 2014b). It states that "After the culture medium has been sampled to control for purity, filtration should be used to separate the medium aseptically from the bacterial mass as soon as possible. A preservative may be added, but phenol should not be used for this purpose. To facilitate filtration, cultures may be centrifuged, provided that suitable precautions have been taken to avoid the formation of potentially hazardous aerosols. A filter aid may be added beforehand. A filter that does not shed fibers should be used."

Consequently several methods can be used for separation of cells from the fermentation medium. Traditionally, this step is achieved by centrifugation or dead-ended depth filtration, but those two methods are time consuming and the step recovery in toxin may be less than 75%. Some manufacturers perform dead-ended depth filtration made out of cellulose and perlite, under a constant differential pressure of 12 psi, in-line with a 0.22 μm membrane sterile filter (Muniandi et al., 2013).

Because of a need for easier operation and a higher yield with reduced risks for operators (the plates of the dead-end filters often plug and must be replaced during manufacturing), manufacturers have recently tried to move forward with using disposable devices, and in particular single-use capsules or self-contained depth filters. While depth filters made of mixed cellulose esters and diatomaceous earths have been successfully used for primary clarification, achieving and in some cases exceeding target process throughputs, single-use devices (i.e. capsules) made of mixed cellulose esters and glass fibers have been shown to also exhibit acceptable throughputs while achieving similar filtrate quality as depth filters. Because of this, they are a viable alternative to the use of depth filters for toxin primary clarification.

Alternately, open-channel microfiltration modules and hollow fibers have been used to achieve similar results. Because of the consistency of a membrane from one lot to another, this method can be easier to validate into a GMP environment and can advantageously and economically compete with depth filters. In a recent publication, Muniandi et al. (2013) related the use of PVDF 0.22 μm membrane in open channel TFF modules for the routine primary clarification of WHO prequalified tetanus vaccine. They used cross flow of 16 L/min/feed channel with an average TMP of 0.8 bar, which allowed the toxin to pass through the membrane and retained the cells concentrating it by 10 times. They also emphasized that for six years, the same five modules of 20 $ft.^2$ (i.e. a total area of 9.29 m^2) were used over 150 batches to clarify 400 L of fermentation broth, with a consistent tetanus toxin recovery (>96%) and an antigenic purity meeting WHO requirements. Traditional TFF cassettes with PVDF 0.22 μm membrane and retentate channel screens can be used as well at 6–8 L/min/m^2 crossflow and 0.5 to 1 bar TMP leading to similar performance.

3.2.3.2. Diphtheria vaccine. Diphtheria vaccine is based on a toxoid. Traditional production steps include growth of toxin producing *C. diphtheriae* in liquid media, then the suspension is centrifuged and the exotoxin-containing supernatant is membrane filtered before the addition of formalin to convert the toxin into toxoid.

Separation of supernatant from the producer cells to purify diphtheria toxin by TFF is very common. Cross-flow filtration allows processing very large volumes of culture within a relatively limited timeframe, is easy to scale up and can be perfectly monitored and fine-tuned. The use of 0.65 μm PVDF open channel TFF devices has been reported by Rao et al. (1992). They mentioned that a total area of 0.58 m^2 was sufficient to clarify 56 L of *C. diphtheriae* harvest within 3 h with no detectable loss of toxin. More recently, Sundaran et al. (2002) evaluated and compared the use of traditional TFF cassettes and open-channel flat sheet devices containing thermally bonded membrane to isolate cells from supernatant. Both devices contained 0.45 μm hydrophilic PVDF membranes. With the open-channel module, the permeate flux was controlled by a pump, while the TMP alone was driving the flux with traditional cassettes. In this study, the open-channel modules (50 $ft.^2$ total) were used to clarify batches of 300 L of cell suspension. During the trials, the TMP was kept constant at 1 bar by adjusting both the permeate pump speed and valve on the retentate line. For traditional TFF cassettes, the TMP was set around 8–10 psi and the recirculation flow rate at 480 L/h for a membrane of 25 $ft.^2$. Whatever the type of TFF device tested, the recovery yield was consistently over 98%. But it has been demonstrated in the study that some toxin was retained on the top surface of the membrane, which was easily recovered by introducing a wash step followed by 10 times volume concentration to increase toxin recovery.

3.2.4. Plasmid DNA vaccines

Plasmids DNA vaccines are in use for animal health purposes and several plasmid DNA vaccines for human use are under different stages of development and clinical evaluation (Ghanem et al., 2013). After *E. coli* fermentation, bacteria are harvested and lysed to release plasmid DNA. Removal of cell debris is typically performed by centrifugation or filtration. This topic is extensively described in a recent publication (Xenopoulos and Pattnaik, 2014). In this publication, the current upstream, downstream and formulation pDNA processes and challenges are addressed. The authors also give insight into gaps at each step of the typical pDNA manufacturing process and potential future innovations and/or current technology gaps that could lead to further process optimizations.

Plasmid DNA vaccine process has two steps of clarification: first, removal of bacterial cells from the culture broth and second, removal of cell debris after cell lysis. Depending on the scale, either centrifugation or TFF microfiltration is used for cell harvesting. Disk stack centrifuges operating at high speed with intermittent ejection can cause poor supercoiled plasmid yields because of shear damage during discharge (Kong et al., 2008). If centrifugation has to be used, solid-bowl centrifuges are preferable. Open-channel, flat-sheet TFF devices with 0.1 or 0.2 μm microfiltration membranes or hollow fiber devices work well. Sometime preference is given to hollow fiber devices due to their higher solid loading capacities. Commonly these processes operate 3–5 fold concentration followed by 3–5 volume diafiltration. To minimize shear and have better control on membrane polarization, permeate controlled operation is highly recommended (Raghunath et al., 2012). Though centrifugation is more cost-effective at large scale commercial operations, smaller scale processes prefer the use of filtration due to portability and ease of operation. There is an increasing trend towards preference of filtration at all scales. Flocculation agents have been used to facilitate processing, but it may cause product loss (Pearson et al., 2004). Some also proposed use of inert diatomaceous earth particles followed by bag filtration (Prazeres and Ferreira, 2004).

Cell lysis generates viscous product with large particles, cell debris, soluble impurities, fine colloidal particles along with pDNA. Removal

of such fine solids is a difficult separation due to complexity of material. Graded density depth filters or membrane filters with open pore structure (>0.45 μm) can work well for cell debris removal. As the cell debris is highly plugging in nature, filtration at low flow rate or low pressure is preferred. TFF based microfiltration has been used at this step and industrial-scale bag filters as well. It was reported that static (in stirred vessels) versus continuous (with in-line static mixers) lysis requires different filters (Antoniou, 2010). Older processes are known to use woven nylon bag filters or MAVAG — FUNDA®-Filter Systems or bucket type centrifugation. More recent approaches use flocculation or bubble column with 3/0.1 μm depth filters for separation of cell debris (Xenopoulos and Pattnaik, 2014).

3.3. Case study: comparison of centrifugation, NFF and TFF methods for tetanus toxin clarification

Muniandi et al. (2013) compared three different methods for the clarification of tetanus toxin and toxoid from fermentation broth, namely centrifugation, depth filtration (NFF), and TFF. Test material was produced in a 400 L fermenter through the use of Modified Mueller Miller (MMM) medium. For the centrifugation study, the cells were centrifuged at 4000 rpm for 60 min in 6 × 1 L containers. Samples of the supernatant were taken and tested for toxoid recovery. For the depth filtration method, the fermentation broth was clarified using 0.45 μm and 0.22 μm depth filters containing diatomaceous earth and cellulosic material. This process was done at 35 °C at 12 psi. The TFF method employed open channel flat sheet TFF modules thermally bonded with 0.22 μm PVDF membrane. The TFF-based clarification process was carried out at a cross-flow rate of 2000 L/h at 23 °C. The clarified filtrate was then concentrated using traditional TFF cassettes with 30 kD PES membranes at a cross-flow rate of 1000 L/h at 25 °C. The clarified broth (approximately 6 L) was concentrated 10 times in this ultrafiltration process. Samples of the concentrated retentate were tested for tetanus toxoid to assess product recovery. The comparison of each of the clarification methods is shown in Table 6. Depth filtration resulted in ~89% product recovery where as TFF device resulted in more than 97% product recovery. The microfiltration and ultrafiltration processes consistently yielded higher product recovery than the NFF method. These results were based on flocculation test (Lf) (Muniandi et al., 2013).

4. Clarification of polysaccharide vaccines

4.1. Considerations for polysaccharide vaccine clarification

The manufacturing process of both non-conjugated/free polysaccharide and conjugated polysaccharide vaccines starts from cultivation of host bacteria in fermenter. At the end of fermentation the bacteria cells could be treated with detergents such as DOC (sodium deoxycholate), Triton® X-100 or other suitable agents to disrupt the bacteria and facilitate the release of the polysaccharides. Due to high packed cell volume, direct harvest through NFF is not economically feasible for scale up as the throughputs could be very low. Hence the ideal choice is to use centrifugation for separation of cell mass. Microfiltration range of TFF could also be used (Gonçalves et al., 2003; Kothari et al., 2010; Macha et al., 2014). The cell-free centrate/permeate containing polysaccharides of interest is further clarified through NFF depth filter train followed by bioburden reduction filtration prior to proceeding into downstream processing for further purification.

4.2. Strategy for polysaccharide vaccine clarification

4.2.1. Primary clarification step

Centrifugation is one of the most common technologies used for separation of cell mass from fermentation broth. Depending on the scale, continuous or batch centrifugation could be selected. Takagi et al. (2008) reported clarification of *Haemophilus influenzae* type b culture broth (7.6 L) by centrifugation at 17,725 ×*g* at 4 °C for 30 min. It is important to note that proper optimization of centrifugation condition and its operation is critical for successful downstream purification. Edmonds-Smith (2013) reported that 4 out of 19 batches of Pneumococcal polysaccharide vaccine fermentation batch (15 L) could not be processed further due to improper clarification using centrifugation. Though traditional polysaccharide vaccine processes primarily used centrifugation for clarification, more modern processes prefer microfiltration (MF) TFF as an alternative for this step. When selecting a specific TFF membrane and pore size it is important to keep in mind the molecular weight of polysaccharides that are typically large and complex in structure with molecular weight ranging from approximately 500 kDa to over 1000 kDa. Due to such a large open pore size, MF membranes (e.g. 0.22 μm, 0.45 μm, 0.65 μm) are preferred to assure successful recovery of PS molecule in permeate.

Gonçalves et al. (2007) reported separation of *Streptococcus* cells from the broth by tangential microfiltration (0.22 μm PVDF membrane). A peristaltic pump was used to drive the suspended broth through the membrane at TMP of 10 psi. Full recoveries (100%) were reported for both capsular polysaccharides (CPS) of interest CPS-23F and CPS-6B clarified through the MF TFF 0.22 μm PVDF membrane, as measured by a specific method for methyl pentoses using rhamnose as a standard. In a recent study on Typhoid Vi polysaccharide vaccine, Kothari et al. (2013) explained clarification of formalin inactivated culture broth by use of TFF device containing 0.45 μm membrane. They reported 7 fold concentration of cells followed by 10 volume diafiltration that resulted in a recovery between 75% and 82% Vi antigen in the permeate pool measured by Vi ELISA. These results were obtained from diafiltration against water, but the yield increased to 99–100% when diafiltered against 1 M NaCl.

4.2.2. Secondary clarification step

The clarity/turbidity of cell-free fermentation broth arriving onto the secondary clarification step is dependent on the specific bacteria, lysis type, individual serotype and the technology used for the primary clarification step (Lages et al., 2003). The turbidity of centrate could range from approximately 50 NTU to 150 NTU (Lander et al., 2005). Positively charged graded density depth filters made out of packed cellulosic fiber impregnated with diatomaceous earth can be used for clarifying the centrate and reducing its turbidity down to <5 NTU (Wang et al., 2006). The volumetric throughputs on such depth filters can range from approximately 150 L/m^2 to 250 L/m^2 (Laska et al., 2005). Typically, the depth filter clarified product fluid is then filtered through a subsequent 0.45 μm bioburden reduction grade or 0.22 μm sterilizing grade membrane to remove any remaining cellular particles and colloids and potential microorganisms (Prasad, 2011).

Table 6
Comparison of tetanus toxin clarification methods.

Method used	Filter(s)/devices	Processing parameters	Area	Volume processed (L)
Centrifugation	Model-Sorvall™ RC 3B plus	4000 rpm for 60 min	N/A	6 × 1 L bottles
Depth filtration	0.45 μm T500 Seitz® filter pads 0.22 μm EKS Seitz® filter pads	12 psi, 35 °C	20 × 20 cm 20 × 20 cm	80–100 L
TFF — clarification	0.22 μm PVDF Prostak®	2000 L/h (cross-flow), 23 °C	5 × 1.9 m^2	800–1000 L
TFF — concentration (10×)	30 kD Biomax® Pellicon® 2	1000 L/h (cross-flow), 25 °C	4.6 m^2	800–1000 L

4.3. Case study: clarification of post centrifuge centrate of Streptococcus pneumoniae fermentation broth

The *S. pneumoniae* serotype 8 fermentation broth (20 L) treated with the addition of 0.1% (v/v) Triton® X-100 was separated from the cell mass by continuous centrifugation. The collected centrate was filtered through two individual positively charged and diatomaceous earth containing cellulosic fiber depth filters. The individual depth filter filtrates were subsequently filtered through a bioburden reduction grade PVDF 0.45 μm membrane. All the filtration tests were performed in a constant flow mode using a peristaltic pump. Filtration tests were performed with charged depth filter and *S. pneumoniae* serotype 8 fermentation broth resulted in reduction of turbidity from approximately 120 NTU down to 3 NTU. The tests were run at a flow rate of 140–150 $L/m^2/h$ and endpoint pressure differential of 20–25 psi achieving volumetric throughput of approximately 180–200 L/m^2 (Yavorsky et al., 2003).

Similar filtration testing was also performed with *S. pneumoniae* serotype 19A fermentation broth. The post-centrifuge 19A broth was clarified through charged depth filter resulting in reduction of turbidity from approximately 40 NTU down to 3 NTU. The test was run at a constant flow of approximately 140–160 $L/m^2/h$ achieving volumetric throughput of 200–230 L/m^2 at endpoint pressure of approximately 15 psi. The HLPC analysis of the product samples collected in the course of the filtration evaluation tests revealed no apparent yield losses associated with depth filtration or 0.45 μm (or 0.22 μm) membranes (Ravenscroft et al., 2014).

5. Conclusion

The development of a clarification process requires integrating several unit processes such as centrifugation, TFF–MF, depth filtration, and sterile filtration. Optimization of the clarification process requires an understanding of the effects of different unit operations on each other. The challenges are to select technology and tools (equipment and device) that meet the increasing complexity of process fluids produced by today's more efficient bioreactors. Increases in upstream productivity (virus titer, cell density, etc.), cell debris, and cell lysis products add difficulty to the clarification process and confound the selection of separation and filtration devices. As process-scale selections are made, consideration should be given to equipment design, ease-of-use, and cleanability. This will ensure efficient changeover and operator safety in handling spent filters. To develop a clarification process, a robust integration of clarification steps is important to ensure the cost-efficient processing of upstream harvests. A range of filter devices is readily available to facilitate laboratory trials, pilot production, and full-scale processing. Through the implementation of a well devised scale-up work plan that assesses several clarification options, one can confidently select and size clarification filters to protect downstream unit operations while reducing operating costs.

Clarification of vaccines presents several challenges. Typically, the filtration process needs to be customized to the process and not necessarily the vaccine due to production system, inactivation or splitting agent, and antigen presentation. Traditional vaccine processes commonly employed centrifugation for primary clarification of vaccines. Modern vaccines with varied technology platforms and smaller processing volumes have made vaccines more amenable to membrane based technologies for clarification. Newly developed vaccines using modern cell lines and expression systems along with the use of more defined cell culture conditions make many vaccine processes more conducive to filtration. Nevertheless, heterogeneity of the antigenic component or "target antigen" for vaccine products adds complexity to clarification by filtration. The antigens are a wide range of sizes, surface chemistry and charge. These characteristics impact yield and recoveries of the antigen. Vaccines mostly due to their macromolecular size pose unique challenges for clarification. This compounded with capacity issues surrounding the clarification, which adds the need for guidance in process development strategies.

The volume and scale of commercial scale operation of vaccine manufacturing has a strong impact on the selection of clarification technology. Being located far in the upstream of the process, proper optimization of clarification is critical to success of downstream unit operations maximizing yield, recovery and robustness of the process. Though centrifugation is still a viable technology option for primary clarification, open channel microfiltration devices (TFF) for primary clarification and fine depth filters or membrane filters for secondary clarification are gaining acceptance in the vaccine industry. This change is triggered by the need for faster processing, quick process development, portable processes and single use implementation. NFF offers an economical process fit as single use options at small to large scale. The availability of gamma irradiated pre-sterile devices, or modules designed for autoclaving has facilitated quicker adaptation of NFF or TFF based technology due to evolving regulatory needs.

Many of the classical vaccine processes consisted of an evolution of clarification unit operations, largely due to regulatory constraints and the associated high cost of re-validation and re-submission or clinical trials. Platform processes employing filtration based clarification schemes have been widely used in several biologics with a high degree of success. Examples and cases outlined in this document show promise for vaccine producers to achieve that level of robustness, economic feasibility, and single use utility by following a template approach. Additional advantages of filtration over centrifugation can be seen with shear sensitive viruses, or viruses prone to aggregation by air interfaces. As device manufacturers bring new products to the marketplace, vaccine producers will continue to be better outfitted for clarification processes.

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