Development of fibro-porous polymeric membranes for WBC syringe filter by electrospinning

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SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES &

TECHNOLOGY

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DECLARATION

I, **ATHIRA K R**, hereby declare that I had personally carried out the work depicted in the thesis entitled, "**Development of fibro-porous polymeric membranes for WBC syringe filter by electrospinning"** under the joint guidance of **Dr P RAMESH, SCIENTIST G, Division of Polymeric Medical Devices and Dr ANUGYA BHATT, SCIENTIST F, Division of Thrombosis Research** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "**Development of fibro-porous polymeric membranes for WBC syringe filter by electrospinning"** submitted by Athira K R in partial fulfilment for the degree of Master of Philosophy in Biomedical Research to be awarded by this Institute. The entire work was done by her under our joint supervision and guidance at Division of Polymeric Medical Devices, and Division of Thrombosis Research, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram.

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Development of fibro-porous polymeric membranes for WBC syringe filter by electrospinning

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For the degree of

Master of Philosophy in Biomedical Technology

Of

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ATHIRA K R

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ABBREVIATIONS

AABB	American Association of Blood Banks
ACD	Acid Citrate Dextrose
APS	Ammonium Persulfate
BP	Benzophenone
CMV	Cytomegalovirus
ESEM	Enviornmental Scanning Electron Microscope
EVAL	Poly ethylene-co-vinyl alcohol)
GVHD	Graft Versus Host Disease
HLA	Human Leukocyte Antigen
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphneyl tetrazolium bromide
NHFTRs	Non hemolytic febrile transfusion reaction
NVP	N-Vinyl Pyrrolidone
PBS	Phosphate Buffer Saline
PC	Phosphoryl choline
PCR	Polymerase chain reaction
PEO	Poly ethylene oxide
PLA	Poly lactic acid
PVP	Poly vinyl pyrrolidone
RBC	Red blood cell
TRALI	Transfusion related acute lung injury
TRIS	Transfusion related immune suppression
WBC	White Blood Cell

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SYNOPSIS

White Blood Cell (WBC) syringe filters can be considered as a simple and effective method for the removal of leukocyte and other blood components from small volume of blood. The main objective of the present work is to develop a filter material (filter media) by electrospinning technique which can be used as WBC syringe filter with high filtration efficiency.

This thesis has been divided into four chapters; the first chapter briefly discusses about the problems in blood transfusion due to leukocyte containing blood, the need for leukodepletion syringe filter, currently available techniques for leukodepletion and types of filtrations, structure of leukodepletion syringe filter and the polymers used for fabricating the filter membrane is also mentioned here. Review of literature along with significance of developing a leukodepletion syringe filter with high efficiency is also given under first chapter.

The second chapter deals with materials and methodology employed in the study, including the fabrication of 3D printed syringe filter prototype, fabrication of filter membranes by electrospinning of modified EVAL, and different crosslinking techniques employed. The fabricated filter membranes are characterized by different physico-chemical techniques and *in-vitro* cytotoxicity assay. The physico-chemical properties of the fabricated membranes were analyzed by Fourier transform infrared spectroscopy (FTIR), Universal testing machine (UTM), Dynamic mechanical analysis (DMA). The surface morphology of the electrospun membranes were analyzed by Scanning Electron Microscopy (SEM).

The extraction studies of the filter membranes were evaluated with UV-visible spectrometer.

Third chapter deals with the results and discussion of the present study. The results of the experiments conducted categorized into four sections. In the first section we are discussing about the fabrication of modified EVAL membranes and optimization of their crosslinking under different conditions. The second section under chapter 3 deals with the comparative analysis of physico-chemical characterizations done in various membranes. Third section discusses the leukodepletion efficiency and hemocompatibility of the fabricated membrane and compared that with commercially available filter. The *in-vitro* cytotoxicity evaluation of the filter membranes was discussed in the fourth section.

Fourth chapter concludes that *in-situ* polymerized and grafted NVP on EVAL is showing better leukodepletion on comparing with PALL filter membrane. The future aspect of the present study is also stated.

CHAPTER 1

INTRODUCTION

1.1 Need for leukoreduction

India urges a huge burden of patient population requiring multiple transfusions. As per the National AIDS Control Organization, around 12.5 million units of blood required in our country annually, and this includes the hemato oncology patients requiring different types of blood component support and patients needed regular transfusions. The donor blood leukocytes may be detrimental to multi-transfused patients in some special circumstances of blood transfusion. In 1950s it is identified that the administration of leukocyte rich blood induce collateral problems like non-hemolytic febrile transfusion reactions (NHFTRs), decreased immune defense to infections, alloimmunization to human leukocyte antigens (HLA Alloimmunization), transfusion related acute lung injury (TRALI), transfusion related immune suppression (TRIS), graft versus host disease (GVHD) or transfer of viral infections such as those caused by cytomegalovirus (CMV) or human T-cell leukemia virus I (HTLV-I) etc.

Non-hemolytic febrile transfusion reactions (NHFTR) are commonly associated with the assembly of leukocyte antibodies, especially in multi transfused patients. These reactions can be prevented by the transfusion of leukocyte depleted red cells. Transfusion-associated graft-versus-host disease (TA-GvHD) caused by the engraftment of viable donor leukocytes, mainly T cells, following a transfusion of cellular products. At present the prevention of transfusion associated graft-versus–host disease is achieved by irradiation of

blood products. The adequate reduction of leukocytes will reduce the risk of TA-GvHD. The recipient's immune response to these infused cells generally prevent TA-GvHD, but when the recipient is immune suppressed, or when there is HLA haplo identical match between donor and recipient, the infused lymphocytes can proliferate in vivo and attack the host tissues (Williamson & Warwick, 1995). Removal of those cells before transfusion has been proposed to scale back the incidence of TA-GvHD (Chun *et al*., 2020; Akahoshi *et al*.,1992). These adverse effects can be minimized if the leukocyte counts are below a threshold of 10^6. The American Association of Blood Banks (AABB) recommends this count to be less than 5×10^6 /unit after leukoreduction (3 log reduction giving 99.9 % leukocyte removal) with almost 85 % of other blood components remained. Another independent study also strongly supports that leukoreduction to a level of $< 5 \times 10^6$ of residual level per transfusate can prevent primary HLA alloantibody formation (V. Novotny *et al*., 1995). At present the simplest leukoreduction are often achieved with the assistance of 3rd and 4th generation leukofilters, both in laboratory and patient bed side.

There has seen a big paradigm shift since the past fifty years within in the provision of allogenic blood products. For more than half a century ago, most of the blood transfused was whole blood. However, since the 1960s, blood has been separated into its various components like RBCs, platelets, and plasma. The latter has been further subjected to various manufacturing processes so that individual plasma proteins are often purified and made available to specific patients with specific plasma protein deficiencies.

1.2 Need for Leukodepletion Syringe Filter

White Blood Cell syringe filter is single use filter cartridge attached to the end of a syringe. It consists of a housing with filter membrane. Syringe filter can be considered as a simple

and efficient technique to remove WBC from blood. Less than 15 mL of blood is filtered by this method. Application includes direct analysis of trapped WBCs, or for removal of WBCs in most antibody-based diagnostics to efficiently analyze leukocyte protein extracts for expression profiling by specific antibodies. The trapped WBC's can be cultured and converted to useful materials in the diagnostic and therapeutic applications for various autoimmune diseases and cancer. It can also be used to extract nucleic acid which can then be prepared for Polymerase chain reaction (PCR) applications It is also used in antigen modulation studies, cell receptor analysis, lymphocyte recovery for cell therapy ,monoclonal antibody development and stem cell research applications(Nicholson *et al*., 2020). The growing need for syringe filters to accomplish the above-mentioned purposes pave the way to carry out research in this field, which is of very social and clinical significance in this day.

1.3 Leukodepletion and Leukoreduction

The terms, leukoreduction and leukodepletion are sometimes used synonymously in literature, leukoreduction implies removal of leukocytes by gross removal method, whereas, leukodepletion is removal of leukocytes with the help of certain specific filters or devices.(Sharma & Marwaha, 2010)

1.4 Leukodepletion filters

Centrifugation techniques are not ideal because it leads to the loss of excessive red cells. Sedimentation with high relative molecular mass polymers produce excellent leukocyte depletion and little red cell losses. Filtration is both relatively straightforward and comparatively cheap, and leads to very effective red cell depletion with acceptable red cell losses. Freezing and thawing of red cells is extremely effective but is costly.

Modern leukocyte removal filters have been developed after years of modifications in design. Modern filters are composite filters in which synthetic microfiber material is arranged as a nonwoven web. To improve performance of the filter material it can be surface modified to alter the surface tension or charge. The filter design effectively promotes contact of blood with the filter material and reduces shear forces. Small-pore size leads to barrier phenomena that let retention of individual cells and increase the entire adsorptive area of the filter. Modifications in surface charge affect cell attraction to the fibers. Optimum interfacial surface tensions between blood components and filter fibers not only permit effective blood flow through small fiber pores, but also facilitate cell contact with the fabric. Barrier retention operates for all modern leukocyte removal filters and applies to all leukocyte subtypes. Barrier retention does not depend on cell viability, barrier retention is provided by retention by adhesion. Blood components differ in their relative adhesion to the filter fibers.(Dzik, 1993)

1.5 Types of filtrations

Filtrations can be performed in two different ways using leukodepletion filters. They are pre-storage filtration and post-storage filtration. Pre-storage filtration is done with the freshly collected blood from the voluntary donor immediately after the collection. After filtration, the filtered blood will be either stored or processed for further component separation. The post-storage filtration is performed with the stored blood or blood components only when required for the patient (Sharma and Marwaha, 2010), therefore post-storage filtration is also called as bed-side filtration. The pre-storage filtration is always preferred over post storage filtration because pre-storage filtration eliminates

cytokine accumulation and risk of leukotropic virus transmission. During pre-storage filtration the blood components can be thoroughly studied.

1.6 Structure of Leukodepletion syringe filter

Leukodepletion syringe filter consists of filter membranes assembled in an outer case. The filter will be equipped with two openings – inlet and outlet. The inlet directs the blood to be filtered, while outlet used collects the filtered blood. Based on the structure and properties of the membranes, the syringe filters are classified into two different types

(1) Symmetric syringe filter

(2) Asymmetric syringe filter

In Symmetric syringe filter the pore size of all the membranes will be uniform where as in asymmetric filter, there will be a steady decrease in the pore sizes of different layers from top to bottom (or within the direction of flow of blood). (Bruil *et al*., 1995)

Comparative studies on evaluation of these two types of syringe filters suggest that asymmetric type of filter is preferred to symmetric filter, because in symmetric syringe filter, there will be more chance for cell clogging on the membranes which ultimately results in pore plugging and prevents the retention of other components. However, in asymmetric syringe filter, the chances for pore plugging will be minimum thus ensuring maximum retention of other functional and viable blood components.

1.7 Polymers used for Leukodepletion

Virgin or chemically modified versions of various polymeric materials have been employed in the manufacture of leukocyte depletion filters. These include polyethylene

terephtalate (PET), polyamide (PA), polyester, polypropylene (PP), polyvinyl alcohol (PVA), and cellulose-based materials.(Mayuri. *et al*., 2021a)

1.8 Methods of fabrication

1. Melt blowing
2. Solvent casting and particulate leaching
3. Electrospinning

1.8.1 Melt blowing

It is a one-step process in which high-velocity fluid, normally air blows molten thermoplastic resin from an extruder die tip onto a conveyor or substrate to form a fine fibered self-bonded web.(McCulloch, 1999). The melt blowing process produce fibers of less than the magnitude of fibers produced by conventional melt spinning. Melt-blown membranes have high surface area and good barrier properties which make them excellent candidates for various filtration applications.(Hassan *et al*., 2013; Shambaugh, 1988)

1.8.2 Solvent casting and particulate leaching

Solvent casting is a simple method. It can control the pore size and porosity by selecting the particle size and the amount of added salt particles. The porous membrane prepared by solvent casting have thickness of less than 4mm.(Sin *et al*., 2010; Stevens *et al*., 2008)

1.8.3 Electrospinning

Electrospinning is a unique technique using electrostatic forces to produce fine fibers from polymer solutions or melts and the fibers thus produced have a thinner diameter (from nanometer to micrometer) and a larger surface area than those obtained from conventional spinning processes. It works mainly based on the principle that strong mutual electrical repulsive forces overcome weaker forces of surface tension in the charged polymer liquid.

Polymers are dissolved in some solvents before electrospinning, and when it (Bhardwaj &Kundu, 2010) completely dissolves forms polymer solution. The polymer fluid is then introduced into the capillary tube for electrospinning. In the electrospinning process, a polymer solution held by its surface tension at the end of a capillary tube is subjected to an electric field and an electric charge is induced on the liquid surface due to this electric field. When the electric field applied reaches a critical value, the repulsive electrical forces overcome the surface tension forces. Eventually, a charged jet of the solution is ejected from the tip of the Taylor cone and an unstable and a rapid whipping of the jet occurs in the space between the capillary tip and collector which leads to evaporation of the solvent, leaving a polymer behind. The jet is only stable at the tip of the spinneret and after that instability starts. Thus, the electrospinning process offers a simplified technique for fiber formation

1.9 Requirement of Leukodepletion syringe filters

WBC syringe filter represents a simple and efficient method to isolate blood cell components which has variety of applications inantigen modulation studies, cell receptor analysis, lymphocyte recovery for cell therapy, antibody development and somatic cell research applications. Compared to buffy coat density gradient and large size filter methods WBC syringe filter is most practical method for leukocyte depletion.

To best of our knowledge, WBC Acrodisc® syringe filter from Pall Laboratory is the only filter available in the market and they use patented Leukosrb^{TM}media. The filter material is either surface modified to alter surface tension or charge to improve performance. The standard price of the imported filter materials is comparatively higher therefore most of the hospitals and laboratories of India use conventional ways to removal of leukocytes from

blood samples. The main objective of the current work is to develop a filter material with high filtration efficiency to develop WBC syringe filter using cost-effective methods like electrospinning.

The effectiveness of leukodepletion by filters depends upon physico-chemical properties of the membranes, blood parameters and filtration parameters. Membrane parameters include material chemistry/composition or surface chemistry, surface charge, pore diameter, fiber diameter, porosity, wettability etc. (Mayuri *et al*., 2021b)

1.10 Electrospinning as a feasible method of fabrication

Electrospinning is a highly versatile technique where the surface topography, fiber morphology and orientation are largely influenced by solution properties and operating conditions. Since the rheology of the polymer solution is important to the fiber formation process, solution properties such as polymer molecular weight and concentration directly affect fiber properties. Conductivity of the polymer solution is additionally known to vary properties of the resultant fibers. Fiber properties also are directly suffering from operating conditions like applied voltage, solution flow and tip-collector distance. Ambient conditions like temperature and humidity of the electrospinning chamber also can alter fiber morphology.(Ahmed *et al*., 2015)

LITERATURE REVIEW

State of the art techniques for leukocyte recovery or depletion use several methods to enhance the overall efficiency of leukodepletion filter. Cellular blood components have variable numbers of donor leukocytes. Passenger leukocytes in red blood cell and platelet transfusions have been associated with adverse effects in recipients. These effects include alloimmunization to leukocyte antigens, febrile reactions after transfusion, pulmonary dysfunction, and refractoriness to platelet transfusion, and the development of graft-versus-host disease. In addition, the transmission of certain infectious agents may be caused exclusively by leukocytes that are present in transfused cellular blood components. Several studies have been carried out to control the undesirable blood transfusion reactions caused by the circulation of donor leukocyte.

Alexander Flemming in 1926 discovered a method for leukocyte depletion of blood by cotton wool filtration. The goal of his research was to prepare a diluent suitable for the investigation of the effect of leukocyte on bacterial growth. This was the basis for the preparation of WBC-reduced red cell (RBC) concentrates for transfusion. In 1972, Diepenhorst *et al*. described the primary WBC-reduction filter, a column crammed with tightly packed cotton fibers that provided a network with equally distributed pores. The original cotton wool was soon replaced by cellulose acetate fibers, because of convenience and the greater possibility of controlling the quality of the fibers. The more recent availability of nonwoven materials has led to the event of flatbed filters with coarse layers for the removal of gross blood clots and with medium and fine layers for the removal of WBCs.(Diepenhorst *et al*., 1972)

There are various methods to remove leukocytes from blood, and among them filters shown to be more efficient. In 1995, Bruil *et al*. discussed about the development leukocyte filters, the various mechanism of filtration, and mathematical model to describe the leukocyte filtration. (Bruil *et al*., 1995a)

Several factors need to be considered when evaluating the techniques used for leukocyte depletion of blood in the laboratory. The first is the degree of leucocyte depletion which can be achieved. Blood containing less than $0.5*10^9$ induce febrile reactions in alloimmunized patients.(Perkins *et al*., 1966)

Patients with a high titre of anti-leukocyte antibodies, may require a greater degree of leukocyte depletion for the prevention of NHFTR, while others, perhaps with a lower titre, may tolerate transfusion of greater numbers of incompatible leucocytes. Second, the effectiveness of platelet depletion should be known as NHFTR can occasionally be caused by antibodies against platelets. In addition, the degree of red cell loss should be analysed as it may be an important factor when comparing techniques that are equally effective in depleting blood of leukocytes.(Decary *et al*., 1984; Thulstrup, 1971)

A variety of techniques have been developed to prepare leukocyte-poor blood components. Although many methodological variations and combinations of these techniques exist, five basic methods can be distinguished; (1) differential centrifugation, (2) sedimentation, (3) cell washing, (4) freezing and thawing, and (5) filtration.

Differential centrifugation of blood to reduce the leukocyte count is the earliest method and still remains as the most frequently used one. However, this method consists of open system handling and gives poor leukocyte removal. Hence various modifications to this

basic technique were also applied and include inverted centrifugation, double centrifugation, upright centrifugation etc. Centrifugation techniques are not ideal because it leads to the loss of excessive red cells.(Meryman & Hornblower, 1986)

Sedimentation with high relative molecular mass polymers produce excellent leukocyte depletion and little red cell losses. Filtration is both relatively straightforward and comparatively cheap, and leads to very effective red cell depletion with acceptable red cell losses. Freezing and thawing of red cells is extremely effective but is costly.

The different methods of leukocyte depletion include buffy coat removal, and deglycerolisation of red cells and apheresis.(Kikugawa & Minoshima, 1978; V. M. Novotny *et al*., 1995). The initial standards for leukodepletion required removal of a minimum of 70% white blood cells and retention of 70% of the first red cells(V. M. Novotny *et al*., 1995). These values have been under review constantly, and blood is considered leukodepleted if the total leukocyte content per unit of blood is $<5 \times 10^9$ and the red cell product should have retained at least 85% of the original red cells (Novotny *et al*., 1995). Sedimentation with high relative molecular mass polymers produces excellent leukocyte depletion and little red cell losses. Filtration is both relatively straightforward and comparatively cheap, and leads to very effective red cell depletion with acceptable red cell losses. Freezing and thawing of red cells is extremely effective but is costly.

Among these, filtration serves as the most addressed method due to its simplicity and efficiency(Bruil *et al*., 1995a). Thus, membrane-based filtration of blood has been accepted as the best methodology for leukocyte reduction since 1990s.

Leukocytes can be removed by using a filter consists of nonwoven fabric material. A study investigated the use of phosphorylcholine (PC)containing polymers as a coating for

leukocyte filters and found that by the use of such coated filters the platelet recovery would be enhanced by around 30%.(Lewis *et al*., n.d.)

Other polymers used as membranes for Leukodepletion filters are poly ethylene terephthalate, poly butylene terephthalate, poly propylene (Cao *et al*., 2012; Gérard *et al*., 2011)

The most common mechanisms that are involved in leukocyte filtration include blocking or straining, bridging, interception, and adhesion (Figure 1). (Bruil *et al*., 1995a)

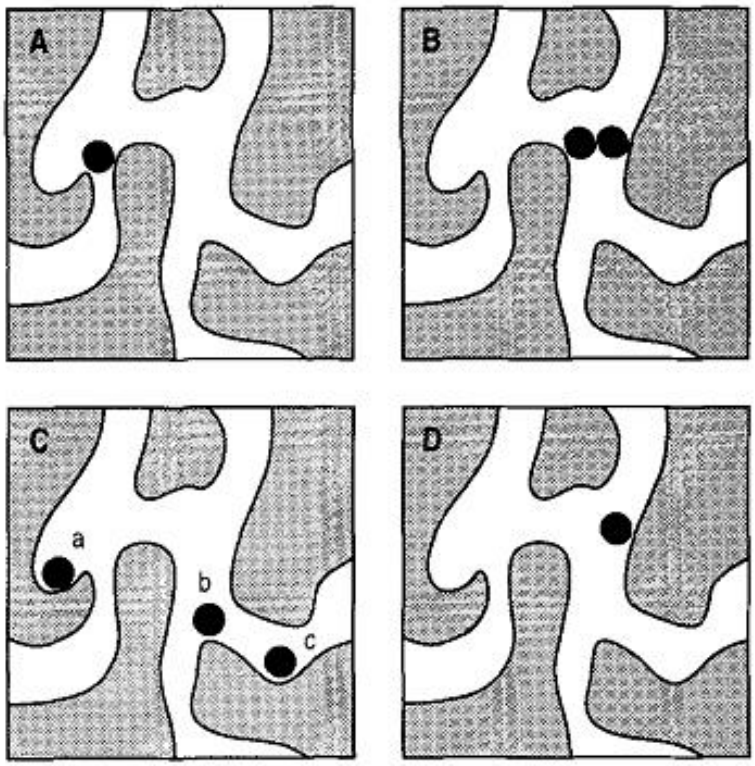


Figure 1: Mechanism of leukocyte removal by filtration. (A)Blocking when particle size is larger than pore diameter, (B) Bridging at high particle concentration, (C) Interception trapping at filter sites other than small pores, (D) Adhesion predominant mechanism .(Bruil *et al*., 1995b)

Stenekar *et al* found that the WBC and platelet removal is partially due to the formation of cell clusters on the surface of the layers and the major mechanism was trapping or mechanical sieving of the cells by the pores in the fibrous networks rather than adhesion.(Pietersz *et al*., 1992)

Recently, research activities are being done to develop filter membranes for syringe filter setup. Nicholson *et al* have evaluated the performance of PALL acrodisc syringe filter by means of WBC adhesion and recovery. They have focused to develop a filter tool for filtering small quantity of blood in order to help laboratory scientists (Nicholson *et al*., 2020).

Objective of the study

1. Synthesis of fibro porous polymeric membranes by electrospinning EVAL with different modifications.
2. Morphological study of the synthesized membranes using scanning electron microscopy.
3. Study on the mechanical properties of the material by universal testing machine.
4. Study on wetting characteristics.
5. Study on cytocompatibility of the material.
6. Study on the filtration characteristics.

Hypothesis

Would hydrophilic modifications of EVAL membranes be used to improve WBC filtration for syringe filter?

CHAPTER 2

MATERIALS AND METHODS

In this part of the study, efforts were made to 1) fabricate modified poly(ethylene-co-vinyl alcohol) filter membranes by electrospinning 2) perform different crosslinking techniques and its optimization 3) study physico-chemical characterization of the fabricated filter membranes 4) carry out *in-vitro* cytotoxicity evaluation of the membranes 5) accomplish whole blood filtration studies using various filter membranes developed and 6) execute *in-vitro* hemocompatibility studies.

This work focus on fabricating modified membranes of poly(ethylene-co-vinyl alcohol) with the incorporation of hydrophilic polymers like polyethylene oxide and polyvinyl pyrrolidone and also performing *in-situ*polymerisation of N-vinyl pyrrolidone with poly(ethylene-co-vinyl alcohol) and further electrospinning. A prototype of syringe filter was developed using 3D printing. The methodology of printing syringe filter prototype is discussed in section 2.2.1. The section 2.2.2 discusses about the preparation and fabrication of modified filter membranes. The methods of crosslinking employed to crosslink the blended systems is discussed in section 2.2.3. Evaluation of chemical characterization, porosity, surface morphology of electrospun fiber, surface wettability, static mechanical properties, dynamic mechanical properties, *in-vitro* cytotoxicity, whole blood filtration of various membranes and its comparison and *in-vitro* hemolysis is presented.

2.1 Materials

Poly (ethylene-co-vinyl alcohol) (EVAL) with 32 mol % of ethylene content, Polyvinyl pyrrolidone (PVP) (Mw= 3,60,000), Polyethylene oxide (PEO) (Mw = 6,00,000), N-vinyl pyrrolidone (NVP) and Ammonium Persulfate (APS) were purchased from Sigma Aldrich (USA). Benzophenone (diphenyl ketone) extrapure crystals were supplied by Sisco Research Laboratories Pvt. Limited (SRL chem), India. Isopropanol was obtained from Merck Life Science Pvt. Limited, Bangalore, India. Gluteraldehyde solution (25 %) was procured from Aldrich chemical company, Inc. Dulbecco modified Eagle's medium (DMEM) and Antibiotic-antimycotic solution were procured from Cellclone^{TM} (India). MTT reagent, 3-(4, 5- Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide A.R. was purchased from Himedia (India)..Commercially available leukodepletion syringe filter was purchased from PALL India Pvt. Limited, India (Figure 2).

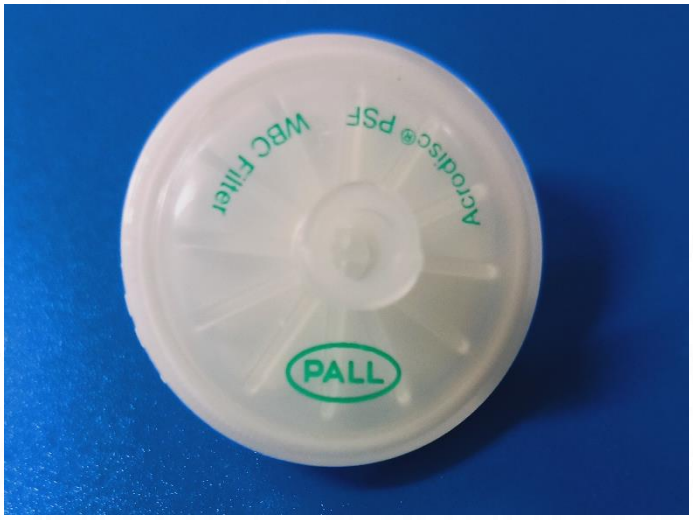


Figure 2: Commercially available leukodepletion syringe filter from PALL Corporation

The chemical structures of polymers and chemicals used in this work used are given in Figure 3.

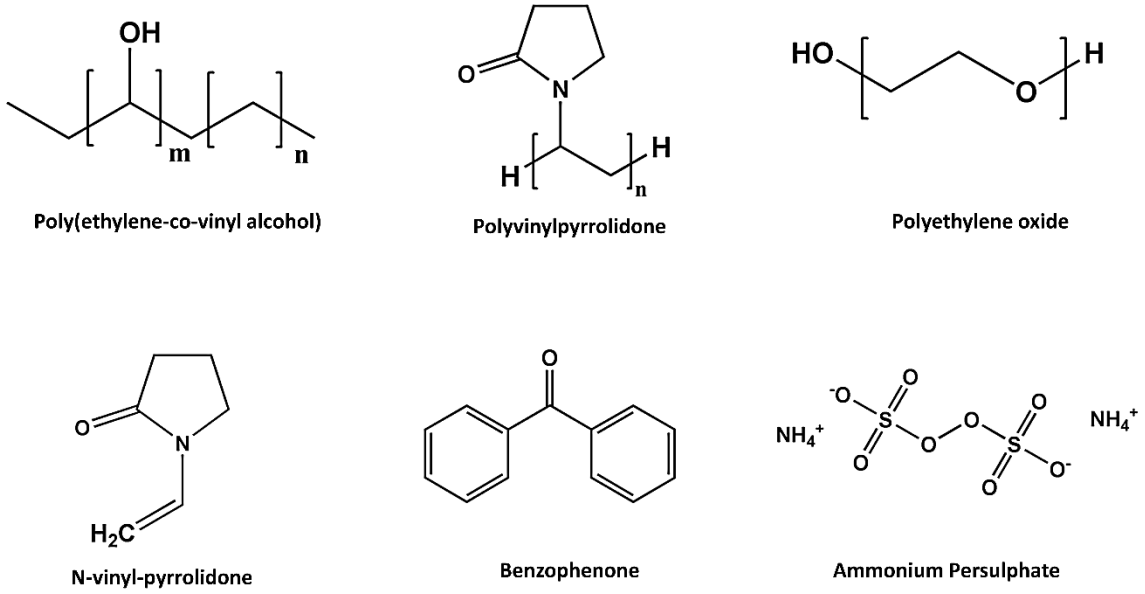


Figure 3: Chemical structures of Polymers and compounds

2.2 Methods

2.2.1 Fabrication of 3D printed syringe filter prototype

Customized mould was 3D printed using Olivetti S2 3D printer. A commercial polylactic acid (PLA) filament with diameter 1.75 mm was used for printing. CAD designing of the mould was performed by SolidWorks and UltimakerCura 4.8.0 was used as the slicing software. The CAD files were sliced into layers with 0.1mm resolution. A nozzle with diameter 500 µm was used for printing. The print and bed temperatures were maintained at 225°C and 60°C respectively. A support structure was enabled to maintain the geometry of the printed part. CAD design of syringe filter is given in Figure 4. Figure 5 is the image of 3D printed syringe filter prototype.

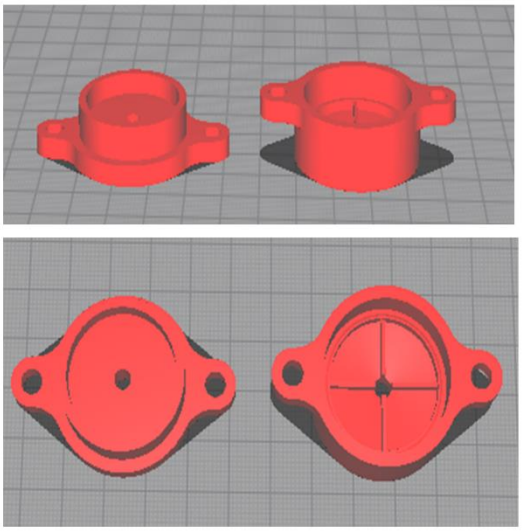


Figure 4: CAD design of 3D printed syringe filter prototype

Parameters set for the 3D printing are mentioned in Table 1.

Table 1. Processing parameters of 3D printing

Processing parameter	Value
Layer height (mm)	0.1
Wall count	2
Infill pattern	Concentric
Infill percentage (%)	100
Print speed	30 mm/s
Infill speed	30 mm/s
Support pattern	Lines
Support infill percentage (%)	50
Overhang angle	45˚

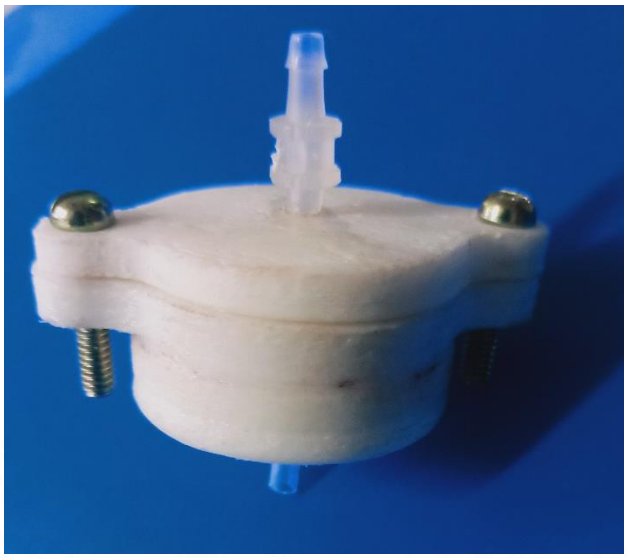


Figure 5: 3D printed syringe filter prototype

2.2.2 Fabrication of leukodepletion membranes of modified Poly(ethylene-co-vinyl alcohol) by electrospinning.

2.2.2.1Fabrication of Poly(ethylene-co-vinyl alcohol) (EVAL) membranes

2.2.2.1.1 Preparation of electrospinning solution

Poly (ethylene-co-vinyl alcohol) (EVAL)solution was prepared in 7:3 isopropanol/water mixture with a concentration of 9% (w/v). The polymer solution was prepared at 70 – 80 º C temperature in oil bath (Xu *et al*., 2011)and 400 rpm stirring was given.

2.2.2.1.2 Electrospinning of EVAL membrane

Electrospinning of the solution was done using 20 mL syringe. The solution was electrospun using 18G size needle and pumped using KD Scientific Pump. The flow rate of electrospinning was set to 8 mL/h and a voltage of 10-12 kV was applied using the high voltage power supply (Zeonics, Bangalore, India). The mandrel was rotated at a speed of

500 rpm in anticlockwise direction. During electrospinning the distance between rotating mandrel and syringe needle was maintained at 10 cm.

2.2.2.2 Fabrication of Polyvinylpyrrolidone (PVP) blended membrane of Poly(ethylene-co-vinyl alcohol) (EVAL)

2.2.2.2.1Preparation of electrospinning solution

Poly (ethylene-co-vinyl alcohol) (EVAL)solution was prepared in 7:3 isopropanol/water mixture with a concentration of 9% (w/v). The polymer solution was prepared at 70 – 80 º C temperature in oil bath and 400 rpm stirring was given. To the above solution, 1% and 2% (w/w) of Polyvinylpyrrolidone (PVP) was added and separate solutions were prepared for respective concentrations of PVP. The mixing of PVP and EVAL was again carried out at 70 – 80 º C temperature in oil bath and under 400 rpm stirring.

2.2.2.2.2 Electrospinning of PVP blended membrane of EVAL

Electrospinning of the solution was done using 20 mL syringe. The solution was electrospun using 18G size needle and pumped using KD Scientific Pump. The flow rate of electrospinning was set to 8 mL/h and a voltage of 10-12 kV was applied using the high voltage power supply (Zeonics, Bangalore, India). The rotating mandrel was rotated at a speed of 500 rpm in anticlockwise direction. During electrospinning the distance between rotating mandrel and syringe needle was maintained at 10 cm.

2.2.2.3 Fabrication of Benzophenone(BP) initiator incorporated Polyethylene oxide (PEO) blended membrane of Poly(ethylene-co-vinyl alcohol) (EVAL)

2.2.2.3.1 Preparation of electrospinning solution

Poly (ethylene-co-vinyl alcohol) (EVAL)solution was prepared in 7:3 isopropanol/water mixture with a concentration of 9% (w/v). The polymer solution was prepared at 70 – 80 ^{o} C temperature in oil bath and 400 rpm stirring was given. To the above solution, 1% and 2% (w/w) of Polyethylene oxide (PEO) and 10% (w/w of PEO) of Benzophenone (BP were added and separate solutions were prepared for respective concentrations of PEO. The mixing of PEO/BP with EVAL was carried out at 50^{o} C temperature in oil bath and under 400 rpm stirring.

2.2.2.3.2 Electrospinning of PEO/BP blended membrane of EVAL

Electrospinning of the solution was done using 20 mL syringe. The solution was electrospun using 18G size needle and pumped using KD Scientific Pump. The flow rate of electrospinning was set to 8 mL/h and a voltage of 10-12 kV was applied using the high voltage power supply (Zeonics, Bangalore, India). The rotating mandrel was rotated at a speed of 500 rpm in anticlockwise direction. During electrospinning the distance between rotating mandrel and syringe needle was maintained at 10 cm.

2.2.2.4 Fabrication of Ammonium persulfate initiator incorporated N-vinyl pyrrolidone (NVP) grafted membrane of Poly (ethylene-co-vinyl alcohol) (EVAL)

2.2.2.4.1 Preparation of electrospinning solution

9 % (w/v) Poly (ethylene-co-vinyl alcohol) (EVAL) was taken in 7:3 isopropanol/water mixture. To the above solution, 2% (w/w) of N-Vinyl pyrrolidone (NVP) and 10% (w/w

of NVP) of Ammonium persulfate (APS) were added. EVAL, NVP and APS were mixed in the solvent at 80º C until thorough dissolution in oil bath and under 400 rpm stirring.

2.2.2.4.2 Electrospinning of NVP/APS blended membrane of EVAL

Electrospinning of the solution was done using 20 mL syringe. The solution was electrospun using 18G size needle and pumped using KD Scientific Pump. The flow rate of electrospinning was set to 3 mL/h and a voltage of 18-20 kV was applied using the high voltage power supply (Zeonics, Bangalore, India). The rotating mandrel was rotated at a speed of 500 rpm in anticlockwise direction. During electrospinning the distance between rotating mandrel and syringe needle was maintained at 10 cm.

2.2.3 Methods of crosslinking of fabricated membranes

Polyvinylpyrrolidone and Polyethylene oxide are hydrophilic polymers which could easily leach out and dissolve in aqueous media. In order to prevent the leach out, crosslinking has to be performed. The membranes were crosslinked after electrospinning by means physical methods like thermal and UV treatment.

2.2.3.1 Thermal crosslinking of PVP blended membrane of EVAL

Electrospun membranes of EVAL/PVP were crosslinked under 120ºCfor 6h. After electrospinning, the membranes were kept in JEIO Tech vaccum oven (OV 11), Korea at 120 ºC for 6h.

2.2.3.2 UV crosslinking of PEO/BP blended membrane of EVAL

Benzophenone (BP) is a photo initiator and reported to crosslink PEO polymer chains also. Hence, UV crosslinking is done with BP incorporated EVAL/PEO membranes by keeping

the membranes under UV light for 3h. UV light of intensity 180 μW cm^{-2} (Toshiba FL15sbl, 350 nm,15 W) is irradiated over the membraneby keeping it in a UV chamber.

2.2.4 Physico-chemical characterization of electrospun membranes

2.2.4.1 Chemical characterization using FTIR

The extent of crosslinking under different conditions and the extent of polymerization were evaluated from Fourier Transform Infrared Spectrometer using ATR method. Samples were evaluated by recording the spectra in the range of 4000 – 400 cm^{-1} in PerkinElmer Series Spectrum two (USA).

2.2.4.2 Surface Morphological Analysis Using Scanning Electron Microscopy (SEM)

The surface morphological analysis of electrospun EVAL and modified EVAL membranes were done in scanning electron microscope (SEM, FEI Quanta 200, Netherlands) at an accelerating voltage of 15kV after gold coating over the polymer surface. The calculation of fiber diameter and pore size was done using SEM images using ImageJ software.

2.2.4.3 Surface Wettability studies

The surface wettability of EVAL and modified EVAL systems were done by coating casted polymer films over a glass slide in 6 x 2 cm^2 dimension. The contact angle was determined using sessile drop method in OCA 15 Plus optical contact angle instrument. The images were obtained from SCA 20 software. Five measurements were recorded for each sample and average value was taken.

2.2.4.4 Porosity evaluation

Porosity evaluation of the electrospun membranes were done by density method of calculation. The method calculates the bulk density and apparent density of the membranes

by measuring thickness and weight of samples cut in disc shape with 2 cm diameter(Remya *et al*., 2018).

The porosity is calculated using the equation

$$\boldsymbol{Porosity}\ (\%) = \left(\mathbf{1} - \frac{\boldsymbol{d}}{\boldsymbol{D}}\right) \times \mathbf{100} \qquad \textbf{(1)}$$

Where d and D represents apparent density and bulk density expressed in g/cm^3 respectively.

$$\boldsymbol{d} = \frac{\boldsymbol{Weight\ of\ membrane}}{\boldsymbol{Area}\ (\boldsymbol{\pi r^2}) * \boldsymbol{h}} \qquad \textbf{(2)}$$

$$\boldsymbol{D} = \frac{\boldsymbol{Sum\ of\ weight\ of\ each\ component\ of\ the\ membrane}}{\Sigma\left(\boldsymbol{Weight\ of\ component} / \boldsymbol{Density\ of\ component}\right)} \qquad \textbf{(3)}$$

2.2.4.5 Static Mechanical Property Analysis using Universal Testing Machine (UTM)

Static mechanical properties of the membranes were evaluated using Universal Testing Machine (UTM), Instron 3345, single column (UK). A 100N load cell was used for the analysis and the sample loaded on the crosshead was moved with a speed of 10 mm/min. Samples were cut in dumbbell shape (ISO 527-2 Type 5B) with a middle rectangular portion *with* length 15 mm and width 3 mm. Six replicates were tested for each sample. Tensile strength, maximum stress developed in the material is calculated from the stress-strain curve of the material.

2.2.4.6 Dynamic Mechanical Analysis (DMA)

The dynamic mechanical properties of the membranes were done in Dynamic Mechanical Analyzer (Triton 2000 DMA, UK). The test was conducted in tension mode with a frequency of 1 Hz and a heating rate of 2 ^{o}C/min. Temperature scanning is done in the

range -80 to 80 ^{o}C for EVAL/NVP 2 % membrane and -80 to 100 ^{o}C for EVAL membrane. The storage modulus and tan delta were plotted.

2.2.5 Whole Blood Filtration Studies

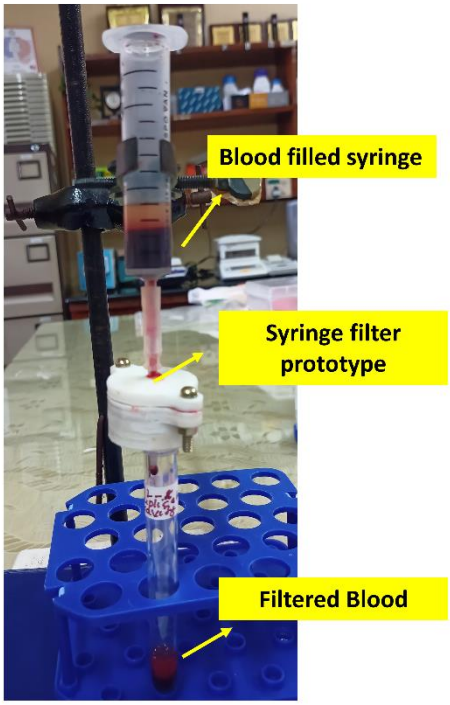


Figure 6: Blood Filtration Setup

To perform these filtration membranes were cut in 3 cm diameter disc shape and enclosed in a customized syringe filter 3D printed prototype. Blood was collected from human volunteer (**SCT/IEC/1366/APRIL-2019**) into anticoagulant acid-citrate dextrose (ACD). Single membrane of thickness0.2- 0.4 mm was placed inside the filter and tightly closed. The blood was filled in a syringe and connected to the inlet of prototype. The blood was allowed to flow down and pass through the membrane by gravity and the filtered blood was collected from the outlet tube of the prototype (figure 6). Two replicates of each membrane were used for the filtration study. Blood samples before and after filtration were subjected to WBC, RBC and Platelet counting using Haematology analyzer. Percentage of

adhesion of WBC on the filter membrane was calculated from the below equation(Cao *et al*., 2012).

$$\boldsymbol{WBC\ adhesion}\ (\%) = \frac{\boldsymbol{WBC\ count\ before\ filtration - WBC\ count\ after\ filtration}}{\boldsymbol{WBC\ count\ before\ filtration}} \times \mathbf{100} \quad \textbf{(4)}$$

RBC recovery percentage was calculated by the below equation.

$$\boldsymbol{RBC\ recovery}(\%) = \mathbf{1} - \left(\frac{(\boldsymbol{RBC\ before\ filtration - RBC\ after\ filtration})}{\boldsymbol{RBC\ before\ filtration}}\right) \times \mathbf{100} \quad \textbf{(5)}$$

Platelet adhesion percentage was also calculated from the below equation.

$$\boldsymbol{Platelet\ adhesion}\ (\%) = \frac{\boldsymbol{Platelet\ before\ filtration - Platelet\ after\ filtration}}{\boldsymbol{Platelet\ before\ filtration}} \quad \textbf{(6)}$$

After filtration, the WBC adhered membranes were studied using ESEM.

2.2.5.1 In-vitro haemolysis assay

Estimation of total haemoglobin in blood is done by in-vitro haemolysis assay in Haematology Analyzer. 1000 µL/ 3000 µL of 0.01 % Na_2CO_3 was taken in microtubes. A 100 µL/ 300 µL of test plasma was added to it. It is mixed well and taken for reading in the instrument. The absorbance was measured at 380 nm, 415 nm and 450 nm. 0.01 % Na_2CO_3 is kept as blank. % Haemolysis is calculated from the below equation(Sawant *et al*., 2007).

$$\%\ \boldsymbol{Haemolysis} = \left(\frac{\boldsymbol{Free\ Hb}/\boldsymbol{Total\ Hb\ in\ blood}}{\mathbf{1000}}\right) \times \mathbf{100} \quad \textbf{(7)}$$

2.2.6 *In-vitro* cytotoxicity studies

In-vitro cytotoxicity studies of the fabricated membranes were done in L929 fibroblast cells. Both qualitative and quantitative estimation of cytotoxicity were done. Qualitative estimation of cytotoxicity was done by direct contact assay and MTT assay was performed to quantify the cytotoxicity.

2.2.6.1 Direct contact Assay

Direct contact assay was done to study the cytotoxic effects of the electrospun membranes. 2 x 10^4 L929 cells were seeded in a well plate in Dulbecco modified Eagles Medium supplemented with 10 % Fetal Bovine Serum and 1 % Anti biotic-anti mycotic solution. Materials were cut in disc shape with a 4 mm diameter. After achieving a confluency of cell monolayer in the well plate, triplicates of materials were placed on the top of the cell monolayer after UV sterilization. The cell-material system was incubated at 37^{o}C for 24 h under 5 % CO_2 and 95 % humidity conditions. Cell monolayer without material placed over it was considered as the negative control. After 24 h, the wells were observed under phase contrast microscope (Leica, DMI 3000B). The appearance of the cell monolayer and the morphology of cells were observed to analyse the cytotoxicity of the material(Prasad *et al*., 2015).

2.2.6.2 MTT Assay for cell viability studies

MTT assay is a colorimetric assay for assessing the metabolic activity of cells. The assay measures the ability of metabolically active cells to reduce tetrazolium salt to formazan complex. MTT is a yellow-colored tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphneyl tetrazolium bromide which will be reduced to purple colored water insoluble formazan complex by the NADP-H oxidoreductase enzymes. 0.5 x 10^4 L929 cells were seeded in a 96 well plate in Dulbecco modified Eagles Medium supplemented with 10 % Fetal Bovine Serum and 1 % Anti biotic-anti mycotic solution. Extract of materials in PBS was collected, by keeping sterilized materials of surface area 6 cm^2 in PBS for 24 h. The PBS extracts of materials were added to a confluent cell monolayer by diluting in DMEM media (10X dilution). After adding the extracts, the cell-extract system was incubated at

37°C under 5 % CO_2 and 95 % humidity. Cell alone wells were considered negative control and wells with diluted phenol added over the cell monolayer were considered as positive control. After 24h of incubation, extract containing medium was replaced with fresh medium and added with 20 µL MTT reagent. MTT reagent was prepared in PBS with 5 mg/mL concentration. The MTT reagent added well plate was again incubated for 4h at 37°C under 5 % CO_2 and 95 % humidity. After 4h, the reagent was removed. The purple-colored formazan complex formed was solubilized in DMSO by adding 200 µL of the same. The absorbance was measured at 570 nm using a Microplate reader (Biotek)(Kumar *et al*., 2018).

The percentage of cell viability was calculated from the equation below.

$$\textbf{\textit{Cell viability}}\ (\%) = \frac{\textbf{\textit{Absorbance of material added cells}}}{\textbf{\textit{Absorbance of control cells}}} \times \mathbf{100} \qquad \textbf{(8)}$$

2.2.7 Statistical Analysis

Statistical analysis of the data was done by student t test performed in Microsoft Excel. The analysis of significant difference on fiber diameter, pore size, porosity, tensile strength, porosity and percentage of cell viability were analysed by student t test. The results were considered significant when p value < 0.05. The results were stated '*' for p value < 0.05, '**' for p value < 0.005, '***' for p value < 0.0005 and '****' for p value < 0.00005 and below.

CHAPTER 3

RESULTS AND DISCUSSION

This chapter discusses the observations and results of the experiments conducted in the work. Results are categorized in four sections- (1) fabrication of EVAL and modified EVAL electrospun membranes and optimization of their crosslinking under different conditions (2) comparative analysis of physico-chemical characterizations done in various membranes (3) study and comparison of membranes based on leukodepletion efficiency, hemocompatibility and also comparing the fabricated membranes with the commercially available filter (PALL syringe filter) (4) *in-vitro* cytotoxicity evaluation of the electrospun leukodepletion filter membranes.

3.1 Electrospinning and crosslinking

3.1.1 Fabrication of modified EVAL electrospun membranes

Poly(ethylene-co-vinyl alcohol) is a polymer known to perform leukodepletion in an efficient way just like commercially available filter materials(Mayuri *et al*., 2021). Solvent mixture of isopropanol and water is a preferred solvent system for electrospinning EVAL being non-toxic and ease of electrospinning(Xu *et al*., 2011).

3.1.1.1 EVAL/PVP and EVAL/BP/PEO electrospinning

Both polyvinylpyrrolidone and polyethylene oxide are synthetic hydrophilic polymers which can be electrospun easily (Eskitoros-Togay *et al*., 2019; Tort *et al*., 2019).

Polyvinylpyrrolidone of molecular weight 3,60,000 and polyethylene oxide of molecular weight 6,00,000 were used in this work. Since PVP and PEO dissolves well in isopropanol/water mixture, the blending was done by simple mixing of EVAL with these polymers.

In this case, high molecular weight polymers are used. Incorporating more than 2% (w/w) of PVP or PEO turns the polymer blend solution too viscous in such a way that electrospinning becomes difficult. Even incorporating more than 2% (w/w of EVAL) also made electrospinning tough as polymer solution was found dripping at the needle tip at all voltage and flow rate combinations. Therefore, in this work 1 and 2 % (w/w) of both PVP and PEO were considered as the optimized concentration to blend with 9 % (w/v) of EVAL.

PVP is capable of crosslinking under thermal treatment and do not need an initiator to kick start crosslinking(Roy & Saha, n.d.). But it is different in the case of PEO.PEO requires an initiator molecule to initiate the crosslinking reaction. Benzophenone is a well-known photo-initiator and (Teixeira *et al*., 2013) have reported that BP initiated crosslinking of PEO chains under photo irradiation. Therefore, 10 % (w/w of PEO) of BP is added with the polymer blend solution of EVAL/PEO.

3.1.1.2 EVAL/NVP electrospinning

N-vinyl pyrrolidone (NVP) is an organic compound that is the monomer of PVP. Ammonium persulfate (APS) is also a well-known thermal initiator and some literatures have discussed their role in initiating polymerization of NVP (Du *et al*., 2018). Therefore, *in-situ* polymerization of EVAL polymer chains with NVP in isopropanol/water mixture at 80 ^{o}C was tried. Mixing of NVP and APS with EVAL at 80 ^{o}C and overnight stirring at

400 rpm facilitated the *in-situ* polymerization of NVP to PVP. While preparing the polymer solution, concentration of NVP was chosen to be 2 % (w/w of EVAL) and that of APS was 10 % (w/w of NVP). During electrospinning it was observed that incorporation of higher concentrations of NVP did not facilitate electrospinning and resulted in electrospraying. Therefore, NVP concentration was fixed at 2 % (w/w).

3.1.2 Optimization of crosslinking of modified EVAL membranes

3.1.2.1 EVAL/PVP and EVAL/BP/PEO membranes

As already discussed, the crosslinking methods employed for EVAL/PVP and EVAL/BP/PEO membranes are thermal and UV treatments respectively. As incorporation of 2 % (w/w) of PVP and PEO could enhance the hydrophilicity of the membranes

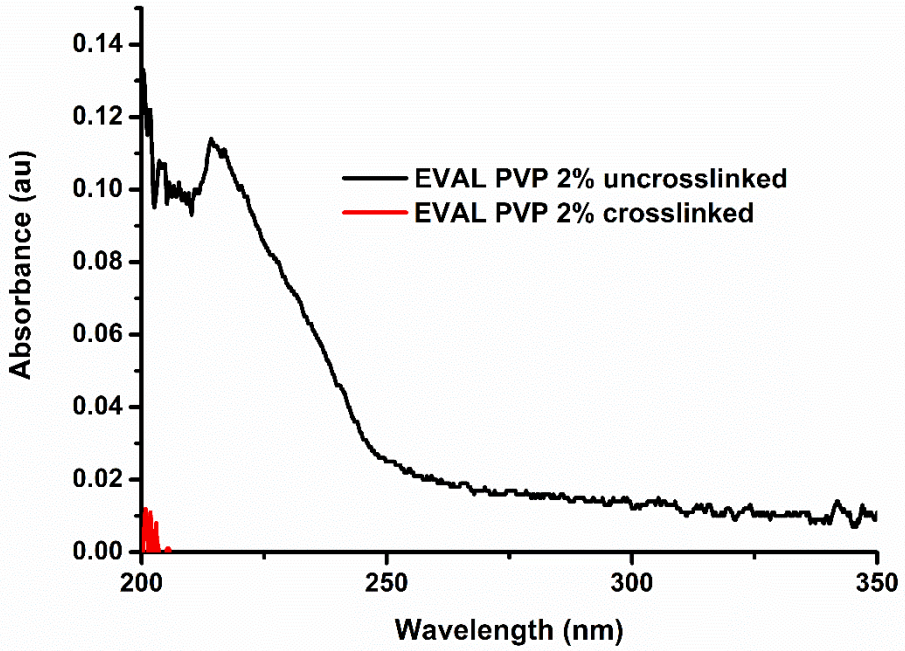


Figure 7: UV Absorbance spectra of PVP extracted from crosslinked and uncrosslinked EVAL/PVP 2 % membrane

compared to 1 % (w/w) concentration, all the following studies were done with EVAL/PVP 2% and EVAL/BP/PEO 2% membranes.

Polyvinylpyrrolidone is a hydrophilic polymer, and there is a chance of leach out of PVP into aqueous system. To prevent the leaching out, thermal crosslinking was done at 120 ^{o}C for 5 h and the extent of leaching was verified by UV Visible Spectroscopy. For that, 1 x1 cm^2 sample of EVAL/PVP 2% membranes before and after crosslinking were dipped in 5mL PBS for 10 min and the UV spectra of the extract was taken. It is evident from the Figure 7 that thermal crosslinking of PVP at 120^0 C has resulted in significant reduction in leaching of PVP compared to uncrosslinked sample.

EVAL/BP/PEO membranes were UV treated for 3 h, 5 h and 10 h. The crosslinking was optimized by carrying out the PEO extraction studies from the membranes. For that, 1 x 1 cm^2 samples were cut from membranes crosslinked for different time periods and immersed in 5 mL Phosphate Buffered Saline (PBS) for 10 min. The UV-Visible Spectroscopy (Schimadzu UV 1800) of the extract was taken in the range of 200 – 400 nm. An absorption maximum was obtained at 213 nm, which corresponds, to PEO leaching out from the matrix. It is found that the absorption maxima disappear for 3 h crosslinked system compared to uncrosslinked membrane (Figure 8).

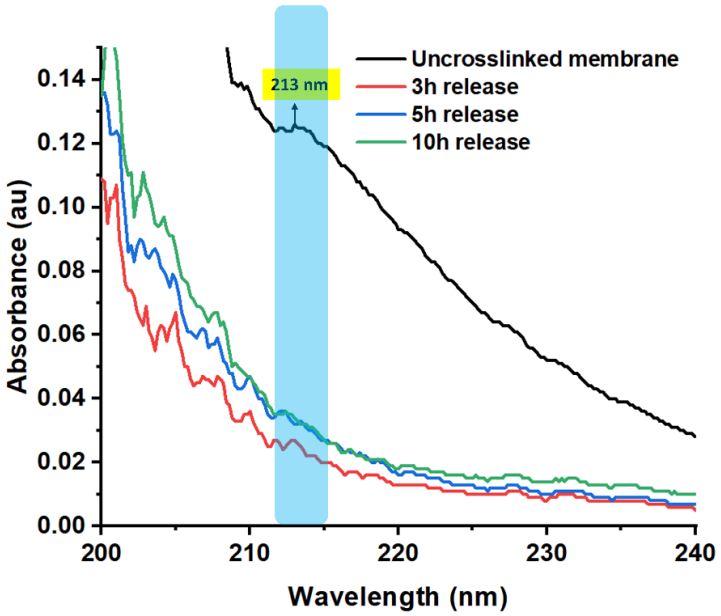


Figure 8: UV spectra of PEO extraction study done for different time period UV crosslinked membranes of EVAL/BP/PEO 2 %.

The proposed mechanism of crosslinking under thermal treatment of PVP and UV treatment of PEO are discussed in Figures 9 and 10 respectively.

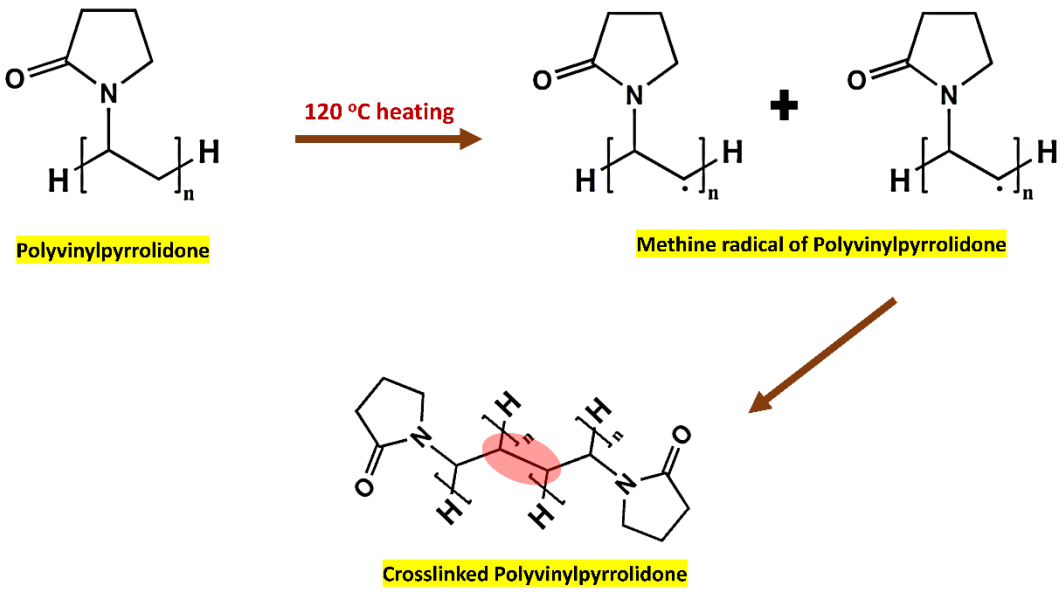


Figure 9: Proposed mechanism of crosslinking of PVP under thermal treatment.

During thermal treatment, a radical is formed by the dissociation of methine of main polymer chain and it is the most stable radical to be formed. Two such methine radicals in two polymer chains could combine and crosslink the polymer system(Tan *et al*., 2014).

Benzophenone is a photoinitiator which is capable of undergoing n-> π* transition under UV irradiation. This proceeds to the formation of formation of radical by dissociation of methine group in PEO backbone. Two such macroradicals could combine and result in the formation of carbon-carbon bond between main chains and thus establish crosslinking(Doycheva *et al*., 2004).

3.1.2.2. Optimization of polymerization of EVAL/NVP membranes

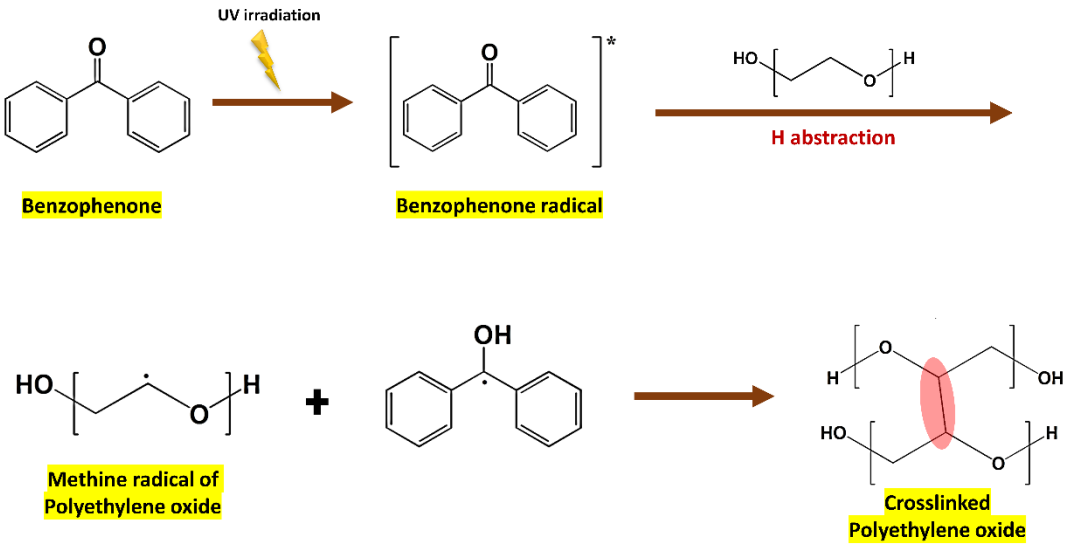


Figure 10: Proposed mechanism of crosslinking of PEO under UV irradiation with benzophenone initiation.

NVP is completely miscible with water and is expected to leach out from the membrane in contact with water. But surprisingly, UV spectra of extract of EVAL/NVP 2% membrane

after 10 min extraction in PBS showed a peak maximum which matches with the same of APS. So, its concluded that NVP or the polymerized NVP is not leaching from the membrane and complete polymerization has taken place. Along with polymerization of NVP in the presence of APS, it is also possible that NVP or polymerized NVP could graft on the polymer chains of EVAL. A schematic representation of expected *in-situ* polymerization and grafting of NVP in EVAL solution is depicted in Figure 11.

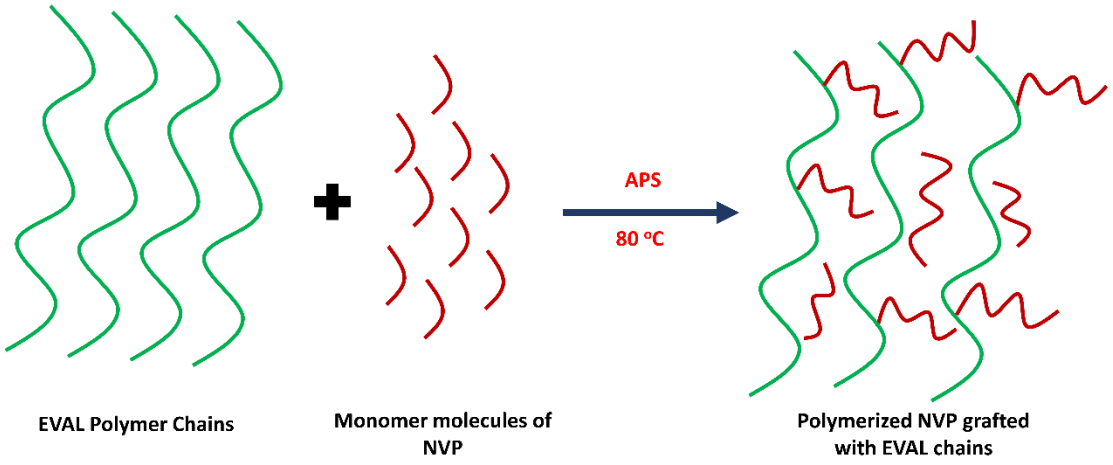


Figure11: Schematic representation of APS initiated *in-situ* polymerization and grafting of NVP in EVAL solution.

Physico-chemical characterization

3.2.1 Chemical Characterization using FTIR

3.2.1.1 FTIR Spectra to confirm crosslinking in EVAL/PVP 2

Fourier Transform InfraRed spectra of EVAL/PVP 2 % membranes before and after thermal crosslinking at 120 °C was taken to confirm crosslinking. As expected from the mechanism of crosslinking, the mechanism of crosslinking explains each -CH2 group in polymer chain as the point of crosslinking. Therefore, -CH2 asymmetric vibrations will be affected and constrained after crosslinking. -CH_2 asymmetric stretching vibrations are

observed at wavelength 2930 cm^{-1}(Zidan *et al.*, 2019) (Figure 12). Vibrations at this wavelength is visible in both uncrosslinked and crosslinked membranes. But the intensity of the peak was observed to be less in crosslinked membrane as verified by vertical cursor gadget in Origin Pro 2021 software. This decreased intensity of vibrations at 2930 cm^{-1} confirms at PVP crosslinking with the proposed mechanism.

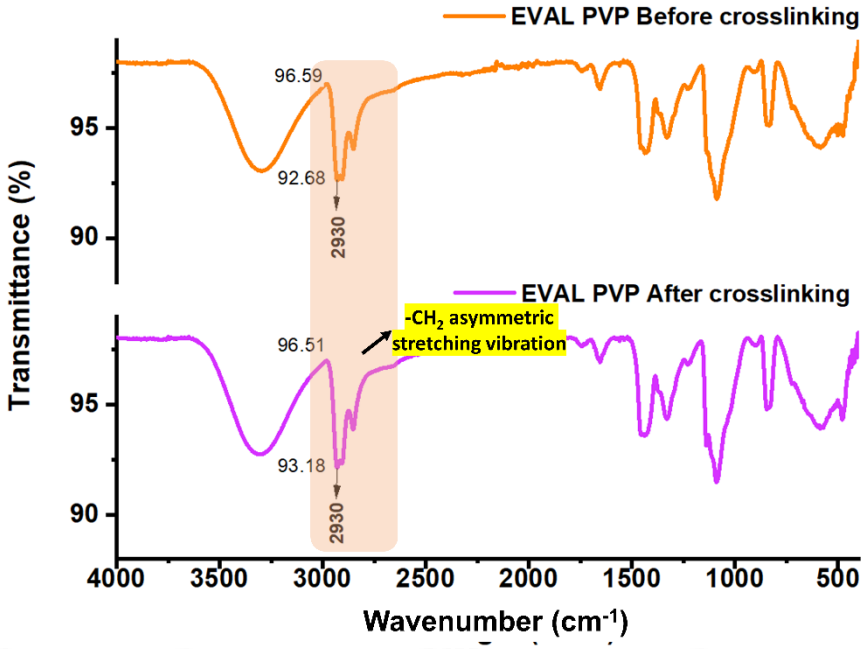


Figure 12: FTIR spectra of EVAL/PVP 2 % membranes before and after crosslinking

3.2.1.2 Spectra to confirm crosslinking in EVAL/BP/PEO 2%

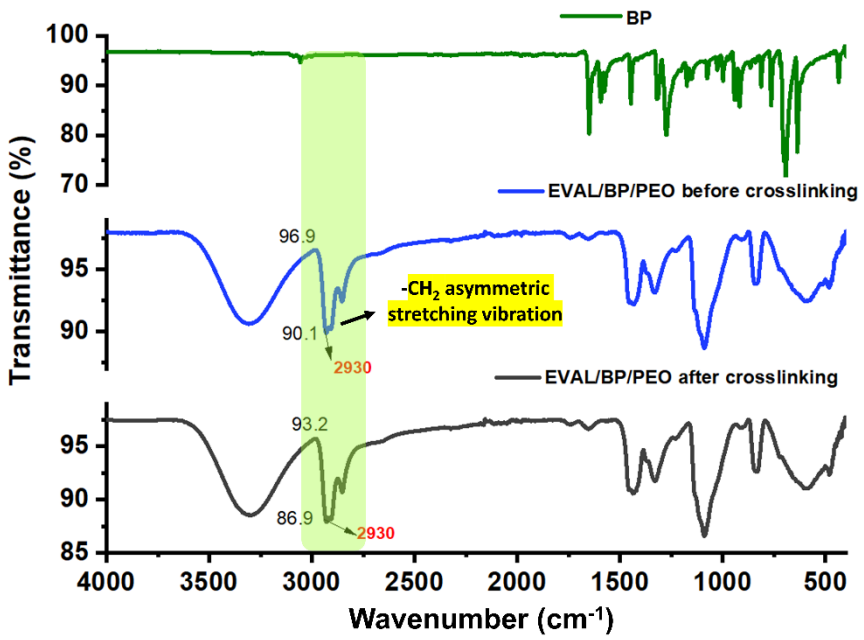


Figure 13: FTIR spectra of EVAL/BP/PEO 2 % membranes before and after crosslinking in comparison with BP spectra

The FTIR spectra of EVAL/BP/PEO 2 % membrane before and after UV crosslinking was compared with that of EVAL/PEO membrane and BP. BP was added to the system in trace amount so that no characteristic peak of BP could be observed in the FTIR spectra of EVAL/BP/PEO 2 %. The proposed mechanism of PEO under UV irradiation is again $-CH_2$ group crosslinking in two polymer chains. Hence the expected observation in FTIR spectra of EVAL/BP/PEO membrane is decreased intensity in $-CH_2$ asymmetric stretching frequency at 2930 cm^{-1}(Ammakutti@Sridevi *et al*., 2012) in crosslinked membrane. As expected, decreased intensity is observed at 2930 cm^{-1}in crosslinked membrane which is analysed by vertical cursor gadget in Origin Pro 2021 (Figure 13) and that confirms PEO crosslinking under UV irradiation under BP initiation.

3.2.1.3 FTIR Spectra to confirm *in-situ* polymerization of NVP in EVAL/NVP 2 %

In-situ polymerization of NVP in the presence of APS in EVAL solution was confirmed using FTIR.Incorporation of vinyl pyrrolidone group in to the system was confirmed by the presence of a peak at 1660 cm^{-1} which corresponds to -C=O stretching. FTIR peak observed at 1334.5cm^{-1} in EVAL corresponds to -C-O stretching vibration. But it is noticed that this peak got vanished in EVAL/NVP membranes with the appearanceof two other split peaks at 1342.5 cm^{-1} and 1284.5 cm^{-1}. These peaks correspond to -C-N stretching vibration and wagging vibrations of $-CH_2$ groups. New appearance of C-N vibration confirms the presence of vinyl pyrrolidone group. Unlike the FTIR spectra of EVAL, an additional peak at 1200 $cm^{-1}$was observed which corresponds to -C-O-C stretching. This indicates a grafting occurred in the presence of APS (Sutirman *et al*., 2017). APS might

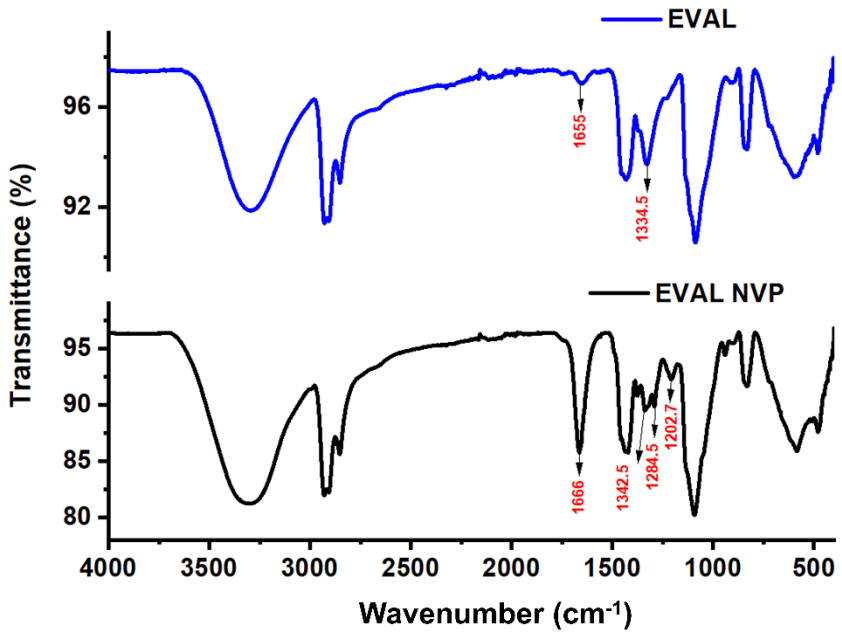


Figure 14: FTIR spectra of EVAL/NVP 2 % membrane in comparison with EVAL membrane

have initiated grafting between terminal alkene group of NVP or polymerized NVP with hydroxyl group of EVAL. Another interesting observation in FTIR spectra is the presence of a weak peak at 1655 cm^{-1} which corresponds to – C=C stretching. But the peak is absent in EVAL/NVP membrane. This might be because the *in-situ* polymerized chains of NVP entangle between the chains of EVAL and hinder the vibrations of -C=C functional groups (Figure 14). No other evidence could be obtained from the FTIR spectra regarding the polymerization of NVP though the presence of NVP can be confirmed.

3.3. Surface Morphology Analysis

3.3.1 SEM Imaging

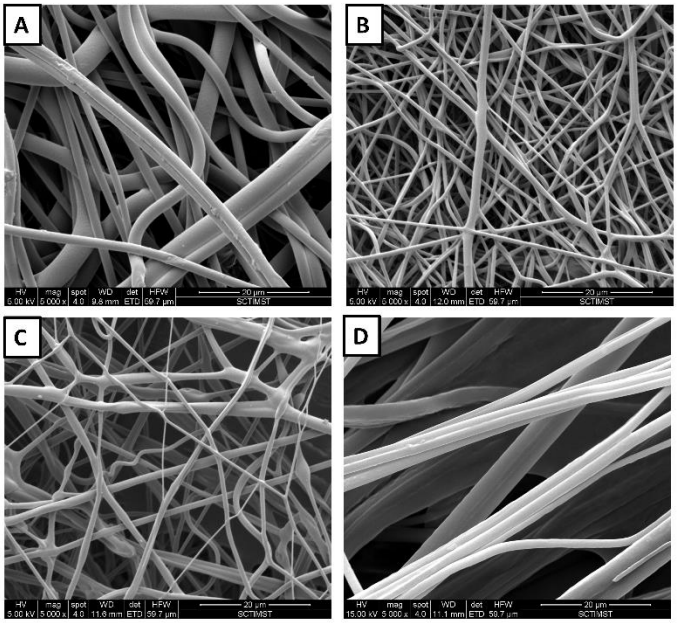
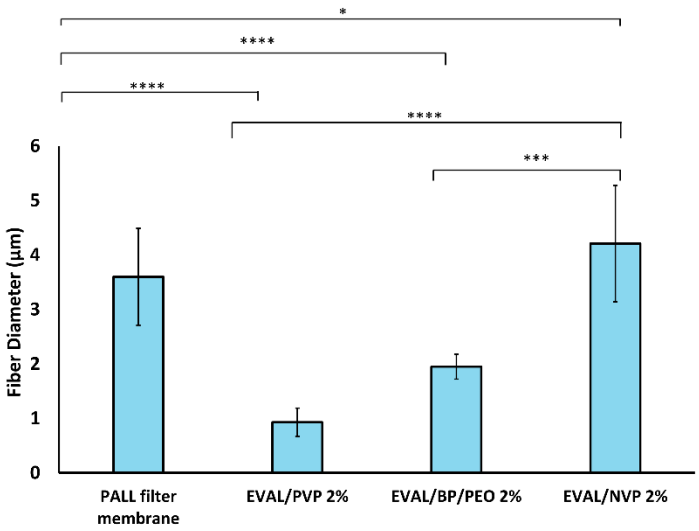


Figure 15: Scanning Electron Microscopy images of A) PALL filter membrane B) EVAL/PVP 2% C) EVAL/BP/PEO 2% D) EVAL/NVP 2 % electrospun membranes

Surface morphology analysis of electrospun membranes and commercially available PALL filter membrane was done and evaluated. Figure15 shows the SEM images of the membranes.

3.3.2 Fibre Diameter Evaluation

Fiber diameters of each membrane were analyzed using ImageJ software. PALL filter membrane was also observed fibrous and the fiber diameter was calculated to be 3.6 ± 0.89 µm. It is found to be significantly higher than EVAL/PVP 2 % and EVAL/BP/PEO 2 %

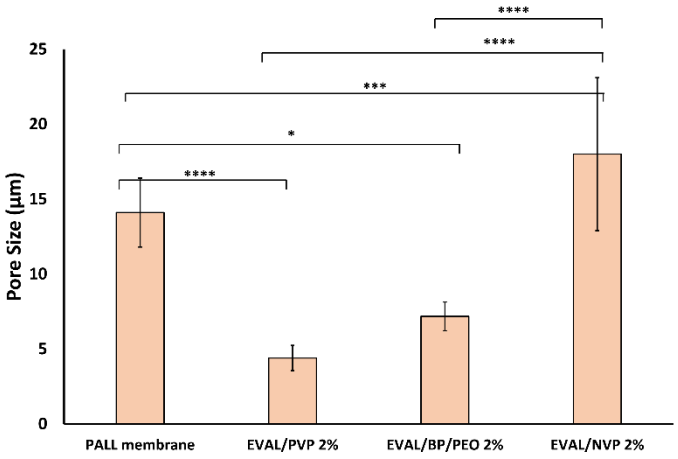


Material	Fiber Diameter (µm)
PALL filter membrane	3.6 ± 0.89
EVAL/PVP 2 %	0.93 ± 0.26
EVAL/BP/PEO 2%	1.95 ± 0.23
EVAL/NVP 2 %	4.21 ± 1.07

Figure 16: Graphical and tabular representation of fiber diameter of various membranes

membrane (p value < 0.00005). The fiber diameter of EVAL/PVP 2 % and EVAL/BP/PEO 2 % membraned were calculated to be 0.93 ± 0.26 µm and 1.95 ± 0.23 µm respectively. The fiber diameter of EVAL/NVP 2 % is significantly higher than EVAL/PVP 2 % membrane (p value < 0.00005) and EVAL/BP/PEO 2 % membranes (p value = 0.0001). Fiber diameter of EVAL/NVP 2 % membranes is noted to be 4.21 ± 1.07 µm. Though it is lesser than that of PALL membrane (p value = 0.019), it is much higher than other modified membranes of EVAL. (Figure 16)

3.3.3 Pore Size Evaluation



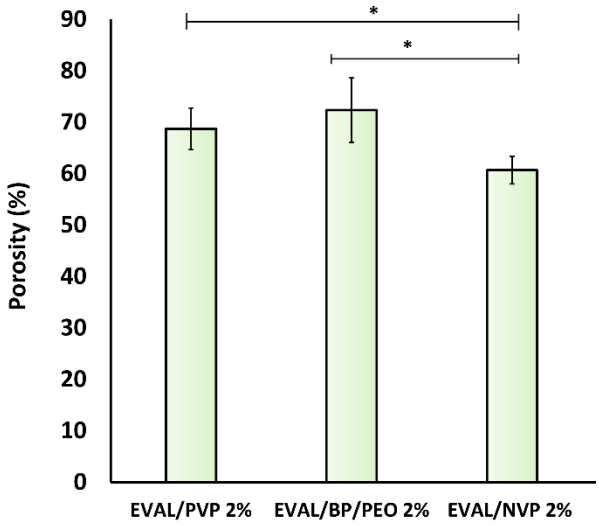
Material	Pore Size (µm)
PALL filter membrane	14.1 ± 2.3
EVAL/PVP 2 %	4.4 ± 0.84
EVAL/BP/PEO 2%	7.18 ± 0.96
EVAL/NVP 2 %	18 ± 5.1

Figure 17: Graphical and tabular representation of pore size of various membranes in comparison with PALL membrane

The fibro-porous membranes of PALL and modified EVAL systems were subjected to pore size evaluation using ImageJ software. The pore size was found to be more for EVAL/NVP 2 % membrane compared all other membranes. Size of pore in EVAL/NVP 2 % membrane is 18 ± 5.1 µm. It is significantly higher than size of pores in PALL membrane, which is 14.1 ± 2.3 µm (p value = 0.0002). The calculated pore size of EVAL/PVP 2 % membrane is 4.4 ± 0.84 µmand that of EVAL/BP/PEO 2 % membrane is 7.18 ± 0.96 µm. These values are significantly lower than EVAL/NVP 2 % membrane pore size (p value < 0.00005). Graphical and tabular representation of pore size evaluated is given in Figure17.

3.3.4 Porosity Evaluation

Porosity evaluation of the membranes was done by density method of calculation. It is found that porosity of EVAL/NVP 2 % membrane is significantly lesser than that of EVAL/PVP 2 % and EVAL/BP/PEO 2 % membranes (p value < 0.05). The calculated porosity of EVAL/NVP 2 % membrane is 60.66 ± 2.67 % and that of EVAL/PVP 2 % and EVAL/BP/PEO 2 % membranes are 68.7 ± 4.06 % and 72.36 ± 6.25 %. The decreased porosity of EVAL/NVP 2% membrane would be because of lower flow rate employed for



Material	Porosity (%)
EVAL/PVP 2 %	68.7 ± 4.06
EVAL/BP/PEO 2%	72.36 ± 6.25
EVAL/NVP 2 %	60.66 ± 2.67

Figure 18: Porosity values given as graphical representation and as table

the preparation of membrane. The membrane was spun with 3 mL/h flow rate whereas all other systems were spun with 8 mL/h flow rate. The values of porosity are given as a table in Figure 18 with a graphical representation.

3.4 Surface Wettability Analysis

The surface wettability analysis was done by measuring the contact angle of each membrane. As already reported in literature, EVAL is having less hydrophilicity due to the presence of ethylene.

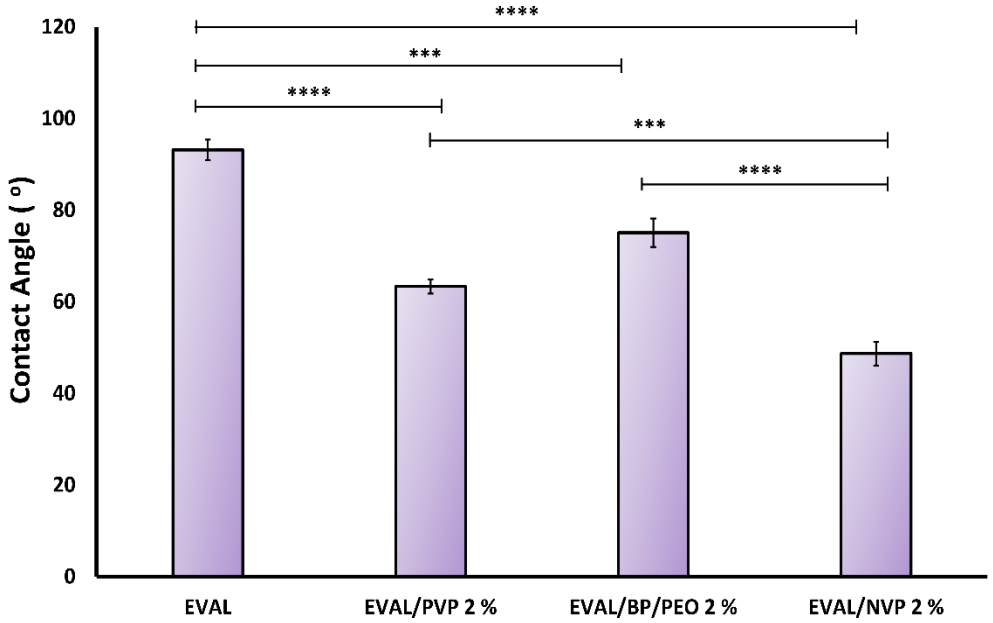


Figure 19: Graphical representation of contact angle values of EVAL and modified EVAL systems

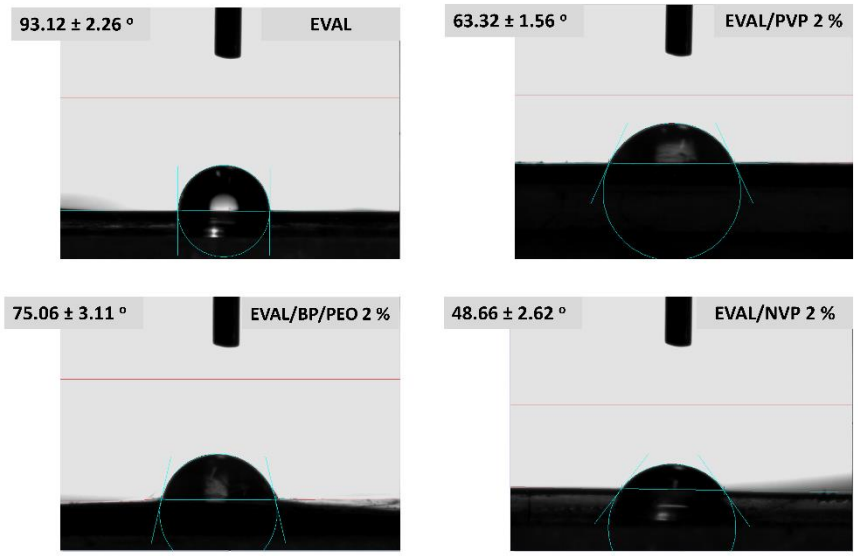


Figure 20: Contact angle images obtained from sessile drop method.

content in the polymer chain(Alghamdi *et al*., 2020) which is again confirmed by the contact angle value of 93.12 ± 2.26 ^{o}. Incorporation and blending with hydrophilic polymers like polyvinylpyrrolidone and polyethylene oxide have caused significant reduction in the contact angle to 63.32 ± 1.56 ^{o} and 75.06 ± 3.11 ^{o}(p value < 0.0005).*In-situ* polymerization and grafting of NVP with EVAL has further reduced the contact angle to 48.66 ± 2.62 ^{o}(p value < 0.00005). The hydrophilicity exhibited by EVAL/NVP 2 % polymer system has been found to be the most wettable system with even significantly less contact angle compared to other EVAL modified systems (p value <0.0005). Figure 19 shows the graphical representation of contact angle values and figure 20 shows the instrument image obtained during wettability study.

3.5 Static Mechanical Property Evaluation

The static mechanical properties of EVAL and modified EVAL membranes were done using Universal Testing Machine (UTM). Evaluation of tensile strength, modulus and elongation at break (%) were done. The result showed that modifications in EVAL hasn't caused any significant change in tensile strength of membranes. Tensile strength of EVAL is 3.65 ± 1.34 MPa and the same for EVAL/PVP 2 %, EVAL/BP/PEO 2 % and EVAL/NVP 2 % membranes are 4.54 ± 2.01 MPa, 4.41 ± 1.28 MPa and 1.94 ± 0.57 MPa respectively.

Nevertheless, there is significant increase in modulus observed in EVAL/PVP 2 % membrane system compared to all other system (p value = 0.008). The modulus values determined using UTM analysis for the samples EVAL, EVAL/BP/PEO 2 % and EVAL/NVP 2 % are 13.30 ± 5.82 MPa, 7.93 ± 2.37 MPa and 9.39 ± 2.60 MPa respectively. On the other hand, the same for EVAL/PVP 2 % membrane system is 33.17 ± 12.34 MPa.

Though it is expected to polymerize NVP to PVP in EVAL/NVP 2 % system and similar result as that of EVAL/PVP 2 % is anticipated. But a contradictory observation of decreased modulus is observed. This might be because of the fact that, though NVP is polymerizing to PVP, the degree of polymerization would be less and polymerizes NVP

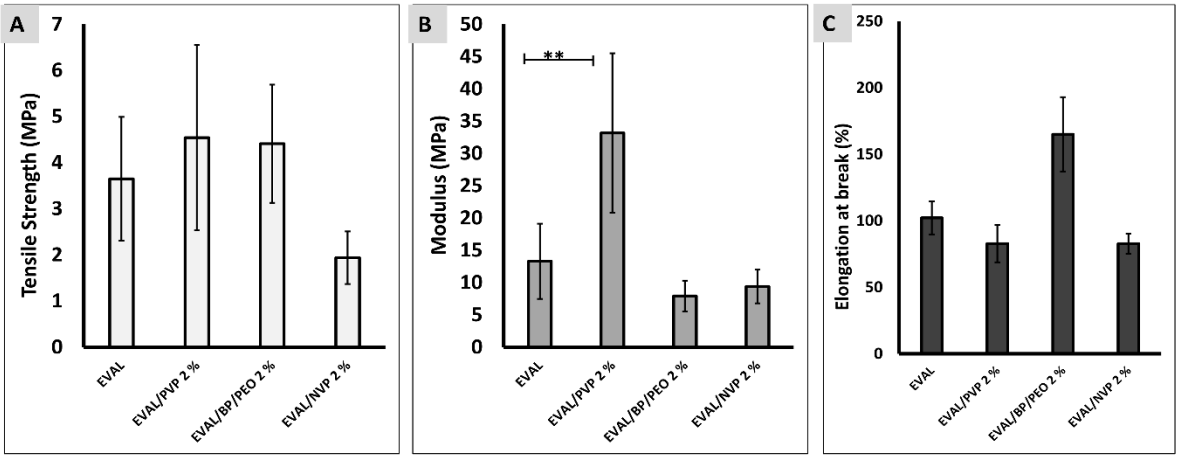


Figure 21: Graphical representation of A) tensile strength B) modulus

C) Elongation at break (%) of EVAL and modified EVAL membranes

chains would be lying between the EVAL chains as a lubricant. The plasticizing effect of polymerized NVP chains might be decreasing the modulus of EVAL/NVP 2 % membrane system.

As far as the elongation at break (%) is concerned, no significant change is observed between EVAL and modified EVAL membrane systems. Figure 21 gives the graphical representation of tensile strength, modulus and elongation at break (%). Table gives the UTM data acquired.

Table 2: UTM data of EVAL and modified EVAL membranes

Material	Tensile Strength (MPa)	Modulus (MPa)	Elongation at break (%)
EVAL	3.65 ± 1.34	13.30 ± 5.82	102.21 ± 12.43
EVAL/PVP 2 %	4.54 ± 2.01	33.17 ± 12.34	82.88 ± 14
EVAL/BP/PEO 2 %	4.41 ± 1.28	7.93 ± 2.37	165.05 ± 27.95
EVAL/NVP 2 %	1.94 ± 0.57	9.39 ± 2.60	82.80 7.50

3.6 Dynamic Mechanical Property Evaluation

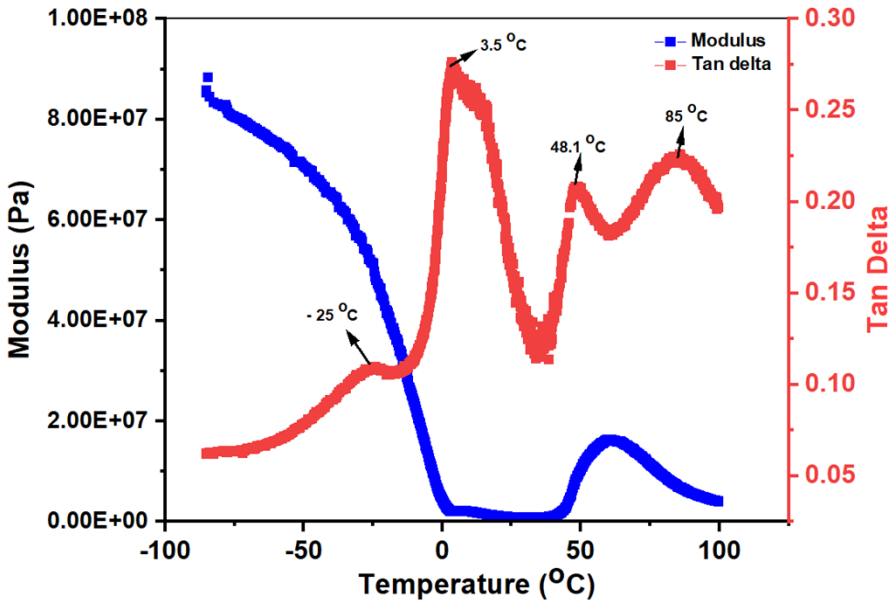


Figure 22: DMA thermogram of EVAL membrane

Dynamic mechanical property determination of EVAL and EVAL/NVP 2 % was done and compared to ascertain the relaxation in mechanical property due to the presence of polymerized NVP. Figures 22 and 23 show DMA analysis curve of EVAL and EVAL/NVP 2 % membranes respectively. Tan delta curve of EVAL shows a first peak maximum at -25^{o}C which corresponds to glass transition of ethylene content of EVAL co-polymer. The next peak maximum is observed at 3.5^{o}C that corresponds to the glass transition of ethylene-vinyl alcohol copolymer. The glass transition of vinyl alcohol is observed at 85

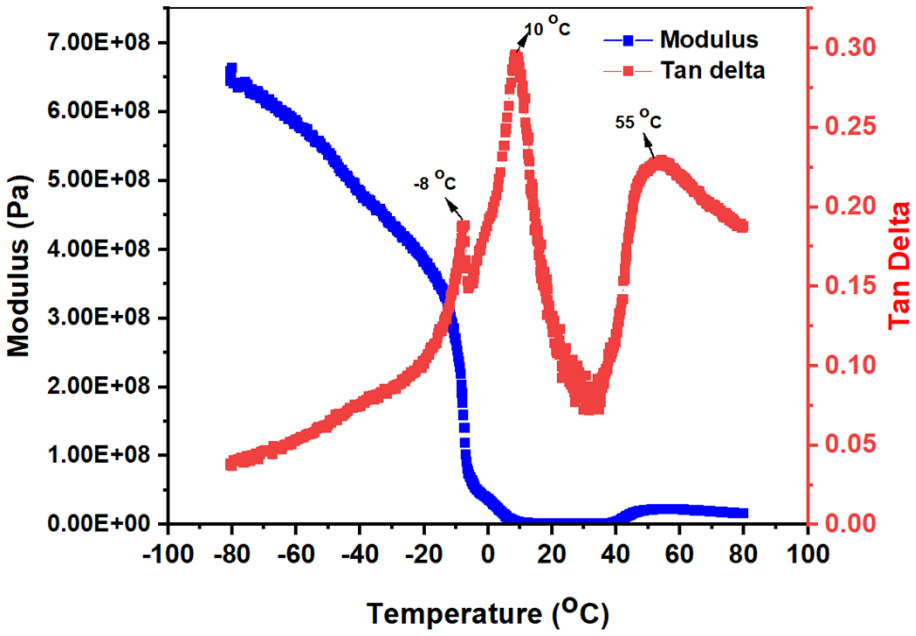


Figure 23: DMA curve of EVAL/NVP 2 % membrane

^{o}C with a shoulder peak at 48^{o}C. It is reported that this shoulder peak corresponds to conformational change on vinyl alcohol side groups (Cassu & Felisberti, 1999).

Unlike EVAL, tan delta curve of EVAL/NVP shows a different pattern. The peak maxima corresponding to ethylene content, vinyl alcohol content and ethylene-vinyl alcohol copolymer were found to be shifted to higher temperature. The shoulder peak at 48^{o}Cwas also missing. Moreover, the transition peak at 85 ^{o}C is found to be much broader. This

relaxation is attributed to the local hydrogen bond interaction in the microenvironment of EVAL and polymerized NVP blend(Cassu & Felisberti, 1999).

3.7 Blood Filtration

3.5.1 Evaluation of Leukodepletion and Haemolysis

Evaluation of efficiency of leukodepletion after blood filtration. Fresh blood collected from human volunteers were filtered through single layer containing syringe filter prototype. RBC, WBC and platelet count were taken before and after filtration of blood.

WBC adhesion ratio was calculated. Statistically no significant difference was observed in the WBC adhesion ratio of EVAL and modified EVAL membranes compared to commercially available PALL membrane. Commercially available PALL membrane was showing a WBC adhesion of 97.63 ± 2.36 %. The WBC adhesion (%) of EVAL, EVAL/PVP 2 %, EVAL/BP/PEO 2 % and EVAL/NVP 2 % are 97.34 ± 0.345 %, 99.30 ± 0.695 % 100 % and 91.86 ± 7.44 respectively (Figure 24).

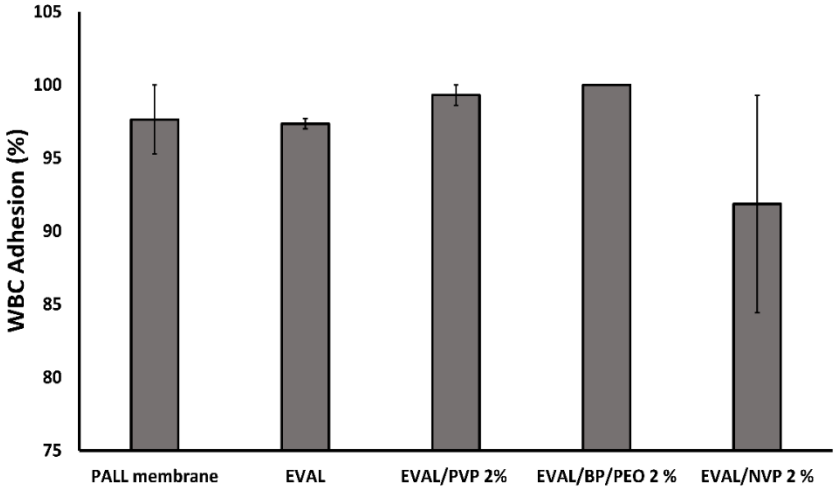


Figure 24: Percentage of adhesion of WBC on various filter membranes- a graphical representation

Surface chemistry has a direct influence on leukodepletion efficiency. Wettability and presence of functional group on the surface affect leukodepletion. A balance between hydrophobicity and hydrophilicity is to be maintained to perform good blood compatibility(Yang *et al*., 2011). Presence of hydroxyl functional group in the polymer is reported to increase the leukocyte adhesion. Hence PEO modified membrane of EVAL is a prescribed system for leukodepletion. PVP without the presence of hydroxyl group, is also a recommended polymer for leukodepletion (Cao *et al*., 2012)..

The analysis of flow time of blood through single layer of filter membrane under gravity showed that modified EVAL membranes have much lesser time of flow per mL of blood compared to EVAL membrane. PALL syringe filter takes around 2 min for 1 mL blood to

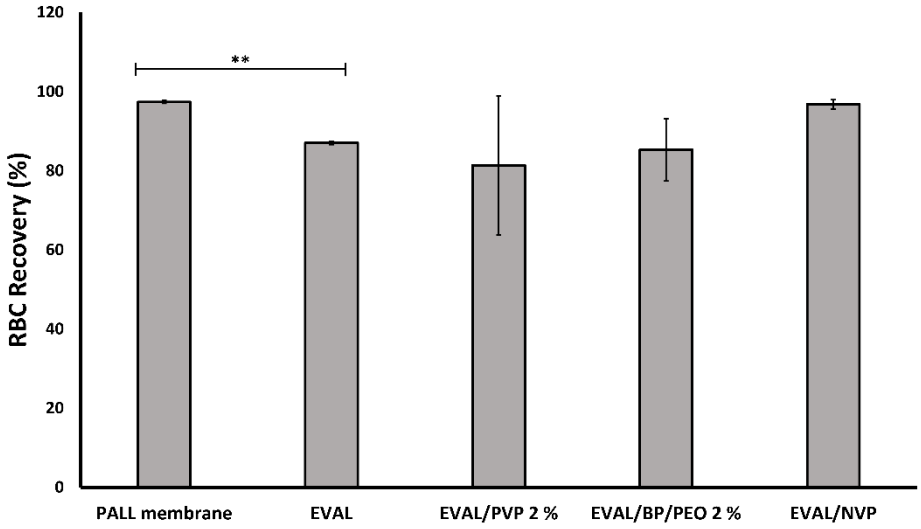


Figure 25: Graphical representation of Percentage recovery of RBC after filtration

flow through it. Whereas, EVAL membrane was taking around 30 min for 1mL blood to flow. This is attributed to the hydrophobic character of EVAL membrane. With less wettability characteristics, it would be difficult for blood to flow through the membrane.

For 1 mL of blood to flow, EVAL/PVP 2 %, EVAL/BP/PEO 2% and EVAL/NVP 2 % membranes take approximately 3 min, 3 min and 2 min respectively. This indicates much closer range of flow time with PALL membrane.

During filtration RBC particles passing through the membrane pores without getting filtered is analyzed. The RBC particle count was taken before and after filtration and calculated the percentage recovery of RBC. It was found that RBC recovery is less in case of EVAL compared to PALL membrane and other modified EVAL membranes (p value = 0.001). Percentage of recovery of RBC in EVAL membrane is 86.99 ± 0.34 %, where as that of PALL membrane is 97.36 ± 0.34 %. EVAL/PVP 2 %, EVAL/BP/PEO 2 % and EVAL/NVP 2% membranes had a percentage of RBC recovery of 81.37 ± 17.50 %, 85.26 ± 7.89 % and 96.76 ± 1.14 % (Figure 25). This change might be due to hydrophobic nature of EVAL.

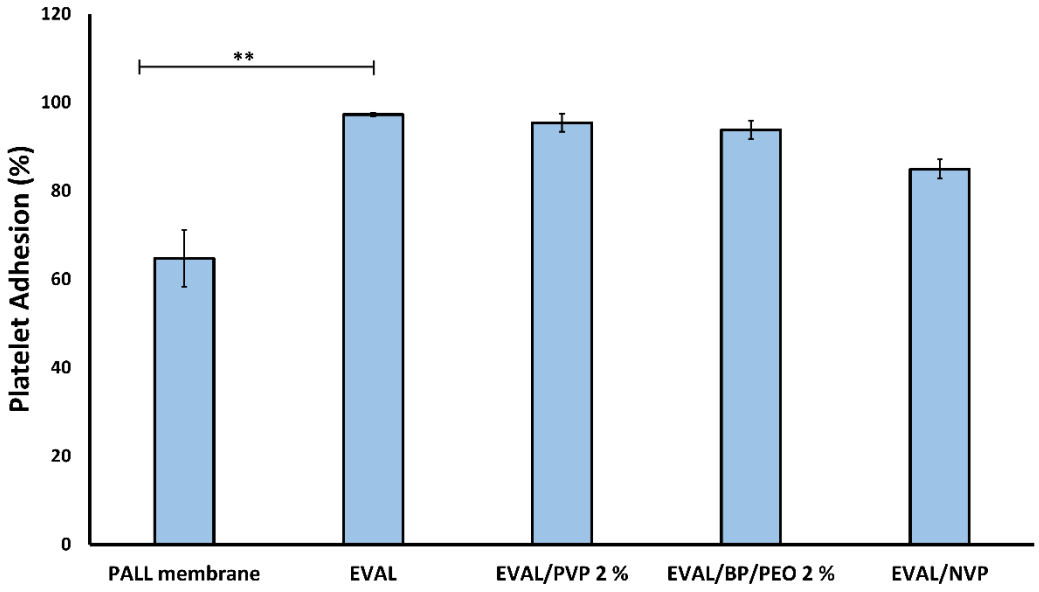


Figure 26: Graphical representation of Percentage of Platelet adhesion after filtration.

Blood filtration data was again analyzed to study the percentage of adhesion of platelet onto the filter membrane. It was found that EVAL, EVAL/PVP 2 % and EVAL/BP/PEO 2 % membranes were having significantly higher percentage of platelet reduction compared to PALL membrane (p value = 0.001). But EVAL/NVP 2 % membrane was having the same percentage of platelet reduction compared to PALL membrane. As leukodepletion filter is particularly designed for the reduction of WBC particles, higher percentage of reduction of platelet is not desirable. PALL membrane and EVAL/NVP 2% have shown a percentage adhesion of platelet of 64.70 ± 6.42 % and 84.93 ± 2.15 %. The same for EVAL, EVAL/PVP 2 % and EVAL/BP/PEO 2 % are 97.27 ± 0.35 %, 95.37 ± 2.01 % and 93.87 ± 2.06 % (Figure 26).

The *in-vitro* hemolysis study done with the blood samples before and after filtration showed that % hemolysis of EVAL and EVAL modified membranes comes in close range with commercially available PALL membrane and is very less. No significant difference between the values was observed between various membranes. A tabular representation of percentage of hemolysis is given below.

Table 3: % haemolysis values of various filter membranes

Material	% Haemolysis
PALL membrane	**0.152 ± 0.069**
EVAL	**0.175 ± 0.104**
EVAL/PVP 2 %	**0.001 ± 0.0004**
EVAL/BP/PEO 2 %	**0.03 ± 0.011**
EVAL/NVP 2 %	**0.056 ± 0.014**

The ESEM images of filter membranes were taken after blood filtration to obtain the images of WBC adhered to the surface of filter membranes and thus to confirm the WBC filtration happening during filtration. Figure 27 shows the images of fibrous membranes with WBC cells. It is visible that RBC is also adhered to the membrane surface but in small numbers.

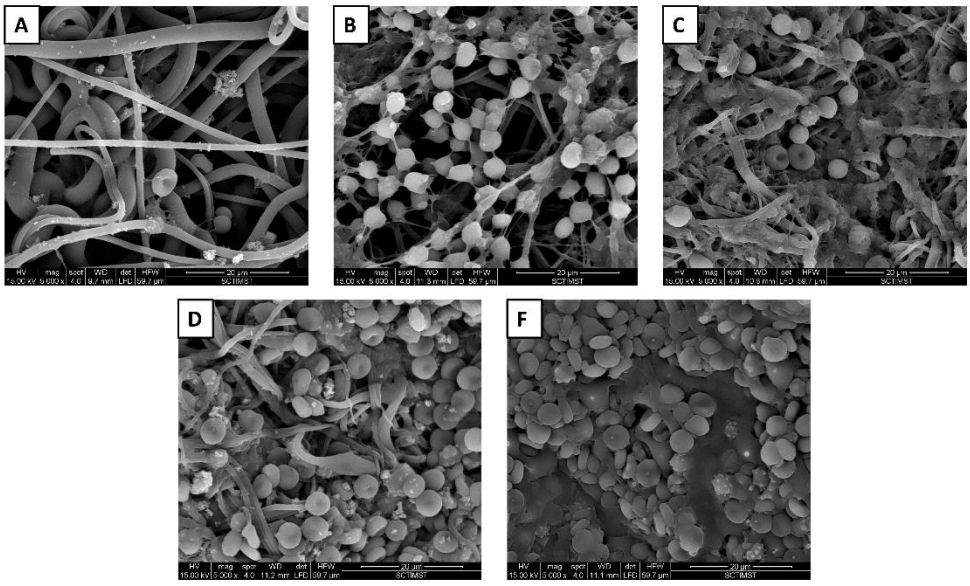


Figure 27: ESEM images of A) PALL membrane B) EVAL C) EVAL/PVP 2 % D) EVAL/BP/PEO 2 % and E) EVAL/NVP 2 %

3.3.2 *In-vitro* Cytotoxicity Evaluation

3.3.2.1 Direct contact Assay

Direct contact assay proved the materials studied as non-cytotoxic. The L929 fibroblast cells were seen live and maintaining the spindle shape morphology even after 24 h contact with materials (Figure 28). EVAL, PVP(Manju & Sreenivasan, 2011) and PEO(Chen *et al*., 2016) are already established non-cytotoxic polymers. Blending of these polymers will not result in any cytotoxicity issues. But incorporation of BP as a photo initiator is expected to cause cytotoxicity. Since BP is added in a trace amount, intending just to initiate

crosslinking, expected cytotoxicity was not found and the EVAL/BP/PEO 2 % membrane was proved as a good biomaterial. Similarly, incorporation of NVP and APS in the EVAL/NVP 2 % membrane is questionable as far as cytotoxicity is concerned. The unreacted NVP left behind and the trace amount of APS present in the membrane haven't resulted in any sort of cytotoxicity. Hence, EVAL/NVP 2 % membrane also proved to be a desirable biomaterial.

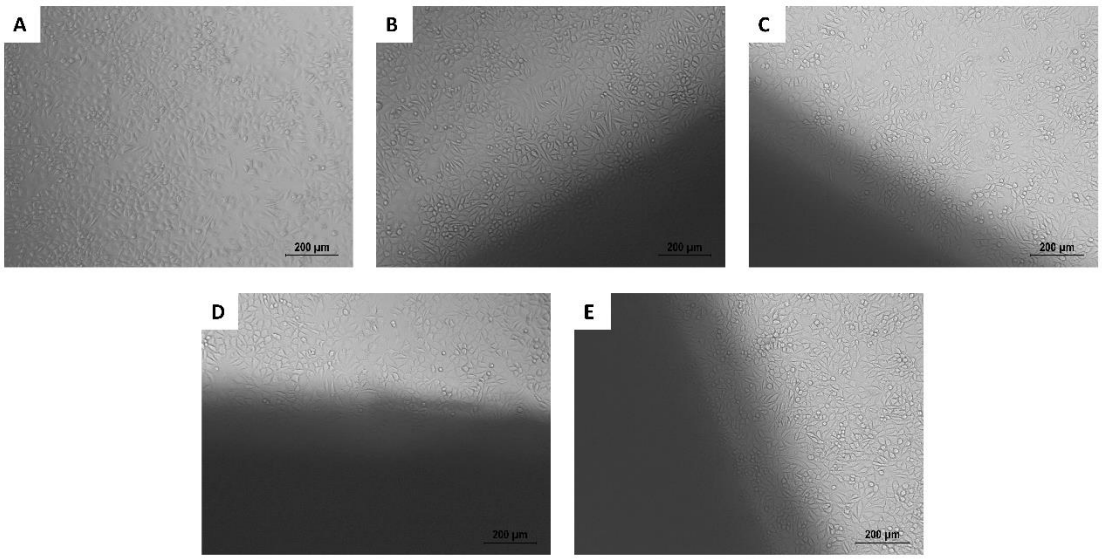


Figure 28: Phase contrast images of direct contact assay in L929 cells of A) control B) EVAL C) EVAL/PVP 2 %D) EVAL/BP/PEO 2 % E) EVAL/NVP 2 %

3.3.2.2 MTT Assay

MTT result also showed higher percentage of cell viability for all materials. It has been already reported in various literature that PVP and PEO are biocompatible polymers with causing higher percentage of cell viability(Haghdoost et al., 2021)(Zange et al., 1998). We also observed the same trend in MTT assay. Incorporation of PEO and PVP has resulted in higher percentage of cell viability. EVAL membrane showed a percentage of cell viability

value of 122.01 ± 16.54 %. The values of other modified materials were almost in the same range as no significant difference observed. Percentage of cell viability is 93.74 ± 7.82 %, 98.73 15.18 % and 115.67 ± 10.09 % for EVAL/PVP 2%, EVAL/BP/PEO 2% and EVAL/NVP 2 % respectively (Figure 29).

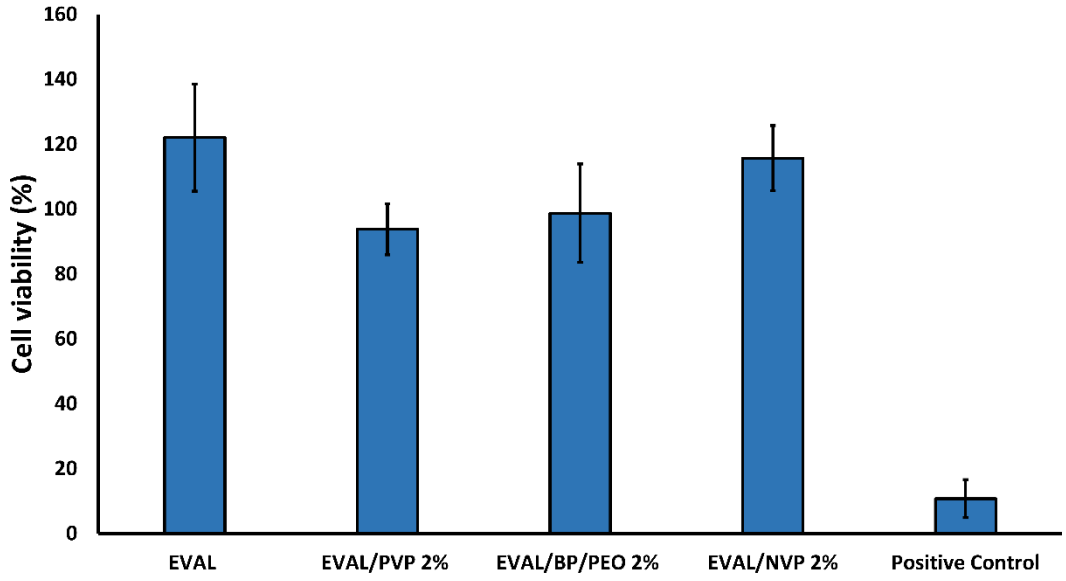


Figure 29: Percentage cell viability result of EVAL and modified EVAL membranes

CHAPTER 4

SUMMARY AND CONCLUSION

Research laboratories and laboratory scientists look forward to develop systems to syringe filter WBC from blood. Such filter setups would be useful for whole blood analysis and flow cytometry sample preparation. Electrospinning enables to fabricate fibrous membranes for leukodepletion filtration purpose. Poly(ethylene-co-vinyl alcohol) is an established polymer with leukodepletion properties. But its poor hydrophilicity makes it unsuitable as an ideal membrane system for leukodepletion filters. Hence, some sort of hydrophilic modifications is needed for improving the efficiency of leukodepletion. The studies made in this work mainly focused on comparing the properties of modified and unmodified EVAL electrospun filter membranes with that of commercially available PALL filter membrane.

EVAL was modified with PVP, PEO and *in-situ* polymerized NVP. Different crosslinking techniques like thermal treatment and UV irradiation were done. Pore size analysis using Image J software showed the values to be 173.01 ± 34.69 µm, 274.08 ± 61.11 µm and 499.78 ± 92.16 µm respectively for EVAL/PVP 2%, EVAL/BP/PEO 2 % and EVAL/NVP 2 % membranes. Wettability analysis evaluated by measuring contact angle showed the values as 63.32 ± 1.56 ^{o,} 75.06 ± 3.11 ^{o} and 48.66 ± 2.62 ^{o} respectively for EVAL/PVP 2%, EVAL/BP/PEO 2 % and EVAL/NVP 2 % membranes. Whole blood filtration study helped to conclude WBC adhesion (%), RBC recovery (%), Platelet adhesion (%) and Hemolysis

(%). For EVAL/PVP 2% membrane WBC adhesion (%), RBC recovery (%), Platelet adhesion (%) and Hemolysis (%) are 99.30 ± 0.695 %, 81.37 ± 17.50 %, 95.37 ± 2.01 % and 0.001 ± 0.0004 % respectively. The same for EVAL/BP/PEO 2 % membrane are 100 %, 85.26 ± 7.89 %, 93.87 ± 2.06 % and 0.03 ± 0.011 % respectively. WBC adhesion (%) of EVAL/NVP 2 % membrane is 48.66 ± 2.62 %. RBC recovery (%) of EVAL/NVP 2 % membrane is 96.76 ± 1.14 %. Platelet adhesion (%) is 64.70 ± 8.42 %. Percentage hemolysis of EVAL/NVP 2 % membrane is 0.056 ± 0.014 %. Evaluation of *in-vitro* cytotoxicity evaluation done in L929 cells proved that all the membranes are non-cytotoxic.

From these results it can be concluded that EVAL modified with grafting of *in-situ* polymerized NVPis showing better leukodepletion which go on par with PALL membrane. The essential features of a filter membrane are better wettability characteristics, porosity, large fiber diameter, pore size, higher percentage of WBC adhesion with maximum RBC recovery and least platelet adhesion. EVAL/NVP modified membrane has shown least value of contact angle compared to other systems. SEM images have shown that EVAL/NVP membrane has large sized fibers with large sized pores. Blood filtration studies also proved that EVAL/NVP membrane exhibited better WBC adhesion and filter blood within short period of time (2 min). Approximately 100 % recovery of RBC was also observed with 0.056 % hemolysis. These results were found to be comparable to that of PALL membrane. This justifies the efficiency of EVAL/NVP membrane as a leukodepletion membrane system for syringe filter application. The currently available filter membranes and even EVAL modified membranes pose the shortcoming of platelet adhesion. Platelet adhesion has to be further reduced to get an ideal leukodepletion membrane. In that case, the final conclusion of the study is that, EVAL/NVP electrospun

membrane is a potential marketable membrane for leukodepletion syringe filter with all adequate properties.

Future perspective

With the increasing demand in the laboratory, new options are required to accommodate the testing needs. Leukocyte recovery or depletion requires approaches that can process large numbers of samples in a shorter interval of time under optimized laboratory conditions.

Future studies in this area include;

- Electrospinning of semi interpenetrating EVAL/NVP polymer network.
- To reduce time of flow of filtration.
- To reduce the platelet adhesion rate.
- To study WBC recovery and viability

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