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멤브레인 크로마토그래피에 의한 바이러스 정제 : 리뷰

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Virus Purification by Membrane Chromatography: A Review

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요 약: 바이러스는 생물 의약 산업에서 다양한 응용 분야를 가지고 있다. 그들은 살충제 생산, 백신 생산, 유전자 전달, 암 치료제 등에 사용된다. 바이러스의 하류 처리는 그들의 생물학적 및 의약적 응용을 위한 필수 단계이다. 다양한 과정 중에서 바이러스의 정제는 매우 중요하다. 막 크로마토그래피는 이 과정에서 중요한 역할을 한다. 이온 교환 막 크로마토그래피는 주로 사용되는 방법이지만 크기 배제 및 불충분한 정제에 관한 다양한 제한을 가지고 있다. 또한, 이는 인플루엔자와 같은 빠르게 변화하는 바이러스의 균주에 적용될 수 없다. 이 검토는 막 크로마토그래피의 다양한 개선된 방법 또는 대안을 검토한다. 이는 정제, 바이러스 회수율 및 방법의 확장성에 초점을 맞추고 있다.

Abstract: Viruses have various applications in the biopharmaceutical industry. They are used in pesticide production, production of vaccines, gene transfers, cancer therapeutics, and more. The downstream processing of viruses is an essential step for their biological and pharmaceutical applications. Among the various processes, the purification of viruses is critical. Membrane chromatography plays a vital role in this process. While ion exchange membrane chromatography is a primarily used method, it has various limitations regarding size exclusion and insufficient purification. Also, it cannot be applied to the rapidly changing strains of viruses such as influenza. This review examines various improved methods of membrane chromatography or alternatives. It focuses on purification, viral recovery rates, and scalability of the methods.

Keywords: *membrane, chromatography, virus, influenza A virus*

1. Introduction

In the purification of viruses, filtration is an important step. However, it is not perfectly efficient as it follows a size exclusion principle, therefore particles that are smaller than the virus are retained[1]. Kosiol *et al.* tested different membranes' pore size gradients and concluded that membranes with shallow pore size gradients were less susceptible to fouling[2]. Polymersomes are a new development in the nanomedical and biotechnology field, an essential process in making them functional is encapsulating them with proteins. This process, however, is not easily scalable. Mertz *et al.* presented a scalable model for the same. It used stirred tank reactors and to enhance its efficiency, hydrophobic membrane anchoring peptides were genetically

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fused to the proteins. They found that more hydrophobic anchors provided greater encapsulation[3].

The development of adeno associated viruses (AAV) as a tool for gene therapy led to a demand for more efficient and cost-effective purification methods. Fan *et al.* used a non-woven ion exchange membrane for the separation of AAV2 from a cell culture lysate was not only efficient but cost-effective and scalable. They formed cation and anion exchange membranes, the combination of which resulted in a capsid recovery of 65.3% with an infective recovery of 76%[4]. Kwak *et al.* compared the most common membranes used for anion exchange membrane chromatography–Mustang Q and Sartobind Q. The latter provided a much greater removal of DNA impurities and was found to be superior of the two[5].

Membrane chromatography has been gaining attention in the bioprocessing field. O'Donnell assessed the effects of Sartobind Q anion exchange membranes on viral recovery and system pressure during the loading phase[6]. In a study conducted by Kawaka *et al.*, additionally, the original membrane chromatography device was compared to an improved membrane chromatography device that used a laterally fed membrane chromatography (LFMC) method, LFMC proved to have considerable benefits over the conventional chromatography device[7].

After the purification of viruses, the FDA requires the final product to contain no more than 10% of the host cell DNA. Many fail to meet these numbers, however, a study conducted by performing a clarification step before DNA digestion the desired results can be obtained[8]. This is because during clarification cell debris are eliminated and with it, all the associated DNA particles will also be removed. Thus, an approach that could accommodate non-homologous viruses had to be found. The use of a size-based purification step which includes the processes of ultrafiltration, diafiltration, and sterile filtration was assessed as an alternative for adsorptive separation. It proved to be successful and had a recovery of 80%. We know that influenza viruses can be purified using pseudo membrane affinity chromatography and on comparing the binding capacity of two beaded resins with a membrane adsorber, the adsorber had significant advantages[9,10]. In a study conducted by Yang *et al.*, a depth filtration process was used to replace centrifugation followed by anion membrane chromatography[11]. This process produces a viral recovery of up to 62.5% and a removal of 96.5%. Moreover, the depth filtration process which is a more cost-effective step as opposed to centrifugation produced similar results. Nanofiber membrane with very high surface area and porous morphology enhance efficient kinetics of virus separation. This review discusses virus, AAV, and Influenza A virus purifications in detail.

2. Virus Purification

Ion exchange membrane chromatography is a method used for the purification of viruses and viral antigens. Chung *et al.* evaluate the purification of *Equine arteritis virus* (EAV) by an anion exchange membrane chromatography capsule (AEC)[12]. This purification method was compared with a differential centrifugation purification method using competitive blocking enzyme-linked immunosorbent assay or cELISA. The comparison was conducted based on the relative purity and quality of glycoprotein 5 of EAV which contains the epitope labeled 17B7, and the relative sensitivity of the cELISA with the 2 purified antigens. A western blot was used to analyze the results. The blot showed that 86% of GP5 was found in AEC-purified EAV and less than 29% in differentially centrifuged EAV. The AEC-purified EAC cELISA had an improved sensitivity of 98.2% and specificity of 99.5%. Additionally, a virus neutralization test which detects antibodies of GP5 and is the most sensitive assay to detect the EAV antibodies was conducted and the AEC-purified EAV based cELISA had 30~40% higher agreement with it than the differential centrifugated EAV based cELISA. Therefore, the improved cELISA should be used in harmonization with the World Health Organization (WHO) prescribed virus neutralization (VN) test.

Autographa californica M nuclepolyhedrovirus (Ac*M*NPV) is a baculovirus commonly used for pesticide production and is infectious for lepidopteran insects[13]. Due to its large-scale application the purification of this virus is of great interest. Grein *et al.* evaluated the stability and ion exchange chromatography of a recombinant of Ac*M*NPV by comparing three different adsorptive membranes. The degree of purification was studied by adjusting the pH or ionic conductivity. The infectivity remained constant at a pH range of 6~8 and decreased rapidly at a pH below 5.5. A maximum virus concentration was obtained with a polyether sulfone-based membrane which has quaternary ammonium functional groups. Additionally, filtration with membranes containing similar ammonium groups showed diminished levels of DNA suspension (below 100 pg per 10^5~10^6 pfu). In conclusion, the recovery of infectivity increases with an increase in salt concentrations this implies that it is also possible to increase separation efficiencies at increased salt concentrations

Virus-like particles (VLPs) are proteins that mimic viruses[14]. They are important in the innovation of vaccines against cancer and infectious diseases. The most used unit for the purification of biopharmaceutical products is ion exchange chromatography, however, there have been no reports of *in silico* optimization of VLPs due to mass transfer of components of unknown size and concentration. In this case study the purification of human B19 parvo-VLPs by high-throughput experimentation and UV-absorption based chromatography modeling is studied. A binding capacity of 5.7 mg VLP per mL Q membrane was obtained in a high-output screening in a 96-well format. An *in silico* optimized process enabled the separation of VLPs. The simulated and experimental purities were 88.6% and 81.5 ± 2%. The optimized membrane chromatography had a protein purity, DNA clearance, and VLP recovery of 81.5%, 99.2%, and 59% respectively.

Baculovirus is one of the safest and most efficient gene transfer tools and has been used for the expression of recombinant proteins previously. It is important for prospects in stem cell treatments for its purification to be efficient and provide optimal outputs. There are multiple techniques for the purification of baculovirus including filtration. However, they have certain drawbacks including the dependence on the viral surface composition to allow virus binding. Steric exclusion chromatography provides a good alternative to overcome this challenge as it pursues the size of the target species rather than the specific surface composition. A study conducted by Lothert *et al.* confirms the optimization of yield and product purity and maintained efficacy by using membrane-based steric exclusion for the purification of *Autographa californica*[15]. Stationary phases such as cellulose, glass fiber, and polyamide membranes were evaluated, and cellulose had the highest binding capacity (5.08E + 07 pfu per cm^2 membrane). Other parameters were evaluated based on the design of experiments approach. The resultant recovery yields and impurity removals for host proteins and DNA were greater than 90%, 99%, and 85% respectively. Additionally, the infectivity of the virus particles remained unchanged. Due to the achieved results, it is a great alternative to other purification methods, it also has room for scalability by increasing the membrane diameter.

Monoclonal antibodies (mAbs) are used in the biopharmaceutical industry and are useful therapeutics in cancer and autoimmune disease treatment. The culture broth of mAbs contains various product and process-related impurities, thus it must be purified using the appropriate steps. Anion exchange chromatography (AEX) is an important step to purify mAbs. However recent experiments in the purification of mAb1 resulted in its retention in the AEX resin under standard chromatographic conditions (p*I* − 0.5). This result was hypothesized to be caused due to asymmetric charge distribution on the protein surface. Masuda *et al.* used a membrane adsorber, Natriflo HD-Q AEX, to solve this issue[16]. It was confirmed that the retention of mAb1 was due to asymmetric charge distribution, and a membrane adsorber was able to successfully solve this issue.

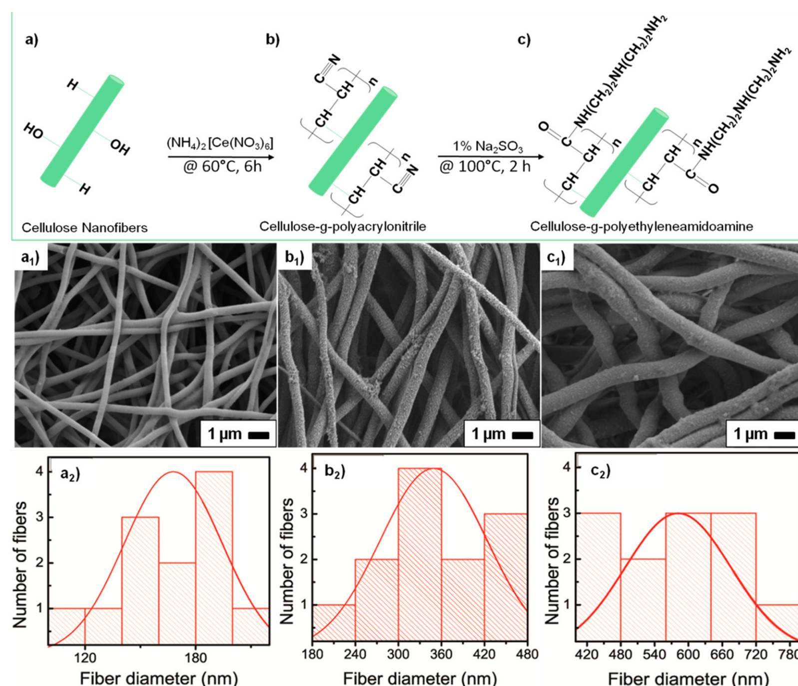

Fig. 1. Schematic of the reaction pathway involved and corresponding morphological changes in the fabrication of the CL-*g*-PEAA anion-exchange membrane adsorber by redox polymerization. (a) Representative image of a single CL nanofiber used as the base material, (b) intermediate step in which CL reacted with an acylonitrile monomer at 60°C for 6 h to form CL-*g*-PAN, and (c) CL-*g*-PAN's final conversion into CL-*g*-PEAA by reaction with diethylenetriamine at 100°C for 1.5 h. (a_1), (b_1), and (c_1), respectively, are the SEM images of nanofibers at synthesis stages (a), (b), and (c). (a_2), (b_2), and (c_2) are the histograms of average nanofiber diameters calculated for images (a_1), (b_1), and (c_1), respectively, using ImageJ software. AN and DETA concentrations of 28.2×10^{-3} and 3.1 M were respectively used for the intermediate and final steps of the CL-*g*-PEAA preparation (Reproduced with permission from Rajesh *et al.*[18], Copyright 2018, American Chemical Society).

Retroviral gene therapy vectors have been used in successful ongoing clinical trials, they must be used at very high purity and must be free of replication-competent viruses[17]. This paper studies the purification of -retroviruses by anion exchange purification and optimization by wash buffers. A Mustang Q membrane was used for the purification. The membrane produced high concentrations of the vector and showed results double that of chromatography. It was found that a buffer of high salt concentration results in greater recovery than loading a buffer of equivalent salt concentration due to the inactivation of virus at high concentrations. Mass spectrometry showed that Bovine Serum Albumin (BSA) was the primary impurity that was removed. An equivalent of 1.27×10^{8} Ifu/mL of ion exchange membrane was successfully bound.

Due to the development of monoclonal antibodies, there is an increasing demand for more efficient downstream purification processes[18](Fig. 1, 2). Membrane adsorbers have proved to be a significant method for the efficient recovery of mAbs. A study developed a cellulose-*graft*-polyethyleneamidoamine (CL-*g*-PEAA) anion-exchange nano-fiber membrane adsorber which was evaluated using BSA. This membrane can separate using ion exchange and size exclusion, at 10% breakthrough it showed a DPBC of 69 mg/g with a residence time of 8 s. It also showed a complete rejection of 100 and 200 nm for model bead particles, only 80 % of 40 nm size and this demonstrated its efficiency in separating viruses. Further, HCCF purification can be completed in a single step with the use of an ion exchange and size exclusion membrane such as the one developed in this study.

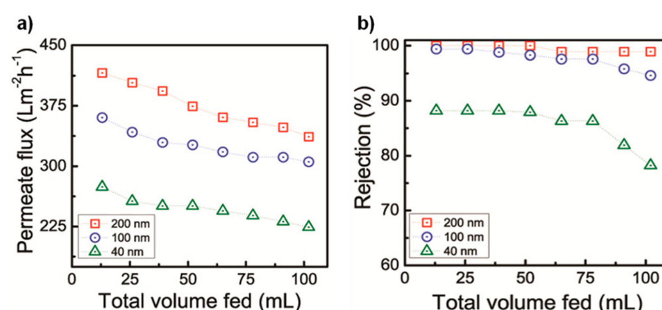

Fig. 2. (a) Permeate solution fluxes and (b) percentage rejection behavior of CL-*g*-PEAA anion-exchange membrane adsorbers throughout the filtration of 200, 100, and 40 nm polystyrene microspheres for 100 mL of solution. Aqueous bead solutions used as the feed have initial concentrations of 0.5 g/L. Solutions were flowed through the membranes under a gravitational pressure effect. CL-*g*-PEAA anion-exchange membrane adsorbers were fabricated with an AN concentration of 18.8×10^{-3} M (reaction time of 6 h), and a DETA concentration of 3.1 M (treatment time of 1.5 h) was used for filtration experiments. A UV–vis spectrophotometer was used to measure the bead solution concentration during the filtration experiments (Reproduced with permission from Rajesh *et al.*[18], Copyright 2018, American Chemical Society).

3. Influenza A Virus

It is a known fact that sulfate cellulose membrane adsorbers (SCMA) are one of the most promising matrices for the purification of virus particles including influenza virus particles (IVPs)[19]. This study demonstrates that pseudo-affinity chromatography– a method used to separate and purify biomolecules based on their reversible interaction with immobilized ligands on a chromatographic substrate–using SCMA can be carried out in a continuous manner using a three-column periodical counter current setup or 3C-PCC. The purification achieved productivity of 22.8 kHAU ml_{memb}^{-1} min^{-1}. The recovery of HA activity and removal of protein was 64.7% ± 1.7% and 67.4% ± 0.6%. In conclusion, this method could be used for other viruses such as SARS-Cov-2.

A new technique for the purification of viral particles called steric exclusion chromatography (SXC) was developed in 2012, it used a non-reactive hydrophilic monolith[20]. This study evaluates the purification of influence A virus particles obtained from suspension Madin Darby canine kidney employing SXC using a regenerated cellulose membrane adsorber instead of a monolith. The product recovery and protein depletion obtained were above 95% and 92.4% respectively. These results proved that it was a better method as opposed to the previous purification methods. Apart from the results it also has various advantages including scalability and increased productivity.

4. Adeno-Associated Virus (AAV)

The development of AAVs for gene therapy demands an efficient purification process. This study developed a nonwoven ion exchange membrane for the separation of AAV2 from a cell culture lysate[4](Fig. 3, 4).

Two membranes were prepared to achieve this, nonwoven membranes grafted with glycidyl methacrylate (GMA) were functionalized with triethylamine (TEA) and iminoadiacetic acid (IDA) to form an ammonium anion exchange membrane (AEX-TEA) and a cation exchange membrane (CEX-IDA) respectively. AEX-TEA showed a binding capacity of 9.6×10^{13} capsids/mL, and when exposed to the clarified Sf9 culture it was 2.4×10^{13} capsids/mL. To improve the results a combination of AEX-TEA and CEX-IDA was used at different pH levels. This combination resulted in a net capsid recovery of 65.3% and an infective recovery of 76%. Moreover, the total impurity protein clearance obtained was 3.3 LRV. The low cost and flexibility of this method make room for scalability.

During the process of producing AAVs the capsids

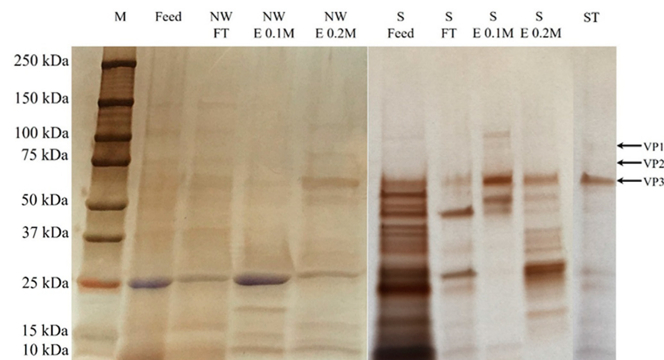

Fig. 3. SDS-PAGE analysis of AAV2 purification from the Sf9 lysate via AEX-TEA nonwoven membrane ("NW") and Sartobind Q membrane ("S") conducted at a binding residence time (RT) of 1 min and steady pH of 8.0. M: marker; FT: flow-through; E 0.1 M: eluate by 0.1 M NaCl elution buffer; E 0.2 M: eluate by 0.2 M NaCl elution buffer; ST: AAV2 standard (full capsids) (Reproduced with permission from Fan *et al.*[4], Copyright 2022, MDPI).

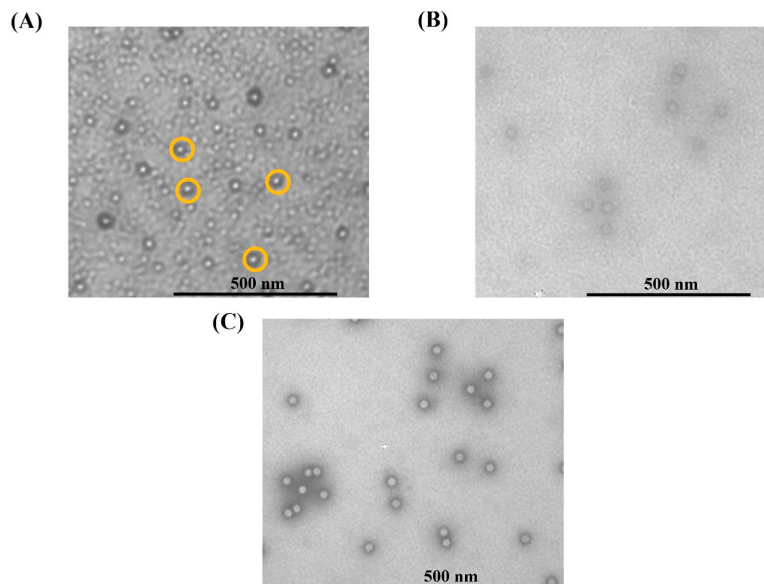

Fig. 4. TEM imaging of (A) feed, the purified AAV2 after two-step membrane process (B), and the standard full AAV2 capsids (C). The yellow cycles indicate viral particles (Reproduced with permission from Fan *et al.*[4], Copyright 2022, MDPI).

produced may or may not contain the gene of interest[21]. Although various methods such as analytical ultracentrifugation are available the problem lies in scalability. This study devises another method to produce full AAV capsids which uses an anion exchange chromatography elution and various conductivity steps. The method used AAV stereotype 5 and successfully showed high flow rates of up to 10 MV per minute. It also produced 50~60% full capsids at a pH of 9. Due to its low costs, it is easily scalable. It may be applied to other AAV stereotypes.

The development of adenoviruses has led to increasing discoveries of downstream purification systems[8]. While effective and scalable methods like membrane chromatography (MC) have been discovered, they show high degrees of sample dilution due to their radial flow configuration. This paper compares MC and an improved MC device–laterally fed membrane chromatography (LFMC) method. The comparison was done with the same type and amount of anion exchange membrane by using computational fluid dynamics (CFD) simulations. A 74% percent viral recovery with

4% and 1% residual protein and DNA respectively was got using LFMC. 100% viral recovery with less than 10% residual protein was obtained with both devices however MC had about 50% higher sample dilution. Other advantages of LFMC include its shorter buffer time and higher and narrower peaks. Therefore, LFMC provides an enhanced performance as opposed to MC.

The use of Adeno associated viruses (AAV) has been integrated into various therapeutic fields[8]. An important step in the processing of these substances is host cell DNA removal, the FDA requires the final product to contain no more than 10% of it. Although efficient purification methods have been created, many fail to achieve this required final product due to pre-purification processes. This study approaches this issue by considering the DNA digestion step and the purification by membrane chromatography. It was found that a clarification step before digestion is useful to eliminate cell debris since DNA associated with debris was also removed, it was done by centrifugation. A design of experiments approach was used with 96 filtered plates were used. The most optimal conditions were found to be an LMFC run in a device containing 1mL of Sartobind Q. This specific process produced a virus recovery of 73% and DNA removal of 93% (6507 ng/dose to 77 ng/dose). Thus, this study successfully produced results that follow the FDA regulations and can be implemented during manufacturing.

5. Conclusions

Various methods were devised to improve the existing purification processes. To combat issues related to small sized impurities, a nano fiber membrane was used which enabled separation based on ion exchange as well as size exclusion. A hydrophilic monolith showed various advantages which made it a better membrane for purification as opposed to others. Various advances were made to purify adeno viruses. An improved MC device, which used a laterally fed membrane chromatography method, showed a shorter buffer time. Further, a cost-effective method was created for the recovery of full capsids which used anion exchange chromatography elution at a pH of 9. All of these improvements provide a ground for future research in the biopharmaceutical industry and further research will only improve these methods and make them more cost effective and scalable.

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