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Affinity chromatography for vaccines manufacturing: Finally ready for prime time?

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ABSTRACT

Affinity chromatography is among the most powerful separation techniques, achieving the finest separation with high yields even in the most challenging feed streams. Incorporating affinity chromatography in vaccine purification has long been attempted by researchers to improve unit yield and purity with the secondary goal of reducing the number of downstream process operations. Despite the success in laboratory-scale proof of concept, implementation of this technique in pilot or cGMP manufacturing has rarely been realised due to technical and economic challenges in design and manufacturing of ideal ligands as well as availability of high-productivity chromatography media. This paper reviews evolving technologies in engineered ligands and chromatography media that are encouraging companies to revisit the possible use of affinity chromatography in larger scale vaccine purification. It is postulated that commercial-scale implementation of high throughput single-use affinity chromatography can significantly simplify process architecture, improve productivity and flexibility, and reduce cost of goods.

1. Rapid, global and affordable access to vaccines

Vaccination is estimated to prevent nearly 6 million deaths annually and brings other societal benefits that include reducing healthcare costs, extending life expectancy, increasing travel safety and mobility, protecting against bioterrorism, and promoting economic growth [1,2]. Despite the tremendous benefits of vaccination, there is great disparity in vaccine availability throughout the world. While vaccines have helped reduce deaths due to communicable diseases in developed countries to less than 10%, approximately 50% of deaths in developing countries are still caused by infectious diseases that are often preventable through vaccination [3]. Vaccine production is characterized by complex, diverse and often aseptic bioprocesses, and exceptional safety demands as healthy individuals are treated, leading to high investment costs in R&D and production facilities which creates barriers in affordable and sustainable global-scale supply of vaccines [4–6]. Beyond the cost concern, recent infectious disease outbreaks have raised questions about rapid and scalable manufacture of vaccines. The technologies used in vaccine manufacturing directly impact the timeline of process development, product quality, ease of process scale-up, and cost of goods (COGs). Current traditional downstream process (DSP) operations in vaccine production are time-consuming and complex with low-productivity and less-than-optimal process capability, often representing the majority of manufacturing cost [7–9]. Substantially simplified and potentially platformable vaccine purification schemes can therefore significantly shorten process development time and reduce COGs, enabling sustainable business models with affordable global prices.

2. Principle and benefits of affinity chromatography

A wide range of expression systems has been employed in vaccine manufacturing owing to the products' vast diversity and complexity. For those expressed in more complex systems (ex. single- or multi-cellular eukaryotes, transgenic organisms), traditional purification schemes typically involve multiple steps including cell disruption, precipitation (ammonium sulfate, calcium chloride, polyethylene glycol, etc.), clarification (filtration, centrifugation, depth filtration, tangential flow filtration), gradient ultracentrifugation (ex. sucrose, cesium chloride, etc.), size exclusion chromatography, and other chromatographic techniques such as ion

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exchange and hydrophobic interaction [10]. Implementation of some of these units (ex. ultracentrifugation) at manufacturing scale is challenging. Complicated purification trains are often costly with low productivities because of long processing times and low yields and constrain traditional facilities due to limited flexibility. Moreover, the diversity of antigen types (polysaccharides, virus-like particles [VLPs], live attenuated viruses, recombinant antigens etc.) demands an array of different purification techniques; the lack of process standardization requires repeated time- and resource-consuming process development activities for almost every vaccine product. New processing paradigms are therefore required to streamline, optimize, and potentially standardize the downstream operations, and be able to adjust to fluctuations in product demand or multi product situations.

Affinity-based development and manufacturing platforms have the potential to address many of the constraints raised above. Affinity chromatography (AC) separates the molecule of interest from the crude process stream based on highly specific interactions between the target and the immobilized ligand (Fig. 1). With the right ligand, sufficient purity can be achieved in one step. This high purification efficiency allows the process architecture to be simplified to a single AC capture step accompanied only by a clarification (before) and a polishing step (after), enabling a platform approach where by coupling a different affinity ligand on the chromatography media, a new molecule can be purified with only minor or no modification to the entire downstream scheme. In principle, a well-established platform has the potential to decrease time-to-market, increase R&D productivity and control costs [11–13]. Monoclonal antibodies (although much simpler and more characterized compared to most vaccine antigens) exemplify the tremendous success of this strategy where recurrent application of different assets provides the preconditions for investing significant R&D efforts. This has contributed to fine-tuned operational performance, and substantial return is achieved from improved cost-efficiency and better product and process characterization. In the vaccine industry, however, the panoply of antigen types has held back the establishment of true manufacturing platforms. In recent years, novel antigen delivery systems such as viral vectors, RNA/DNA vaccines and lipid-based carriers have emerged for which AC can be potentially developed for each delivery system to help build manufacturing platforms. Moreover, affinity-based platforms are also suitable for purifying free and/or conjugated carrier proteins (cross-reacting material [CRM], tetanus toxoid, diphtheria toxoid, *Neisseria meningitidis* and *Haemophilus influenzae* protein D) that are recurrently used in polysaccharide (glycoconjugate) vaccines. Sophisticated affinity ligand design and chromatographic support coupling system can lead to a truly revolutionary paradigm shift in the industry as application of affinity-based purification will enable a low-footprint, intensified and generic downstream process independent of the type or specific antigen of interest. In such approach, most of the focus of downstream development would shift from resource-intensive process development to potentially externalized, fast and cost-effective ligands generation for any given antigen with high probability of success which could be plugged onto the generic DSP.

3. Laboratory-scale proof of concept

Over the past decades, various AC techniques including immunoaffinity, lectin, immobilized metal affinity chromatography (IMAC), and heparin were investigated for purification of different types of vaccines at laboratory scale. Promising purification performance along with higher yield compared to other separation methods such as ultracentrifugation demonstrated that AC is a simple and specific method for vaccine purification. Table 1 highlights examples of virus applications for the most popular types of affinity chromatography along with their advantages.

Besides the four major types of AC mentioned above, other affinity ligands have also been explored. Dye ligands have been

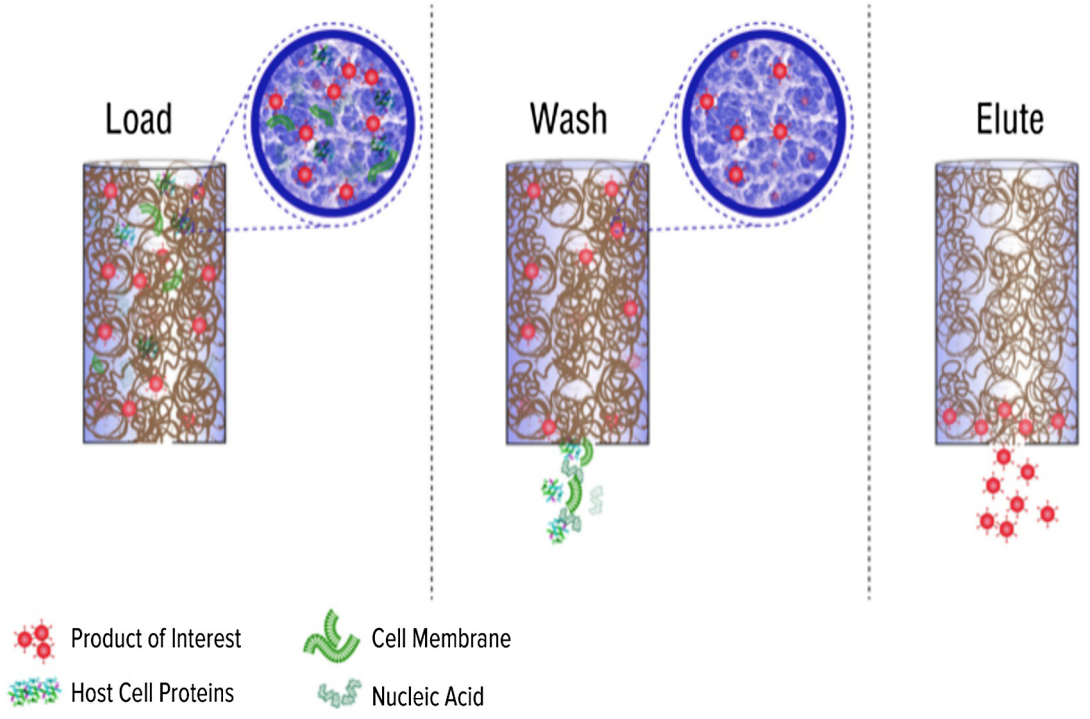

Fig. 1. Principle of affinity chromatography. Affinity chromatography separates the product of interest from a mixture based on highly specific interactions between the target and the immobilized ligand. Under binding condition, the target molecules bind to the ligands while impurities including cell membrane components, host cell proteins and nucleic acids flow through the column. Optimized washing condition removes non-specifically bound impurities. Bound target molecules are then released from the affinity media under elution condition.

Table 1
Application of affinity chromatography techniques for lab-scale vaccine purification.

Affinity mechanism	Applications in vaccine purification	Advantages
Immunoaffinity chromatography (IAC)		
Specific antigen-antibody interactions	**Viruses** – Aleutian mink disease virus [14] – Hepatitis A Virus [15] – Poliovirus type 1 [16] – Canine parvovirus (CPV) [17] – AAV-2 capsids [18] – AAV serotype 1, 2, 3 and 5 [19] – Measles[20] – Infective mumps virus particles from non-infectious particles [21] **Toxins** – *Clostridium perfringens* enterotoxin [22,23] – *Clostridium perfringens* β-toxin [24] – Tetanus toxin [25] – Chimeric diphtheria toxin [26] **Proteins** – *Mycobacterium tuberculosis* antigen 5 [27] – Hepatitis B surface antigen (HBsAg) [28] – Respiratory syncytial virus fusion protein [29,30] – Recombinant *Bordetella pertussis* fusion protein [31] – Chimeric polyepitope protein [32]	– High specificity for target molecule and therefore high purification efficiency
Lectin affinity chromatography		
Lectin ligands bind to specific carbohydrates via carbohydrate recognition domains (CRD)	**Viruses** – Influenza A [33] – Baculovirus [34] – Herpes Simplex Virus type 1 (HSV-1) [35] **Proteins** – Influenza virus glycoproteins [36] – Parainfluenza virus glycoproteins [36] – Viral haemorrhagic septicaemia virus (VHSV) glycoprotein [37] – Hepatitis B surface antigen (HBsAg) [38] – Mycobacterial glycoproteins [39] – *Mycobacterium bovis* BCG Tokyo antigens [40]	– Ligand binds to specific glycoproteins, such as haemagglutinin (HA) and neuraminidase (NA), which are major components of many virus lipoprotein envelopes – Mainly used for purification of intact viruses or glycosylated viral subunits
Immobilized metal affinity chromatography (IMAC)		
Ligands made up of immobilized metal ions (Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, or Fe^{3+}) interact with electron donor groups (particularly histidine) on the surface of the target molecule.	**Viruses** – Baculovirus [41] – Herpes Simplex Virus Type 1 [42] – Adeno-associated virus [43] – Foot-and-mouth disease virus [44] – Retrovirus vector [45] – Influenza virus A [46] – Lentiviral vectors [47] **Toxins** – Tetanus toxoid [48] – Adenylate cyclase toxin [49] **Proteins** – Hepatitis B core antigen (HBcAg) [50] – Nucleocapsid protein of NDV [51] – West Nile virus envelope proteins [52] – Influenza antigens [53] – Tuberculosis antigens [54] – N19 polyepitope for meningococcal vaccine [55] – Clumping factor A for *Staphylococcus aureus* vaccine[56] **DNA Vaccines** – Single-stranded nucleic acid [57]	– Target can be engineered with affinity tags enabling binding to the IMAC column – High ligand stability – Low cost
Heparin and heparin sulfate pseudo-affinity chromatography		
Heparin and heparin sulfate ligands are naturally occurring glycosaminoglycans that resemble substrates for which biomolecules have affinities.	**Viruses** – Herpes Simplex Virus [58] – Hepatitis B virus [59] – Hepatitis C virus [59] – Adeno-associated virus vectors [60–64] – Retrovirus vectors [65] – Lentivirus vectors [66] – Vaccinia Ankara virus [67,68] – Foot-and-mouth disease virus [69] – Porcine reproductive and respiratory syndrome virus [70] – Baculovirus [71] **Proteins** – Hepatitis B surface antigen [72] – Human papillomavirus type 16 VLPs [73]	– Ligand has stable structure, can be autoclaved and tolerates high concentrations of denaturing agent – Ligand has high affinity for a wide variety of molecules

widely evaluated for purifying viruses (HIV-1 and HBV) and toxins (diphtheria, pertussis, and staphylococcal enterotoxin A) [74–79]. Certain sugars such as D-galactose and β-lactose can also be used as ligands for purifying toxins (Shiga toxin 2e, *Escherichia coli* heat-labile enterotoxin, cholera toxin, and *Clostridium botulinum* type A and B toxin) [80–83]. Mucin affinity column was used to purify AAV-4 and AAV-5 vectors as they bind to the sialic residues on mucin [84]. Engineered AAV-1, 2, 3, 4, and 5 with biotin accep-

tor peptides can be purified using an avidin affinity column [85]. Virus-binding peptides have been discovered for AAV, porcine parvovirus, norovirus, rotavirus and poliovirus [86–90]. Triple-helix, peptide, and amino acid-DNA affinity chromatography have been studied for pDNA purification [91–93]. RNA and DNA aptamers have been reported to recognize vaccinia virus, HIV, HCV, and influenza virus [94–96]. Glutathione affinity column has been reported to purify GST-rPL as a carrier for pneumococcal type 18C conjugate vaccine [97].

4. Scale-up challenges and progress towards industrial-scale implementation

The proven success of AC for lab-scale vaccine purification triggered many researchers to consider and investigate its advantages for processing large volumes of vaccine products [16,98,99]. However, currently there are only few GMP manufacturing processes utilizing AC (mostly heparin AC) for viral vector production for clinical trials [100–103]. The fact that AC techniques have rarely been implemented in manufacturing-scale vaccine purification despite proven success in laboratories reflects several remaining barriers to employing this technique for industrial production. Major challenges for industrial implementation include restrictions in design of engineerable ligands, large-scale ligand production and raw material traceability for commercial supply as well as a lack of high-productivity chromatography media that would be compatible with high efficiency processes and standard procedures for cleaning in place (CIP) and column sanitization.

4.1. Technical and economical restrictions of affinity ligands

A high-performance AC step that fits in the platformable approach for vaccine purification requires ligands that have a high degree of specificity and tunable affinity towards the target to achieve robust impurity clearance at mild elution conditions that preserve the structure and viability of the product. Until today, ligands used in AC for vaccine purification are mostly existing molecules that are discovered, rather than designed or engineered, to have specific interactions with the target or engineered target (ex. HIS-tagging). For example, most of the immunoaffinity ligands are antibodies isolated from infected animals or patients, whereas heparin or heparan sulfate are natural cell receptors for many viruses. Because most of these naturally existing, non-proteinaceous ligands such as heparin can only be derivatized to a limited extent, many of their characteristics such as specificity and dissociation constant cannot be sufficiently tuned to better match the requirements of vaccine purification. Antibodies on the other hand, provide high specificity but are complex molecules which often lack the required stability and are associated with high production cost. Furthermore, clinical products purified with tag-based systems are required by regulation to demonstrate total tag cleavage and clearance, which usually requires additional processing steps and analytical assessment. It is postulated that all these limitations impose significant challenges and are responsible for the limited industrial implementation of AC.

Among the four most extensively studied affinity ligands, lectin and heparin AC cannot offer the same specificity, hence purity, as IMAC and IAC. Lectin chromatography relies on the interaction between lectins and specific carbohydrate structures; therefore in addition to the product of interest, a lectin ligand can also bind to other glycosylated proteins with the same carbohydrate structure. Another consequence of this nature is that performance of lectin affinity is dependent on the host cell and cultivation conditions of the process as both can affect the glycosylation patterns of the target and contaminant proteins [104]; therefore, the lectin AC lacks the required robustness for efficient and consistent purification. Heparin or heparan sulfate ligands used in heparin AC are highly-sulfated, negatively-charged glycosaminoglycans. The charged nature of these ligands makes it possible to have ionic protein-ligand interactions on top of affinity interactions, leading to non-specific binding [105]. Moreover, affinity binding to heparin may be serotype dependent for some viruses as the research from Wu et al. and Mietzsch et al. demonstrated that only AAV-2, -3, -6, and -13 are natural heparin-binding serotypes [64,106].

Another challenge in the application of AC in vaccine purification is the necessity of harsh elution conditions for the currently existing ligands. The affinity interaction between the ligand and target is very strong so harsh dissociation conditions such as high acidity (for immunoaffinity) or high ionic strength (for heparin and IMAC) are required to reverse the binding. However, such elution conditions are detrimental to target stability and infectivity and limit the applications of AC [17,21]. Recently, Brgles et al. attempted to develop native elution conditions using amino acids and demonstrated that the majority of mumps virus can be recovered in high-concentration amino acid buffer around neutral pH [21]. Buffer manipulation using co-solvents such as $MgCl_2$ have also been shown to improve dissociation of antibody–antigen interactions at milder pH [107]. These studies open new possibilities to address the challenge in elution conditions for large-scale vaccine purification. Besides exploring new buffer conditions with current affinity methods, the challenge can also be tackled by developing new ligands that can reverse affinity interaction under mild elution conditions.

The cost of affinity ligands is another factor that can discourage industrial use of AC [108]. Compared to more inexpensive traditional chromatography media (ion exchange, hydrophobic interaction and size exclusion), affinity ligands, particularly immunoaffinity ligands which require costly industrial antibody manufacturing capabilities, can cost several hundred dollars per milligram [109]. Moreover, antibodies and lectins are biologically active proteins and their fragility usually prohibits extensive multi-cycling [110]; hence, oversized columns are often required, resulting in escalated capital expenses. Given that the Phase 1 Likelihood of Approval for vaccine candidates is only 16.1% (n = 238) between 2006 and 2015, pharmaceutical companies can hardly justify the extensive capital investment in AC, and vendors are unlikely to initiate massive production of these ligands unless major return will accrue from their use [111].

Besides the challenges mentioned above, there are many ligand-specific issues with the currently existing ligands. For example, the proteases used to cleave the tags used for IMAC create additional burden for subsequent purification processes and QC/QA requirements [112], while for IAC there is a concern that different affinity ligands have to be developed for each strain of a virus because the affinity interaction is too specific [10]. Commercial heparin ligands are mostly of animal origin and production of immunoaffinity ligands are usually mammalian cell culture-based [113,114]. This is bringing significant challenges in raw material traceability and management of supply chain security, validation and risks, as highlighted by the recall incident of contaminated porcine heparin in 2008 [115].

4.2. Engineered ligand solutions

Given the limitations of current affinity approaches discussed above, one can deduce an ideal profile for a next generation ligand technology that is compatible with the technical and commercial requirements of a modern production process. Such ligands have to combine a number of properties as listed in Table 2.

Table 2
Selection criteria for ligand technology.

Criterion	Comment
Purification Performance	
Specificity	High and reproducible purity is a key goal of the purification process
Capacity	Ligands have sufficient capacity for optimized process economics by avoiding unit oversizing and extensive cycling
Engineerability	Ligand properties can be tailored to process requirements such as multimerization or tunable affinity for mild elution conditions
Ligand Stability	Ligands are stable across the operational pH and salt concentration range and under common CIP conditions, allowing reproducible process and ensuring product critical quality attributes
No need for target engineering	The affinity interaction is between the ligands and naturally-presenting proteins/domains on the target. Amino acid modifications of the target are avoided
Ligand Development and Screening	
Time Required	Development and screening for customized ligands is completed in a short time frame
Quantity of target required	Quantity of target required for ligand screening is acceptable
Platformability	The ligand design platform and screening process can be applied to any given target
Ligand Production	
Homogeneity	Homogenous ligands can be supplied sustainably
Cost effectiveness	Both generation and subsequent production of ligands are at acceptable costs
Scalability	Ligand production can support commercial supply from lab to GMP vaccine manufacturing scale
Animal-free production	Manufacturing processes avoid the use of mammalian cells and animal-based components. Microbial production or chemical synthesis is ideal

4.2.1. Scaffold-based ligands

The tight constraints mentioned above have led to the application of in vitro methods for the generation of designed, target-specific ligands rather than relying on animal-derived antibodies or engineering of various types of lectins. In particular, a class of proteins, so-called scaffolds, qualify for such an approach. In general, the scaffold approach utilizes very small, highly stable, single-domain proteins with no posttranslational modifications, isolated from microorganisms, plants or humans. They can be engineered to introduce de novo, specific binding properties not present in the parent molecule [116]. Such ligands provide sufficient interaction area for the target built onto a rigid molecule and thus enable similar specificities and affinities as antibodies while avoiding a number of their disadvantages. Although an appreciable number of molecules have been utilized for this approach including Protein A (Affibodies), Ubiquitin (Affilin), Cystatin A (Affimer), Fibronectin 10th type III domain (Adnectins), Fyn SH3 domain (Fynomers), Sac7d (Affitin), Lipocalin (Anticalin) and others, only a small number of these are applied for technical purposes such as affinity ligands while most others focus on drug development [117–123].

The process of identifying scaffold-based binders starts with the generation of libraries with complexities in excess of 10^{10} molecules in which ~10–15 surface residues are fully randomized (Fig. 2). Progress in gene synthesis leading to improved library quality and complexity and a good choice for the starting scaffold have increased success rates for identifying suitable candidates. Phage display or ribosome display are preferred methods to isolate binders from the libraries [124,125]. Partial automation and parallelization of selections have increased throughput and shortened timelines. Similarly, introduction of fully automated high-throughput screening in a miniaturized format enables testing of tens of thousands of variants per screen to narrow down on the best performing candidates. Taken together, quality improvements as well as process optimization now enable efficient lead generation. Furthermore, due to their small size (most are under 100 amino acids) and simple structure, scaffold-based binders can be expressed cost-effectively at high-levels in *E. coli* therefore mitigating the cost concerns regarding an affinity approach. Some are even small enough to allow chemical synthesis as an alternative. The small size, simple *E. coli* expression as well as fast and efficient genetic methods allow quick and efficient engineering of the molecules to further tune the properties or coupling formats if needed.

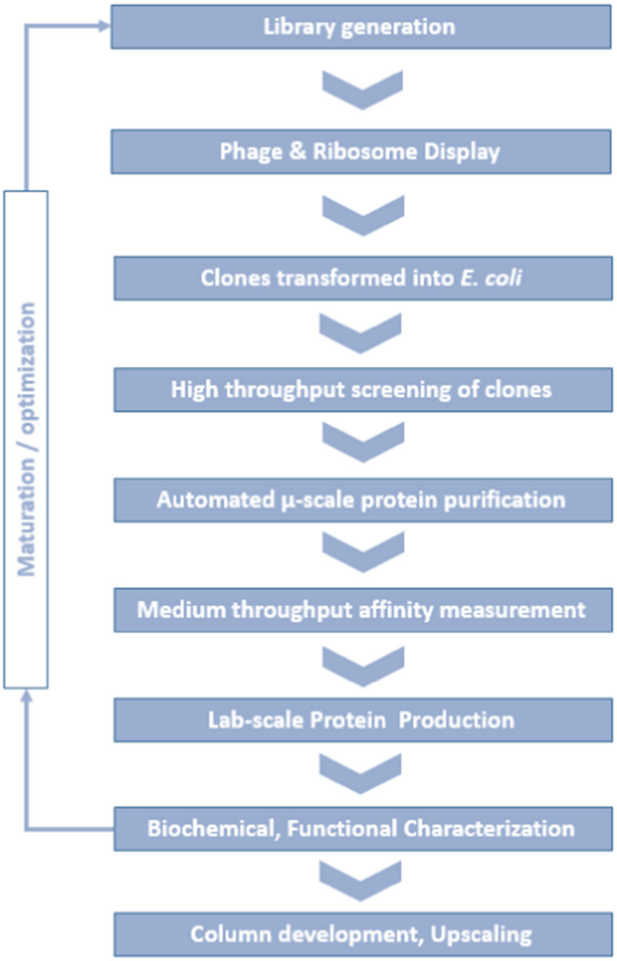

Fig. 2. Work flow for the identification of ligands. Efficiency is realized by a seamless integration of all steps and a high degree of automation allowing high throughput hit generation and characterization.

4.2.2. Other ligand technologies

Taking the approach of size and complexity reduction from antibodies to scaffolds even further, one can also apply short peptides or peptidomimetics as affinity ligands [126]. These peptides

are usually 5–15 amino acids long and do not form a stable secondary structure but are rather flexible unstructured entities. Lead identification is based on similar technologies described for scaffold-based ligands above, i.e. display and screening methods or combinatorial chemistry approaches [127]. Advantages of short peptides include their amenability to chemical synthesis at comparatively low costs, the potential to easily incorporate non-natural amino acid functionalities, and high stability to caustic cleaning conditions. However, due to the smaller interaction area and the flexible structure, affinity and specificity may be limited compared to more extended and rigid scaffold paratopes [128]. A further potential disadvantage is the sensitivity of unstructured peptides to proteolytic degradation and measures have to be taken to reduce this effect [129].

Molecularly imprinted polymers (MIPs) represent yet another approach to generate chromatography material with specific affinity for target molecules. MIPs are generated by polymerization of an appropriate mixture of monomers in the presence of the target molecule which is then partially surrounded by the polymer as it forms during the polymerization reaction. After polymerization the target molecule is extracted leaving behind cavities which resemble a mould specific for the target. Although the basic approach dates back to the 1970′s, applications to more complex targets such as proteins are more recent [130–132]. Although examples of MIPs for purification of monoclonal antibodies have been described which get close to requirements of an efficient purification, it will still take significant development efforts and time until MIPs reach the maturity and up-scalability required for an industrial process [133].

4.2.3. Benefits and challenges of current ligand technologies

As mentioned in previous sections, preserving the physical and biochemical properties of the virus particles throughout the process is of high importance. In particular, elution from an affinity chromatography resin is often performed under harsh conditions. Determination of acceptable elution conditions should therefore be performed with great care by extensive buffer screens, considering competitive elution, complexation of bivalent metal ions (often involved in binding), and addition of detergents, etc. With current ligand engineering technologies, design of the elution mode should be included in the ligand selection strategy. Ideally, bind/elute conditions are incorporated into the ligand identification process. In phage display, for example, buffer conditions during incubation of phages with target should resemble bind-conditions and removal of phages should resemble elution conditions, respectively. Furthermore, ligand optimization can be performed by histidine-scanning, i.e. mutating of residues within the paratope to histidine which has a pK around 6, thus providing a change in protonation status in a pH-range which is compatible with virus integrity in most cases.

Besides the ligand-associated advantages, engineering ligands also brings additional benefits to facilitate product characterization and development of characterization assays which are critical for regulatory filing as well as product development and management in the long term. Knowledge gained during ligand screening can be used to develop affinity-based assays for assessing quality, stability, potency and comparability. Better understanding of the product in this aspect can facilitate the establishment of analytical target profile while a ligand-assay co-development approach will significantly improve the overall vaccine development time.

Although ligand-based affinity chromatography provides all the advantages discussed above it does not come without risks. One is the impact of chaotrope denaturation (of either the target or the ligand) on binding efficiency, which depends on the type of epitope (conformational or not) and the relative stability of ligand versus target under these conditions. Another risk is the carry-over of any trace contaminations from the ligand fermentation into the vaccine product. Therefore, stringent process controls have to be applied to ligand fermentation and purification as well as production of the affinity media in order to avoid any such carry-over. An advantage of the scaffold-based ligands is the possibility to produce these in animal-free, chemically defined minimal media in E. coli, rather than the more complex mammalian cell based fermentations required for e.g. antibody production. Leaching of ligand or fragments thereof into the vaccine product poses additional potential risk. Highly sensitive assays have thus to be developed and validated for the ligand molecule class to monitor potential contamination. However, both issues, carry-over and leaching, are well-known and handled for protein A-based antibody purification. Monitoring for ligand-based processes can therefore benefit from the experience gained since decades in the antibody area.

4.3. Vaccines manufacturing technologies

4.3.1. Limits of traditional strategies

Although AC has rarely been incorporated in industrial vaccine purification, ion exchange and hydrophobic interaction chromatography (HIC) columns have been widely used in manufacturing-scale processes in combination with other separation techniques such as filtration, precipitation and ultracentrifugation [10]. Porous resins have dominated as the solid support for most of the chromatographic media. However, there are many major downsides to resin media, such as the necessity for slow flow rates and pore exclusion for large viruses, which all create barriers for the implementation of AC at manufacturing scale.

Resin columns depend heavily on diffusion so that target molecules can access the functional affinity ligands within the pores of the resin beads and be captured (Fig. 3) [134]. Low feed stream linear velocities are necessary to maximize the binding capacity. As a result, resin columns often need long processing time which decreases the productivity of the purification train and can be detrimental to unstable molecules. Oversizing the column in order to run at faster volumetric flow rates reduces the run time but at the expense of wasted capacity, larger production skids, and increased capital expenditure and operating costs [135].

Another major drawback of resin columns is reduced binding capacity for large viruses due to pore exclusion. Pore diameters are typically less than 100 nm, therefore most viruses can only access the binding sites at the external resin surface but not the inner binding sites within the pores. Compared to protein adsorption, resin capacity for adenovirus (virion diameter = 70–100 nm) is at least 10-fold lower [136,137]. Visualization of virus binding to resin suggested that the adsorption of large virus on conventional resins is mostly restricted to a thin shell on the outer surface of the beads [138,139]. This compromised virus binding capacity due to pore exclusion, therefore reducing the productivity of the AC even further.

4.3.2. Paradigm change in chromatography

The above discussion of limitations of resin chromatographic support suggests that an ideal chromatographic media for vaccine purification should enable high process throughput through rapid mass transfer and promote high binding capacity via high functional ligand density and large accessible area. Newer media such as monolith and membrane supports have proven their capability through many well-established chemistries including ion exchange and hydrophobic interaction. Compared to resin beads, monolith and membrane matrices have larger pore size and use convective transport, making them suitable for processing large molecules at higher flow rates without compromising on binding capacities (Fig. 3). Besides increased productivity, the shortened

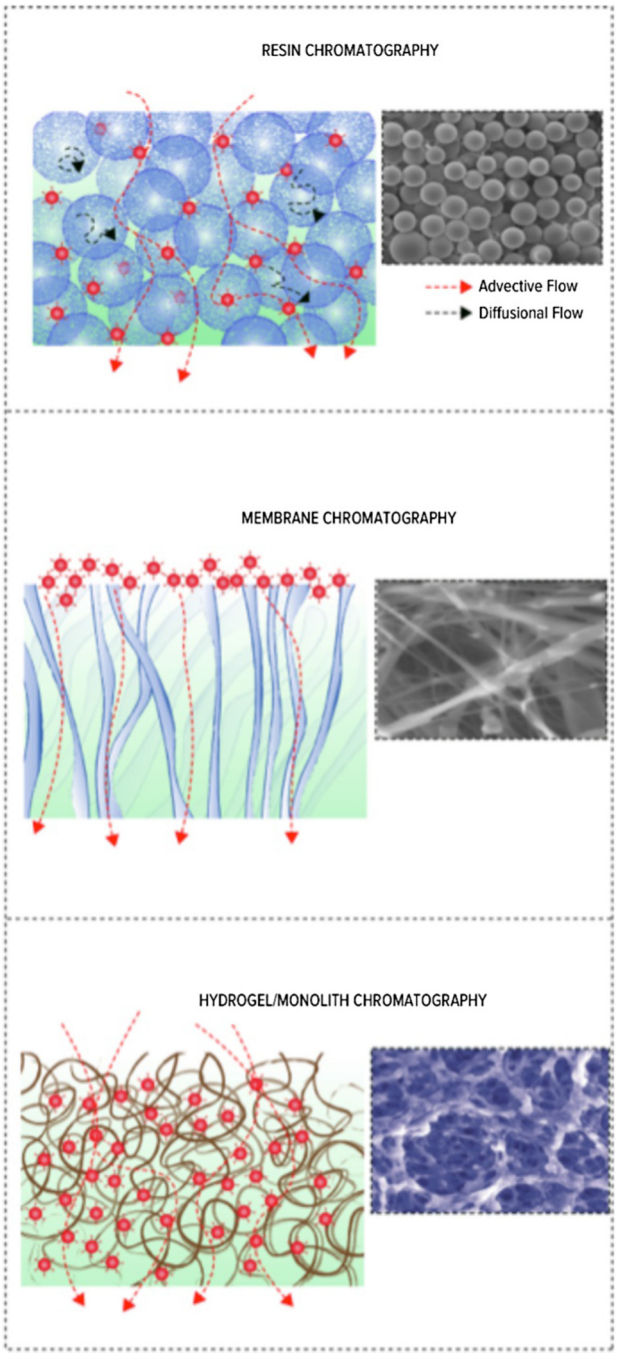

Fig. 3. Comparison of different chromatographic supports. Functional ligands are internalized inside the pores for resin media, leading to slow diffusive mass transfer and significant size exclusion effect for large molecules. Traditional membrane technology immobilizes functional groups at the surface of the porous base membrane. The large pore size permits high flow rate and minimizes size exclusion effects, but the usually low ligand density translates into inferior binding capacity. Monolithic supports are continuous interconnected channels with macropores or both macro and mesopores in which functional ligands are located. The highly interconnected network of large diameter channels enables fast mass transfer and minimizes size exclusion effect. Hydrogel membrane technology features the formation of 3-D structured hydrogel within the porous polymeric base membrane. Functional groups are immobilized in high density within the hydrogel structure to promote binding capacity while the large pore size permits fast mass transfer.

processing time using monolith or membrane chromatography columns is also beneficial for live and attenuated viruses that are labile under the purification process conditions.

Monolithic chromatography columns have been an attractive option for vaccine purification because of their large diameter channels (ex. 1–2 μm). The highly porous structure provides large adsorption area for dynamic binding capacity with fast flow rates, leading to significant improvement in unit productivity compared to resin columns [140]. Successful purification of various viruses, VLPs and bacteriophages using ion exchange or HIC monolith columns have been reported [141–156]. The study from Forcic et al. demonstrated that purification of 145 mL of rubella virus suspension can be completed at around 1 h using a disk-shaped anion-exchange monolithic column compared to >10 h with only 10 mL of viral suspension loading using anion exchange resin [142,157]. Ion exchange monolith columns have been reported to be implemented in clinical-scale production for influenza vaccine and plasmid DNA [158,159]. Affinity monolith columns have been available for analytical use for various biomolecules and lab-scale pDNA, virus and VLP purification, but there is little information on their application in industrial processes [47,92,160–164]. This is partly due to the manufacturing complexity associated with pore distribution consistency, risk of cracking, and high production cost [165]. Ion exchange and hydrophobic interaction monolith columns up to 8L have been commercialized (ex. CIMmultus columns from BIA Separation) but affinity monolith > 1L remains unavailable [165].

Membrane chromatography represents the other purification alternative to resin columns. Membrane columns possess the same advantages as monoliths: faster flow rate and less pore exclusion over resins but with proven scalability and lower production costs [166]. Ion exchange membrane adsorbers such as Sartobind and Mustang have been evaluated for purifying many viruses and VLPs including adenovirus, AAV, baculovirus, densonucleosis virus, alpha herpes virus, influenza A virus and rotavirus VLP [167–175]. Nevertheless, traditional membranes are often associated with lower binding capacity and non-optimized flow distribution due to ineffective device design compared to resin columns which limits their application in bind-and-elute mode that dominates the vaccine purification strategy [166]. Nanofiber technology using macroporous electrospun polymeric nanofibers can offset the negative impact of low binding capacity on productivity by enabling rapid bind-and-elute cycles with even higher flow rate [135,176]. Hydrogel membrane technology can improve the productivity further by boosting the binding capacity with high functional group density in a three-dimensional structure. Together with improved column design, membrane chromatography is enabled for bind-and-elute applications [177]. The hydrogel technology is versatile in that different ligands can be coupled to the same base matrix, enabling a platform approach to affinity membrane design that accelerates product development and facilitates regulatory filing [178]. The higher binding capacity combined with fast mass transfer translates into less chromatography media required and allows right-sizing the chromatography operations to the process scale. The miniaturization of the process and the rapid cycling capability of the membrane technology enables high productivity output [135,177]. Unlike resin and monolith columns, membrane and nanofiber columns are usually designed for disposability and single-use applications that promote savings in developing and validating cleaning and sanitization procedures. Moreover, this disposable purification strategy also reduces the risk of cross contamination between batches.

Depending on the feedstreams' particulate profile, diverse clarification techniques may need to be implemented to protect and allow efficient use of chromatography media. Filtration-based clarification schemes have been gaining popularity. A variety of microfiltration and depth filtration products are commercially available for designing an adequate clarification train for any cell culture harvest including the more challenging ones [179,180]. In the most

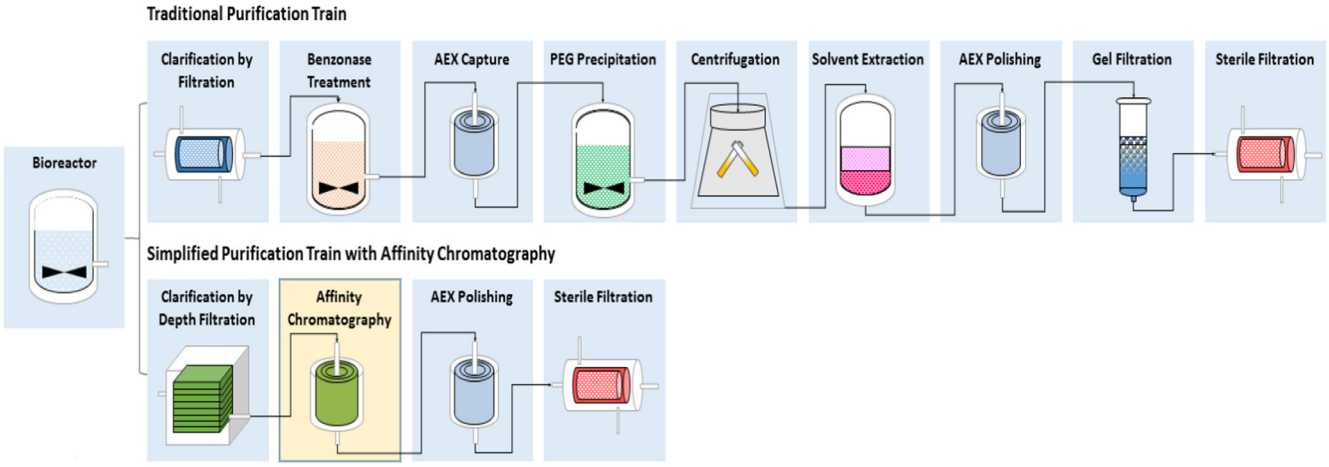

Fig. 4. Implementation of affinity chromatography can simplify and standardize vaccine purification process. Traditional purification train for Hepatitis A vaccine is shown as an example in the upper panel [184]. When using AC, highly specific interactions between the target and the affinity ligand translates into unparalleled purification efficiency which eliminates the need for multiple unit operations to achieve desired purity. Less purification units can significantly improve product yield, shorten process development and operational time, improve productivity and reduce capital and operational costs. Availability of diverse affinity ligands can enable a platformable purification strategy for product-independent purification schemes. Platformable strategies improve cost savings further, promote flexibility to support multi-product facility and adapt to fluctuations in market demands.

optimized scenario, implementation of AC can simplify the process architecture to a single chromatography capture step accompanied only by a clarification step and a polishing step (ex. AEX for DNA removal, or gradient purification with CsCl or iodixanol to separate empty particles from full particles) (Fig. 4). AC can also enable a platform approach where a universal downstream scheme is suitable for purifying multiple products by switching AC columns. When the membrane is packed into highly efficient, single-use columns, process throughput can be significantly improved and substantial savings in infrastructures, operations and quality procedures can be realized [135]. Other solutions including multi-column systems, advanced Process Analytical Technologies (PAT) (ex. biolayer interferometry [181], dynamic light scattering [182], near infrared spectroscopy [183]), portable clean rooms, automation and in-market/for market production may further improve the impact of these newer tools.

5. Current trends

Information gathered from the latest literature, patents and suppliers data files, as well as multiple conferences suggests that there are currently four traditional affinity technologies that can be supplied at commercial scale, but proof of concept with new modalities are becoming more common. CaptoDevirS uses dextran sulfate ligand that is a derivative of branched glycan dextran and exhibits an heparin affinity-like behaviour for various viruses including different strains of influenza virus, yellow fever virus, Japanese encephalitis virus, dengue virus and West Nile virus [185]. It uses a traditional approach, relying on naturally existing ligands with their limited efficiency and agarose beads that suffer the limitations of resins previously described. Cellufine media uses sulphated cellulose which also mimics the heparin affinity behaviour for purifications of various viruses including human and avian influenza viruses, Moloney murine leukaemia virus, dengue virus, and West Nile VLPs [186–189]. AVB Sepharose High Performance is an immunoaffinity media developed by BAC BV using a 14-kDa Camelid-derived single domain antibody fragment expressed in yeast. It is designed to purify AAV-1, 2, 3 and 5 but experiments with AAV-1 through AAV-8 have been conducted [190]. This media exhibits serotype-dependent affinity and recovery (high towards AAV-1, 2, 3 and 5 while low towards AAV-8 and 9), which can be improved by epitope modification [191,192]. CaptureSelect AAV8 and AAV9 use a 13-kDa single-domain fragment comprising 3 complementarity-determining regions (CDRs) that form the antigen-binding domain for AAV-8 and AAV-9 serotypes [193]. The engineered BAC BV ligand is produced in *Saccharomyces cerevisiae* and grafted to the Poros bead technology that has increased throughput to constitute the most modern solution commercially available. Genethon (Evry, France) has reported purification of AAV9 using CaptureSelect AAV9 at 10L and 50L scale with >80% recovery. Process scale-up to 200L is in progress [194].

Industry, government and funding agencies recognize the need for and feasibility of industrial AC strategies for vaccine purification to overcome technical and cost challenges faced in the current manufacturing scheme. DiViNe is a project funded by the European Union involving iBET, Affilogic, Aquaporin, Merck KGaA, GenIbet Biopharmaceuticals, and GSK. The ultimate goal of the project is to develop custom AC solutions for vaccine production that can enable affordable and environmental-friendly purification processes and promote access to vaccines in developing countries [195]. A first result of the project was communicated at World Vaccine Congress 2016 demonstrating identification and resin conjugation of customized ligand against CRM197 carrier protein for conjugate vaccines production [196]. More recently, The Bill and Melinda Gates Foundation awarded The Grand Challenge "Innovations in Vaccine Manufacturing for World Markets" to an industry consortium composed of Univercells, Natrix Separations, Navigo Proteins and Batavia Biosciences. This project aims at developing an integrated platform for the low cost manufacture of vaccines for global health, using affinity membrane chromatography as an essential component of the strategy [197]. Proof of concept using hydrogel affinity membrane and engineered ligands for single-step virus purification in simplified process architectures have been presented at several conferences but still need to be published in peer-reviewed journals [198–202].

6. Conclusion and perspectives

The powerful impurity reduction of AC has long been proven at laboratory scale. However, implementation at manufacturing-scale vaccine production has been hampered due to technical and economic restrictions in ligand generation and limitations of chromatographic support. Advancements in synthetic technology now

allow ligands to be engineered with specific characteristics and properties to accommodate vaccine purification requirements. New chromatographic media now offer higher capacity and higher productivity than columns and are also single-use per batch and provide additional operational benefits. All these improvements enable a new generation of AC that is ready for industrial-scale implementation. There will be a cost concern associated with ligand development and affinity columns for this particular unit operation. However, from a holistic point of view COGs can still be significantly reduced as simplified purification workflow eliminates capital and operational expenses of unneeded DSP units and associated process development requirements, enables downsizing of USP thanks to improved recovery, and improves flexibility and overall facility utilization. Currently there is no published economic comparison between affinity-based versus traditional vaccine purification process at manufacturing scale. Economic feasibility is a critical consideration and should be evaluated for each individual case. Overall, modernization of legacy vaccine DSP and new vaccine applications enable new manufacturing paradigms such as:

- Cost-efficient production that can be deployed for in-country for-country manufacture
- Streamlined and platformable process and facility that can be operational in a few months to address pandemics or bioterrorism rapid response
- Application to "small markets" that were once considered not economically viable
- Application to other fields such as gene therapy and oncolytic virotherapy that require supply of very large quantities of ultrapure viruses.

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Conflicts of interest

All authors have declared the following interests: Mochao Zhao, Melissa Vandersluis, James Stout and Renaud Jacquemart are all employed by Natrix Separations, a supplier of chromatography membrane columns, but report no financial conflict of interests. Ulrich Haupts reports no financial conflict of interests. Matthew Sanders is an employee of the GSK group of companies and he reports ownership of shares in the GSK group of companies.

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