DEVELOPMENT OF FUNCTIONALIZED MEMBRANE BASED TECHNOLOGY FOR ISOLATION OF VIRUS

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by

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under the supervision of

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I, **Ayushi Agarwal** (Enrollment No. **16556003**)**,** hereby declare that this dissertation entitled **DEVELOPMENT OF FUNCTIONALIZED MEMBRANE BASED TECHNOLOGY FOR ISOLATION OF VIRUS** represents my original work carried out as a postgraduate student of IIT Roorkee and, to the best of my knowledge, it contains no material previously published or written by another person, nor any material presented for the award of any other degree of IIT Roorkee or any other institution. Any contribution made to this research by others, with whom I have worked at IIT Roorkee or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the section "Bibliography". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

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ABSTRACT

Virus filtration is a critical component of the downstream purification process during the production of important biotherapeutics. However, it is a challenging and economically expensive process. This study has been directed towards the development of reusable and efficient functionalized polymeric membrane for the isolation of virus from process stream. Theoretical analysis of the commercially available virus filters revealed that despite high LRV value, these membranes compromise in terms of permeability, which is in the order of 10^{-11} $m^3/(m^2\text{-s-Pa})$. This ultimately decreases the productivity and consequently increases the processing cost. In this work, functionalized membrane was developed using Layer-by-Layer technique, which offered 2 fold increase in permeability with additional benefit of flux recovery.

MS2 was used as a surrogate virus model. Studies have shown that bacteriophages are similar to some human viruses, which occur naturally in the human and animal intestinal tract and grow faster. Therefore, they are well suited as model system for experiments. Quantitative data for the virus filtration was obtained through the functionalized membrane. In addition, flux recovery was also examined by mitigating the fouling in the membrane with Triton X detergent. Results show LRV value of 1.1 with permeability value of 2×10^{-10} $m^3/(m^2\text{-s-Pa})$ at 34.5 kPa (5psi). Flux recovery was achieved by dislodging the two bilayers of PEI-PSS form the functionalized membrane, keeping the PAA backbone intact. This, in turn enables the reusability of the membrane. In addition, the number of bilayers within the membrane can be chosen based on the size of target virus.

Keywords: Functionalized membrane, Layer-by-Layer (LbL) technique, virus filtration, permeability.

1. INTRODUCTION

Removal of infectious agents (bacteria, protozoans, viruses) from therapeutic products has been a major and foremost concern for all biopharmaceutical industries. Pathogen safety of products especially those of biological origin (protein based biopharmaceuticals, plasma derived blood products) is an essential part during downstream purification process [1]. Among these pathogens, viral clearance is a tedious process, especially during continuous operations, because of their small size, non-viability outside the host cells, and chemically resistant nature. Recombinant therapeutic products such as monoclonal antibodies, and proteins, are usually expressed in mammalian cell lines, which are susceptible to several viruses. Viral contamination can be either due to endogenous viruses, which originate within the cultured cell lines (size ≈ 80-120nm) or adventitious viruses, which enter through media, equipment or environment (size ≈ 20nm) [2]. Viral contamination possesses a major safety concern for the end users because, after administration of these drugs through various routes (intravenous, intramuscular, subcutaneous injections) the human digestive system becomes vulnerable. To ensure the safety and virus inactivation, multiple virus clearance steps like physical inactivation (use of dry heat treatment), chemical inactivation (use of solvents/detergents), adsorptive processes (chromatographic techniques), and size based separation (use of membrane filters), are employed depending upon the characteristics of process stream. Current FDA regulations also require the use of at least two viral clearance steps as a part of downstream process during production of biotherapeutics [3–5]. In addition to this, good facility design in compliance with the GMP is another approach to tackle these safety issues. However, existing industrial techniques for virus clearance either reduce/destroy the normal functions of labile blood components present in the manufacturing solution or are expensive (especially chromatography), which ultimately increase the final price of the drugs.

Although membrane filtration has gained considerable importance in virus filtration in the recent years, however there are number of challenges in this experimental work. Researches are being done mostly with the ultrafiltration membrane, reverse osmosis membrane, and nanofiltration membrane[6]. All these membrane filters operate at a high pressure drop, offering permeability value of 10^{-11} $m^3/(m^2\text{-s-Pa})$. As permeability reflects the ability of the membrane to process a certain volume feed stream per unit time per unit area and per unit pressure drop, it is directly correlated to the productivity of the process.

Therefore, development of efficient bioprocess for virus removal from therapeutically relevant product streams is need of the hour.

2. LITERATURE REVIEW

2.1. Membrane

Membrane is a semi-permeable barrier, which under the influence of a driving force allows selective transportation of substances from one side (feed) to another (permeate) (Figure 2.1). The driving force is due to the difference in the chemical or electrical potential on the two sides of membrane and is usually expressed in terms of pressure, concentration, or electrical potential gradient. Membranes can be porous or non-porous and varies depending on the pore size, starting from 10μm for microfiltration membrane to 1 A^0 for reverse osmosis membrane [7]. Although the history of use of synthetic polymeric membrane goes back to the beginning of nineteenth century, its commercial application started in late 1960s. However, one of the most important developments in this field was the discovery of asymmetric membrane in 1964 [8]. As compared to symmetric membranes, this type of configuration gained more popularity in process industries because a thin layer of separating barrier supported on a polymeric backing allowed to operate at a much lower pressure drop [9]. Modification of membrane by incorporation of chemical groups, known as functionalized membrane, added another milestone to the research.

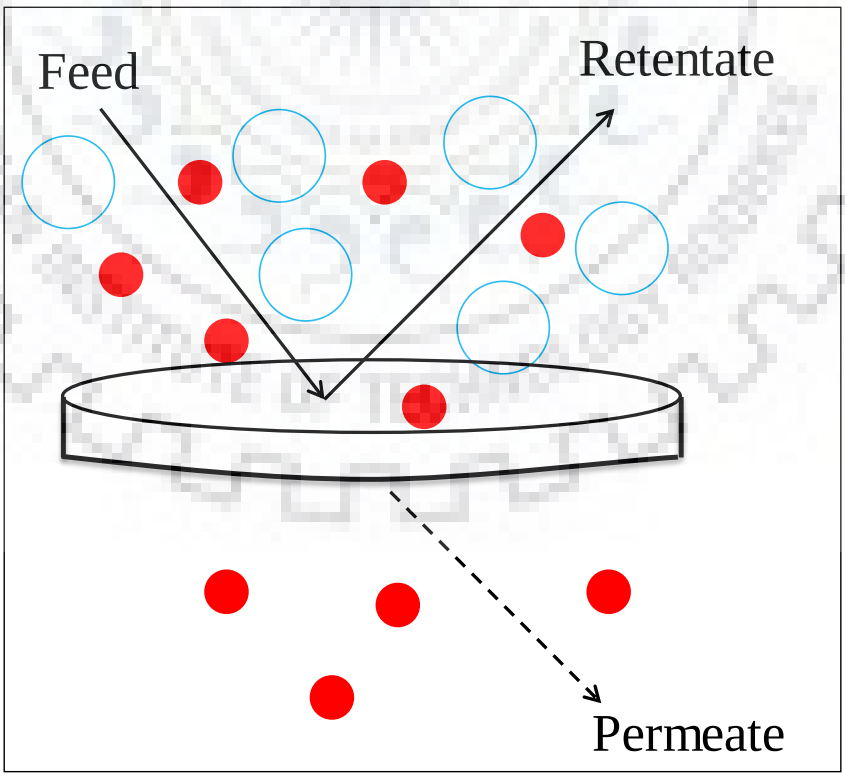


Figure 2.1: Different operating streams in membrane separation process.

2.2. Functionalized membrane

Functionalized membranes offer great versatility of the active groups, which can be exploited depending upon the application. Membrane functionalization is used to enhance not only the separation capabilities, but also to extend the spectrum of application to new areas such biosensors, drug delivery, biofuel cells, heavy metal capture, etc [10,11]. Porous polymeric membranes (UF or MF membrane) are used as a base for creating functionalized membrane as they serve two advantages over non-porous membranes. Firstly, they allow functionalization inside the pores which increases the membrane capacity as pore surface area is couple of orders of magnitude greater than membrane external surface area. Secondly, it aids in minimizing mass transfer resistance as molecules of interest can permeate under convective flow conditions. Studies have reported use of different base materials as constituents of starting membrane viz. cellulose, regenerated cellulose, nylon, polypropylene, PVDF [12–14]. This dissertation uses PVDF as a base material for all the studies.

Polyvinylidene fluoride (PVDF), repeating unit $–CH_2-CF_2$, is one among the most favoured organic polymers used in studies by the researchers despite its highly hydrophobic nature. This is because it possesses excellent thermal stability and resistance to the aggressive reagents like acids, bases, organic solvents [15]. However, high hydrophobicity increases the risk of membrane fouling, thereby reducing the flux and increasing capital cost. Thus, membrane functionalization is used to impart hydrophilicity to it. Literature reveals that Polyacrylic acid (PAA) has been used extensively for this purpose [16]. For this study, *in situ polymerization* of acrylic acid within the membrane matrix was carried out as per the protocol already reported by Gabriel and co-worker [17], followed by Layer-By-Layer (LBL) technique for the attachment of polyelectrolytes.

Layer-By-Layer (LBL) technique: This technique offers an easy and inexpensive process to create multi layers within the membrane matrix. It is an adsorption technique where alternative attachment of polycations and polyanions is conducted within the membrane matrix. The technique was first conceived by Decher [18], however Hollman and Bhattacharyya first implemented it for membrane pore functionalization [19]. Alternation of the attached charges leads to the formation of continuous assembly of positive and negative charges, creating versatility and variability for various applications. Since most biomolecules (like proteins, virus) contain charged groups on their surface, LBL electrostatic assembly finds application in separation of Biomolecules [20]. Lvov et al. have exploited this technique to form

an alternate assembly between the charged polyelectrolyte and virus [21]. This study deals with isolation of virus particles from fermentation/ media on the basis of charge of its outer protein capsid and charge deposited on membrane through polyelectrolyte layers.

2.3. Viral clearance

The general approaches employed for viral clearance are inactivation and chemical treatment. Chemical treatment involves use of harsh reagents/solvents in the feed stream, which ultimately destroys the activity of the macromolecules (especially proteins) present. Heat, radiation, low pH are some techniques used for inactivation, however they are dependent upon other parameters like time of exposure, temperature of the system and are less effective [22]. Chromatography is yet another method employed for virus removal on the basis of either electrical charge (i.e. ion-exchange chromatography), or hydrophobicity (hydrophobic interaction chromatography), or affinity (affinity chromatography). In the recent years, studies have shown that virus filtration is a robust technique that removes both enveloped & non enveloped particles on the basis of size, complementing other unit operations [23]. This is because unlike other methods, this technique poses a minimum risk in reducing or destroying the properties of fermentation media/labile blood components. Initially virus filters were developed for use in the tangential flow filtration, where the feed flow is adjacent to the upper skin layer of the asymmetric membrane, but the current operations are performed in normal flow filtration due to process simplicity and low capital cost and are designed in such a way that the product of interest (proteins) passes through the membrane pores while retaining at least 99.9% of virus on the filter [24–26]. Moreover, dead end filtration increases the log reduction capacity as the open support region serve as a prefilter, removing protein aggregates and foulants, thereby protecting the virus retentive layer over the membrane [27].

Commercially available filters specifically designed for virus retention are classified on the basis of size of targeted virus: retrovirus filters, for 80-110 nm size viruses; parvovirus filters, for 22-26 nm size viruses. Although virus filtration generally operates on the basis of size, literature reveals that virus retention can vary significantly in response to changes in the operating conditions such as pH of the solution, type of ions, salt concentration, transmembrane pressure, preconditioning of the membrane used[28–31]. During filtration, different mechanisms such as adsorption, electrostatic repulsion coexist. The relative

contribution to the overall viral retention depends upon the size of virus compared to the membrane pores and the characteristics of both, the virus and membrane.

Several studies have reported a significant decline in virus retention during the course of filtration involving conventional ultrafiltration membranes available exclusively for virus, like Ultipor DV20 membrane (Millipore Corp.) and, Viresolve NFP membrane (Pall Corp.) [23]. Researchers have hypothesized that decline in virus retention is due to preferential blockage of the smaller pores of the membrane, with the fluid flow re-directed to the larger (non-retentive) pores [32]. Experiments have been conducted to demonstrate that virus passes through the abnormally large pores of the membranes, reducing the log reduction value. Process disruptions, e.g., switching between feed tanks or using a buffer flush to recover residual product, have led to large differences in virus retention behaviour, although proper scientific explanation was not offered. In contrast to the reduction in the virus retention, it has been found out that preconditioning of membrane with the solutions of Bovine Serum Albumin (BSA) increased virus retention due to formation of cake layer on the surface of membrane, which served as an additional resistance layer in virus rejection[33].

In summary, most of the virus retention studies are inclined towards the separation through the difference in pressure or concentration. They usually make use of membrane pore size to remove virus from process stream. Few membranes separation processes are designed such that they introduce special selective recognition mechanism for the viruses on the basis of its properties. Incorporation of molecular brushes with polymeric cationic/anionic side chains on the membrane is one such example. It is a method through which virus particles can be removed efficiently with minimal removal of proteins [6]. Both fundamental understanding and practical implementation of virus filter performance continues to develop and build upon existing literature.

2.4. Virus surrogates

Surrogate viruses are those which are expected to mimic the viruses they represent. Although it is required that the validation studies should be carried out with the mammalian viruses, but initial proof-of-concept experimental studies can be performed using surrogate viruses [34]. Bacteriophage is generally used as surrogate model virus. Bacteriophage is a class of virus that infects bacteria. They are available in wide range of shape, charge and size with physical properties similar to those of mammalian viruses. Most commonly used bacteriophage models include MS2, φX174, and pp7. They are non-pathogenic to humans,

easier to enumerate as compared to enteric viruses, and their small size makes them suitable for filtration studies [35]. Also, working with mammalian virus requires biosafety level 2 (BSL2) or higher in the laboratory, whereas BSL1 is sufficient to work with bacteriophage. For the present study, data is obtained using MS2 as a model phage. It is an icosahedral, non enveloped, single stranded RNA phage, with an outer capsid diameter of 27.4 nm and isoelectric point (pI) of 3.5. MS2 is specific to the bacteria which contains F plasmid. MS2 genome contains 3569 nucleotides, encoding coat protein (cp), maturation protein, replicase subunit (rep) and a lysis protein (lys) [36] (Figure 2.2).

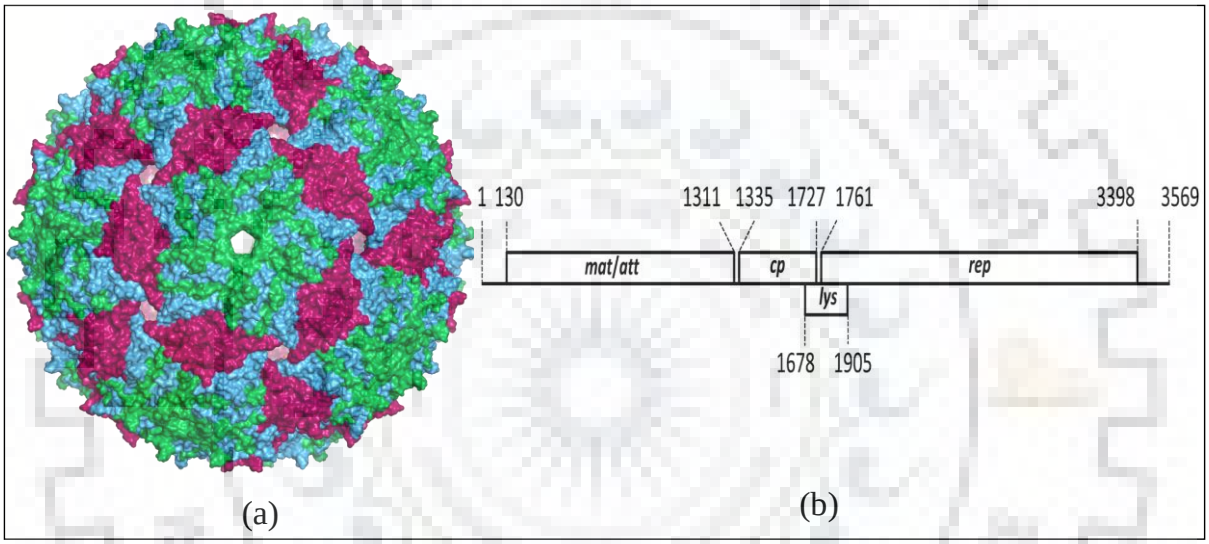


Figure 2.2: (a) Structure of MS2 bacteriophage (b) Genomic sequence of MS2. Reference: google photos

3. OBJECTIVE

The objective of this study is to develop a functionalized membrane based process for effective filtration of virus, overcoming the existing limitations. Earlier studies have demonstrated that membrane filters designed especially for the virus retention show variations in flux and viral retention with little change in the process conditions. Moreover, the filters are usually operated at a very high pressure drop which ultimately increases the cost of the process. We hypothesize that *in situ* polymerization of acrylic acid to form polyacrylic acid (PAA) within the microfiltration membrane matrix followed by subsequent attachment of poly-electrolyte layers above it will aid in virus removal at comparatively lower pressure drop. MS2 retention was studied and quantified using PFU Assay, providing a Log Reduction Value to validate the results.

4. MATERIAL & METHODS

4.1. Equipment

Experiments were conducted in Amicon stirred Ultrafiltration cell (model no. 8200) from Millipore. To ensure complete mixing of the components inside cell, magnetic stirrer was used. Virus filtration experiments were conducted inside the biological safety cabinet to avoid contamination. Figure 4.1 represents the schematic of the experimental set up. Virus filtration experiments (in triplicates) were performed inside biological safety cabinet to avoid contamination of the undesired microbes. Functionalized PVDF-PAA-$(PEI-PSS)_n$ membrane (as described in section 4.3.1) was used to conduct the experiments. Effective surface area of the membrane used was 33.16 cm^2. Magnetic stirrer was employed to ensure proper mixing of broth during the course of filtration. All the experiments were performed at a constant pressure of 34.5 kPa (5 psi), which was maintained through continuous N_2 supply. Permeate collected was used to count the number of virus particles along with the feed and the retentate as explained in section 4.3.3.

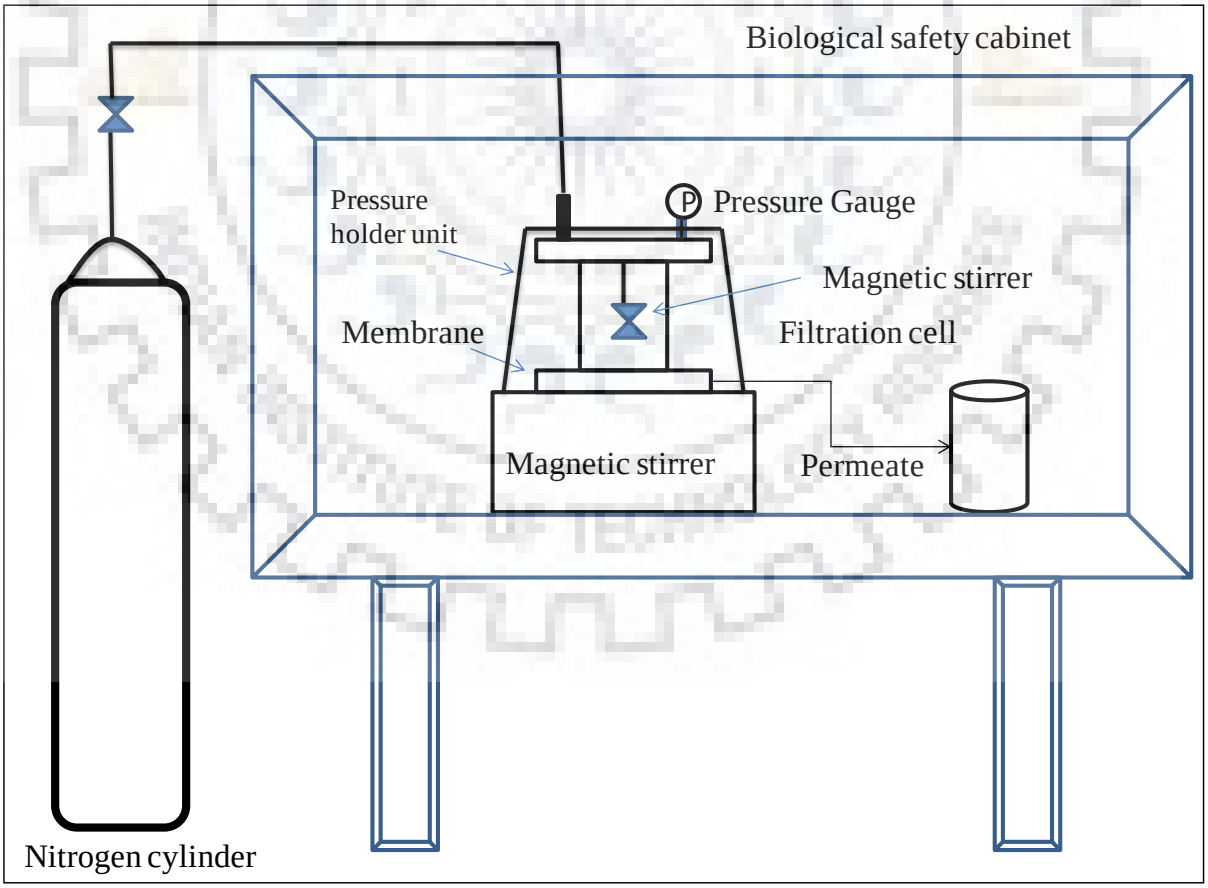


Figure 4.1: Schematic of equipment set up.

4.2. Materials

Durapore® PVDF hydrophobic membrane filters (membrane diameter 90 mm, pore diameter 0.22 μm, average thickness of 125 μm), used for all the experiments, were purchased from Millipore Corporation (Product No. GVHP09050). Acrylic acid (product no. 147320, MW 72), Trimethylolpropane triacrylate (TMPTA, Product No. 246808), Polyethlenimine (PEI, Product No. 408727, MWt. 25000), Poly (sodium 4-styrenesulphonate) (PSS, Product No. 243051, MWt. 70000, MWt. of repeat units 206) were purchased from Aldrich. Toluene (Product No. T0130) was procured from Rankem Laboratories (Gurgaon, India) while Benzoyl peroxide (Product No. RM3184) was obtained from HiMedia Laboratories, India. Luria-Bertani (LB) Broth (Product No. 29817) was purchased from Sisco Research Laboratories Pvt. Ltd., India.

4.3. Experimental methods

4.3.1. Functionalization of membrane

A method reported by X et al. [17] was adopted to functionalize hydrophobic PVDF membrane with PAA via *in situ* polymerization of acrylic acid monomer. The polymerization solution consisted of 70% toluene, 30% acrylic acid, 0.5% benzoyl peroxide, and 1.2% TMPTA (by weight). Benzoyl peroxide was used as free radical polymerization initiator. TMPTA acted as the cross-linker to increase the stability and density of the polymer network inside the membrane pore (Figure 4.2). The membrane was dipped into the polymerization solution for 30 s, and then it was sandwiched between two teflon plates and clipped tightly. It was then kept inside an oven at 90 °C for 4 hours under controlled N_2 environment. The membrane was then washed with a copious amount of DIUF water, under convective flow conditions, to remove the impurities. The membrane obtained at this point was functionalized with the hydrogen form (H-form) of PAA. It was converted to Na-form by permeating 200 mL of 0.1 M NaOH solution. The Na-form resists any drastic change in pH that generally occurs in the H-form for the following LbL attachment steps.

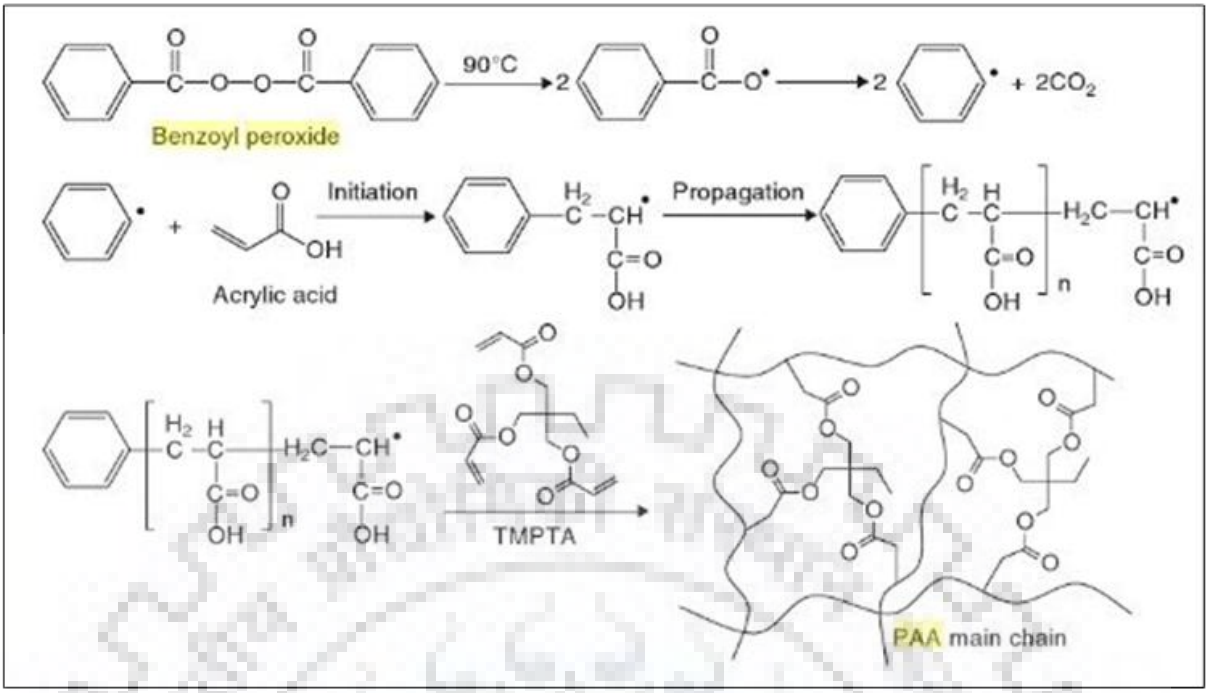


Figure 4.2: Reaction scheme of polymerization of acrylic acid in the membrane[37].

In order to provide a stable anchor for the formation of subsequent functional layers by LbL attachment technique, a bare membrane first needs to be modified. This modification was achieved by polymerisation of PAA within the membrane pores. Subsequently, layers of polyelectrolytes were assembled within the membrane pore by alternative electrostatic attachment of cationic and anionic polyelectrolytes as represented in Figure 4.3. All the layer formation steps were carried out under convective flow and constant stirring conditions. N_2 gas was supplied to maintain the convective flow conditions. Layer by layer deposition was achieved mainly within the membrane pores because of higher pore surface area as compared to the external membrane surface area. First layer of PEI was attached by permeating 200mL of 0.15mM solution in DIUF water at pH 6.5 in presence of 0.1 M NaCl. Salt was used to promote non-stoichiometric attachment of the charged groups of the polymers. Attachment of PEI resulted in a functionalized membrane (PVDF-PAA-PEI) with net positive charge.

Consequently, PVDF-PAA-PEI membrane was further modified by electrostatic attachment of poly (sodium 4-styrenesulphonate) (PSS) to obtain net negatively charged membrane. The layer of PSS was attached by permeating 100 mL of 0.2 mM PSS solution at pH 6.5. Positive charged amine groups of PEI were attached to the negatively charged sulphonic acid groups, of PSS, thereby forming net negatively charged membrane.

Depending on the requirement, number of subsequent bilayers (in this study, one bilayer has been referred to PEI-PSS) can be added to impart the desired characteristics. We have evaluated the performance of functionalized membranes for virus removal with up to 3 bilayers, i.e. Membrane-PEI-PSS-PEI-PSS-PEI-PSS (Figure 4.3). Permeate flux of water at different stages of functionalization was studied.

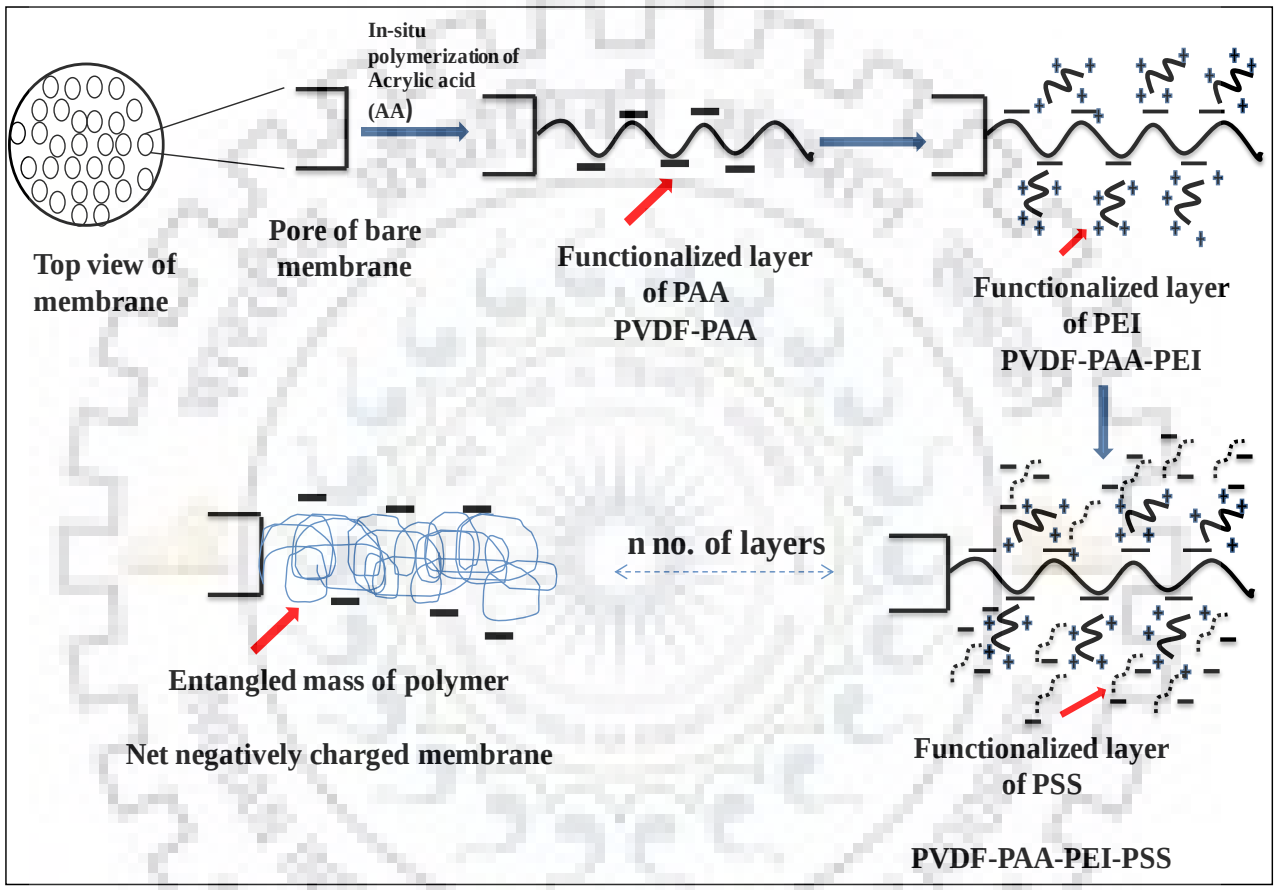


Figure 4.3: Schematic of formation of polyelectrolyte layers inside PVDF membrane pore using LbL technique.

Functionalized PVDF membrane (PVDF-PAA-PEI) was characterized by studying the morphology using Field Emission-Scanning Electron Microscope (Fe-SEM, Carl Zeiss, Ultra *plus*, Germany). The images were taken in high vacuum conditions at 20kV. To determine the elemental composition of the functionalized membrane, energy dispersive X-ray (EDX) was performed. Attachment of PSS was quantified by Ultraviolet-Visible (UV-Vis) Spectrophotometer (Jenway, Genova Bio) at 261nm.

4.3.2. Growth of host

Escherichia coli (ATCC® 15597™) was grown as bacteriophage host according to ATCC protocol. ATCC medium 271 was prepared in deionized water as specified (Tryptone 10.0 g/l, Yeast Extract 1.0 g/l, NaCl 8.0 g/l, Glucose 1.0 g/l, $CaCl_2$ 0.294 g/l, Thiamine 10.0 mg/l) and was autoclaved. Using a single tube of #271 broth (5 to 6 mL), approximately 0.5 to 1.0 mL was withdrawn with a Pasteur or 1.0 mL pipette. The entire pellet was rehydrated. This aliquot was aseptically transferred back into the broth tube and mixed well. Several drops of the suspension were used to inoculate a #271 plate. The tubes and plates were incubated at 37°C for 24 hours. Also, the culture of the host was preserved at -80˚C in 60% glycerol stock solution.

4.3.3. Propagation of Bacteriophage-

Escherichia coli bacteriophage MS2 (ATCC® 15597-B1™) was propagated as per the ATCC instructions, using Double Agar Layer overlay method (DAL method). Briefly, an actively growing broth culture (6-24 hours old culture, depending upon the growth) of the recommended host strain was prepared from a frozen stock before opening the phage specimen. Approximately 1.0 mL of the recommended broth was added to a freeze dried phage vial. Plates of the recommended medium (containing 1-1.5% solidified agar) were pre-warmed in an incubator. The surface was overlayed with 2.5 mL of melted 0.5% agar (same medium) containing one drop of the 6 hour or a 24 hour old host. The soft agar was maintained at 43°C to 45°C till ready to pour. The overlay was allowed to harden. The rehydrated phage was serially diluted by passing 0.5 mL of the phage into a tube containing 4.5 mL of the broth medium as desired. 100 µL of each dilution was spotted on the surface of the prepared plates & allowed to dry. After 24 hour of incubation, lysis was visible and individual plaques were countable at the higher dilutions.

In addition to the DAL method used for determining MS2 concentration, Transmission Electron Microscopy (TEM, Techni G2G20) was done to characterize virus culture in terms of shape and size. Samples were applied to the carbon coated grids, stained with 2% (w/v) ammonium molybdate for 30s, dried and visualized at 200kV, under focal length of 135mm.

5. RESULTS & DISCUSSION

5.1. Characterization of functionalized membrane

In order to provide a stable anchor for the formation of subsequent functional layers by LbL attachment technique, hydrophobic PVDF membrane was modified with acrylic acid, which also converted it into hydrophilic membrane. *In situ* polymerization of acrylic acid (pk_a ~ 4.2) formed a network of negatively charged carboxylic acid groups (-COOH) within the membrane pores. Deprotonation of the –COOH groups resulted into electrostatic repulsion between the negatively charged groups ($-COO^-$) and transformed them from contracted form to elongated form, which led to increased effective pore coverage. Hydrophilicity of PAA coated PVDF membrane was observed physically and was characterized by contact angle goniometer. The results are represented in Figure 5.1. Small contact angle is observed when water spreads on the surface, depicting its hydrophilicity, while hydrophobic surface shows a large contact angle as water tries to avoid the surface, thereby reducing the contact area with the surface. Figure 5.1 demonstrates that the angle decreased from 73.7˚ to 37˚ as hydrophobic PVDF membrane was converted to PAA coated PVDF membrane, representing hydrophilic nature of the membrane.

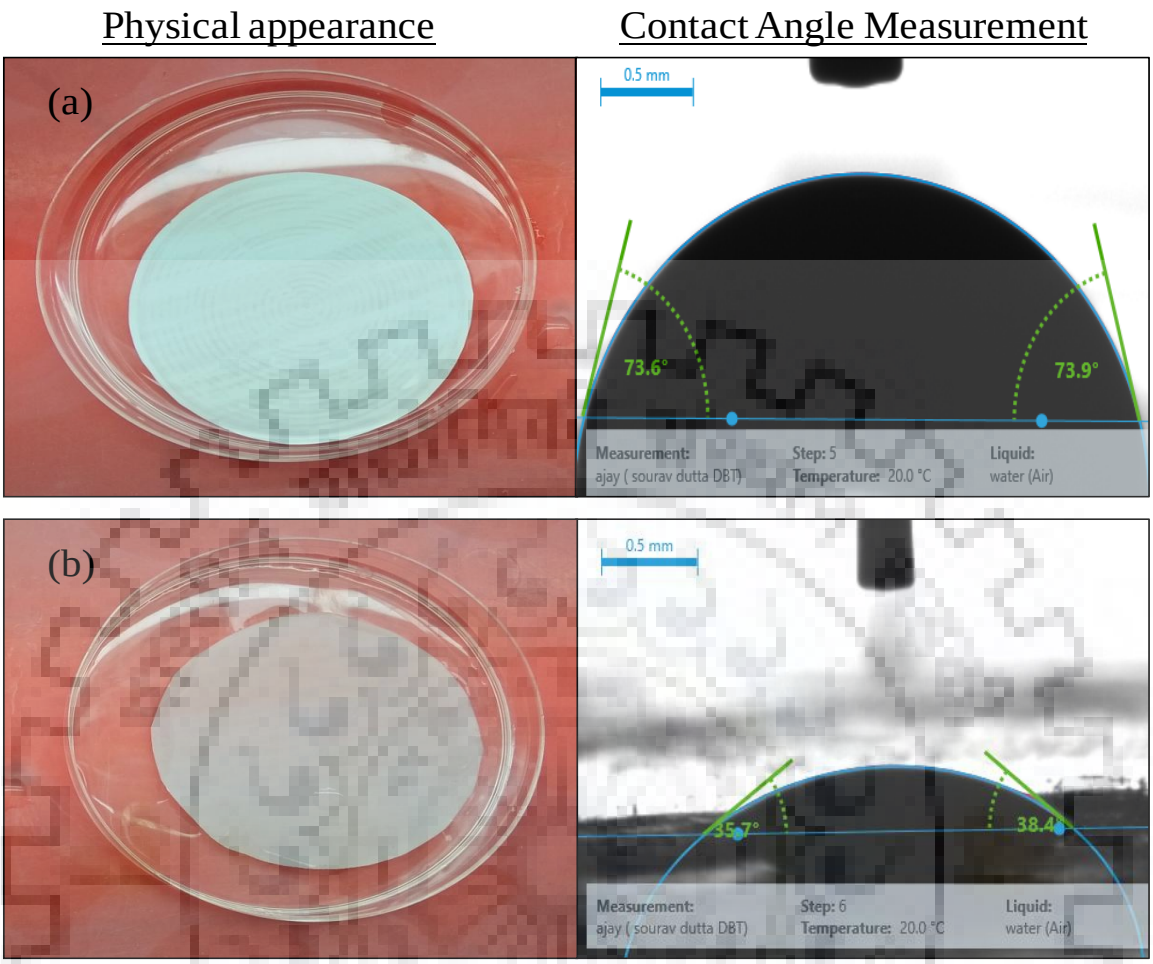


Figure 5.1: Physical appearance and Contact angle measurement of (a) Hydrophobic PVDF membrane (b) Hydrophilic PVDF-PAA membrane.

Non-stoichiometric attachment of subsequent layers was characterized by Fe-SEM and EDX analyses as depicted in the Figure 5.2. Since each layer was equipped with excess of opposite charges as compared to previous layer, electrostatic attachment has governed the LBL process. Figures 5.2(a-c) show the representative SEM images of the membrane surface after each functionalization step, while Figures 5.2(d-f) represent EDX spectra for the chemical analysis of the membranes at different steps of functionalization. A significant difference in morphology was observed between bare PVDF membrane (Figure 5.2a) and PVDF-PAA membrane (Figure 5.2b). The red arrows show the deposition of polymer within the membrane pore. The corresponding EDX spectrum (Figure 5.2e) confirmed the presence of oxygen (O) (~7% by weight), arising from the carboxylic acid groups of PAA in addition

to carbon(C) and fluorine (F) present in bare PVDF (Figure 5.2d). Similarly, Figure 2c shows difference in morphology after functionalization with PEI. The membrane surface turned smooth with narrow pore openings due to attachment of polymer layers. EDX spectrum also revealed the presence of additional peak of nitrogen (N) (~11% by weight) along with O, C and F arising from the amine groups of PEI (Figure 5.2f).

Third layer of PSS was attached to the membrane and was quantified by measuring the absorbance of feed, permeate and retentate directly at 261nm, using UV-Vis spectroscopy (Figure 5.3). The amount of PSS attachment was 0.1366 μmol. The net charge after attachment of three bilayers of polymer i.e. [PVDF-PAA-(PEI-PSS)$_n$, n=3] used in this study was negative.

Water flux was measured through bare PVDF (Q_{w0}) membrane and through the functionalized membrane (Q_w) after each step. Effective cumulative thickness after addition of n^{th} layer (δ_n) was calculated using Hagen-Poiseuille's equation as mentioned below:

$$r_n = r_0 \left(\frac{Q_{wn}}{Q_{w0}}\right)^{0.25}$$

$$\delta_n = r_n - r_0$$

Where, r_n= pore radius after addition of three bilayers, r_0= pore radius of bare membrane (0.22μm)

Permeability (P_n) after n^{th} layer of attachment, in $m^3/(m^2\text{-s-Pa})$, was calculated as per the below mentioned equation:

$$P_n = \frac{Q_{wn}}{A_m \Delta P}$$

Where, Q represents volumetric flow rate (m^3/s), r represents the pore radius of membrane, A_m is the effective area of membrane = 33.16×10^{-4} m^2, and ΔP is the trans-membrane pressure drop

SEM micrographs SEM-EDX analysis spectra

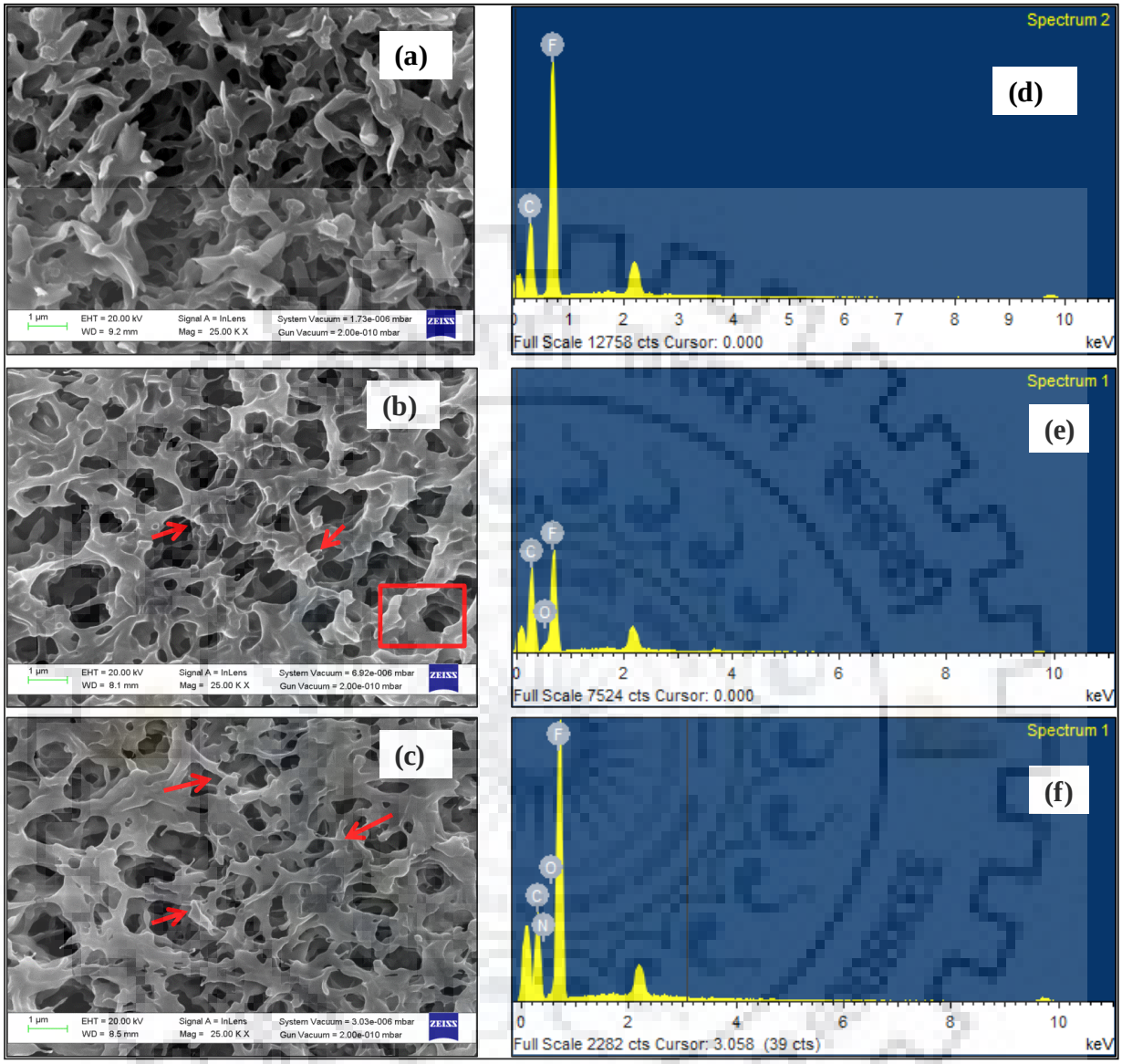
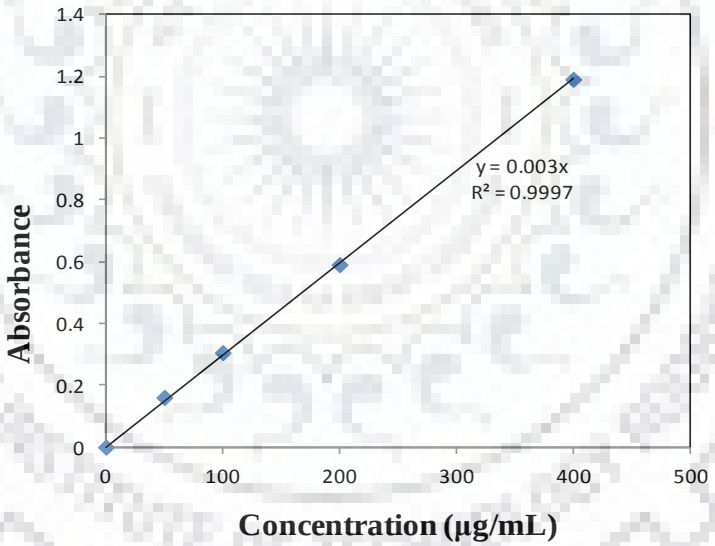


Figure 5.2: SEM images of (a) bare PVDF membrane (b) PAA functionalized PVDF membrane (c) PVDF-PAA-PEI membrane. The figure also depicts SEM-EDX analysis of different elements present in (d) unmodified PVDF membrane (e) PVDF-PAA membrane (f) PVDF-PAA-PEI membrane.

Figures 5.4 and 5.5 represent the change in water permeability at different stages of membrane functionalization. Conventionally, deposition of polymers within the membrane pores creates resistance in water flow, which decreases the flux at a particular pressure drop, i.e. decrease in permeability. However, as illustrated in Figure 5.4, incorporation of PAA within the membrane matrix increased flux at a particular pressure drop as compared to bare PVDF membrane. This increase is ascribed to the increase in membrane hydrophilicity, which in turn improved the water transport characteristics through the membrane. Subsequent addition of bilayers PVDF-PAA-$(PEI-PSS)_n$ however, decreased the flux drastically as the effect of increase in resistance due to additional layers have superseded the effect of increase in hydrophilicity (Figure 5.5).

It can be observed that cumulative thickness of layers increased as subsequent layers were added to the membrane. This created the reduction of 44% in pore radius, making effective pore diameter of 125 nm for further study of virus filtration.

(a)



(b)

	Absorbance	Concentration (µg/mL)
Feed	1.275	425
Permeate	0.698	232.7

Figure 5.3: (a) Standard curve of PSS at 261 nm (b) Concentration of PSS in feed and permeate as calculated from standard curve.

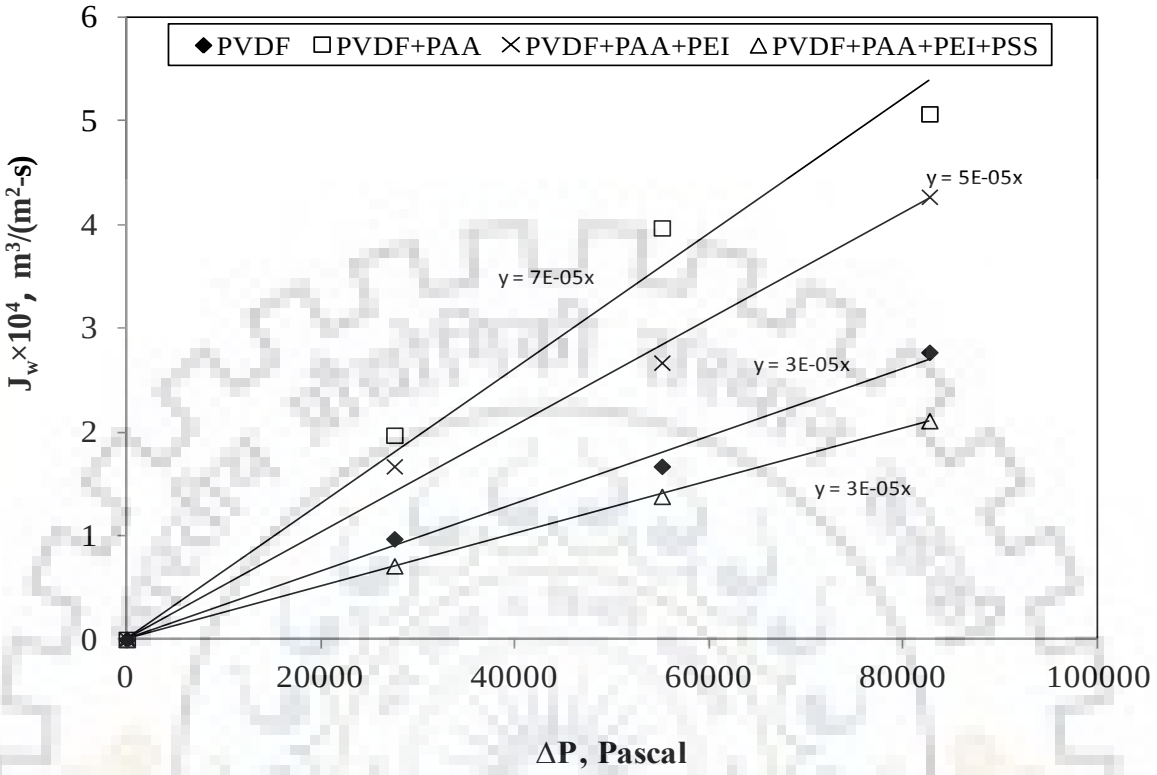


Figure 5.4: Comparison of permeability values at various stages of functionalization of PVDF membrane.

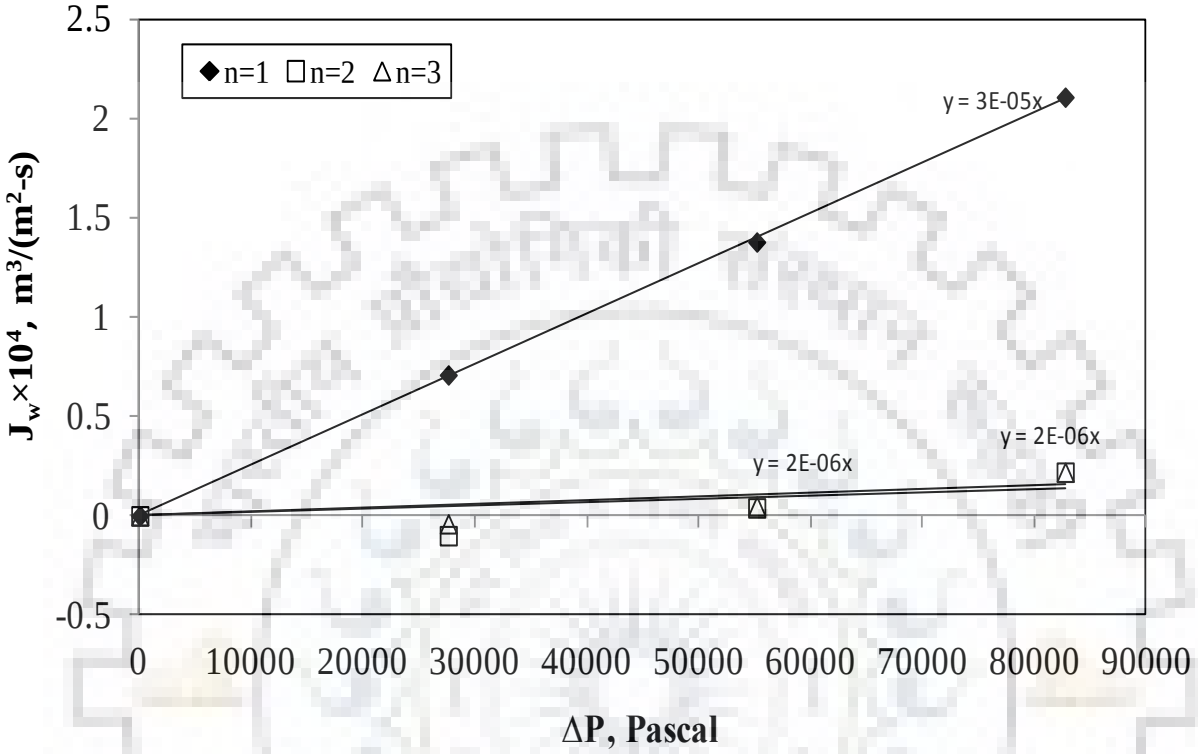


Figure 5.5: Comparison of permeability values after addition of different bilayers $(\text{-PEI-PSS})_n$ on PAA functionalized PVDF membrane.

5.2. Characterization of bacteriophage

In order to verify the cultures obtained, in terms of shape and size, TEM image was obtained as shown in Figure 5.6. The red arrow indicates the icosahedral symmetry of phage. The measured diameter of the MS2 phage is 28nm, which is in good agreement with the reported literature [37].

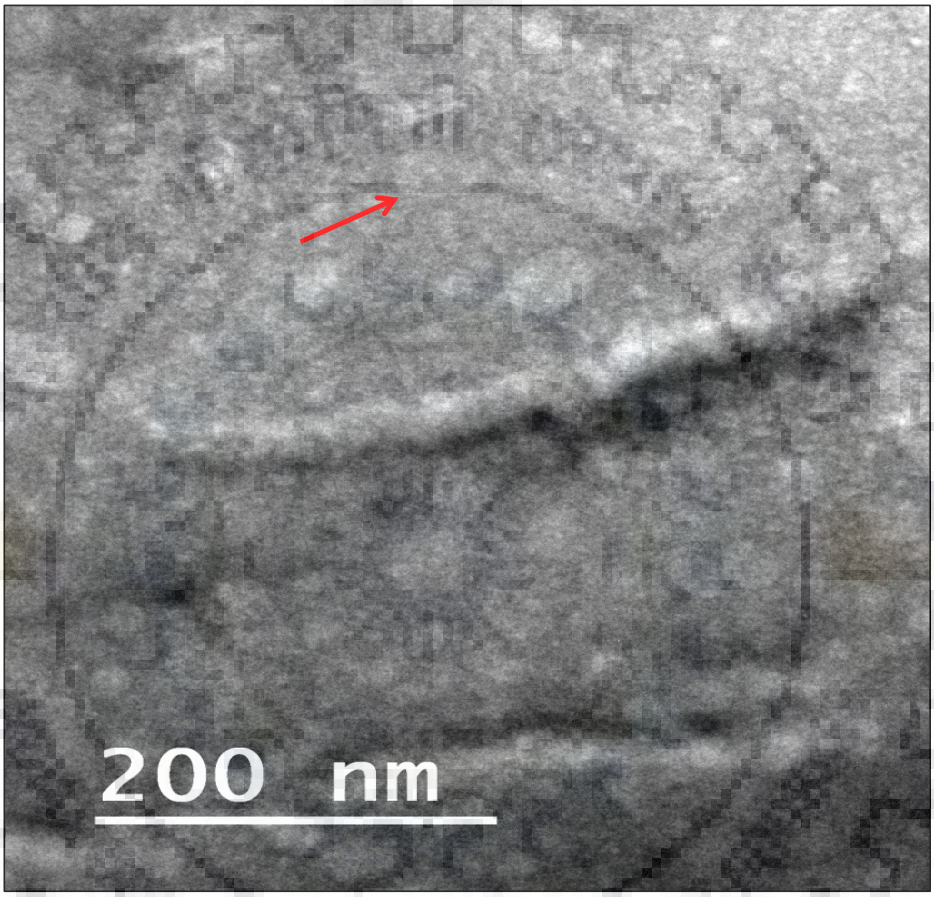


Figure 5.6: TEM image representing MS2 phage.

5.3. Bacteriophage filtration

The filtration experiments were conducted with the functionalized PVDF membranes. The mechanism of virus rejection was governed by the electrostatic repulsion between the negatively charged functionalized membrane and the negatively charged virus particles. The pH of the buffer stream used for the study was maintained at near neutral, i.e. 6.5. Phage became negatively charged as pI of the virus (=3.5) is less than the operating pH

(6.5). Further studies were carried out using feed concentration of 1.2×10^{6} pfu/mL of MS2 in media. Approximately, 100mL of the phage broth was filtered through the membrane. The concentration of MS2 in the filtrate samples was determined through plaque forming unit (PFU) assay. As depicted in Figure 5.7, PFU assay of the permeate showed very few colonies as compared to the feed and the retentate. This is because virus particles in the feed were primarily rejected by the functionalized membrane. Very few particles permeated through the membrane, whereas majority of the particles were rejected and ended up in the retentate.

Separation performance of the membrane was investigated by monitoring the flux decline profile during virus filtration. Figure 5.8 illustrates that the initial filtrate flux for first cycle was 5.48×10^{-10} $m^3/(m^2\text{-}s)$, which decreased to 3.26×10^{-10} $m^3/(m^2\text{-}s)$ over 60 min of filtration. This decrease is attributed to the deposition of foulants present in the media (protein molecules) during the process. With the second filtration cycle, flux decreased to a low value of 0.98×10^{-10} $m^3/(m^2\text{-}s)$, thereby requires washing.

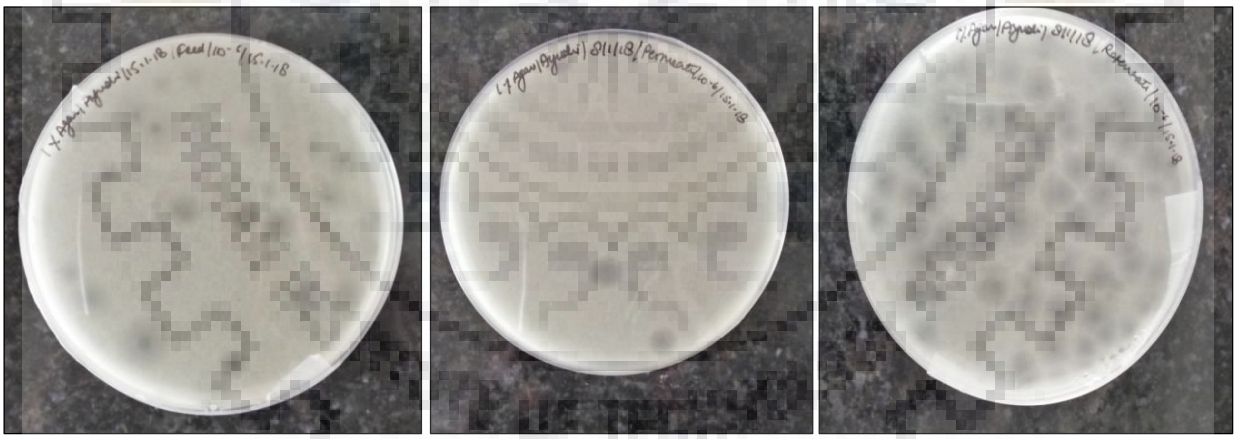


Figure 5.7: Image of agar plates used to determine number of plaque forming units of feed, permeate and retentate.

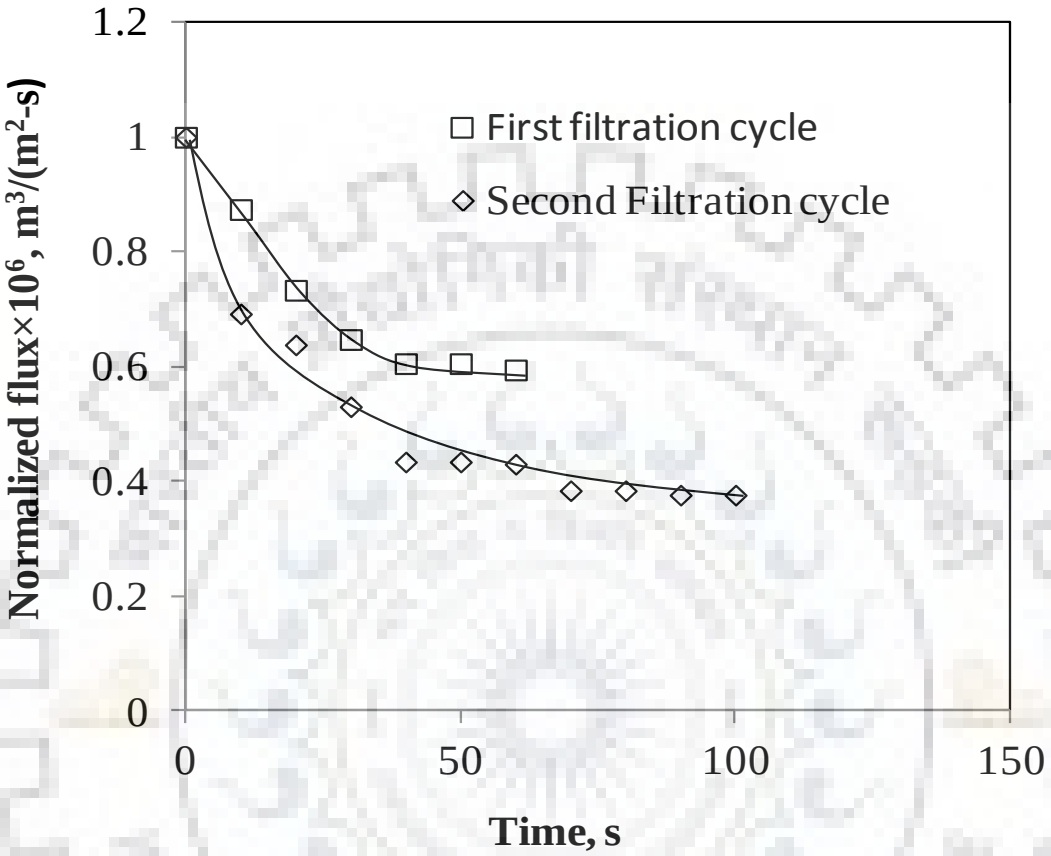


Figure 5.8: Comparison of flux values during the course of filtration during the first and second cycle from PVDF-PAA-$(PEI-PSS)_3$ membrane.

To evaluate the effect of number of bilayers on virus filtration performance, experiments were also conducted with one, two and three bilayers i.e. PVDF-PAA-(PEI-PSS)$_1$, PVDF-PAA-(PEI-PSS)$_2$ and PVDF-PAA-(PEI-PSS)$_3$. The performance of virus filtration is typically expressed by the Log Reduction Value (LRV), which is defined as a measure of ability of the treatment process to remove microorganisms. LRVs are determined as per the below mentioned equation:

$$LRV = -\log_{10} \frac{Concentration_{filtrate}}{Concentration_{feed}}$$

LRV value of 1 indicates 90% removal of the microbe, LRV of 2 is equivalent to 99% removal, LRV of 4 is corresponds to 99.9% removal and so on. Therefore, higher value of LRV indicates better rejection characteristics of the membrane.

Table 5.1 shows that the LRV values for functionalized membranes with one, two and three bilayers were 0.85, 0.98 and 1.1, respectively. The commercially available virus filtration membranes, such as Ultipor DV20 has reported LRV values of 2.66×10^{-11} $m^3/(m^2\text{-s-Pa})$[38]. So, the performance of the functionalized membranes was close to that of the commercial membranes. Besides, the LRV can be improved with addition of more bilayers. Table 5.1 also reveals that the LRV value increased with increase in the number of bilayers within functionalized membrane. The observed increase was because of the higher amount of negative charges with more number of bilayers, which accounted for the strong repulsion between the virus particles and the negatively charged groups. The greater pore coverage with more bilayers also attributed to the increase in the LRV. In contrary, as expected, permeability showed decline with deposition of more bilayers due to the additional resistance offered by the layers. However, only marginal difference in permeability was observed after addition of third bilayer. These results clearly indicate a trade-off between the LRV and the permeability. With increase in number of layers, the LRV increases, but the permeability decreases. Therefore, optimum value for the number of bilayers should be found out to maintain a balance between the required LRV (quality of product) and the acceptable permeability (quantity of product). Although, the permeability decreased with increase in number of bilayers, it was almost 2 orders of magnitude higher than the reported permeability value of the commercially available virus filters, which was of the order of 10^{-11} $m^3/(m^2\text{-s-Pa})$ [38]. Moreover, the pressure drop (energy) requirement was significantly lower than the commercially available ultrafiltration (UF) or nanofiltration (NF) membranes, as the

experiments were conducted at 5psi (34.5 kPa) trans-membrane pressure, whereas the existing virus filters are operated at 30psi (210 kPa) trans-membrane pressure. This offers an additional advantage of lower energy requirement, making the overall process cost effective. Operation at lower pressure also retains the bioactive components of the process stream. Thus, the functionalized membranes developed in this study were superior to the commercially available membranes in many aspects of virus filtration. In addition, opportunities exist to further improve the performance of the functionalized membranes.

Number of bilayers (n)	Sample	PFU/mL	LRV	% reduction	Permeability ($m^3/(m^2$-s-Pa))
1	Feed	8.5×10^6	0.85	85.88	3×10^{-9}
	Permeate	1.2×10^6			
	Retentate	1.07×10^7			
2	Feed	1.9×10^6	0.978	89.5	2×10^{-10}
	Permeate	2.0×10^5			
	Retentate	6.7×10^6			
3	Feed	1.2×10^6	1.079	91.67	2×10^{-10}
	Permeate	1.0×10^5			
	Retentate	1.7×10^6			

Table 5.1: Log Reduction Value (LRV) of MS2 phage through various levels of functionalization on PVDF-PAA-$(PEI-PSS)_n$ membrane.

5.4. Flux recovery

During the course of virus filtration, layers of foulant molecules (mainly media proteins) were adsorbed on the membrane matrix. Fouling affects membrane performance, therefore efficient cleaning strategies have to be adopted depending upon the properties of the foulant molecules. In this study, two strategies were adopted to mitigate fouling from the membrane matrix. Mild washing was carried out by permeating 0.5M NaCl solution through the membrane. There was significant flux recovery without any change in LRV value, suggesting the removal of the foulant molecules without damaging the functional bilayers deposited on the membrane are intact (Figure 5.9).

In another approach, reusability of the membrane matrix was investigated by cleaning it under harsh conditions, such as with detergent Triton X. Since the functionalized architecture is created by reversible electrostatic interaction among the subsequent layers, they can easily be detached by simple manipulation of operating conditions, such as pH. This is particularly useful when the membrane is significantly fouled due to the non-specific deposition of the biomolecules and lost its activity. In order to reuse a significantly fouled membrane, the functional layers can be detached by keeping the initial PAA backbone intact. Triton X (0.1% w/v, pH=10.5) was permeated through the membrane under convective flow for 45 min and flux data was tabulated. Figure 5.9 reveals that after cleaning with Triton X, permeability has increased significantly. It can be speculated from the final flux data that cleaning under harsh conditions dislodged two bilayers attached to the membrane.

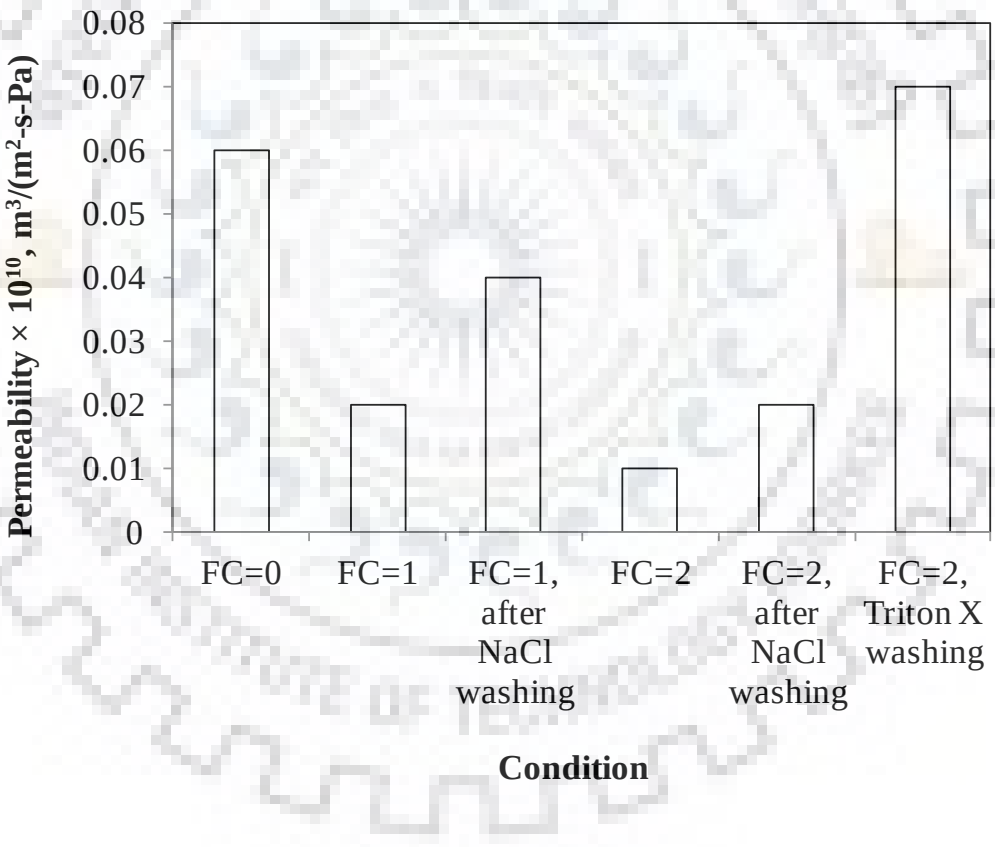


Figure 5.9: Representative plot showing flux recovery after washing the membrane under different conditions. FC= Filtration cycle with the MS2.

6. CONCLUSION

The study emphasized on the development of polymeric membrane by alternative attachment of polycations and polyanions within the membrane matrix, i.e. by layer-by-layer attachment technique. *In situ* polymerization of PAA onto the hydrophobic PVDF membrane was done to provide a stable anchor for the layer formation. The experiments have clearly demonstrated the change in morphology of membrane after PAA attachment, making it hydrophilic. Three bilayers of PEI-PSS were deposited on the membrane, making it net negatively charged. MS2 phage (pI = 3.5), which was negatively charged at pH 6.5 in the media, was rejected by the negatively charged membrane accounting for virus retention.

Virus filtration was quantified by counting the number of plaques in the permeate, the feed and the retentate through DAL method. The PFU assay revealed the Log Reduction value (LRV) of 1.1 which accounts for 90 % virus removal. Although the existing technologies have reported LRV>4, they are energy intensive, require high operational cost and also compromise with the membrane performance in terms of flux. The membrane developed in this study showed 2 fold increase in the permeability value. Also, the process is conducted at much lower pressure drop, thereby making the process cost effective and also retaining the bioactive properties of the components.

In order to check the reusability of the membrane, membrane was washed with Triton X, at pH = 10.5 to mitigate the fouling deposited during the course of filtration. This detached the layers of polyelectrolytes attached to the membrane (-PEI-PSS) along with the foulant layers present within it. The PAA backbone, however, remained intact, thereby providing an opportunity to rebuild the functional layers and reuse the same membrane. In summary, functionalized membrane developed by layer-by-layer attachment technology demonstrated significant improvements in performance for virus filtration. With further tuning of the membrane functionalization technique and process optimization, this technology promises to deliver an efficient bioprocess for biopharmaceutical industries.

7. FUTURE ASPECTS

- Optimization of number of bilayers to be attached to attain greater LRV value.
- Optimization of process conditions (pH, ionic strength, flow rate).
- Incorporation of some model proteins in the media mimicking the actual process stream of biopharmaceutical industries.

REFERENCES

1. Ireland, T., Lutz, H., Slwak, M. & Bolton, G. Viral filtration of plasma-derived human IgG. *Pharm. Technol. Eur.* **18,** 43–49 (2006).

2. Rabenau, H., Ohlinger, V., Anderson, J., Selb, B., Cinatl, J., Wolf, W., Frost, J., Mellor, P. & Deorr, H. W. Contamination of Genetically Engineered CHO-cells by Epizootic Haemorrhagic Disease Virus (EHDV). *Biologicals* **21,** 207–214 (1993).

3. Brorson, K., Krejci, S., Lee, K., Hamilton, E., Stein, K. & Xu, Y. Bracketed generic inactivation of rodent retroviruses by low pH treatment for monoclonal antibodies and recombinant proteins. *Biotechnol. Bioeng.* **82,** 321–329 (2003).

4. Kern, G. & Krishnan, M. Virus removal by filtration: Points to consider. *BioPharm International* **19,** 32–41 (2006).

5. Dishari, S. K., Venkiteshwaran, A. & Zydney, A. L. Probing effects of pressure release on virus capture during virus filtration using confocal microscopy. *Biotechnol. Bioeng.* **112,** 2115–2122 (2015).

6. Ohwada, T., Makoto, O., Tatebe, K., Ohmura Y. & Shimura, K. Pathogenic substance removing materal and a blood filter. US 5,547,576 (1996).

7. Mudler, M. Basic principles of membrane technology. *Kluwer Academic Publishers, Dordrecht, Netherlands* (1996).

8. Loeb, S. & Sourirajan, S. High Flow Porous Membranes for separating water from saline solutions. US 3,133,132. 9 (1964).

9. Hollman, a. M. & Bhattacharyya, D. New Insights into Membrane Science and Technology: Polymeric and Biofunctional Membranes. [In *Membrane Science and Technology* **8,** 329–352 (2003)].

10. Hestekin, J. A., Bachas, L. G. & Bhattacharyya, D. Poly(amino acid)-functionalized cellulosic membranes: Metal sorption mechanisms and results. *Ind. Eng. Chem. Res.* **40,** 2668–2678 (2001).

11. Datta, S., Cecil, C. & Bhattacharyya, D. Functionalized membranes by layer-by-layer assembly of polyelectrolytes and in situ polymerization of acrylic acid for applications

in enzymatic catalysis. *Ind. Eng. Chem. Res.* **47,** 4586–4597 (2008).

12. Schisla, D. K., Carr, P. W. & Cussler, E. L. Hollow Fiber Array Affinity Chromatography. *Biotechnol. Prog.* **11,** 651–658 (1995).

13. Liu, Y., Suen, S., Huang, C. & Changchien, C. Effects of spacer arm on penicillin G acylase purification using immobilized metal affinity membranes. *J. Memb. Sci.* **251,** 201–207 (2005).

14. Hollman, A. M. & Bhattacharyya, D. Controlled permeability and ion exclusion in microporous membranes functionalized with poly(L-glutamic acid). *Langmuir* **18,** 5946–5952 (2002).

15. Oh, S. J., Kim, N. & Lee, Y. T. Preparation and characterization of PVDF/TiO2 organic-inorganic composite membranes for fouling resistance improvement. *J. Memb. Sci.* **345,** 13–20 (2009).

16. Xu, J., Dozier, A. & Bhattacharyya, D. Synthesis of nanoscale bimetallic particles in polyelectrolyte membrane matrix for reductive transformation of halogenated organic compounds. *J. Nanoparticle Res.* **7,** 449–467 (2005).

17. Gabriel, E. M. & Gillberg, G. E. In situ modification of microporous membranes. *J. Appl. Polym. Sci.* **48,** 2081–2090 (1993).

18. Decher, G. Fuzzy nanoassemblies: Toward layered polymeric multicomposites. *Science* **277,** 1232–1237 (1997).

19. Hollman, A. M. & Bhattacharyya, D. Pore assembled multilayers of charged polypeptides in microporous membranes for ion separation. *Langmuir* **20,** 5418–5424 (2004).

20. Ariga, K., Hill, J. P. & Ji, Q. Layer-by-layer assembly as a versatile bottom-up nanofabrication technique for exploratory research and realistic application. *Phys. Chem. Chem. Phys.* **9,** 2319 (2007).

21. Lvov, Y, Hass, H., Decher, G., Mohwald, H., Mikhailov, A., Mtchedlishvily, B., Morgunova, E. & Vainshtein, B. Successive Deposition of Alternate Layers of Polyelectrolytes and a Charged Virus. *Langmuir* **10,** 4232–4236 (1994).

22. Farshid, M., Taffs, R. E., Scott, D., Asher, D. M. & Brorson, K. The clearance of viruses and transmissible spongiform encephalopathy agents from biologicals. *Curr Opin Biotechnol.* **16**, 561-567 (2005).

23. Prowse, C. & Robinson, A. E. Pathogen inactivation of labile blood components. *Transfusion Medicine* **11,** 147 (2001).

24. Lute, S., Bailey, M., Combs, J., Sukumar, M. & Brorson, K. Phage passage after extended processing in small-virus-retentive filters. *Biotechnol. Appl. Biochem.* **47,** 141 (2007).

25. Schreiber, G. B., Busch, M. P., Kleinman, S. H. & Korelitz, J. J. The risk of transfusion-transmitted viral infections. The Retrovirus Epidemiology Donor Study. *N. Engl. J. Med.* **334,** 1685–90 (1996).

26. DiLeo, A. J., Vacante, D. A. & Deane, E. F. Size exclusion removal of model mammalian viruses using a unique membrane system, part II: Module qualification and process simulation. *Biologicals* **21,** 287–296 (1993).

27. Brough, H., Antoniou, H., Carter, J., Jakubik, J., Xu, Y. & Lutz, H. Performance of a novel Viresolve NFR virus filter. *Biotechnol. Prog.* **18,** 782–795 (2002).

28. Oshima, K. H., Evans-Strickfaden, T. T., Highsmith, A. K. & Ades, E. W. The use of a microporous polyvinylidene fluoride (PVDF) membrane filter to separate contaminating viral particles from biologically important proteins. *Biologicals* **24,** 137–145 (1996).

29. Yokoyama, T, Murai, K., Murozuka, T., Wakisaka, A., Tanifuji, M., Fujii, N. & Tomona, T. Removal of small non-enveloped viruses by nanofiltration. *Vox Sang.* **86,** 225–229 (2004).

30. Herath, G., Yamamoto, K. & Urase, T. Removal of viruses by microfiltration membranes at different solution environments. in *Water Science and Technology* **40,** 331–338 (1999).

31. Wickramasinghe, S. R., Stump, E. D., Grzenia, D. L., Husson, S. M. & Pellegrino, J. Understanding virus filtration membrane performance. *J. Memb. Sci.* **365,** 160–169 (2010).

32. Troccoli, N. M., McIver, J., Losikoff, A & Poiley, J. Removal of viruses from human intravenous immune globulin by 35 nm nanofiltration. *Biologicals* **26,** 321–329 (1998).

33. Oshima, K. H., Evans-Strickfaden, T. T., Highsmith, A. K. & Ades, E. W. The use of a microporous polyvinylidene fluoride (PVDF) membrane filter to separate contaminating viral particles from biologically important proteins. *Biologicals* **24,** 137–45 (1996).

34. Miesegaes, G., Lute, S. & Brorson, K. Analysis of viral clearance unit operations for monoclonal antibodies. *Biotechnol. Bioeng.* **106,** 238–246 (2010).

35. LaCasse, D, Genest, P., Pizelli, K., Greenhalgh, P., Mullin, L. & Slocum, A . Impact of process interruption on virus retention of small-virus filters. *Bioprocess Int.* **11,** 34–44 (2013).

36. Fiers, W., Contreras, R., Duerinck, R., Haegeman, G., Iserantant, D., Merregaert, J, Min Jou, W., Molemans. F., Raeymaekers, A., Volckaert, G & Ysebaert, M. Complete nucleotide sequence of bacteriiphage MS2 RNA: primary and secondary structure of the replicase gene. *Nature* **260,** 500–507 (1976).

37. Aher, A., Papp, J., Colburn, A., Wan, H., Hatakeyama, E. & Prakash, P. Naphthenic acids removal from high TDS produced water by persulfate mediated iron oxide functionalized catalytic membrane , and by nanofiltration Naphthenic acids removal from high TDS produced water by persulfate mediated iron oxide functionalized catalytic membrane , and by nanofiltration. *Chem. Eng. J.* **327**, 573-583 (2018).

38. Kuzmanovic, D. A., Elashvili, I., Wick, C., Connell, C. O. & Krueger, S. Bacteriophage MS2 : Molecular Weight and Spatial Distribution of the Protein and RNA Components by Small-Angle Neutron Scattering and Virus Counting. *Structure* **11,** 1339–1348 (2003).