MISCIBILITY, MORPHOLOGY AND BIOCOMPATIBILITY STUDIES OF NOVEL HEMODIALYSIS MEMBRANES WITH ENHANCED ANTI-OXIDANT AND ANTI-INFLAMMATORY PROPERTIES

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MISCIBILITY, MORPHOLOGY AND BIOCOMPATIBILITY STUDIES OF NOVEL HEMODIALYSIS MEMBRANES WITH ENHANCED ANTI-OXIDANT AND ANTI-INFLAMMATORY PROPERTIES

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ABSTRACT

The prolonged contact of blood with the synthetic polymeric surface in hemodialysis leads to two very important long term complications viz., dialysis induced oxidative stress (DIOS), and membrane induced inflammation (MII). Therefore an attempt was made in this dissertation to fabricate hemodialysis membranes that were modified with phytochemicals, which could potentially reduce both DIOS and MII. Phytochemicals are non-nutritive plant-derived chemicals that are reported to have multiple disease preventing properties. Mangiferin, a phytochemical derived from mango tree and genistein, which is a soy-bean derived phytochemical were chosen based on superior anti-oxidant and anti-inflammatory properties. Membranes were decided to be modified by solution blending of phytochemicals with the polymer solution (physical modification) used to fabricate the membranes.

Miscibility characteristics of poly(amide)/poly(vinyl pyrrolidone)/mangiferin and poly(ether sulfone)/poly(vinyl pyrrolidone)/mangiferin blends were established. Membranes were cast in form of film via non-solvent induced phase separation process. Here DMSO was employed as solvent and water was employed as non-solvent. Unmodified membranes typically showed a dense skin layer while the cross section showed finger-like channel which progressively increased in diameter along the thickness. This gradient morphology ensures sufficient pressure gradient for the toxins to
be removed from the blood to the dialysate stream. Mangiferin modification of PA membranes led to an increase in surface porosity of the membranes whereas PA/PVP/mangiferin membranes not only showed microporous surface but also network structure. Membrane modification was also performed with genistein. Even at very low genistein concentration embedded crystals were observed on the membrane surface. As the concentration of genistein increased large spherulitic structures evolved on the membrane surface.

The anti-oxidant properties of pure phytochemicals, unmodified membranes, as well as modified membranes were measured. The author volunteered himself for donating the blood samples. The unmodified membranes were demonstrated to increase the reactive oxygen species (ROS) generation, which lent support towards the idea of DIOS. DHR assay with pure mangiferin showed an increase in the ROS levels at low concentration range (25–200 µg/ml) and a reasonable decrease (20%) at very high concentration range (500–1000 µg/ml). As a result, the mangiferin modified membranes (both PA/mangiferin as well as PA/PVP/mangiferin membranes) showed enhanced intracellular oxygen radical generation compared to that of the unmodified membranes. On the other hand, genistein modified membranes showed suppression of ROS levels in a dose dependent manner. It was hypothesized and experimentally confirmed that the presence of glucose in mangiferin might be one of the reasons for the observed increase in ROS levels. Measurement of cytokine profiles revealed that genistein modified membranes were effectively able to suppress the levels of IL-1β, IL-6, and TNF-α compared to that of mangiferin modified membranes.
DEDICATION

In memory of my Father, Late. Mr. J. Chandrasekaran
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CHAPTER I
INTRODUCTION

Polymers have evolved from simple trash-bag materials to sophisticated biomaterials that save human lives. Currently, the field of biomaterials has been one of the fastest growing amongst interdisciplinary research frontiers [1]. Though synthetic biomaterials cover a wide spectrum of materials including metals and ceramics, most of the biomaterials/devices have been polymeric in nature. By virtue of some valuable properties such as viscoelasticity and biocompatibility, polymers have become an inseparable part of tissue engineering and regenerative medicine applications, which are considered as future of medical field by experts [2,3,4]. These polymers are non-exotic and commercially available, which are employed in conventional processing such as extrusion, blow molding, injection molding, and film casting. The fact is well amplified through a huge difference in cost between commercial and medical grade polymers. Some of the biomedical applications for polymers include but not limited to cardiovascular stents, drug delivery vehicles, contact lenses, scaffolds for tissue engineering and dialysis membranes.

In the broadest sense, this dissertation is focused on miscibility, morphology, and biocompatibility studies pertaining to hemodialysis membranes. In hemodialysis, a small volume of blood is constantly pumped out of the human body into a machine called the
dialyzer, the main component of which are micron-sized hollow fiber membranes made out of synthetic polymeric materials [5]. The dialyzer has a shell and tube type geometry. The shell side is filled with the dialysate fluid which is made up of essential elements like sodium, calcium, acetate, bicarbonate, potassium, and dextrose dissolved in ultrapure water. The main function of a dialyzer is to remove toxins like urea from blood while retaining valuable proteins and other components of blood plasma. There exists a concentration gradient for the toxins between the blood and dialysate streams that provides the driving force for mass transfer from blood to dialysate. Some of the commonly used polymeric membranes are made of cellulose, cellulose acetate, polyamide, polyether sulfone, and polysulfone.

In general, hemodialysis is prescribed for acute or chronic renal failure (i.e. a condition where the function of kidney is partially or fully compromised in terms of toxin removal from blood). These patients are referred to as ‘end stage renal disease’ (ESRD) patients, who will eventually be prescribed to dialysis or for kidney transplantation. Hemodialysis enables these patients to sustain life. In principle, dialysis membranes achieve the function of glomerulus (the smallest filtration units) of kidney in terms of purification of blood, but these membranes are far from resembling kidney in terms of biocompatibility, which are in turn dictated by physico-chemical properties of the membranes. The main disadvantage of any cellulosic or synthetic polymer membranes is the induction of adverse alteration of blood components which causes serious complications in dialysis patients. Alteration of blood pressure and body temperature, coagulatory state of blood, hyperoxidative state of patients, and reduction in phagocytic action are a few important complications, which are caused by the direct contact of blood
with dialysis membranes. The aforementioned complications are caused by virtue of the “non-self” (something that does not belong to or originate from body) nature of the membranes, which induces protein adsorption, reactive oxygen species (ROS) generation, platelet and complement activation, and cytokine release. Constant research initiatives have been taken over the years to reduce these complications and design dialyzers that resemble glomerulus filtration [6].

One of the major long term problems associated with hemodialysis is oxidative stress, which is triggered due to direct blood-membrane contact and serves as a precursor for pathogenesis of many diseases and complications in hemodialysis patients. In general, ROS molecules (pro-oxidant) serve as signal transduction molecules in order to execute various fundamental processes at a cellular level and also to fight against microorganisms. This multi-purpose role of ROS molecules necessitates the requirement of a delicate balance between pro-oxidant and anti-oxidant molecules. This delicate balance is achieved by virtue of built-in natural anti-oxidant mechanisms in healthy human beings. However, in dialysis patients the tender balance is shifted in favor of pro-oxidant molecules (oxidative stress), owing to prolonged contact of blood with hemodialysis membranes. In general, the dialysis patients’ blood contacts the membranes approximately 3-4 sessions per week and 3-4 hours per session. This state is referred to as “dialysis induced oxidative stress” (DIOS). Hence a major therapeutic approach is to employ an anti-oxidant drug that will retain the balance. An engineering approach is to solve the problem at source, which is the blood-membrane interface. This dissertation focuses on a research plan to develop a modification process using phytochemicals in order to modify the blood-membrane interface. Phytochemicals are plant-derived
chemicals that are reported to possess multiple disease preventing properties. Some of the distinctive advantages of phytochemicals such as natural origin, abundance availability, lower toxicity levels, and easiness of extraction combined with the disease preventing properties have significantly contributed towards the recognition of phytochemicals as alternative and complimentary medicine. The phytochemicals are proposed to be incorporated with the polymer membranes by directly blending with the feed solution used to form the membranes.

A membrane is the heart of the complex dialysis setup. In this study, non-solvent induced phase separation process is used to prepare the membranes, which consists of three components system containing a polymer, a solvent, and a non-solvent. The necessary conditions to form a membrane includes complete miscibility of the solvent/non-solvent pair, complete miscibility of polymer/solvent pair, and partial or complete immiscibility of the polymer/non-solvent pair. Usually, it is highly desired to construct a ternary phase diagram for the polymer/solvent/non-solvent system before attempting to fabricate membranes. Phase diagrams serves as an excellent guide in order to fabricate membranes on a consistent basis with less trial and error procedures. In order to form a membrane, a homogeneous polymer solution consisting of a polymer and a solvent is cast in form of a film on a glass substrate and immersed into a non-solvent bath. Solvent/non-solvent exchange occurs across the interface where the solvent leaves the film and the non-solvent enters the film. As a result, the solution becomes thermodynamically unstable and begins to phase separates into polymer-rich (matrix) and polymer-poor (pores) region. The resulting membrane properties are a direct consequence
of competing thermodynamic and kinetic processes, which can be tailored by suitable manipulation of process variables. The membranes can also be formed as hollow fibers.

This study can be divided into three parts: miscibility studies of the polymers with the phytochemicals, membrane formation and modification, and *in vitro* blood compatibility studies. Chapter 2 deals with the background literature review of different parts of this study. Blood compatible materials are designed by adopting ideas related to general biocompatibility. Hence general issues related to biocompatibility and material requirements in this regard are elaborated. Since the origin of this study stems from problems associated with dialysis, a glossary of terminologies used in dialysis is provided followed by details about dialysis processes and literature review of recent advances. Phytochemicals are introduced and their advantages are highlighted in light of material improvement and specifics are provided for two such phytochemical called ‘mangiferin’ and ‘genistein’. The final part of Chapter 2 is dedicated to the description of concepts related to thermodynamics of polymer solutions, binary and ternary phase diagrams, dynamics of morphology evolution, and membrane fabrication. The criteria for the selection of polymers and phytochemicals are described in Chapter 3 along with experimental and instrumental techniques employed in this study. Miscibility studies pertaining to the chosen polymer/phytochemical blends are described in Chapters 4, and 5. Chapter 6 and 7 describe the aspects of poly(amide)/poly(vinyl pyrrolidone) membrane formation and modification using both mangiferin and genistein respectively. Chapter 8 highlights the outcome of *in vitro* blood compatibility studies while Chapter 9 is devoted for important conclusions of this study and recommendations for future work.
CHAPTER II
BACKGROUND AND LITERATURE REVIEW

2.1. Introduction to Biomaterials

A biomaterial is defined as “a material intended to interface with biological systems to evaluate, treat, augment, or replace any tissue, organ, or function of the body” [7]. The main function of a biomaterial is to interact with the human body in a specified way. The body’s immune system identifies a biomaterial as foreign body and acts against it. Due to this reason, most of the biomaterials induce a non-specific stereotypic biological response. This means that it is necessary to design biomaterials which can stealth itself from body’s immune system and provide the intended function. The comprehension of mechanisms involved with host-material interactions is crucial for the design and development of novel biomaterials. A few important requirements for a successful biomaterial are described below.

Toxicology: A biomaterial should not be toxic, unless specifically designed, like controlled release of drugs to fight cancer. In general, a biomaterial is not supposed to loose anything from its mass. Toxicology deals with substances that migrate out of a biomaterial. For example low molecular weight polymer fragments that leach out from soft tissue implants may exhibit some physiological activity and cell damage.
Biocompatibility: Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a specific application [1]. In other words, materials that are biocompatible for one application may not be considered biocompatible for other applications. Here the term ‘compatibility’ is not defined in a strict thermodynamic sense but rather broadly describes the ability of a material to render a specific function/functions for a given application without eliciting harmful effects.

Healing: There is considerable difference between the mechanisms by which biomaterials heal compared to an injured tissue. Further, the intensity and duration depends on anatomical site involved. An understanding of differences in physiological and biomaterial healing mechanisms is essential for designing good biomaterials.

Mechanical and performance requirements: Any biomaterial needs to have some bulk strength and integrity to serve properly for a specified period of time. This time can vary from few days to few years. For example, a hip prosthesis must be rigid and tough whereas dialysis membranes must be flexible. Biomaterials should retain these properties over the intended life span of the material or device. As it can be seen from the aforementioned requirements, the field of biomaterials research is highly interdisciplinary, which requires fundamental knowledge of biology, chemistry, physics, materials science, chemical and mechanical engineering.

2.2. Classification of Biomaterials

Overall the field of biomaterials can be classified into “hard-tissue replacement” and “soft-tissue replacement”. Hard-tissue replacement materials are typically made of metals and ceramic and used as orthopedic and dental materials whereas soft-tissue
replacement materials are typically made of polymers and used in cardiovascular grafts, and scaffolds for tissue engineering. Biomaterials can also be classified based on the duration of contact with physiological medium. Though this is not a strict classification, it provides an estimate of the expected life of the material. The following table [1] gives a wide range of biomaterials and corresponding applications.

Table 2.1: Some applications of synthetic and natural materials in medicine

<table>
<thead>
<tr>
<th>Application</th>
<th>Type of materials</th>
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<tbody>
<tr>
<td><strong>Skeletal system</strong></td>
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<tr>
<td>Joint replacement</td>
<td>Titanium, stainless steel, poly(ethylene)</td>
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<tr>
<td>Bone plate</td>
<td>Stainless steel, cobalt-chromium alloy</td>
</tr>
<tr>
<td>Bone cement</td>
<td>Poly(methyl methacrylate)</td>
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<tr>
<td>Bony defect repair</td>
<td>Hydroxylapatite</td>
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<tr>
<td>Artificial tendons and ligaments</td>
<td>Teflon, Dacron</td>
</tr>
<tr>
<td>Dental implant for tooth fixation</td>
<td>Titanium, Ti-Al-V alloy, stainless steel, polyethylene, alumina, calcium phosphate</td>
</tr>
<tr>
<td><strong>Cardiovascular system</strong></td>
<td></td>
</tr>
<tr>
<td>Graft</td>
<td>Dacron, Teflon, polyurethane</td>
</tr>
<tr>
<td>Heart valve</td>
<td>Reprocessed tissue, stainless steel, carbon</td>
</tr>
<tr>
<td>Catheter</td>
<td>Silicone rubber, Teflon, polyurethane</td>
</tr>
<tr>
<td><strong>Organs</strong></td>
<td></td>
</tr>
<tr>
<td>Artificial heart</td>
<td>Polyurethane</td>
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</tbody>
</table>
**Skin repair template**
- Artificial kidney (hemodialyzers)
- Heart and lung machine

<table>
<thead>
<tr>
<th>Silicone-collagen composite</th>
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<tbody>
<tr>
<td>Cellulose, polyacrylonitrile</td>
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<td>Silicone rubber</td>
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**Senses**
- Cochlear implants
- Intraocular lenses
- Contact lenses
- Corneal bandage

<table>
<thead>
<tr>
<th>Platinum electrodes</th>
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<tbody>
<tr>
<td>Polymethyl methacrylate, silicone rubber,</td>
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<tr>
<td>Silicone-acrylate, hydrogel</td>
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**Short term biomaterials:** These materials are required to function only for a few hours to a few days. Examples include dialysis membranes and catheters. Though dialysis membranes can be reused, the contact time per cycle is only 3-4 hours. Catheter may only have to perform for a few days.

**Medium term biomaterials:** The functional period of these materials ranges from a few days to a few months. Examples include bone plates and scaffolds. Typical time period is approximately 6 months.

**Long term biomaterials:** The functional period of these materials ranges from a few months to few years. Examples include joint replacements and pacemakers both of which are designed to last for at least 10 years.

The broad focus of this dissertation is to improve the properties of hemodialysis membranes in order to suppress some long term complications. Hemodialyzers (or artificial kidney) are not implanted devices and are classified as extracorporeal devices. Other examples for extracorporeal devices include membrane oxygenators and heart and
lung machines. However, ideas related to general biocompatibility are adopted for the design of materials used in these devices. These ideas originate from the interactions between physiological medium and a foreign body (i.e. something that is not a part of or belong to the body). Hence a general idea of biocompatibility will be established by comparing physiological wound healing and biomaterial wound healing and general implications are presented in the following.

2.3. Physiological Wound Healing

Wound healing is a complex process during which an injured tissue is repaired. It involves inflammation, re-epithelialization, neo-angiogenesis, and connective tissue cell

![Figure 2.1: A schematic representation of events associated with physiological wound healing [8]. (Reprinted with permission from Cambridge University Press)](image-url)
activation with subsequent extra cellular matrix (ECM) degradation and re-synthesis eventually resulting in a scar tissue as shown in Figure 2.1 [8]. All these processes are regulated by cell-ECM interactions and by cytokines and growth factors.

The first response of the host upon injury is to flood the injured area with blood. Fibrinogen within the blood is cleaved into insoluble fibrin which forms a clot that promotes platelet adhesion and aggregation. Platelets derived growth factors (PDGF), transforming growth factors (TGF) like TGF-β initiates coagulation which results in the formation of coagulation matrix. The wound area is rich in cytokines and growth factors which dictates cell migration and proliferation.

The stromal remodeling is made up of three phases. The inflammatory phase is characterized by increased vascular permeability, local release of cytokines and growth factor. Neutrophils clear the injured area from bacteria. Cytokines act as chemotactic agents for neutrophils and macrophages, which in turn secrete tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1). Influx and activation of macrophages are critical to the wound healing process. They participate in matrix synthesis and degradation, and are able to mediate angiogenesis via secretion of cytokines.

During the proliferatory phase fibroblast like cells become activated and migrate to the wound area where the provisional matrix acts as reservoir of cytokines and fibronectin provides the scaffolding for contact guidance. Mesenchymal cells are activated by PDGF and TGF-α produced by the platelets and macrophages. After migration to the wound area the fibroblasts and macrophages begin to produce proteins for ECM and convert fibrin clot into highly vascularized (presence of blood vessels) granulation tissue. Newly formed blood vessels provide nutrients and oxygen. Collagen
synthesis in deep dermis begins few days after injury. Transformation of granulation tissue fibroblasts into myofibroblasts results in the contraction of wound with subsequent diminishing of wound area.

During the *remodeling phase*, which is the longest phase of wound healing, formation of new blood vessels recede and loose provisional matrix is gradually replaced by collagen fibers, the main constituent of scar tissue. Despite the long modeling phase the scar tissue cannot possess the originality of normal tissue [9]. Formation of new blood vessels is the hallmark of physiological wound healing process.

2.4. Biomaterial Healing

Most of synthetic biomaterials heal by a process called as “*foreign body reaction*”. A biomaterial upon contact with a physiological medium elicits non-specific protein adsorption of various forms and conformations. This leads to adhesion of various cells to the biomaterial surface including platelets and monocytes which play an important role in wound healing process. Thus non-specific protein adsorption acts as a precursor for foreign body reaction which does not occur in normal physiological healing. Cell adhesion increases cytokines levels and subsequent pro-inflammatory process. Macrophages cannot phagocyte (i.e., engulf and destroy) the biomaterial because the physical size of biomaterial is larger than their size. As a result the “frustrated” macrophages fuse together to form multi-nucleated giant cells around the biomaterial which lasts for the life time of the biomaterial. The end process of biomaterial healing is the formation of an avascular (absence of blood vessels) collagen fibrous tissue of approximately 50-100 µm thick as shown in Figure 2.2. [4].
Thus the major differences between physiological and biomaterial healing can be summarized as follows.

Table 2.2: Differences between physiological and biomaterial healing

<table>
<thead>
<tr>
<th>Physiological healing</th>
<th>Typical biomaterial healing</th>
</tr>
</thead>
<tbody>
<tr>
<td>No non-specific protein adsorption</td>
<td>Non-specific protein adsorption</td>
</tr>
<tr>
<td>Formation of fibrin and later conversion of fibrin into highly vascularized tissue</td>
<td>Formation of avascular fibrous tissue surrounding the biomaterial</td>
</tr>
<tr>
<td>Acute inflammation</td>
<td>Foreign body reaction</td>
</tr>
</tbody>
</table>

Figure 2.2: Events associated with typical healing of a biomaterial

2.5. Current research trends in biomaterials

In general, the biological response induced by contemporary biomaterials is not controlled. Hence an engineering approach is needed to control the biomaterial
physiological interface. The following section outlines some of the current research trends that pursue this goal.

The surface chemistry and morphology of biomaterials influence the \textit{in vitro} proteins and cell interactions with the surface. However, the \textit{in vivo} response seems to be unaffected. In other words, polymers, ceramics, metals, and composite materials elicit the same response \textit{in vivo}. The main reason for this response is considered to be non-specific protein adsorption, which is a chaotic phenomenon. In living organisms the interactions between surfaces and physiological medium are complex. Hence simple surface chemistries and topographies of biomaterials are insufficient to control the interface. Therefore one of the engineering approaches is to modify the surface of biomaterials to reduce non-specific protein adsorption. There has been a significant research effort to make protein resistant surfaces called non-fouling surfaces. Recent research initiatives have focused on decorating the biomaterial surface with various peptide molecules that in turn dictate the protein adsorption process.

Another actively pursued area of research is to reduce biomaterial infection. A major barrier in the long-term use of medical devices is development of infection. \textit{Staphylococcus epidermidis} is one of the most common bacterial strains that cause these infections and eventually forms a biofilm. Currently, anti-biotics are used as the main form of therapy. However with the emergence of staphylococcal resistance, this form of therapy is quickly becoming ineffective. Anti-bacterial surface treatments, non-fouling surfaces, and anti-biotic controlled release are some of the engineering strategies aimed to prevent bacterial infections.
2.6. Introduction to Hemodialysis [10]

**Acute Renal Failure (ARF):** A reversible condition where abrupt decrease in renal function results in declination of glomerular filtration rate and retention of nitrogenous waste such as blood urea, nitrogen, and creatinine.

**Anti-coagulation:** The contact of blood with the extracorporeal circuit during dialysis initiates clotting which is routinely prevented by anti-coagulation with heparin (an anionic sulfated mucopolysaccharide of variable molecular weight). Heparin inactivates thrombin and other coagulation factors.

**Blood flow rate** \( (Q_b) \): The blood flow rate is defined as the volume of blood which passes the extracorporeal circuit in a certain period of time. It is generally measured in ml/min. In general, typical blood flow rates used during hemodialysis range from 250-400 ml/min.

**Clearance:** Clearance is a measure for the removal of products from blood. It is reflected by the amount of blood cleared of certain products of metabolism such as creatinine or urea in a certain period of time. It is usually expressed in ml/min. The principal determinants of the whole blood clearance during hemodialysis are blood and dialysate flow rate.

**Complement:** Complement components are a complex series of cascading plasma enzymes and proteins which are involved in the immune response and are capable of cell lysis (destruction of cells). Dialysis membranes activate the complement system depending on their composition. In general, hydrophobic membranes (i.e., synthetic membranes) have been shown to minimize this effect.
**Creatinine:** Creatinine is generated in muscle from the conversion of creatine and phosphocreatine. Its generation is proportional to muscle mass. Creatinine is mainly excreted by the kidney and accumulates in renal failure. Creatinine clearance is routinely used to estimate the glomerular filtration rate and also to characterize the performance of a dialyzer.

**Dalton:** Dalton is a non-SI unit of mass (symbol Da), equal to the unified atomic mass unit (atomic mass constant). It is defined to be one twelfth of the mass of an unbound atom of carbon-12 at rest and in its ground state. It is often used in biochemistry and molecular biology to represent the unit mass of a molecule. Larger molecules are expressed in kilo Daltons (kDa).

**Dialysate:** A solution of similar electrolytic composition to blood, the dialysate consists of highly purified water with sodium, potassium, calcium, and magnesium. Bicarbonate and acetate are other ingredients of the dialysate. During hemodialysis, the dialysis solution is passed in a counter current fashion in the dialysate compartment of the dialyzer. Small molecular uremic toxins pass from blood to dialysate during the hemodialysis process and are discarded.

**Dialysate flow rate (Q_d):** The dialysate flow rate is defined as the volume of dialysate solution per unit of time that passes through the dialyzer. It is given in ml/min. The standard dialysis solution flow rate is set to 500 ml/min. Increasing the flow rate will result in increased efficiency of diffusion of urea (and other waste products) from blood to dialysate solution.
**Dialyzer:** A dialyzer shell is a cylindrical cartridge with four ports. Two ports communicate with a blood compartment as a part of the extracorporeal blood circuit and two with a dialysate compartment. Both compartments are separated by a semi-permeable membrane consisting of cellulose, substituted cellulose, or synthetic polymers. To increase blood and dialysate contact area, a large number of hollow fiber tubes are bathed in a dialysate solution. Typical number hollow fibers within a dialyzer range from 7,000-14,000.

**Dialysis membrane:** The dialysis membrane separates the blood compartment from the dialysate compartment in the dialyzer shell. It can be viewed as a micron sized cylindrical tube with porous surface. Water and solutes that are small enough can pass through the membrane pores in both directions, but solutes larger than the membrane pores (such as proteins) will be held back. The membrane acts as a sieve. Solute transport occurs by two different mechanisms: diffusion and convection/ultrafiltration. The membrane materials currently used in dialyzers can be subdivided into three groups: cellulose, synthetically modified cellulose, and synthetic polymers.

**Endotoxin:** Endotoxins are components of the bacterial cell wall which become toxic when released into the blood stream. Endotoxins have high biological activity which induces the uncontrolled production and release of immune mediators such as cytokines. Increased levels of cytokines may result in a harmful systemic inflammatory response in the patient. To avoid the transfer of cytokine-inducing endotoxins from contaminated dialysate to the patient’s blood stream during dialysis therapy, ultrapure dialysate should be used.
**Ethylene Oxide:** Ethylene oxide is a highly toxic gas used for the sterilization of dialyzers and other medical devices. In rare cases, serious anaphylactic reactions have been observed in patients who were treated with ETO sterilized dialyzers. The risk of such anaphylactic reactions can be reduced if the dialyzers are degassed after sterilization by ETO for a sufficient time and if the dialyzers are accurately pre-rinsed with ultrapure water before dialysis. Alternative methods to sterilization by ETO are steam sterilization and irradiation by gamma ray or electron beam.

**High-flux dialysis:** Dialysis using high-flux dialyzers are characterized by membrane permeability of 20-60 ml/hr/mm Hg and ability to remove large sized toxins (approximately 20-30 kDa). In the U.S., high-flux dialysis is often combined with high efficiency dialysis, where blood flow rates is as high as 500 ml/min and dialysate flow rates up to 700-800 ml/min.

**Hollow fiber:** Hollow fibers (also called capillary membranes) are the most common membrane type used in modern dialyzers. A normal dialyzer contains up to 14,000 hollow fibers tightly bound in a fiber bundle. The dialyzer is designed so that the patient’s blood flows inside the hollow fibers, which have an inner diameter of about 180 – 200 µm. The dialysate flows around the outside of the membranes. The wall thickness of the hollow fibers depends on the membrane polymer and varies from 6–8 µm in cellulosic membranes to 30–50 µm in synthetic membranes.

**Kt/V:** In hemodialysis, Kt/V is a dimensionless number which signifies the adequacy of dialysis operation. ‘K’ is the dialyzer clearance for urea, ‘V’ is the patient’s body water volume, and ‘t’ is dialysis time. The US National Kidney Foundation’s hemodialysis Kt/V target is 1.3.
Low-flux dialysis: Dialysis using low-flux dialyzers, characterized by a membrane permeability of < 10 ml/hr/mm Hg and ability to remove toxins as high as 1 kDa. From the size of solute, it can be inferred that these membranes contains very small pore sizes. Low-flux dialysis represents the standard dialysis procedure with a dialysate flow of 500 ml/min and a blood flow of 200-350 ml/min.

Middle molecules: Class of uremic toxins with a molecular weight between 300 and 12,000 Dalton which are not efficiently removed by conventional low-flux hemodialysis. The accumulation of middle molecules may play an important role in the morbidity of patients on long-term dialysis. For example, dialysis-associated amyloidosis is a well-known clinical syndrome in which the accumulation of the middle molecule β2-microglobulin (11,800 Da) may be of particular significance.

Morbidity: A state of being diseased.

Mortality: A state susceptible to death.

Thrombogenicity: Thrombogenicity is the property of an extracorporeal treatment procedure to induce thrombus (clot) formation in the blood.

Ultrafiltration coefficient (K_{UF}): Hemodialysis membranes are traditionally classified according to water flux, a term synonymous with water permeability. The clinical parameter used to characterize the water permeability of a dialyzer K_{UF}. It is defined in terms of ml/hr/mm Hg. It is usually derived from in vitro experiments in which bovine blood is ultrafiltered at varying trans-membrane pressures (TMP). Dialyzer K_{UF} is defined by the slope of the linear portion of this ultrafiltration rate versus TMP curve.
**Uremia:** A clinical syndrome with a variety of symptoms resulting from profound loss of renal function with excretory failure and impairment of metabolic and endocrine functions.

**Urea:** Urea is the end product of protein catabolism. Urea is primarily synthesized in the liver and excreted by the kidneys where it is filtered by the glomerulus and reabsorbed by the proximal and distal part of nephron (the smallest unit of kidney which filters blood). Urea accumulates in case of renal failure. Urea clearance is a useful measure of the performance of a dialyzer.

2.6.1. Hemodialysis Process and Membrane Materials

In blood purification, a small volume of blood is constantly pumped from the patient into the dialyzer unit where urea, uric acid and other waste materials are removed from blood. The dialyzer unit is also referred to as an artificial kidney. A schematic representation of dialyzer unit is drawn in Figure 2.3. Inside the dialyzer unit, blood travels through many tiny tubules called hollow fiber membranes, which are made up of polymers. A large number of such hollow fibers of specific length are potted using an epoxy at both ends and inserted into a shell in such a way that the dialyzer assembly has a shell and tube type configuration. Blood flows inside the hollow fibers while dialysate flows outside the hollow fibers (in the shell side) in a countercurrent fashion. Countercurrent flow promotes continual solute removal by maintaining an adequate diffusion gradient. The dialysate fluid is introduced into the shell at the blood outlet end, where the concentration of solutes has begun to fall (due to removal from the blood). The dialysate fluid flows towards other end where blood enters the dialyzer. Water and
solute can pass across this semi-permeable membrane. Any fluid or solutes that enter the dialysate compartment are drained out as waste.

Figure 2.3 (a) Dialyzer assembly showing different components and (b) cross sectional view of the dialyzer assembly showing hollow fibers filled with blood and surrounding dialysate solution

Although similarities exist between an actual kidney and an artificial kidney, the artificial kidney has limited capabilities. In a fully functional kidney, most of the water and electrolytes are reabsorbed. Unlike kidney, the artificial kidney cannot reabsorb water or solutes that enter the dialysate stream. To overcome the inability to reabsorb water and solutes following removal from the blood, the artificial kidney is manipulated to restrict the actual removal to only surplus water and wastes. This is done by adjusting composition of dialysate solution and ultrafiltration rates. If more water or solutes are removed than desired, it is necessary to replace the lost fluid via intravenous infusions. Blood can be purified by utilizing different modes of operation having different driving forces viz., hemodialysis, hemofiltration and hemodiafiltration [11].
**Hemodialysis:** The hemodialysis processes is carried out in isobaric conditions where the only available driving force for solute removal from blood to dialysate is the concentration gradient. Hence hemodialysis is a diffusion controlled process where thickness of the membrane needs to be as small as possible since solute transport occurs through the thickness of the membrane. Small molecular weight solutes are easily removed by diffusion (e.g. glucose, urea, and creatine). Solute move across the semipermeable membrane until the solute concentrations in blood and dialysate become equal. The hemodialysis process is shown in Figure 2.4. In this figure, $Q^{Bi}$ and $Q^{Bo}$ are the inlet and outlet flow rates of blood stream (measured in ml/min), $Q^{Di}$ and $Q^{Do}$ are the inlet and outlet flow rates of dialysate stream (measured in ml/min), $C^{Bi}_i$, $C^{Bo}_i$ are concentration of component ‘$i$’ in the inlet and outlet blood stream, $C^{Di}_i$, $C^{Do}_i$ are concentration of component ‘$i$’ in the inlet and outlet dialysate stream [11].

![Figure 2.4: A schematic representation of hemodialysis representing various streams](image)

**Hemofiltration:** In hemofiltration, hydrostatic pressure difference is applied across the membrane to facilitate the transport of fluid from the blood to the dialysate. The difference between the blood pressure in hollow fibers and the surrounding pressure is
referred to as transmembrane pressure (TMP), which determines the ultrafiltrate production. Solutes are transferred across the membrane via convection into the filtrate. This mode leads to volume change due to filtration that necessitates pre-dilution or post-dilution (with suitable solution called as “replacement fluid”) to make up for the lost volume. In pre-dilution, the replacement solution is added to the blood before it reaches the filter, diluting the blood in the hollow fibers. In post-dilution, the replacement fluid is added to the blood after the filter (but before returning to patient). Predilution dilutes the blood in the filter, reducing clot formation whereas post dilution concentrates the blood enhancing clearance. Prerequisites for hemofiltration are the use of high-flux dialyzers and blood flow rates of at least 250 ml/min. Hemofiltration process is schematically shown in Figure 2.5.

The terminologies for the blood stream remain the same as hemodialysis. $Q^F$ and $C_i^F$ represent the flow rate of filtrate and concentration of component ‘$i$’ in the filtrate stream. Though dialysis effectively removes small to mid size molecular weight solutes, the pore size limits the ability for diffusion of middle sized molecules. One way to increase the clearance of mid sized molecules is to transport large quantities of water across the semi-permeable membrane, “dragging” additional solutes by convection. While increased ultrafiltration rates during hemofiltration help to remove molecules too large to travel by diffusion, hemofiltration can also lead to excessive removal of small molecules. Consequently, electrolyte removal can be increased beyond that produced by the diffusion gradient alone. When hemofiltration rates are high, careful monitoring is required to maintain normal electrolyte balance. Replacement fluids may need to be
adjusted to keep serum electrolyte levels within range [11]. For example, a hemofiltration rate of 250 ml/min and a fluid removal rate of 60 ml/min \( (Q^F) \) require 310 ml/min \( (Q^{Bi}) \) of blood to be removed from patient and 250 ml/min \( (Q^{Bo}) \) of replacement fluid to be given back.

Figure 2.5: A schematic representation of hemofiltration representing various streams

**Hemodiafiltration:** Hemodiafiltration is a combination of hemodialysis and hemofiltration as shown in Figure 2.6. The use of high-flux dialyzers is a prerequisite for hemodiafiltration. The driving forces are both concentration gradients and hydrostatic pressure difference. The main purpose of blood purification is to purify the blood of waste substances like urea and uric acid. The purification of blood stream is defined by the term called ‘clearance’, which is the rate at which solutes are cleared from the blood. Clearance is abbreviated by the letter \( K \). The clearance of a solute is the volume of blood from which the solute is completely removed per unit time. For example, if a dialyzer has the ability to clear 170 ml/min of urea at a blood flow rate of 200 ml/min, it means that for every 200 ml of blood that flows through the filter, 170 ml will be returned urea free. The remaining 30 ml will have the same concentration of urea as the blood entering the filter. The following formula can be used to calculate the clearance of a solute in ml/min.
The following formula can be used to calculate the clearance of a solute from blood stream [5].

\[
K = \frac{Q^B_i C^B_i - Q^B_o C^B_o}{C^B_i} \quad (2.1)
\]

Since there is no net loss of fluid \( Q^B_i = Q^B_o \), the Eq. 2.1 can be simplified as

\[
K = \frac{Q^B_i (C^B_i - C^B_o)}{C^B_i} \quad (2.2)
\]

Figure 2.6: A schematic representation of hemodiafiltration representing various streams

Eq. 2.1 is applicable strictly for hemodialysis process. For example, when \( Q^B_i = 150 \) ml/min, \( C^B_i = 0.90 \) mmol/lit and \( C^B_o = 0.30 \) mmol/lit, then \( K = 100 \) ml/min, i.e., 100 ml of blood returned per minute is solute free, the remaining 50 ml has a solute concentration of 0.9 mmol/lit.

For hemofiltration clearance is defined as

\[
K = \frac{C^F}{C^B_i Q^F} \quad (2.3)
\]
A second parameter that needs to be defined for any membrane separation process is the flux. For hemodialysis where concentration gradient is the primary driving force, flux of component ‘$i$’ is defined by Fick’s law of diffusion as follows:

$$J_i = -D_i \frac{dc}{dz}$$  \hspace{1cm} (2.4)$$

$D_i$ is the diffusion coefficient of component ‘$i$’ at the membrane interface; $\frac{dc}{dz}$ is the concentration gradient across the membrane. For diffusion of small solutes through liquid filled porous of a membrane, Eq.2.4 can be modified as

$$J_i = \frac{\varepsilon}{\tau} D_i \frac{C_i^{MB} - C_i^{MD}}{\Delta z}$$  \hspace{1cm} (2.5)$$

where $\varepsilon$ is the porosity of the membrane; $C_i^{MB}$, $C_i^{MD}$ are the concentration of component ‘$i$’ blood membrane interface and dialysate membrane interface respectively; $\tau$ is the tortuosity factor and $\Delta z$ is the thickness of membrane. In simple terms, Eq.2.5 means that flux of component ‘$i$’ is directly proportional to concentration gradient across the membrane and porosity but inversely proportional to tortuosity.

For hemofiltration where convection is the primary mode of transport, flux is directly proportional to the applied pressure gradient. The most commonly used model to describe flow rate is based on Poiseuille equation for laminar flow through pores, which are assumed as cylindrical channels (Eq. 2.6):
\[ Q = \frac{\Delta P \pi r^4}{8\eta L} \]  

(2.6)

\( r \) is the radius of membrane pores; \( \Delta P \) is the pressure difference across the membrane and \( \eta \) is the viscosity of solution through the pores.

The artificial kidney does not replace other important kidney functions, including stimulation of red blood cell production, maintenance of blood pressure and sodium regulation. The important factor contributing to the inferior nature of artificial kidney is the bio-incompatibility of the polymer membranes. Biocompatibility is one of the important requirements for any synthetic material to be employed in a physiological setting. Biocompatibility of hemodialysis membranes involves modification of numerous blood components such as complement activation, activation of leukocytes and production of pro-inflammatory cytokines and reactive oxygen species (ROS). These reactions are triggered by direct contact of the membrane with blood and by endotoxin leakage through contaminated dialysate. To prevent clot formation inside the dialyzer, blood is generally mixed with heparin (anti-coagulant) before passing it through the membrane.

The main objective of hemodialysis is to remove the toxic materials like urea, uric acid, creatinine and excess water from the blood stream while retaining valuable blood proteins such as albumin, and ovalbumin. Hence the selection of membrane material plays a very important role in hemodialysis. The membrane should be able to separate the small toxins and retains the large proteins. This is made possible by making a porous
membrane of a specific porosity, pore size, and pore size distribution. The following are some important characteristics required for the membrane:

(a) An asymmetric structure with a very thin skin having a high porosity and narrow pore size distribution.

(b) High filtration rates for a given area

(c) Highly hemocompatible and should not trigger any immunological response

(d) Less interaction with blood proteins

(e) Good sieving properties and with neutral surface (no surface charges)

2.7. Recent Developments in Hemodialysis Technology

Sustained research efforts have been undertaken over the past decade to improve the overall quality of dialysis. Recently, hemodialysis membranes have been perceived as an active biomaterial rather than a passive filter. Such a shift in perception arose due to certain long term complications associated with hemodialysis such as dialysis induced oxidative stress (DIOS) and membrane induced inflammation in hemodialysis patients which have been primarily attributed to the bio-incompatibility of the blood-polymer interface. Hence, modification of blood-polymer interface would serve as an effective approach to reduce the ill-effects of hemodialysis. Broadly, engineering innovations in hemodialysis can be classified as material-related and flow-related. Material research includes modification of existing membranes and development of novel biocompatible materials while flow-related innovation are primarily focused on improvement of mass transfer characteristics of the dialyzer. A brief outline of these developments are
presented in the following few paragraphs with emphasis on the material modification
and development aspects.

Urea clearance, $K$ is given by the expression

$$K = Q_b \left[ \exp \left( K_0 A \left( \frac{1 - Q_b/Q_D}{Q_b} \right) \right) - 1 \right] \left[ \exp \left( K_0 A \left( \frac{1 - Q_b/Q_D}{Q_b} \right) \right) - \frac{Q_b}{Q_D} \right]^{-1}$$

(2.7)

where $K_0$ is the overall mass transfer coefficient of solute of interest; $A$ is the surface
area of membrane and $Q_b, Q_D$ are blood and dialysate flow rates respectively. Eq. 2.7,
which includes the effects of counter flow, is more complicated than the simple definition
of clearance used in Eq. 2.1.

Increasing dialysis dosage (number of times a patient is prescribed to dialysis
treatment) is one of the ways to reduce mortality rate, which is directly associated with
increasing $Kt/V_{\text{urea}}$ [6]. The parameter that is amenable to this need is the $K$ parameter.
From the above expression it can be seen that $K$ can be increased by increasing $K_0 A$
(which is usually lumped parameter), commonly assumed constant for a dialyzer
irrespective of operating conditions. Increasing dialysate flow rate increases the $K_0 A$
value. But dialysate flow increase is limited by flow systems capability. Hence attempts
have been made to increase either $K_0$ or $A$. $K_0$ is composed of three terms.

$$\frac{1}{K_0} = R_s + R_m + R_D$$

(2.8)
where $R_B$, $R_M$ and $R_D$ are the resistances at blood side, membrane and dialysate side, respectively. Increasing flow rate of the dialysate or blood reduces the boundary layer thickness resulting in higher $K_0$ values. Membrane resistance has been reduced by decreasing the thickness of the membranes. Recently, attempt has been made to perfuse the fiber bundle by incorporating solid ‘spacer yarn’ between the hollow fibers [12]. The rationale behind the perfusion of fiber bundle is to evenly spread dialysate across the hollow fibers and avoid stagnant spots within the dialyzer. Another method to improve flow is to give the fiber bundle a moiré or wavy structure [13]. Blood side resistance can be reduced by using smaller diameter fibers [14] which also increase the removal of larger molecules as shown by vitamin B$_{12}$. Recently, a fixed O-ring has been employed in the dialysate compartment within in the dialyzer to alter the pressure profile, thereby providing mixing and disrupting stagnant boundary layers on dialysate side [15].

To develop better biocompatible membrane it becomes necessary to have a good understanding of characteristics of various types of polymers when subjected to blood contact. Hemodialysis membranes can be classified based on physical and chemical properties, morphology, electrical charges, interaction in physiological environment [16]. Based on water permeability the membrane can be classified into low flux and high flux membranes. For low flux membranes, diffusion is the main mode of solute transport. $K_{UF}$ values for these membranes range from 4 to 8 ml/hr/mmHg. Performance for these kinds of membranes can be improved by decreasing the membrane thickness. For high flux membranes $K_{UF}$ values are greater than 20ml/hr/mmHg and convection is the main mode of solute transport. These membranes also have an asymmetric cross sectional morphology.
Cellulose was the first material employed for hemodialysis purposes. However, these membranes showed high complement activation due to the presence of hydroxyl groups. Recent developments have been aimed at replacing the hydroxyl groups with acetate moieties [17]. This replacement increased the pore size leading to improved water permeability. Complete replacement of hydroxyl groups with acetate resulted in further reduction of complement activation. In the second approach only a small percentage of hydroxyl groups (5%) have been replaced by bulky groups, which sterically hinder the interaction between complement activation products and membrane. Synthetically modified cellulose [18] is an example for this approach. Diffusion is the main mode of solute transport for cellulosic membranes.

One of the main reasons for using synthetic polymeric membranes for blood purification was due to their large pore size and their hydrophobicity, which lead to reduction in complement activation. Lysaght [19] showed that water permeability of a membrane is roughly proportional to the fourth power of pore radius assuming Hagen-Poiseuille flow through cylindrical pores. Hence synthetic membranes were initially used for high-flux hemodialysis and hemofiltration. The mode of transport for these membranes is convection. The membranes can be made very thin thus leading to higher permeability and higher solute removal [20]. In general, two prominent types of cross-sectional morphologies are observed in hemodialysis membranes, viz., sponge-like and finger-like. Membranes with various combinations of these two morphologies are also available. From morphology evolution standpoint, the finger-like morphology is the result of nucleation and growth mechanism combined with the hydrodynamics involved in the membrane formation process. Sponge-like structures arises due spinodal
decomposition mechanism. These mechanisms are explained in greater details later in this chapter. The microscopic morphology of a finger-like structure and sponge-like structures are displayed in Figure 2.7 (a) and (b) respectively.

![Figure 2.7: Typical cross-sectional membrane morphology](image)

(a) Finger-like morphology is formed possibly due to the combined effect of nucleation and growth mechanism and hydrodynamics, and (b) sponge-like morphology.

Hydrophobic polymers are potent adhesion promoters and less complement activators whereas hydrophilic polymers are potent complement activators and less adhesion promoters. Matsuda [21] showed that an optimal membrane surface should contain both hydrophilic and hydrophobic components. This can be achieved by blending relatively hydrophobic polymers like poly(amide) (PA), poly(sulfone) (PS), poly(ethersulfone) (PES) with hydrophilic polymer such as poly(vinylpyrrolidone) (PVP). Generally PVP is used to impart sufficient hydrophilicity to membrane and as a wetting agent, modulates surface tension and viscosity during membrane formation. PVP plays an important in determining overall pore size and pore size distribution.

Development of hydrophilic-hydrophobic microdomain structure was based on observations made on blood-material interactions. In general, omission of nucleophilic
groups leads to the reduced complement activation [22]. This observation was made due to high complement activation by membranes made from cellulose and poly(vinyl alcohol). A second concern was the omission of negative charges on surfaces because the presence of negative charges activates intrinsic pathway of coagulation. This coupled with Matsuda’s observation [21] resulted in the development of microdomain structured polymeric membranes. One of the ways to synthesize microdomain structure is by utilizing the self-assembling characteristics of block-copolymer system. Okano et al [23] used hydroxyl ethylmethacrylate and dimethylsiloxane to form hydrophilic-hydrophobic block copolymer and showed platelet activation as a function of domain sizes. It was concluded that inhibitory effect on platelet activation was pronounced when domain sizes were of the order of 10 nm. Other examples of materials used include poly(propylene oxide)-block-poly(amide) copolymer and segmented polyurethanes, which are used in vascular grafts. Wang et al. [24] showed the factors that relates the chemical structure of a polymer to its biocompatibility. In summary, it was observed that biocompatibility of a polymer is decided by (a) the ability to achieve minimum interfacial free energy, (b) balance between hydrophilic and hydrophobic domains, (c) chemical structure, (d) surface charges, and (e) conformational flexibility and surface topography of the polymer for a given application.

In recent years, hemodialysis membranes are considered as a complex active biomaterial rather than a passive porous filter. This view of thinking is critically important especially when extensive investigations demonstrated that the long term complications observed in hemodialysis patients are predominantly caused by direct blood/membrane contacts. There is also a common acceptance that dialysis induced
oxidative stress (DIOS) and membrane induced inflammation are two of the most prevalent long term clinical complications that act as precursors for the pathogenesis of multiple diseases in hemodialysis patients including atherosclerosis. Oxidative stress is the result of a chemical imbalance between the formation of reactive oxygen species (ROS) and anti-oxidants, which has been suggested to play an important role in many diseases [25]. An anti-oxidant is defined as “a substance that when present at low concentration compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate” [26]. Overproduction of free radicals, free metalloproteins, and complexes of transition elements and defective anti-radical defenses are some of the important causes of oxidative stress. The typical sources of free radicals include Fe-heme containing proteins and lipids in the plasma membrane [27].

During phagocyte activation, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (an important source of ROS) catalyzes the electron transfer from NADPH to molecular oxygen resulting in the formation of superoxide anion ($O_2^-$), which is further dismutated into peroxide ($H_2O_2$) and hypochloride ($HCIO$) by the activity of superoxide dismutase (SOD) and myeloperoxidase, respectively [28]. Apart from ROS, the roles of reactive nitrogen species and reactive chlorine species on inflammatory diseases are well documented [26]. Anti-oxidants have been generally sought to neutralize oxygen radicals. The anti-oxidants defense systems can be classified as enzymatic and non-enzymatic. Vitamin E, vitamin C, β-carotene, protein, and albumin thiols are typical examples of non-enzymatic anti-oxidants, whereas catalase, SOD, and glutathione peroxidase represent of enzymatic anti-oxidants [25].
One of the important biological effects of oxidative stress is cell injury or death. Different extra cellular stimuli induced by oxidative stress trigger apoptosis, specifically proinflammatory cytokines, upon exposure to low concentrations of H$_2$O$_2$, NO and its derivatives [29]. ROS generated either extra- or intra-cellularly through ligand-receptor interactions can function as signal transduction molecules to activate cytokine production and modulate gene expression by specific mechanism of cell activation. The over production of ROS has been implicated in the pathogenesis of cancer, atherosclerosis and participates in defective response of the immune system [30]. Given the significant role of oxidative stress toward the pathogenesis of diverse diseases, it is imperative to understand the biological implications of DIOS. In hemodialysis patients, one of the most important consequences of DIOS is the injury to red blood cells (RBC), i.e., hemolysis. Moreover, DIOS leads to other phenomena such as activation of circulating leukocytes and platelets that result in the liberation of enzymes, heme-containing proteins, and transition metals, which in turn produces ROS that act as a catalyst for DIOS [31]. The most important contributor to DIOS is considered to be the bio-incompatibility of the HD membranes, which are in direct contact with RBCs and hence activate phagocytes and generate ROS [32,33]. Further interactions between the monocytes and endothelium have been attributed to oxidative stress, which eventually has led to the production of atheromatous plaque [34,35].

Two types of therapeutic approaches are currently undertaken to reduce oxidative stress. First one is the dietary supplementation of anti-oxidant molecules. Results showed reduced membrane lipid peroxidation of erythrocytes, platelets and blood mononuclear cells by oral intake of vitamin E [36]. Second approach is a pharmacological anti-oxidant
approach where N-acetylcysteine (a glutathione inducer) showed beneficial results on prevention of oxidant mediated renal injury [37]. One of the recent advances in reducing DIOS is due to vitamin E bonded membranes, owing to its anti-oxidant property [38]. The basic function of vitamin E is to protect cell membrane phospholipids from oxidative damage via radical neutralization (Figure 2.8). A direct correlation between increase in RBC malondialdehyde and decrease in RBC vitamin E suggests that RBC membrane undergoes considerable oxidation in hemodialysis patients and vitamin E supplementation leads to the reduction of oxidative damage to RBC [38].

An additional complication of HD membranes is the activation of complement system leading to the generation of anaphylotoxins C3a and C5a that coat the membrane surface and signals the release proinflammatory cytokines such as IL-1β, TNF-α [39]. According to the United States Renal Data System, there were 506,000 HD patients in 2008; the mortality rate of approximately 40% of this population is contributed by

\[ \text{(a) Structure of vitamin E; (b) radical scavenging mechanism of vitamin E via resonance stabilization mechanism} \]

Figure 2.8: (a) Structure of vitamin E; (b) radical scavenging mechanism of vitamin E via resonance stabilization mechanism
oxidative stress [40]. The aforementioned multifaceted complications in HD patients combined with the enormity of HD population attest to the significant problem at hand and emphasizes the imminent need for a truly multifunctional membrane. The goal of this dissertation is to fabricate such multifunctional hemodialysis membranes using phytochemicals as modifying agents.

2.8. Introduction to Phytochemicals

Phytochemicals are non-nutritive chemicals derived from plants that contain protective, disease-preventing compounds. More than 900 different phytochemicals have been identified as components of leaves, roots, vegetables, and fruits. It is estimated that a single serving vegetables and fruits contain up to 100 different phytochemicals. Phytochemicals are associated with the prevention and/or treatment of at least four of the leading causes of death - cancer, diabetes, cardiovascular disease, and hypertension [41]. They are involved in many biological processes including ones that help prevent cell damage, prevent cancer cell replication, and decrease cholesterol levels. Though the existence of phytochemicals has been known for a long time, only recently they are being recommended for protection against human disease. Alkaloids, flavanoids, hydrocinnamic acids, organosulfides, phenolic acids, carotenes, isoflavones and xanthophylls are some of the commonly occurring phytochemicals. Some common fruits and vegetables that contain phytochemicals include tomato, mango, grape, broccoli, orange, blackberry, pomegranate, and soy. Phytochemicals possess multiple biological properties like anti-oxidant, anti-inflammatory, anti-bacterial, anti-diabetic, anti-tumor, and anti-viral and anti-thrombogenic properties. Some of the distinctive advantages of
phytochemicals such as natural origin, abundance of availability, lower levels of toxicity and easiness of extraction combined with the aforementioned properties have significantly contributed towards the recognition of phytochemicals as alternative and complimentary medicine.

Figure 2.9: Molecular structure common to flavanoid compounds

One of the sub-classes of phytochemicals are called polyphenols, which can be further sub-categorized in to tannins, lignins and flavanoid. Flavanoids are the largest and best studied polyphenols. The molecular structure that is common to flavanoid compounds is shown in Figure 2.9. In this dissertation, two such polyphenols were used viz., mangiferin, and genistein, which are derived from mango tree and soy bean respectively. Details related to the properties of these phytochemicals along with the selection criteria will be discussed in detail in Chapter 3 under materials specification section.

2.9. Membrane Separation

Any membrane is a selective barrier between two streams [42]. Membrane processes can be classified as microfiltration, ultrafiltration, reverse osmosis, and gas separation. Each application imposes specific requirements on the membrane material and membrane structure. For microfiltration and ultrafiltration membranes, the porosity and the pore sizes of the membrane determine the efficiency of filtration. For gas
Figure 2.10: Size range of particles in various membrane separation processes [43]

separation, the selectivity and permeability of the membrane material determine the efficiency of gas separation. Sometimes in gas separation thin nonporous and fragile active layers are often supported on porous support sub layer [44]. The size range and membrane characteristics for each separation process are depicted in Figure 2.10. Any membrane process involves a feed, permeate and retentate streams. In hemodialysis, which is an ultrafiltration process, the feed stream consists of impure blood containing high concentration of urea, permeate stream contains urea that is removed form the blood and retentate stream is the purified blood pumped back into the patients veins.
2.10. Membrane Formation

Porous membranes can be prepared by several methods such as sintering, stretching, track etching and phase separation processes. Majority of the membranes are formed using controlled phase separation processes of a polymer solution into a polymer rich and polymer poor phase. Phase separation can be achieved by the following ways:

1. Thermally induced phase separation (TIPS): A solvent which acts as good solvent at high temperature looses its quality with decreasing temperature leading to phase separation. The solvent is removed by extraction, evaporation, or freeze drying.

2. Air-casting of polymer solution: A polymer is dissolved in a mixture volatile and less volatile non-solvent. After casting the film the more volatile solvent is allowed to evaporate. This increases the less volatile non-solvent concentration leading to reduction in solubility of polymer and thus phase separation takes place.

3. Precipitation from vapor phase: Polymer solution is cast in an atmosphere saturated with non-solvent vapor. Phase separation is induced by vapor diffusion into the polymer solution leading to phase separation.

4. Non-solvent induced coagulation: This is the most commonly used of all the four techniques. Here polymer solution is cast in form of a film or fiber and subsequently immersed into a non-solvent bath (Figure 2.11). Solvent/non-solvent exchange occurs across the interface, thus making the polymer solution thermodynamically unstable leading to the phase separation. Usually, this process is carried out under ambient conditions.
TIPS generally require a binary system where the phase boundaries are shown as a function of polymer composition and temperature. In this proposal membranes will be formed using non-solvent induced coagulation process. Since this process requires polymer, solvent, and non-solvent, isothermal ternary phase diagrams are required to describe the thermodynamics of the system. It should be noted that phase diagram describe only the thermodynamic feasibility of any phase transitions. However kinetic factors dictate the occurrence of particular phase transition favored by thermodynamics. Non-equilibrium processes play an important role during membrane formation [45].

Figure 2.11: Method of membrane formation by non-solvent induced phase separation. Here a polymer solution is cast in form of a film and immersed into a non-solvent bath maintained at room temperature.

2.11. Thermodynamics of Polymer Solutions

Flory and Huggins independently developed a theory, based on lattice model, to explain mixing in polymer solutions [46,47]. It can be described with following
illustration of a lattice model. Consider a solution containing two small molecules. Only one molecule can occupy any given site in the lattice as shown in Figure 2.12. Entropy of a system is the measure of randomness of the system. The increase in entropy due to mixing of two components can be given by Boltzmann's relation

\[ \Delta S_m = k \ln \Omega \]  
\[ \ln \Omega = \frac{N!}{n_1!n_2!} \]

where \( N = n_1 + n_2 \), is the total number of molecules consisting of \( n_1 \) molecules of component 1 and \( n_2 \) molecules of component 2. By applying Stirling’s approximation (\( \ln N! \approx N \ln N - N \)) Eq. 2.10 can be written as

\[ \Delta S'_m = k \left[ (n_1 + n_2) \ln (n_1 + n_2) - n_1 \ln n_1 - n_2 \ln n_2 \right] \]

Substituting \( x_1 = \frac{n_1}{n_1 + n_2} \) and \( x_2 = \frac{n_2}{n_1 + n_2} \) in Eq. 2.11

\[ \Delta S_m = -k \left( n_1 \ln x_1 + n_2 \ln x_2 \right) \]

where \( x_1, x_2 \) are mole fractions of component 1 and 2 respectively.

For a polymer solution Eq. 2.12 needs to be modified because the entropy of mixing decreases due to long chain nature of polymer (Figure 2.13). For equal density system
mole fractions and volume fractions are equal. If $\phi_1$ and $\phi_2$ represents the volume fractions of polymer and solvent and if $r_1$ represents the chain length of polymer then the entropy of mixing for a polymer solution is given by

$$\Delta S_m = -k \left( n_1 \ln \phi_1 + n_2 \ln \phi_2 \right)$$  \hspace{1cm} (2.13)

For an ideal solution enthalpy of mixing is zero, i.e., $\Delta H_m = 0$. Polymers are regular solutions, for which $\Delta H_m \neq 0$. Flory provided following expression for enthalpy of mixing

$$\Delta H_m = z n_1 r_1 \phi_2 \Delta \omega_{12}$$  \hspace{1cm} (2.14)

where $z$, lattice coordination number, gives the number of neighboring cells to a given cell, $r_1$ is chain length of component 1 and $\Delta \omega_{12}$ is internal energy of formation of an unlike 1-2 molecular pair.

$$\Delta \omega_{12} = \omega_{12} - \frac{\omega_{11} + \omega_{22}}{2}$$  \hspace{1cm} (2.15)
where $\omega_{11}, \omega_{22}, \omega_{12}$ are the interaction energy for 1-1, 2-2 and 1-2 contacts respectively.

Substituting $\chi = zr_1 \Delta \omega_2$ into Eq. 2.14

$$\Delta H_m = \chi n \phi_2$$

(2.16)

where $\chi$ is called as Flory-Huggins interaction parameter and has a reciprocal dependence with absolute temperature. An empirical relationship between the interaction parameter and temperature can be given as [48].

$$\chi = A + \frac{B}{T}$$

(2.17)

Figure 2.13: A schematic representation of lattice model for a polymer solution of chain length $r_1$ (filled circles) and solvent (open circles) [48]

Most generalized form of Flory-Huggins interaction parameter is given by Koningsveld to account for deviations from ideality [49]

$$\chi = \left( A + \frac{B}{T} + C \ln T \right) \left( 1 + D\phi + E\phi^2 \right)$$

(2.18)

The general Flory-Huggins equation for a binary system, which is the sum of entropic and enthalpic terms, can be given by
\[
\frac{\Delta G_m}{k_BT} = \frac{\phi_1 \ln \phi_1}{r_1} + \frac{\phi_2 \ln \phi_2}{r_2} + \chi_{12} \phi_1 \phi_2
\]  

(2.19)

2.12. Phase Equilibrium

For a system to be stable, free energy of mixing has to be negative. From the above equation it can be seen that entropy term is always negative due to the appearance of logarithmic terms involving fractions. Hence the stability of the system is decided by the enthalpic term which is in turn decided by the Flory-Huggins interaction parameter \( \chi \). If \( \chi \) is negative, the free energy becomes more negative and favors mixing. This leads to the formation of a single well potential as depicted in Figure 2.14.

![Figure 2.14: A schematic representation of a single well potential](image)

The free energy is concave upwards in the whole composition range. A system will try to minimize its free energy to be stable. In this regard if we consider a mixture represented by two points having a composition \( \phi_1 \) and \( \phi_2 \) on the free energy curve, the
free energy of mixture is given by the point Q' which, is higher than the minimum free energy where the system prefers to be. Hence this mixture will mix to attain a minimum free energy represented by point Q. This is the case when $\chi$ is negative.

When $\chi$ is positive, the summation of enthalpic and entropic term will lead to so called “double well potential” as illustrated in Figure 2.15. In this case any mixture having compositions up to $\phi_1$ and beyond $\phi_2$ will form single phase because in the composition range 0-$\phi_1$ and $\phi_2$-1 the lowest free energy is at $\phi_1$ and $\phi_2$ respectively. However in between $\phi_1$ and $\phi_2$ the free energy curve is concave downwards instead upwards. Hence the system phase separates into two phases the composition of which is given by the line connecting the two points B$_1$ and B$_2$ by a double tangent line. Any point in this line represents the proportions of the two phases that are formed. The free energy of any single point between B$_1$ and B$_2$ is greater than free energy of single phase system. Thus the system is immiscible over this composition range. The point of contact of the double tangent line with the free energy curve defines this composition range of immiscibility. The slope of this line is called ‘chemical potential’ which represent the first derivative of free energy with respect to composition. Hence the condition for phase equilibrium is the equality of chemical potential between two phases. This is mathematically represented as

$$\left(\frac{\partial \Delta G_m}{\partial \phi}\right)_{\phi_1} = \left(\frac{\partial \Delta G_m}{\partial \phi}\right)_{\phi_2}$$ (2.20)

If $\mu$ represents the chemical potential then the condition for phase equilibrium can be written as $\mu_i = \mu_\beta$ where ‘i’ represents the component and ‘$\alpha$’ and ‘$\beta$’ represent the two
phases. This means that chemical potential for component ‘i’ in both the phases should be
same for phase equilibrium. The compositions at which the chemical potential are equal
are called the ‘binodal points’ and their loci of the binodal points at different temperature
forms the ‘binodal curve’ which separates single phase region from two phase region.

The free energy curve changes its slope in between the two points B_1 and B_2 at S_1
and S_2. These two points are called as ‘spinodal points’. In between points B_1 & S_1 and
B_2 & S_2 the system is stable to small changes in local composition and energy is required
to drive the system to phase separate. Hence these regions are called ‘metastable’ regions.
Mathematical condition for spinodal point is that the second derivative of free energy
with respect to composition be zero (i.e. slope at inflexion point = 0). This is given by

$$\frac{\partial^2 \Delta G_m}{\partial \phi^2} = 0$$

(2.21)

The loci of the spinodal points at different temperature form the ‘spinodal curve’.
The region between S_1 and S_2 forms ‘unstable region’ which undergoes phase separation
instantaneously. Figure 2.16 shows a binary phase diagram which of a polymer solution
which is plot of temperature as a function of composition. For small molecule system
both r_1 and r_2 is equal to 1 and hence the entropy of mixing is very large compared to
polymer solvent system. But for a polymer-solvent system r_1 is much larger whereas r_2 is
equal to 1 and for polymer blends both r_1 and r_2 are large numbers. As can be clearly seen
the entropy of mixing has the following order solvent-solvent > polymer-solvent >
polymer-polymer system.
Figure 2.15: A schematic representation of a double well potential

Figure 2.16: A schematic representation of temperature composition phase diagram for polymer solutions
2.13. Flory-Huggins Equations for Ternary System

Flory-Huggins equation for a ternary system can be obtained by extending the equations obtained for binary system. Here subscript ‘1’, ‘2’, ‘3’ represent polymer, solvent and non-solvent respectively.

\[
\frac{\Delta G_m}{k_BT} = \phi_1 \ln \phi_1 + \phi_2 \ln \phi_2 + \phi_3 \ln \phi_3 + \chi_{12}\phi_1\phi_2 + \chi_{23}\phi_2\phi_3 + \chi_{31}\phi_3\phi_1
\]  
\tag{2.22}

where \(r_1\) is the number of lattice sites occupied by one polymer chain. \(\chi_{12}, \chi_{23}, \chi_{31}\) are Flory-Huggins interaction parameters between polymer/solvent, solvent/non-solvent polymer/non-solvent pairs respectively. Interaction parameters are tedious to determine. However, experimental methods are available to determine the same. \(\chi_{12}\) can be determined by light scattering or osmometry measurements [45]. \(\chi_{23}\) is determined from activity data of solvents and non-solvents while \(\chi_{31}\) can determined by swelling measurements [50]. \(\chi_{12}\) and \(\chi_{23}\) can be determined more rapidly using intrinsic viscosity and heat of mixing respectively.

Figure 2.17 shows a typical ternary phase diagram used to form porous membrane. The system consists of three components viz., polymer, solvent, and non-solvent. Each corner of the triangle represents pure components. Each side of triangle represents binary compositions and any point inside the triangle represents ternary composition. The phase diagram consists of a single phase region and a two phase region. The single and two phase regions are separated by binodal lines. Two phase region further consists of two metastable regions, one at low polymer concentration, and one at high polymer concentration which are separated by an unstable region. A spinodal line
separates the metastable region from the unstable region. In most of the membrane forming system, the polymer concentration in the polymer-poor phase is so low that a part of phase diagram is very close to the solvent/non-solvent axis. Ternary phase

![Ternary Phase Diagram](image)

Figure 2.17: An example of ternary phase diagram for typical membrane forming polymer/solvent/non-solvent system

diagram as a function of temperature would result in a temperature axis passing through the plane of paper and the phase diagram would look like a 3-D surface inscribed inside a triangular prism. Figure 2.17 shows one slice of the phase diagram at a particular temperature (mostly room temperature).
2.14. Dynamics of Phase Transitions

In order to form a successful membrane, it is necessary to understand the relationship between phase diagrams and dynamics morphology evolution. The three regions of phase separation are pictured in Figure 2.18 and are labeled as $\alpha$, $\beta$ and $\gamma$. Phase separation in regime $\alpha$ and $\gamma$ takes place through nucleation and growth (NG) whereas in $\beta$ regime phase separation takes place through spinodal decomposition (SD).

In general, when polymer concentration $\phi_{\text{polymer}} > \phi_{\text{critical}}$ phase separation is possible through $\alpha$ and $\beta$ regimes, whereas for $\phi_{\text{polymer}} < \phi_{\text{critical}}$ phase separation is possible through $\gamma$ and $\beta$ regimes. In $\alpha$ regime, NG mechanism leads to the nucleation of polymer-poor domains dispersed in polymer-rich matrix leading to the formation of membranes with good mechanical integrity. On the other hand, NG mechanism in $\gamma$ regime results in the nucleation of polymer-rich phase in polymer-poor matrix. Generally it is hard to get a continuous polymer-rich phase in this regime. But if polymer chains are highly stiff and have high molecular weight it is possible to get an open polymer network of very high porosity. This is also subjected to the condition that the polymer-rich phase behaves as a solid from the time of precipitation to the final stages of membrane formation so that the structures do not disintegrate [51]. Figure 2.19 demonstrates the time evolution of domains and typical NG morphology. $\phi^{'}, \phi^{'\prime}$ represent equilibrium compositions whereas $\phi_0$ is the initial composition. NG mechanism is characterized by down-hill diffusion and the two phases are always at equilibrium composition [52]. The domains grow only in size with time.
SD mechanism in $\beta$ regime leads to the formation of a bicontinuous structure. In general SD mechanism is characterized by a spontaneous generation and continuous

![Graphical representation of a ternary phase diagram showing different regimes of phase separation.](image)

**Figure 2.18:** A schematic of a ternary phase diagram showing different regimes of phase separation

![Graphical representation of nucleation and growth mechanism.](image)

**Figure 2.19:** A schematic of nucleation and growth mechanism (a) time evolution and (b) typical morphology consisting of one discrete phase dispersed in continuous phase.
growth of one phase within another phase. The growth originates from small amplitude sinusoidal composition fluctuations with a certain maximum wavelength [53]. SD is characterized by up-hill diffusion where both wavelength and amplitude grows with time as illustrated in Figure 2.20. An important point to note here is that NG and SD process can be distinguished only during initial stages.

Figure 2.20: A schematic of spinodal decomposition mechanism (a) time evolution and (b) typical morphology consisting bicontinuous structure which is the signature of spinodal decomposition mechanism.

2.15. Role of Mass Transfer during Membrane Formation

Mass transfer model for a ternary system was developed by Cohen et al [54] which were later improved by Reuvers and co-workers [55,56], Tsay and McHugh [57]. The latter two models are frequently used in the literature. These models used basic diffusion equations and continuity equations for both the bath side and film side. Using irreversible processes, the fluxes were calculated and related to space derivatives of chemical potentials of three components. The resulting set of equations described the composition of the film and bath side as a function of space coordinates and time. The input variables to solve these equations were thermodynamic and kinetic parameters. Some of the commonly used assumptions used in this modeling were
(a) No convection occurs in film or non-solvent bath and diffusion is one-dimensional.

(b) Instantaneous equilibrium exists at the interface film and non-solvent bath.

(c) No polymer dissolves in coagulation bath.

Reuvers [56] showed that most of the membrane forming systems can be classified into two categories: systems that undergo instantaneous demixing and systems that undergo delayed demixing. During delayed demixing the polymer solution stayed in single phase region for a long time before entering the immiscibility gap. This resulted in higher polymer concentration at interface during the time of phase separation. On the other hand, during instantaneous demixing the immiscibility gap was reached quickly. The delay time was of crucial importance for membrane formation as it determined gradients in pore size over the membrane thickness and the porosity of the membranes.

Strathmann et al [58] studied the effects of processing variables on morphology of the final membranes. The cross sectional morphology of membranes was related to rate of precipitation. Low precipitation rates resulted in sponge-like cross sectional morphology whereas high precipitation rates resulted in finger type morphology. The initial stages of precipitation at film-bath interface are very rapid thus forming a homogeneous layer of high polymer concentration which then hinders the transfer in the thickness direction. Glass transition temperature of the polymer plays an important role during membrane formation. As solvent/non-solvent exchange occurs, there exists a certain ternary composition at which the mobility of the system becomes very low and the structure of the membrane gets fixed. Hence most of the final membrane morphologies tend to be non-equilibrium morphologies.
2.16. Morphology Evolution and Ginzburg-Landau Equations

So far only a thermodynamic description of binary and ternary systems has been given based on Flory-Huggins equations. Further the mass transfer models available in literature can only track the composition path during phase inversion but cannot describe actual evolution of morphology. Therefore the next step is to utilize these thermodynamic potential to describe the morphology or structure evolution of the system; i.e., to describe a dynamic phenomena. But any dynamic phenomenon requires information about changes in system variable in space and time. The thermodynamic free energy and chemical potential described so far does not provide information about time and space dependence. Time Dependent Ginzburg-Landau (TDGL) equations are the simplest type of equations that supply the required information which relates thermodynamic potential to the system variables by constitutive equations. The general form of TDGL equations is given by

\[
\frac{\partial y_i}{\partial t} = -\Lambda_i \frac{\delta F}{\delta y_i}
\]

where \(\Lambda_i\) is mobility which satisfies Onsager reciprocity [59], \(\frac{\delta F}{\delta y_i}\) is thermodynamic potential, \(y_i\) is system variable and \(F\) is free energy. TDGL equations can be classified as follows.

1. **Model A**: Only one non-conserved parameter is considered, e.g., orientation order parameter. It is also known as Allen-Cahn equation.

2. **Model B**: Only one conserved order parameter is considered e.g. volume fraction. The model B equation is also known as Cahn-Hilliard equation.
3. **Model C**: One conserved and one non-conserved parameter are considered. This results in two simultaneous equations.

4. **Model H**: Two conserved and two non-conserved parameter are considered. This results in two conserved equations and two non-conserved equations.

For system where one conserved system variable is considered combining, TDGL equation with continuity equation results in the following equation

\[
\frac{\partial y_i}{\partial t} = \nabla \left[ \lambda_i \nabla \frac{\delta F}{\delta y_i} \right]
\]  
(2.24)

The total free energy of a system is the summation of local free energy and non-local free energy. The local free energy is given by Flory-Huggins equation.

\[
\frac{f_i}{k_B T} = \sum_{i=1}^{m} \left( \frac{1}{n_i} \right) \phi_i \ln \phi_i + \sum_{i=1, i \neq j}^{m} \chi_{ij} \phi_i \phi_j
\]  
(2.25)

where \(n_i\) is the degree of polymerization of component ‘i’ and \(\phi_i, \phi_j\) are volume fraction of component ‘i’ and ‘j’ respectively and \(\chi_{ij}\) is the Flory-Huggins interaction parameter.

The general form of non-local free energy is given by deGennes [60] as

\[
\frac{f_{nl}}{k_B T} = \frac{1}{36} \sum_{i=1}^{m} a_i \left( \nabla \phi_i \right)^2
\]  
(2.26)

where \(a_i\) is the statistical chain length of component ‘i’

Therefore the total free energy of the system is given by the summation of local and non-local free energy.

\[
\frac{F}{k_B T} = \sum_{i=1}^{m} \left( \frac{1}{n_i} \right) \phi_i \ln \phi_i + \sum_{i=1, i \neq j}^{m} \chi_{ij} \phi_i \phi_j + \frac{1}{36} \sum_{i=1}^{m} a_i^2 \left( \nabla \phi_i \right)^2
\]  
(2.27)
Figure 2.21: (a) Hypothetical phase diagram with $\chi_{12} = 0; \chi_{23} = 0; \chi_{13} = 3$; (b) Formation of concentration bands. The parameters utilized were $\phi_1 = 0.3, \phi_2 = 0.65, \phi_3 = 0.05$, $\alpha_{20}/\Lambda_{23} = -1.0$ and $\alpha_{30}/\Lambda_{23} = 0.0$; (c) Evolution of smooth outer surface with bicontinuous core. The parameters utilized were $\phi_1 = 0.2, \phi_2 = 0.5, \phi_3 = 0.3$, $\alpha_{20}/\Lambda_{23} = -0.1$ and $\alpha_{30}/\Lambda_{23} = 0.001$; (d) Formation of porous fiber. The parameters utilized were $\phi_1 = 0.3, \phi_2 = 0.60, \phi_3 = 0.1$, $\alpha_{20}/\Lambda_{23} = -0.01$ and $\alpha_{30}/\Lambda_{23} = 0.001$ [62] (Reprinted with permission from Elsevier).
For a ternary system, Dayal et al [61], used the TDGL-B equations in order to demonstrate morphology during solvent/non-solvent exchange. The expressions of total free energy and chemical potential along with the continuity equations are given below.

\[
\mu_2 - \mu_1 = (1 + \ln \phi_2) - \frac{1 + \ln \phi_1}{n} + \chi_{12} (\phi_1 - \phi_2) + \chi_{23} \phi_3 - \chi_{31} \phi_3
\]

\[
+ \frac{1}{36} \left[ \frac{a_1^2 (\nabla \phi_1)^2}{\phi_1^2} - \frac{a_2^2 (\nabla \phi_2)^2}{\phi_2^2} \right] + \frac{1}{18} \left[ \frac{a_1^2 (\nabla^2 \phi_1)}{\phi_1} - \frac{a_2^2 (\nabla^2 \phi_2)}{\phi_2} \right]
\]

(2.28)

\[
\mu_3 - \mu_1 = (1 + \ln \phi_3) - \frac{1 + \ln \phi_1}{n} - \chi_{13} \phi_2 - \chi_{23} \phi_2 + \chi_{31} (\phi_1 - \phi_3)
\]

\[
+ \frac{1}{36} \left[ \frac{a_1^2 (\nabla \phi_1)^2}{\phi_1^2} - \frac{a_3^2 (\nabla \phi_3)^2}{\phi_3^2} \right] + \frac{1}{18} \left[ \frac{a_1^2 (\nabla^2 \phi_1)}{\phi_1} - \frac{a_3^2 (\nabla^2 \phi_3)}{\phi_3} \right]
\]

(2.29)

Continuity equations are as follows

\[
\frac{\partial \phi_2}{\partial t} = - (\nabla . J_2^*)
\]

(2.30)

\[
\frac{\partial \phi_3}{\partial t} = - (\nabla . J_3^*)
\]

(2.31)

where,

\[
J_2^* = - \Lambda_{22} \nabla (\mu_2 - \mu_1) + \Lambda_{23} \nabla (\mu_3 - \mu_1)
\]

(2.32)

\[
J_3^* = \Lambda_{23} \nabla (\mu_2 - \mu_1) - \Lambda_{33} \nabla (\mu_3 - \mu_1)
\]

(2.33)

Substituting for \(J_2^* \) and \(J_3^* \) gives in to Eqs. (2.30) and (2.31) we have,

\[
\frac{\partial \phi_2}{\partial t} = \nabla \left[ \Lambda_{22} \nabla (\mu_2 - \mu_1) - \Lambda_{23} \nabla (\mu_3 - \mu_1) \right]
\]

(2.34)

\[
\frac{\partial \phi_3}{\partial t} = \nabla \left[ - \Lambda_{23} \nabla (\mu_2 - \mu_1) + \Lambda_{33} \nabla (\mu_3 - \mu_1) \right]
\]

(2.35)

\[
\Lambda_{22} = \frac{\Lambda_{0,22} (\Lambda_{0,11} + \Lambda_{0,33})}{\Lambda_{0,11} + \Lambda_{0,22} + \Lambda_{0,33}} \quad ; \quad \Lambda_{33} = \frac{\Lambda_{0,33} (\Lambda_{0,11} + \Lambda_{0,22})}{\Lambda_{0,11} + \Lambda_{0,22} + \Lambda_{0,33}} \quad ; \quad \Lambda_{23} = \frac{\Lambda_{0,22} \Lambda_{0,33}}{\Lambda_{0,11} + \Lambda_{0,22} + \Lambda_{0,33}}
\]

(2.36)
where $\Lambda_{0,i}$ is the Onsager kinetic coefficient for pure component ‘$i$’.

Dayal et al. [61,62,63] and Guenthner et al. [64] had used the above dynamic equations to illustrate the morphology development for a variety of problems including polymer fibers undergoing solvent/non-solvent exchange. The results pertaining to latter problem are demonstrated in Figure 2.21. A hypothetical phase diagram was established using only a constant value for polymer/non-solvent interaction parameter, while other interactions were set to zero. It was shown that a variety of morphologies can be obtained by manipulating thermodynamic and kinetic parameters of the system.

Some of the important conclusions from above simulations are listed below.

(a) When the net out flow of solvent was greater than (at least an order of magnitude in these simulations) the inflow of non-solvent the average polymer concentration at the surface of the fiber increased leading to a non-porous dense outer layer as shown in Figures 2.21 (b) and (c).

(b) Quenching the fibers in the metastable region leads to the formation of porous fibers as illustrated in Figure 2.21 (d). This is an important requirement to form porous membranes. Further the solvent/non-solvent exchange rate was adjusted in such a way that the composition of the skin layer would not exit into the single phase region thereby generating porous structure.

(c) Quenching the fibers in the unstable region leads to the formation of bicontinuous structure suggestive of spinodal decomposition (Figure 2.21 (c)). This mechanism might come handy in order to get, the much needed, interconnectivity of pores.
The results of these simulations might serve as a good guidance in order to understand and therefore control the membrane morphology.

2.17. Blood-material Interactions

Unlike biocompatibility, there exists no clear definition for blood compatibility, which might serve as guidance for developing standard testing experiments for blood contacting materials. In other words, no synthetic materials that are available till date can be classified as truly blood compatible. This is referred to as “The blood compatibility catastrophe” [65]. It is called so because of the sheer number of blood contacting devices in use and the lack of proper guidelines to assess the blood-material interactions. One of the major reasons for this paucity is due to complexity of blood components. Blood at least has two ways of producing thrombi (aggregation of blood factors, primarily platelets and fibrin with entrapment of cellular elements). One is a protein-based system in which fibrinogen is cleaved into fibrin which forms red thrombi. Other system is platelet-based, which leads to the formation of white thrombi. Further protein-based systems become active when low shear stresses are involved whereas platelet-based systems become active when high shear stresses are involved. If blood can be considered a tissue, then the mobility of blood further complicates the interactions. Though no definitive standards are available to evaluate blood-material interactions, there exist some methods which give an idea about mechanism of thrombi formation and possible reduction methods. So, in the absence of a clear guidance for blood compatibility, it becomes necessary to depend on some of the evaluation procedures that are currently considered acceptable for blood contacting devices.
One of the important classes of biomaterials employed for blood contact is vascular grafts. Vascular grafts are commonly fabricated from woven or knitted Dacron® or textured polytetrafluoroethylene (Gore-tex®). Smooth walled vascular grafts have generally not been considered for long term application, since smooth surfaces may not permit proper surface healing. Polytetrafluoroethylene (PTFE) (Teflon®) is another widely used material for vascular grafts because of its inert and oxidation resistant properties. For vascular grafts, the essential blood compatibility requirement is the suppression of thrombi formation at low and high shear rates. The other class material that contacts blood is dialysis membranes. Recently, vitamin E-modified polysulfone membrane (PS-ViE) have been used which can take advantage of antioxidant activity of vitamin E [40]. In order to assess the blood compatibility of these membranes the following tests are conducted.

1. Measurement of oxidized lipoproteins (measurement of anti-oxidant property)
2. Cytokine generation (evaluation of anti-inflammatory property)
3. Complement activation test
4. Platelet spreadability

Hence, it becomes clear that each blood contacting material should undergo a battery of tests depending on application to assess the ability of non-thrombogenicity or blood compatibility. In summary, blood compatibility is still not clearly defined and no standard list of tests is available to evaluate for the same. However, some tests like time for coagulation and platelet adhesion provides an idea to assess thrombi resistance and platelet resistance, which forms a part of blood compatibility issues.
3.1. Materials

From Chapter 2, it has become clear that it is necessary to have both hydrophobic and hydrophilic components in hemodialysis membranes. Therefore, poly(amide) (PA) and poly(ether sulfone) (PES) were chosen as hydrophobic polymers whereas poly(vinyl pyrrolidone) (PVP) was selected as the hydrophilic polymer. Mangiferin and genistein were selected as the phytochemicals. The selection criteria for polymer and phytochemical selection along with their physico-chemical properties are highlighted in this chapter.

3.1.1 Hydrophobic and Hydrophilic Polymers

An amorphous PA (commercial name: TROGAMID® T5000) was chosen as a hydrophobic polymer. It was provided by Degussa Corporation (New Jersey, USA). This is an aromatic polyamide, ($M_n = 20,000$ and $M_w = 63,000$) [66] which possesses excellent film forming properties with a reported water absorption of 5.1 wt% under specified conditions [67]. The structure of PA is shown in Table 3.1 and has a glass transition temperature of 144 °C. The diamine structure is a 1:1 mixture of 2,2,4 and 2,4,4-trimethylhexamethylenediamine. PA based hemodialysis membranes are currently...
employed in dialyzers and are manufactured by companies like Gambro Corporation. PA membranes are shown to be less protein adsorbing and complement activating compared to cellulose acetate membranes [68]. In other words, PA membranes are demonstrated to be blood compatible. PA is soluble in highly polar solvents and alcohols and insoluble in most of the commonly used organic solvents.

PES was chosen as the second hydrophobic polymer in this study. It absorbs as low as 1.8 wt% of water under saturated conditions. PES (commercial name: Ultrason® E 6020P) is an amorphous polymer having a weight average molecular weight (Mw) of 46,000 with glass transition temperature of 230 °C and was generously provided by BASF Corporation (Wyandotte, MI, USA) [69]. This PES grade is biocompatible, approved by Food and Drug Administration (FDA) for food and blood contacting applications and commonly employed in dialyzer membrane applications [70]. PES based dialyzers are manufactured by companies such as Baxter International Incorporated. Majority of the hemodialysis membranes that are currently employed serve for single use only, which means that the dialyzer unit is discarded after single use. This is economically unfavorable for the patients considering the fact that each patient needs to purify their blood at least 3 times a week. Owing to higher glass transition temperature, the PES based dialyzer units can be steam sterilized at higher temperatures or electron beam sterilized multiple times without altering the morphology and separation characteristics of the membranes. This attractive feature of PES served as the selection criteria for this study. PES is soluble in highly polar solvents and insoluble in most of the commonly used organic solvents. The structure of PES and physico-chemical properties are tabulated in Table 3.1
As described in Chapter 2, hydrophobic polymers are potent adhesion promoters of proteins and cells and less complement activators whereas hydrophilic polymers are potent complement activators and less adhesion promoters. Hence it is necessary to blend hydrophobic and hydrophilic polymers. For this study, poly(vinylpyrrolidone) (PVP) \( (M_w = 40,000) \) was chosen as hydrophilic polymer and was bought from Sigma Aldrich, USA. In general, PVP is used to impart hydrophilicity and as a wetting agent that modulates surface tension. PVP is highly biocompatible and is used as a binder in pharmaceutical tablets that provides mechanical strength to the tablets. PVP absorbs as high as 40 wt% of water under saturated conditions. Unlike PA and PES, PVP is soluble in almost all solvents and also in water. The structure of PVP and physico-chemical properties are shown in Table 3.1

3.1.2. Solvent and Non-solvent

Some of the PA membranes needed for commercial separation process require strong acids in order to dissolve PA. However, utilization of strong acids in preparing HD membranes warrants caution as they may not be pharmacologically suitable. Hence dimethylsulfoxide (DMSO) was chosen as solvent. DMSO has been shown to be pharmacologically benign [71,72]. Further, DMSO is capable of dissolving PA, PES and PVP and also the phytochemicals which are selected in this study. A reagent grade DMSO, purchased from Sigma-Aldrich, was employed as solvent, and was utilized without further purification. Reverse Osmosis grade water was employed as non-solvent for membrane formation. DMSO is highly soluble in water and traces can be removed from the membranes by repeated washing with water.
Table 3.1: Polymers used in this study and some of their physical properties

<table>
<thead>
<tr>
<th>Properties</th>
<th>PA</th>
<th>PVP</th>
<th>PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical structure</td>
<td><img src="image1.png" alt="Chemical structure PA" /></td>
<td><img src="image2.png" alt="Chemical structure PVP" /></td>
<td><img src="image3.png" alt="Chemical structure PES" /></td>
</tr>
<tr>
<td>Amorphous/</td>
<td>Amorphous</td>
<td>Amorphous</td>
<td>Amorphous</td>
</tr>
<tr>
<td>crystalline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Density (g/cc) at 23</td>
<td>1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1-1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.37&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;g&lt;/sub&gt; (°C) at 5</td>
<td>144&lt;sup&gt;d&lt;/sup&gt;</td>
<td>173&lt;sup&gt;e&lt;/sup&gt;</td>
<td>230&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>°C/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular weight</td>
<td>45,400 [73, 74]</td>
<td>40,000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46,000&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b, c</sup> – From manufacturer’s data
<sup>d, e, f</sup> - Experimental findings from this study.

3.1.3. Properties of Phytochemicals and Selection Criteria

Mangiferin was selected as the first phytochemical in this study. Mangiferin is a naturally occurring glucosyl xanthone (2-C-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone) obtained from barks, leaves and fruits of *Mangifera indica* (Mango tree). It has a molecular formula of C<sub>19</sub>H<sub>18</sub>O<sub>11</sub>, molecular weight of 422 g/gmol and melts at 271° C. Chemical structure of mangiferin is illustrated in Figure 3.1(a). It is known for its anti-oxidant [75,76,77,78] anti-tumor [79], anti-viral [80,81,82], antibacterial and anti-fungal [83], and immunomodulatory [84] activities. Other properties of...
mangiferin include anti-platelet [85], anti-thrombotic [86] and anti-inflammatory [87,88]. Pinto et al. [89] has recently reviewed all the biological activities of xanthone derivatives with emphasis on mangiferin. The procedure for isolation [90] and characterization [91,92] is also available in literature. To justify the use of mangiferin the following section discusses some of the biological mechanism through which these properties are rendered.

![Chemical structures](a) Mangiferin and (b) Genistein

Figure 3.1: (a) Chemical structure of mangiferin demonstrating the presence of four hydroxyl groups and a glucose moiety attached to the xanthone backbone [92]; (b) chemical structure of genistein showing three hydroxyl groups attached to the isoflavone backbone [99].

Mangiferin was the first xanthone ever investigated for pharmacological purposes [93]. A remarkable central nervous system stimulating effect was observed for mangiferin which was due to the inhibition of monoamine oxidase [94]. Mangiferin has the ability to scavenge free radicals [95,96] involved in lipid peroxidation initiation, an activity evidenced by redox properties. Some studies have related the anti-oxidant property of mangiferin with their hepatoprotective effect. It was found to exert protective effect on tissues of heart, kidney, liver, and brain. The anti-inflammatory and immunomodulatory effects of mangiferin are related to the reduction on the expression of inflammation-related genes in macrophages such as TNF-α [97], interleukins 1β [98],
TGF-β [97], colony-stimulating factor, NFκB and secondary mediators such as NO synthase, cyclooxygenase-2 and intercellular adhesion molecule-1(ICAM-1). A study conducted by Lin et al [86] revealed that the inhibition of platelet aggregation of xanthone derivatives was due to the reduction in thromboxane formation and phosphoinositide breakdown. In this study washed rabbit platelet were used and their aggregation was induced by collagen.

During the material selection process, it was thought that the presence of glucose molecule in mangiferin might alter the blood glucose levels of hemodialysis patients (false positive) leading to unwanted complications. As majority of hemodialysis population is diabetic in nature, a phytochemical without glucose molecule was sought which would also possess good anti-oxidant properties. Hence, genistein was chosen the second phytochemical in this dissertation. Genistein (4',5,7-Trihydroxy isoflavone) is a soybean-derived polyphenol that belongs to a class of chemicals called isoflavones (Figure 3.1(b)) that contains three hydroxyl groups per molecule. It has a molecular formula of C_{15}H_{10}O_{5}, molecular weight of 270 g/mol and melts at 306° C. Genistein was shown to reduce H_2O_2 production in HL-60 cancer cells and human polymorphonuclear cells in a dose dependent manner [99]. It also suppressed superoxide anion formation and exogenous production of H_2O_2 [100]. One of the important findings, which served as a rationale towards selecting genistein as one of the polyphenol for this proposal, is its ability to prevent low density lipoprotein (LDL) oxidation in presence of copper ions, superoxide, or nitric oxide radicals. Genistein also effectively protected the human endothelial cells from damage caused by oxidized LDL. These finding suggest that genistein is not only a good antioxidant but also a vascular protective agent [101]. In
addition to its antioxidant properties, genistein was also shown to be a good immunosuppressive agent. Genistein suppressed natural killer cell activation when stimulated by lipopoly saccharides [102]. Atluru et al. showed that genistein inhibited IL-2 production and IL-2 receptor expression without being toxic to the T-cells [103]. Fiedor et al. showed that transplanted pancreatic cells survived longer after genistein treatment. Both these findings indicate that genistein plays a critical role in suppression of lymphocyte activation [104]. From the above literature evidences it becomes clear that polyphenols possess useful multifunctional properties, which can be appropriately utilized towards development of multifunctional biomaterials.

3.2. Experimental Methods

For characterization of pure samples and blends, solutions of pure polymers, phytochemicals and required blends were prepared in DMSO at a polymer concentration of 10 wt% and homogenized for 48 hours followed by solvent removal under vacuum at 150 °C for 24 hours.

3.2.1. Thermo Gravimetric Analysis (TGA)

Thermal stability studies were conducted using a thermo gravimetric analyzer (TGA) (TA Instruments, Model 2050). Approximately 10 mg of the samples were used for each run. As received polymers and phytochemicals as well as various polymer/phytochemical blends were subjected to TGA analysis at a heating rate of 10 °C/min from 25 ºC to 500 ºC in a nitrogen atmosphere with a flow rate of 120 ml/min. The TGA experiments were also performed with solvent cast samples. The temperature at
which the weight loss was more than 5% of the initial weight was regarded as the degradation temperature. Care was taken to ensure that the temperature employed for any further characterization of materials was selected below the degradation temperature.

3.2.2. Differential Scanning Calorimetry (DSC)

Thermal analysis of the samples was conducted at a heating rate of 10 °C/min using TA Instruments (Model 2920) differential scanning calorimeter (DSC) calibrated for temperature and enthalpy using indium standard having a melting point of 165.5 °C. Samples weighing 7–10 mg were sealed in aluminum hermetic DSC pans using a crimping device. A sealed empty aluminum pan was used as a reference. Nitrogen gas was purged to the unit to maintain an inert atmosphere. The second runs of DSC curves were used to avoid the thermal history. For DSC experiments, pure samples and blends were prepared as explained in sample preparation section. The first DSC run was employed in order to eliminate thermal history. The data from second run DSC is used for further analysis.

3.2.3. Fourier Transformed Infrared (FTIR) Spectroscopy and Polarized Optical Microscopy (POM)

For FTIR analysis, the samples were prepared by solution casting on KBr discs using a 5 wt% solution in DMSO. The samples were dried under vacuum at 150 °C for 24 hours to ensure complete removal of any residual solvent. The samples were then stored in desiccators until further use. Infrared measurements were recorded on a FTIR spectrometer (Thermo Scientific Nicolet 380) at a resolution of 4 cm⁻¹ averaged over 32
scans. Since each of the components show characteristic affinity towards moisture, care was exercised to minimize the effects of moisture with the aid of a temperature cell by heating the samples to 150 °C and spectra were recorded after equilibrating at 100 °C to assure the absence of moisture and residual solvent. The samples for POM experiments were prepared under the same conditions as FTIR samples, except that thin films (~10 μm) were cast on glass substrates. All samples were stored in desiccators until further use. An optical microscope (BX60, Olympus) equipped with a 35 mm digital camera (EOS 400D, Canon) and a hot stage (TMS 93, Linkam) was used for the POM study. The images were captured at 200X magnification.

3.3. Phase Diagrams

Ternary phase diagram for polymer/solvent/non-solvent system was experimentally constructed by cloud point measurements. A series of polymer solutions of varying concentrations were prepared by adding specified amounts of polymer (dried at 80 °C overnight for moisture removal) with solvent and mixed thoroughly for 48 hours to obtain homogeneous solutions. Non-solvent was added to each of the solutions that are constantly mixed and were maintained at 25 °C. The amount of non-solvent required for the solution to turn turbid was regarded as the cloud point. Once the solution turned turbid, samples were immediately drawn and morphology of the solution was carefully observed under optical microscope. Cloud point measurements were duplicated to ensure consistency.
3.3.1. Ternary Phase Diagrams for Phytochemical/solvent/non-solvent System

Solvent and non-solvent were mixed at various compositions and maintained in a thermostat maintained at 25 °C in order to remove the heat of mixing. Small quantities of phytochemical were incrementally added to this mixture and sufficient time between additions was provided to ensure equilibrium. Phytochemical addition was continued until saturation point was reached where the solution can no longer dissolve phytochemical. This point was considered as saturation point. Upon reaching saturation, samples were drawn and morphology was observed with an optical microscope. Experiments were duplicated to ensure consistency.

3.3.2. Membrane Casting

Polymer pellets were vacuum-dried at 80 °C for 24 hours and subsequently dissolved in solvent to required concentration and blend composition. In order to prepare phytochemically modified membranes, appropriate amount of phytochemicals were mixed with polymer solution. The solutions were mixed thoroughly for 48 hours and degassed under vacuum at room temperature to remove trapped air. The solutions used to cast membranes will hereafter be referred as ‘feed solution’. The membranes were prepared by casting the homogeneous solutions in form of a film of pre-determined thickness on a pre-cleaned glass plate followed by immersion into non-solvent maintained at 25 °C (reverse osmosis grade water unless otherwise mentioned). The coagulated membranes were peeled-off from the glass plate, rinsed with excess non-solvent, and dried at room temperature.
3.3.3. Scanning Electron Microscopy (SEM)

For the SEM study, dried membranes samples were fractured in liquid nitrogen and then sputtered with silver using a sputter coater (Emitech, Model K575X) and analyzed with JEOL-JSM-7401F field emission scanning electron microscope. The sample surfaces were cleaned gently with compressed air before sputtering. In order to get consistent results, SEM data were collected at three different spots that were representative of the whole sample. An accelerative voltage of 10 kV was employed. For each membrane, micrographs corresponding to the cross sectional and surface morphology were captured.

3.4. *In vitro* Blood Compatibility Studies

Details of some of the *In vitro* blood compatibility experiments will be discussed in details in the following section.

3.4.1. Dihydro Rhodamine (DHR) Assay

Venous blood was obtained from the author who had volunteered to donate the blood samples. Ten milliliter specimens were collected in lithium heparin coated tubes and used within 12 hours of sampling. Stock solution of DHR in DMSO was prepared. Phorbol myristate acetate (PMA) was used to activate the neutrophils in the blood samples. Three 100 μl samples were taken from whole blood specimen and placed in separate polypropylene tubes. These were labeled as stimulated, resting and reagent blank samples. PMA solution was added only to the stimulated samples and phosphate buffer saline (PBS) was added to the resting and reagent blank samples. All tubes were
incubated at 37°C for 15 min followed by subsequent addition of DHR solution to the stimulated and resting samples and PBS was added to the reagent blank. The red blood cells were lysed with a solution containing formaldehyde and diethylene glycol and were added to all tubes. The tubes were centrifuged, the supernatant was discarded, and the cells were resuspended in PBS before being centrifuged again. After the second centrifugation, the supernatant was discarded and replaced with fixing solution to stabilize the cells. The samples were then analyzed using a flow cytometer. The principle behind DHR analysis is that DHR 123 is a non-fluorescence molecule that is readily taken up by the neutrophils. In presence of reactive oxygen species (ROS) DHR 123 is converted to rhodamine which fluoresces green and can be detected using a flow cytometer [105].

3.4.2. Enzyme linked immunosorbent assay (ELISA)

In ELISA, an enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. Such a substrate is called a chromogenic

![Figure 3.2: A schematic representation of principle of sandwich ELISA. An antigen is captured between a primary antibody and an enzyme linked secondary antibody. Upon addition of substrate, the enzyme cleaves the substrate to produce color which is directly proportional to the concentration of antigen.](image-url)

73
substrate. Sandwich ELISA is a modification of the basic ELISA and is employed for quantification of antigen [106]. In this technique, an antibody (called primary antibody) is immobilized on a microtiter well. A sample containing antigen is added and allowed to react with the immobilized antibody. After the well is washed, a second antibody (conjugated with an enzyme) specific for a different epitope (macromolecule that is recognized by the immune system) on the antigen is added and allowed to react with the bound antigen. After the removal of excess secondary antibody by washing, a substrate is added. The enzyme present in the secondary antibody cleaves the substrate and produces a colored product and the optical density of the resulting colored product is measured. A schematic representation of ELISA principle is represented in Figure.3.2. In case of immunofluorescence technique, the secondary antibody is coupled with a fluorescent dye (rather than an enzyme) and no substrate is needed to produce a colored end product. The dye is activated by a light source and the resulting fluorescence is measured.

3.4.3. Flow Cytometry

Flow cytometry rapidly measures complicated physical and biological properties of large numbers of individual cells in a very short time. In this technique, a suspension of cells are prepared and slowly introduced into a flowing stream of saline solution. The fluid encapsulates the cells and by controlling the flow of the fluid, cells can be precisely aligned in front of a laser beam. This process is called as hydrodynamic focusing. This approach allows hundreds or even thousands of cells per second to pass in front of the laser beam [107].
An important requirement for flow cytometry is the need to specifically label the cell constituents with fluorescent molecules, which are then used to identify the cells. Cell constituents can be any of a number of cellular components including DNA, which can be labeled by different dyes. These labels help to identify “positive” and “negative” cells. As cells pass one at a time through the measuring region, each cell carrying a label produces a short flash of fluorescence, the intensity of which is directly proportional to the number of “copies” of the labeled constituent present in that cell. These flashes of fluorescence are then collected by a series of optics (different fluorescent dyes produce fluorescence of different wavelengths) and focused on a sensitive detector (Figure 3.3).

Figure 3.3: A schematic of different functional parts of a flow cytometer. Here a suspension consisting of large number of cells is hydro dynamically focused in front of a laser beam. Specific constituents of the cells are fluorescently labeled. The interaction of laser with fluorescent labels provides information regarding that particular constituent. The advantage of flow cytometry is that thousands of cells can be counted in a very short time [108].
The detector, called a photo-multiplier tube, transforms flashes of light into electric pulses, which are recorded by electronic converters and transferred to a computer for assimilation and interpretation. Therefore a cell carrying one or more copies of the constituent of interest is recorded as “positive” while one that does not have any copy of this constituent is identified as “negative”. While measuring signals resulting from the flashes of fluorescence generated by the different labels, a flow cytometer can also measure two other important criteria of each cell passing within the measuring region. These measurements are the cell size and cell density. Incident laser light intersecting a cell will be scattered forward in front of a detector measuring the degree of light scattering. The degree of forward scattering is directly related to the diameter of the cell and will therefore represent the relative size of each cell in the population of cells being measured.

![Flow Cytometer Dot Plot](image)

**Figure 3.4**: A schematic representing a typical flow cytometer dot plot. Each point represents a cell that has passed through the laser beam.
analyzed. Incident light can also be scattered at a right angle to the light beam hence known as right angle light scatter or side scatter. Scattering of light at a right angle results from the presence of granules or organelles inside the cell i.e., complexity of cells. Size and density are very important criteria used extensively in hematology to distinguish between different lineages of blood cells.

A typical light scatter dot plot representing forward scatter (measure of cell volume) vs. side scatter (measure of cell complexity) is shown in Figure 3.4. One group consists of small less dense cells (bottom left), which contains the majority of lymphocytes. The second group is made up predominantly of larger and denser granulocytes (monocytes and neutrophils) and is therefore to the right and above lymphocytes.

3.4.4. Measurement of Cytokine Profiles

The anti-inflammatory properties of phytochemicals and modified membranes were determined by measuring the levels of pro-inflammatory cytokines in serum. Cytokine profiles were measured using specially designed equipment called Luminex® flow cytometer. The Luminex® flow cytometer is uniquely designed by combining the principles of flow cytometry and immunofluorescence. The system is capable of simultaneously measuring up to 100 analytes (in this case multiple cytokines) in a single microtiter well, using very small sample volumes. The Luminex® assays consist of a family of 100 polystyrene microspheres (5.6 μm in diameter) that are fluorescently dyed which, act as both the identifier and the solid surface to build the assay (Figure 3.5). The second component of Luminex® is essentially a flow cytometer which conveys and aligns
the microspheres (instead of cells) in front a laser beams. The third component is the assays that are designed around the microspheres. The polystyrene microspheres are internally dyed with red and infrared fluorophores. Using different amounts of the two dyes, a family of 100 different microsphere sets can be created. Each bead is unique with a spectral signature determined by the red and infrared dye mixture and can be detected with a red laser. Therefore, multiplexing up to 100 tests in a single reaction volume is possible.

The Luminex® reader detects individual microspheres by flow cytometry. The fluidics system of the reader aligns the microspheres into single array as they enter a stream of flowing fluid and then enter a flow cell. The reader uses a 532 nm green laser ("assay" laser) to excite the streptavidin-phycoerythrin (SAPE) dye of the assay. The 635 nm solid state red laser ("classify" laser) is used to excite the dye inside the microspheres and hence determine their "color". An example to simultaneously measure multiple

![Figure 3.5: A family of polystyrene microspheres that are filled with a specific mixture of infrared and red dyes is used in the Luminex assay that serves as surface to build the assay [109]. (Reprinted with permission from Affymetrix, Inc)](image-url)
cytokines from a single serum sample is schematically explained in Figure 3.6. Here two different microspheres (orange and purple colored) are attached with particular antibodies that can bind to IL-10 and TNF-α respectively. The microspheres are incubated with a serum sample collected from a patient so that IL-10 and TNF-α from the serum can bind to the pre-attached antibodies. Subsequently a fluorescent marker linked secondary antibody is added to the microtiter plate followed by a streptavidin-phycoerythrin (SAPE). As the microspheres pass through the laser beams, the signature and the fluorescent signal of each bead can be discriminated and recorded simultaneously.

Figure 3.6: Schematic of an example to simultaneously measure IL-10 and TNF-α from a serum sample. Two set of polystyrene microspheres are individually attached with IL-10 specific and TNF-α specific primary antibodies. These microspheres were then incubated with the patients serum so that IL-10 and TNF-α gets attached to the primary antibody. Subsequently, secondary antibody specific to IL-10 and TNF-α is added, followed by the addition of a fluorescent label. The suspension of microsphere is passed through a red laser which identifies the color of the microsphere and a green laser which analyzes the fluorescent signal from the label [109] (Reprinted with permission from Affymetrix, Inc).
CHAPTER IV
HYDROGEN BONDING INTERACTIONS AND MISCIBILITY STUDIES OF
POLY(AMIDE)/POLY(VINYL PYRROLIDONE)/MANGIFERIN BLENDS

4.1. Introduction

Past couple of decades has witnessed a tremendous surge in interest on plant-derived, non-nutritive compounds called phytochemicals owing to their multifunctional properties including anti-oxidant, anti-inflammatory, anti-tumor, and anti-microbial properties. Phytochemicals have been under extensive investigation as potential compounds for treatment of major diseases such as cancer, diabetes, cardiovascular disease, and hypertension. Some of the compelling advantages of using phytochemicals include their natural origin in plants, fruits and vegetables, abundant availability, easy extraction, minimal levels of toxicity, and inexpensiveness compared to their synthetic counterparts.

The ultimate goal of this dissertation is to develop multifunctional hemodialysis membranes by incorporating phytochemicals into polymer blends. Mangiferin is a naturally occurring glucosyl xanthone (2-C-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone) derived from barks and leaves of mango tree. Physico-chemical properties of mangiferin have been described in detail in Chapter III. By virtue of anti-oxidant, anti-inflammatory, and anti-microbial properties of mangiferin, it is proposed to
incorporate the same with membrane forming polymers. However, prior to fabricating hemodialysis membranes modified with mangiferin, it is crucial to understand molecular interactions of phytochemicals with its polymer counterparts and their miscibility behavior.

The present chapter focuses primarily on the miscibility aspects of PA/PVP blends with and without mangiferin modifications. Miscibility behavior of binary blends of PA/PVP, PA/mangiferin, and PVP/mangiferin will be characterized by DSC and subsequently by FTIR for determining specific intermolecular interactions such as hydrogen bonding contributing to the blend miscibility. Subsequently, this approach will be extended to their ternary system.

4.2. Experimental Section

Thermal analysis of pure components, binary and ternary blends was conducted with DSC and TGA. The instrumental procedures are described in chapter III. As received PA, PVP and mangiferin were subjected to TGA analysis at a heating rate of 10 ºC/min from 25 ºC to 500 ºC in a nitrogen atmosphere with a flow rate of 120 ml/min. The TGA experiments were also performed with solvent cast samples. The temperature at which the weight loss was more than 5% of the initial weight was regarded as the degradation temperature. For DSC experiments, solutions of pure PA, PVP, mangiferin and their blends were prepared in DMSO at a polymer concentration of 10 wt% and homogenized for 48 hours followed by solvent removal under vacuum at 150 ºC for 24 hours. During the first DSC run, the samples were heated only up to 225 ºC (owing to the
thermal stability of mangiferin) to remove any thermal history followed by a second run in which the samples were heated up to 300 ºC.

The interaction between PA/PVP, PA/mangiferin, and PVP/mangiferin were analyzed by FTIR analysis. The FTIR samples were prepared by solution casting on KBr discs using a 5 wt% solution in DMSO. The samples were dried under vacuum at 150 ºC for 24 hours to ensure complete removal of any residual solvent. The samples were then stored in desiccators until further use. Infrared measurements were recorded on a FTIR spectrometer (Thermo Scientific Nicolet 380) at a resolution of 4 cm⁻¹ averaged over 32 scans. Since each of the components show characteristic affinity towards moisture, care was exercised to minimize the effects of moisture with the aid of a temperature cell by heating the samples to 150 ºC and spectra were recorded after equilibrating at 100 ºC to assure the absence of moisture and residual solvent. The samples for polarized optical microscopy (POM) experiments were prepared under the same conditions of DSC and FTIR, except that thin films (~10 μm) were cast on glass substrates. All samples were stored in desiccators until further use. An optical microscope (BX60, Olympus) equipped with a 35 mm digital camera (EOS 400D, Canon) and a hot stage (TMS 93, Linkam) was used for the POM study.

4.3. Results and Discussion

The results of PA/PVP/mangiferin blends are discussed in detail in the following section.
4.3.1. Thermal Stability and Miscibility Characteristics of PA/PVP Binary Blends

Figure 4.1(a) shows the chemical structures and TGA thermograms of pure PA and PVP indicating both polymers to be thermally stable up to ~350 °C. Visually, the cast
films of PA/PVP blends were transparent and colorless. In the DSC scans, a single $T_g$ was found to move systematically with blend composition (Figure 4.1(b)). Moreover, the widths of these glass transition curves were not wider than those of pure components suggesting that the PA/PVP blends were completely miscible over the entire composition range. In the comparison with the Fox equation, [110] a positive departure was observed (Inset of Figure 1(b)) suggesting possible occurrence of strong specific interactions between PA and PVP, which may be contributing to enhanced miscibility. The POM studies of PA/PVP blends revealed no evidence for either phase separated regions or crystalline textures, which are in good agreement with the findings from the DSC experiment.

FTIR experiments were carried out in order to determine the source of this enhanced miscibility of the PA/PVP blends. To prevent moisture absorption, the samples were first heated to 150 °C, and then FTIR spectra were acquired after equilibrating at 100 °C. Figure 4.2(a) depicts the FTIR results of these PA/PVP blends in the 1550-1800 cm$^{-1}$ range. The 1640 cm$^{-1}$ band of PA corresponds mostly to self-associated amide I groups (i.e., hydrogen bonding within the same species) [111, 112, 113]. As PVP is added to PA, a new shoulder corresponding to free C=O appears, which is probably due to release of some of the self-associated amide I groups. Any further addition of PVP shifts the amide I band to higher frequencies implying the release of more self-associated amide I groups. A corresponding effect can be seen in the N-H stretching region (Figure 4.2(b)), where some of the self-associated (intra-molecular hydrogen bonds) N-H vibrations are released, which shifts this band (3322 cm$^{-1}$) to a higher frequency. While the released N-H groups should contribute to an increase in intensity of the free N-H band (3444 cm$^{-1}$),
Figure 4.2: FTIR spectra recorded at 100 °C (a) in the region of 1550–1800 cm$^{-1}$ for PA/PVP blends demonstrates hydrogen bonding formation as shown by the systematic movement of carbonyl peak to higher frequencies and formation of free carbonyl band and (b) in the 2750–3550 cm$^{-1}$ range illustrating the release of some of the self-associated N-H groups of PA due to the addition of PVP.
the trend shows some discrepancy. This seemingly inconsistent trend may be interpreted as a possibility that the released N-H groups readily undergo inter-molecular hydrogen bonding with the carbonyl groups of PVP. The net result is that the free N-H band shifts to a lower frequency despite marginally. Thus, FTIR results confirm our hypothesis that the enhanced miscibility is due to cross-hydrogen bonding (i.e., hydrogen bonding between dissimilar species) between the N-H group of PA and the C=O group of PVP.

4.3.2. Thermal Stability of Pure Mangiferin and its PA Binary Blends

Figure 4.3 exhibits the overlaid plot of DSC and TGA thermograms of neat mangiferin along with its chemical structure. It can be clearly noticed that there is a

Figure 4.3: Overlay of DSC and TGA thermograms for mangiferin illustrating the decomposition of mangiferin occurring at its melting point. The inset shows the chemical structure of mangiferin.
significant weight loss of mangiferin that coincided with the crystal melting peak. It may be envisioned that the thermal stabilization of the mangiferin molecules can be improved through interactions such as hydrogen bonds upon blending with its polymer counterparts. The solvent cast PA/mangiferin samples were transparent to naked eyes, but tinted yellowish owing to the inherent color of mangiferin. As evidenced in Figure 4.4(a), the degradation temperature of PA/mangiferin blends move towards elevated temperature with PA loading. This improvement is insignificant at low loading level (say 10 wt% of PA), but it becomes more pronounced with further increase of PA content. The DSC thermograms of PA/mangiferin are displayed in Figure 4.4(b). A single $T_g$ can be clearly discerned upon addition of mangiferin and it is striking to observe its shift to higher temperature. The increase in the $T_g$ of the PA/mangiferin blends may be attributed to the rigidity of the heterocyclic backbone of mangiferin (please see the chemical structure of xanthone in Figure 4.3) and its polyphenolic nature containing numerous hydroxyl groups, which are capable of interacting with the amide groups of PA chains. The presence of crystal melting peak at higher mangiferin concentrations ($> 40$ wt % of mangiferin) signifies the occurrence of liquid-solid phase transition and also underscores the solubility limit of mangiferin in PA. Another interesting feature of PA/mangiferin DSC thermograms is the virtually invariant nature of the $T_m$ of mangiferin (~267 °C) at low PA concentrations, but a slight depression of $T_m$ occurs at higher PA concentrations (say 50 wt % of PA).

Figure 4.5(a) exhibits the FTIR spectra of PA/mangiferin blends in the 1500-1800 cm$^{-1}$ range. It should be emphasized that both PA and mangiferin are self-associating components, capable of forming intra-molecular hydrogen bonding. With the addition of
Figure 4.4: (a) TGA thermograms for PA/mangiferin blends showing the improved thermal stability of mangiferin due to the addition of PA. The horizontal dashed line corresponds to the 5% weight loss. (b) Second run DSC thermograms for PA/mangiferin showing single $T_g$ and its systematic movement to higher temperature at lower mangiferin concentrations whereas an endothermic crystal melting peak at higher mangiferin concentrations.
Figure 4.5: FTIR spectra recorded at 100 °C (a) in the range of 1500–1800 cm⁻¹ for PA/mangiferin blends demonstrates hydrogen bonding formation as shown by the systematic movement of amide I band of PA and carbonyl band of mangiferin to lower frequencies and amide II band of PA to higher frequencies due to blending and (b) in the 2700–3600 cm⁻¹ range for PA/mangiferin blends.
mangiferin, it is seen that the amide I band of PA exhibit a marginal shift to lower frequencies due hydrogen bonding with the hydroxyl groups of mangiferin. In contrast, the aromatic C=C band does not show any movement since it is not involved in any specific interaction. It is more difficult to analyze band movements in the N-H stretching region due to the overlap of O-H and N-H stretching bands. Nevertheless, there is a movement of the hydrogen bonded N-H band to the higher frequencies (figure 5(b)) due to the release of some of the hydrogen bonded N-H groups of PA upon interacting with hydroxyl groups mangiferin. In addition, the amide II band (1540 cm⁻¹) shifted to a higher frequency upon addition of mangiferin, which is consistent for systems involving stronger N-H interactions [114]. All these observation confirm the occurrence of hydrogen bonding between PA and mangiferin. The possible interactions, in these blends include N-H…C=O (self), N-H…C=O (cross), N-H…O-H (cross) and O-H…O-H (self) interactions. The new shoulder that appears for PA/mangiferin (around 1670 cm⁻¹) cannot be entirely assigned to the free carbonyl group due its overlap with that of the carbonyl of mangiferin.

4.3.3. Thermal and Miscibility Characteristics of PVP/mangiferin Binary Blends

There were some similarities and differences between PA/mangiferin and PVP/mangiferin blends. Visually, all PVP/mangiferin blends were transparent but tinted yellowish. TGA thermograms of PVP/mangiferin blends (Figure 4.6(a)) illustrate the improved thermal stability of mangiferin due to the addition of PVP. DSC thermograms of PVP/mangiferin samples also showed single T_g, which consistently shifted to higher temperatures with increasing mangiferin concentration (Figure 4.6(b)). No liquid-liquid
Figure 4.6: (a) TGA thermograms of PVP/mangiferin blends showing the improved thermal stability of mangiferin due to the addition of PVP. The horizontal dashed line corresponds to the 5% weight loss. (b) Second run DSC thermograms of PVP/mangiferin blends showing a similar trend as that of PA/mangiferin blends except for larger melting point depression suggesting enhanced miscibility of the PVP/mangiferin blends as compared with the PA/mangiferin blends.
phase separation was observed in the entire composition range, but large spherulitic structures developed at higher mangiferin concentrations signifying the isotropic liquid + crystal coexistence regions. However, unlike PA/mangiferin blends, PVP/mangiferin blends showed isotropic-crystal transition at a much higher mangiferin composition (80 wt% vs. 30 wt%). One of key differences between PVP/mangiferin and PA/mangiferin blends is related to the melting point depression of mangiferin crystals. It can be clearly seen that the depression of melting point of PVP/mangiferin blends is significantly more pronounced as compared to PA/mangiferin blends in which the melting peaks remain virtually stationary except for high PA contents where a slight depression is noticeable. This observation combined with the larger isotropic composition range for PVP/mangiferin blends suggests that mangiferin is seemingly more miscible with PVP than with PA. The reason for PVP to show higher affinity towards mangiferin is probably due to strong interactions between PVP and glucose molecule present in mangiferin. The present finding is consistent with literature involving strong hydrogen bonding interactions between PVP and various saccharide moieties [115,116,117].

It should be noted that PVP does not self-associate, but contains functional groups (C=O) that are capable of forming hydrogen bonds with mangiferin via electron donor-acceptor type of interaction. Figures 4.7 (a) and (b) illustrate the FTIR spectrum of PVP/mangiferin blends in the 1500-1800 and 2700-3600 cm⁻¹ range, respectively. These spectra were acquired at 100 °C to minimize moisture absorption. Pure mangiferin shows a broad band at 3366 cm⁻¹ corresponding to the O-H stretching, whereas PVP has no such band in the corresponding region. The FTIR analysis of the present PVP/mangiferin
Figure 4.7: FTIR spectra recorded at 100 °C for PVP/mangiferin blends (a) in the range 1500-1800 cm$^{-1}$ demonstrates hydrogen bonding formation illustrated by the systematic movement of C=O band of the blends to lower frequencies. The aromatic C=C band of mangiferin doesn’t show any shift and (b) in the 2700-3600 cm$^{-1}$ range showing the shift of O-H band of mangiferin bands to lower frequencies.
system is relatively straightforward because only mangiferin can self-associate. The addition of PVP is expected to free up some of the self-associated hydroxyl groups of mangiferin due to hydrogen bonding between C=O of PVP and O-H of mangiferin. The decreasing trend of the O-H stretching band of mangiferin with PVP loading seems to be governed by the competition between hydroxyl-hydroxyl (self) of mangiferin and hydroxyl-carbonyl (cross) interactions between the constituents. As the concentration of PVP is increased the hydroxyl band becomes even broader and shifts to lower frequencies while reducing its magnitude. The corresponding effect can be explicitly seen in the carbonyl spectral region in which the carbonyl peak of PVP shifts to lower frequencies due to strong hydroxyl-carbonyl interactions. These trends are consistent with literature for interactions involving PVP and hydroxyl groups [118,119]. Further, the CH₂ stretching band (2950 cm⁻¹) becomes well defined. This band is very weak in pure mangiferin relative to the hydroxyl band, whereas it is more pronounced in PVP. The intensity of C=C aromatic stretching vibrations (1615 cm⁻¹) gradually reduces due to dilution effect, but this band shows no shift. Judging from the extent of band shift, the miscibility of the PVP/mangiferin seems to be more pronounced as compared to the PA/mangiferin.

4.3.4. Miscibility Characteristics of PA/PVP/mangiferin Ternary Blends

Varying extents of miscibility for the PA/PVP, PA/mangiferin, and PVP/mangiferin, does not guarantee similar miscibility trends for their ternary case. The preferential affinity of mangiferin to PVP relative to PA can be manifested in the ternary
blends of PA/PVP/mangiferin. Upon addition of mangiferin to the PVP rich-blends (e.g., 25/75 PA/PVP), a single glass transition is evident up to 80 wt% of mangiferin, but it

![Figure 4.8: Second run DSC thermograms for PA/PVP/mangiferin ternary blends with (a) 25/75 PA/PVP ratio exhibiting a single T_g shifting systematically to higher temperature with mangiferin loading and (b) 50/50 PA/PVP ratio showing liquid-liquid phase separation at lower mangiferin concentrations but liquid-solid phase segregation at higher mangiferin concentrations.](image-url)

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becomes less distinct with further increase of mangiferin concentration due to the interference arising from the depressed melting point (Figure 4.8(a)). However, in case of 50/50 PA/PVP blends, the addition of only 10 wt % of mangiferin has led to liquid-liquid phase separation as manifested by the appearance of dual glass transition temperatures corresponding to those of individual phases (Figure 4.8(b)). This phase separated trend continues with further increase of mangiferin concentration in which the dual glass transitions shifted to elevated temperatures. At very high mangiferin loadings, these $T_g$ transitions were interfered by the crystal melting peaks of mangiferin, indicating a competition between liquid-liquid phase separation and liquid-solid phase transition. A competing trend of liquid-liquid phase separation with liquid-solid phase transition can be discerned in the PA rich-blend such as 75/25 PA/PVP (data not shown).

The phenomena of liquid-liquid phase separation and solid-liquid phase transition can be confirmed by an independent approach such as POM. The PA/mangiferin and PVP/mangiferin blends were transparent to naked eyes showing no indication of liquid-liquid phase separation at low mangiferin loading. Further increase in mangiferin concentration resulted in crystallization of mangiferin showing the large spherulitic structures in the continuum of isotropic liquid signifying the isotropic-crystal (liquid-solid) coexistence phase. At very high concentrations of mangiferin, the entire field of optical microscope view was filled with solid crystals. In the case of PA/PVP blends, the addition of mangiferin revealed liquid-liquid phase separated domains in addition to isotropic single phase and crystal + liquid coexistence phase.

To better appreciate this complex phase behavior, a ternary morphology phase diagram for PA/PVP/mangiferin ternary blends was mapped out based on information
gathered from DSC along with the optical micrographs of the POM experiments (Figure 4.9). Different types of shading were employed to delineate various coexistence regions. The symbols represent the data points obtained from the DSC showing a single phase (I) (open symbols), crystal solid (C) (filled symbols), two liquid coexistence phases (L + L)

Figure 4.9: Ternary morphology phase diagram, mapped out with the aid of POM and DSC data for the PA/PVP/mangiferin blends, showing various coexistence regions (labeled by different shading). Polarized and unpolarized of optical microscope images representing typical morphology of each region (except the crystal region) are shown in relation to the ternary phase diagram. In the crystal region, the left image shows 10/90 PVP/mangiferin blend whereas the right image shows that of the neat mangiferin crystals. Keys for labels are: I = isotropic (unfilled symbols), C = crystal (completely filled symbols), (I + C)_{PVP} = coexistence of isotropic and crystals for PVP rich compositions (left-half-filled symbols), (I + C)_{PA} = coexistence of isotropic and crystal for PA rich compositions (right-half-filled symbols), L + L = liquid + liquid (bottom-half-filled symbols), L + L + C = liquid + liquid + crystal (hour glass-filled symbols).
(half-filled) and the liquid + liquid + crystal solid phases (L + L + C) (hour glass-filled symbols). The phase boundary of these coexistence regions was drawn by hand based on the morphology observation by POM. Typical bicontinuous morphology of a liquid-liquid phase separated region is evident corresponding to the L + L biphasic region in Figure 9. This co-continuous structure is a typical signature of spinodal decomposition. In the region marked ‘L + L + C’ region, spherulitic (crystal) structures were found to be dispersed in a continuum of liquid-liquid phase separated matrix, which is in good agreement with the preceding DSC results. Finally, in the crystal region, the whole field of view of POM is filled with solid crystals of mangiferin.

4.4. Conclusions

In this chapter, the miscibility behavior of PA/PVP blend without and with mangiferin has been demonstrated. The PA/mangiferin and PVP/mangiferin blends exhibited a single phase at low mangiferin loading, but large spherulites developed at higher mangiferin concentrations signifying the solid-liquid coexistence phase. The FTIR results revealed varying extents of hydrogen bonding in the binary blends of PA/PVP, PA/mangiferin, and PVP/mangiferin, which are responsible for miscibility of these systems. More specifically, mangiferin has preferential hydrogen bonding to PVP relative to PA. The addition of mangiferin to a completely miscible PA/PVP blends led to liquid-liquid and liquid-solid phase separations in a composition dependent manner as demonstrated in their ternary phase diagram.
CHAPTER V
MISCIBILITY CHARACTERIZATION IN RELATION TO PHASE MORPHOLOGY
OF POLY(ETHER SULFONE)/POLY(VINYL PYRROLIDONE)/MANGIFERIN
BLENDs

5.1. Introduction

Multiple use hemodialysis membranes are highly desired primarily for two reasons. First of all, multiple exposures of patients to the same dialyzer potentially suppress DIOS and inflammation associated complications compared to new dialyzer. In addition, multiple use dialyzers are economically favorable compared to new dialyzers. Recent introduction of poly(ether sulfone) (PES) has paved way towards the development of multiple use dialyzers. PES is a high performance engineering thermoplastic with a high glass transition temperature (230 °C) having superior chemical and thermal resistance. Moreover, PES is highly hemocompatible [120] and exhibits profound hydrophobicity [121,122]. Consequently, PES found its way to the hemodialysis membrane applications via blending with hydrophilic PVP [123,124]. It is intriguing to examine whether or not PES is preferable to PA in blending with PVP and mangiferin in the development of multifunctional hemodialysis membranes. As a sequel to the results of chapter IV, the miscibility characterization in reference to the phase diagram of the PES/PVP/mangiferin blends are presented in this chapter. Miscibility behavior of binary
blends of PES/PVP, PES/mangiferin, and PVP/mangiferin was characterized by
differential scanning calorimetry (DSC) and polarized optical microscopy (POM).
Subsequently, Fourier transform infrared spectroscopy (FTIR) experiment was carried
out to probe occurrence of any specific intermolecular interactions such as hydrogen
bonding or dipole-dipole interaction among the blend components. The ternary phase
diagram of PES/PVP/mangiferin was constructed and compared with that of the
PA/PVP/mangiferin system.

5.2. Experimental Section

The glass transition temperature of the blends and crystal melting temperature of
mangiferin were determined by using differential scanning calorimeter (Model 2920, TA
Instruments). For DSC experiments, homogeneous solutions of pure components and
blends were prepared in DMSO followed by solvent removal under vacuum at 150 ºC for
24 hours. Approximately 10 mg of the samples were used for each run. During the first
DSC cycle, the samples were heated from room temperature to 225 ºC to remove any
thermal history and subsequently ramped for a second time from ambient temperature to
300 ºC. Thermal stability studies of the solvent cast samples were conducted using a
thermogravimetric analyzer (TGA) (TA Instruments, Model 2050). In the TGA
experiments, the samples were heated at a rate of 10 ºC/min from 100 ºC to 500 ºC in a
nitrogen atmosphere with a flow rate of 120 ml/min. The temperature at which the 5%
weight loss occurred was regarded as the degradation temperature.

Samples for FTIR analysis were prepared by solution casting on KBr discs from a
5 wt% DMSO solution of the neat components and their blends. After drying under
vacuum at 150 °C for 24 hours, the samples were stored in a desiccator until further use. Infrared spectrum was acquired on a FTIR spectrometer (Thermo Scientific Nicolet 380) at a resolution of 4 cm⁻¹ averaged over 32 scans. In order to minimize the effects of moisture, the samples were initially heated to 150 °C and then equilibrated at 100 °C for data acquisition. The samples for POM experiments were prepared under the same conditions of DSC and FTIR, except that thin films (~10 µm) were cast on glass substrates. An optical microscope (BX60, Olympus) equipped with a 35 mm digital camera (EOS 400D, Canon) and a hot stage (TMS 93, Linkam) was used for acquiring the POM images.

5.3. Results and Discussion

The results of PES/PVP/mangiferin blends are discussed in detail in the following section.

5.3.1. Thermal Stability and Miscibility Characteristics of PES/PVP Blends

Figure 5.1(a) shows the TGA thermograms labeled by the chemical structures of neat PES and PVP. It is seen that PES is more thermally stable than PVP, which may be attributed to the highly aromatic backbone of PES. The DSC thermograms of PES/PVP blends revealed a single Tg, exhibiting a systematic movement with blend composition (Figure 5.1(b)). Moreover, the widths of these glass transition curves of the blends appear comparable to those of pure components suggesting that the PES/PVP blends are miscible over the entire composition range. A positive departure of the experimental Tg from the trend of Fox equation [110] (Inset of Figure 5.1(b)) implies possible specific
interactions occurring between PES and PVP, which may be contributing to the blend miscibility. In visual examination, the cast films of PES/PVP blends were transparent and

Figure 5.1: (a) TGA thermograms of pure PES and PVP demonstrating the thermal stability of pure components. Insets show the chemical structure of the two polymers. (b) DSC thermograms of binary PES/PVP illustrating the complete miscibility of the blends. The inset shows the plot of Tg of blends as a function of weight fraction of PVP.

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colorless to naked eyes. The POM investigation of these PA/PVP blends showed neither phase separated regions nor crystalline textures in conformity with the findings of the DSC experiments.

FTIR experiments were carried out in order to examine the occurrence of any specific interactions between PES and PVP. To prevent moisture absorption, the FTIR spectra were acquired at 100 °C. Figure 5.2(a) depicts the FTIR spectra of various PES/PVP blends in the 1550-1800 cm\(^{-1}\) range. Several characteristic bands of the neat PES can be identified; viz., aromatic C=C band at 1578 and 1485 cm\(^{-1}\), asymmetric and symmetric stretching of O=S=O at 1325 and 1150 cm\(^{-1}\), and 1240 cm\(^{-1}\) (assigned to aromatic ether) [125]. The characteristic peaks along with the band assignments for the PVP includes 1682 cm\(^{-1}\) (C=O stretching), 1461 cm\(^{-1}\) (CH\(_2\) bending), 1428 cm\(^{-1}\) (CH\(_2\) deformation), and 1288 cm\(^{-1}\) (C–N stretching) [126,127].

As can be seen in Figure 5.2(a), the carbonyl band of PVP and O=S=O band (both asymmetric and symmetric) remained virtually constant with little or no spectral shift when PVP was added to PES. In view of the absence of complementary electron donor and acceptor in the chemical structures of both PES and PVP, one can preclude any hydrogen bonding between the two species. In literature, however, PES and PVP were reported to form a miscible pair due to the interactions involving O=S=O of PES and N-C=O of PVP [124]. Moreover, according to an \textit{ab initio} calculation [128], the sulfone groups of PES can be characterized by a net positive charge on the sulfone group. On the other hand, PVP is a tertiary amide, which is highly electronegative in nature. The electrostatic dipolar interaction between the sulfone of PES and tertiary amide group of PVP leads to miscibility of their blends. Figure 5.2(b) illustrates the FTIR spectra in the
Figure 5.2: FTIR spectra recorded for PES/PVP blends at 100 °C (a) in the region of 1000–1800 cm⁻¹ and (b) in the region of 2750–3550 cm⁻¹. No movement of any characteristic band in this region illustrates the absence of any specific interactions between PES and PVP.
2700–3300 cm\(^{-1}\) range showing the characteristic bands of 3069 and 3095 cm\(^{-1}\) (C-H asymmetric and symmetric stretching) for PES and 2950 cm\(^{-1}\) (C-H stretching) for PVP, respectively. The lack of any observable spectral shift in the 2700-3300 cm\(^{-1}\) range implies that the above dipolar interaction is presumably not strong enough to cause any noticeable spectral movement.

5.3.2. Thermal and Miscibility Characteristics of PES/mangiferin Blends

Mangiferin is a small crystalline molecule with a sharp melting peak (~ 267 °C). The solvent cast PES/mangiferin sample tinted yellowish owing to the inherent color of mangiferin. Figure 5.3(a) demonstrates the improvement in thermal stability of PES/mangiferin blends due to the addition of PES. DSC thermograms and the effect of mangiferin addition on \(T_g\) and \(T_m\) of the blends are demonstrated in Figure 5.3(b) and its inset respectively. All the PES/mangiferin samples exhibited single \(T_g\). The addition of mangiferin lowered the glass transition temperature of the blends, but only up to a certain composition range (10-40 wt % mangiferin), above which liquid-solid phase transition took place [129]. This finding suggests that there is a solubility limit for mangiferin in PES. It was difficult to identify the glass transition of the blends once mangiferin started to crystallize. The PES/mangiferin blends also exhibited a slight melting point depression at low concentrations of PES (10 wt % PES). No further depression is discernible with further increase of PES concentration (20-50 wt % PES) and the melting peak virtually remained constant. In addition, no liquid-liquid phase separation was noticed in the entire composition range for PES/mangiferin blends. According to literature [130,131], various drug molecules can exert plasticizing effect on polymers depending upon the rigidity of
Figure 5.3: (a) TGA thermograms for PES/mangiferin blends showing the improved thermal stability of mangiferin due to the addition of PES. The horizontal dashed line corresponds to the 5% weight loss. (b) A plot of T_g and T_m of PES/mangiferin blends as a function of mangiferin concentration showing systematic movement of single T_g to lower temperature up to 40% mangiferin concentration and endothermic crystal melting peak at higher mangiferin concentrations. The melting peaks of blends remain virtually stationary owing to very marginal melting point depression.
the drug molecule and its molecular interactions with the polymer counterpart such as hydrogen bonding. Typical outcomes include the depression of $T_m$ if the drugs were crystalline; otherwise enhanced flexibility of the blends reflecting the reduced $T_g$.

Figure 5.4(a) shows the FTIR spectra of PES/mangiferin blends in the 1500-1800 cm$^{-1}$ range. It should be emphasized that mangiferin molecules can self-associate among themselves via self-hydrogen bonding (i.e., intermolecular hydrogen bonding among the same species), while PES, although contains functional groups, cannot self-associate but are capable of interacting with mangiferin (i.e., cross-hydrogen bonding). With the addition of mangiferin, it is seen that the asymmetric, symmetric stretching bands of O=S=O and aromatic ether of PES shifts to lower frequencies due cross-hydrogen bonding with the hydroxyl groups of mangiferin [132]. As a result, some of the self-hydrogen bonded groups in mangiferin may be released resulting in red shift of the carbonyl band of mangiferin (1656 cm$^{-1}$). In contrast, the aromatic C=C band does not show any movement because the C=C stretching is not involved in any specific interactions. Figure 5.4(b) shows the FTIR spectra of PES/mangiferin blends in the 2700-3600 cm$^{-1}$ range. The broad absorption band of mangiferin at 3366 cm$^{-1}$ indicates the presence of numerous hydroxyl groups including those forming self-hydrogen bonds. When PES is added to mangiferin, the broad peak maximum of 3366 cm$^{-1}$ band shows a low frequency shift, suggesting the cross-hydrogen bonding between PES and mangiferin. Further, the aromatic stretching peak of PES gradually reduces its intensity due to the dilution effect exerted by mangiferin concentration. All of the above observations lend support the conclusion that cross-hydrogen bonding occurs between PES and mangiferin. The possible interactions in these PES/mangiferin mixtures include

Figure 5.4: FTIR spectra of PES/mangiferin blends recorded at 100 °C (a) in the range of 1000–1800 cm⁻¹ demonstrating hydrogen bonding formation as shown by the systematic movement of O=S=O and aromatic ether bands of PES to lower frequency and carbonyl band of mangiferin to higher frequency (b) in the 2700–3600 cm⁻¹ range demonstrating low frequency shift of the broad hydroxyl band of mangiferin.

Figure 5.4: FTIR spectra of PES/mangiferin blends recorded at 100 °C (a) in the range of 1000–1800 cm⁻¹ demonstrating hydrogen bonding formation as shown by the systematic movement of O=S=O and aromatic ether bands of PES to lower frequency and carbonyl band of mangiferin to higher frequency (b) in the 2700–3600 cm⁻¹ range demonstrating low frequency shift of the broad hydroxyl band of mangiferin.
5.3.3. Thermal and Miscibility Characteristics of PVP/mangiferin Blends

The miscibility characteristics of PVP/mangiferin were already demonstrated in chapter IV and thus only the pertinent highlights of the outcome are presented here. Visually, PVP/mangiferin samples were transparent but tinted slightly yellowish arising from the inherent color of mangiferin. The thermal stability of mangiferin was improved in the blends due to the cross-hydrogen bonding with PVP. The PVP/mangiferin blends exhibited a single glass transition temperature shifting to a higher temperature with increasing mangiferin, which is in sharp contrast to the PES/mangiferin blends, where the T_g was found to decline. The neat mangiferin is highly crystalline and thus its T_g is generally not observable in the DSC experiments. Although a single T_g was observable in the low mangiferin compositions (< 40 wt %), the T_g was presumably hidden in the high mangiferin compositions (> 60 wt %) due to the highly crystalline nature of mangiferin. Another important observation is that the addition of PVP to mangiferin caused a significant melting point depression as compared to that of the PES/mangiferin blends where the melting peaks remain virtually stationary. This observation implies that mangiferin is seemingly more miscible with PVP than with PES. Moreover, the FTIR experiments [126] revealed strong hydrogen bonding between PVP and mangiferin as manifested by the appreciable blue shift of the C=O band. Hence, it may be inferred that the highly aromatic xanthone backbone of mangiferin combined with the strong cross-hydrogen bonding between PVP and mangiferin might be responsible for the enhancement of the glass transition temperature of these blends. The possible interactions for the PVP/mangiferin blends include hydroxyl-hydroxyl (self) and hydroxyl-carbonyl (self) of mangiferin, and hydroxyl-carbonyl (cross) interactions.
5.3.4. Miscibility Characteristics of PES/PVP/mangiferin ternary blends

Even though the binary systems showed varying extents of miscibility for the PES/PVP, PES/mangiferin, and PVP/mangiferin systems, there is no assurance that the same miscibility trend will hold for their ternary case because of the preferential affinity of mangiferin for PVP relative to PES. The 50/50 and 75/25 PES/PVP ternary blends containing mangiferin showed the single phase character in low mangiferin concentrations, especially in the PVP-rich compositions. However, the 75/25 PES/PVP at intermediate mangiferin loading of 40–60 wt % exhibited dual glass transition temperatures, suggestive of liquid-liquid phase separation between PES/mangiferin and PVP/mangiferin phases. At much higher mangiferin concentrations, the DSC thermograms were further complicated by the depressed crystal melting peaks of mangiferin, reflecting the competition between liquid-liquid phase separation and liquid-solid phase transition. To identify various coexistence regions, POM experiments were performed on the ternary PES/PVP/mangiferin blends.

In order to better appreciate this complex phase behavior, a ternary morphology phase diagram was mapped out for the PES/PVP/ mangiferin blends on the basis of thermal transitions (i.e., \( T_g \) and \( T_m \) by DSC) along with the morphological evidence from the POM experiments (Figure 5.5). Different types of shading were employed to delineate various coexistence regions. The symbols represent the data points obtained from the DSC and POM studies showing isotropic (I) (open symbols), crystal (C) (filled symbols), liquid + liquid (L + L) (hourglass-filled symbols), and isotropic + crystal (I + C) (half-filled symbols) phases. The phase boundary of these coexistence regions was drawn by hand with the guidance of DSC and POM data. At low mangiferin loading, the
PES/mangiferin and PVP/mangiferin blends were optically transparent and showed no indication of liquid-liquid phase separation. Moreover, it is noticed that there is a clear preferential interaction for mangiferin with PVP relative to PES. On the other hand, the strength of interactions in PES/PVP blends is the weakest among the three constituent pairs, although this pair is completely miscible. Judging from the DSC and the FTIR results, the strength of molecular interactions among the three binary pairs can be categorized in the following sequence, i.e., PVP/mangiferin > PES/mangiferin > PES/PVP. Hence, it may be inferred that the phase behavior of the present ternary system is dominated by the hydrogen bonding interactions between the PVP/mangiferin and PES/mangiferin pairs. As manifested by a large single-phase region in Figure 5.5, the cross-hydrogen-bonding between PVP and mangiferin is much stronger relative to that of PES/mangiferin, especially in the PVP-rich compositions.

With increasing mangiferin, the system exhibits a signature of liquid-liquid phase separated morphology, albeit only in the limited ternary compositions shaded by the horizontal lines (see the left bottom pictures in Figure 5.5). The morphology of the liquid-liquid phase separated domains resembles a sea-and-island type, i.e., suggestive of nucleation and growth. However, caution should be exercised in this simplistic interpretation because one can distinguish the nucleation and growth versus spinodal decomposition only during the early stages of liquid-liquid phase separation. That is to say, the possibility of late stage ripening of spinodally decomposed structure cannot be ruled out. Further increase in mangiferin concentration resulted in crystallization of mangiferin, showing the large spherulitic structures in the continuum of isotropic liquid, signifying the isotropic + crystal (I + C) coexistence phase (see the central bottom and the
Figure 5.5: Ternary morphology phase diagram, mapped out with the aid of POM and DSC data for the PES/PVP/mangiferin blends, showing various coexistence regions (labeled by different shading). Polarized and unpolarized of optical microscope images representing typical morphology of each region (except the crystal region) are shown in relation to the ternary phase diagram. In the crystal region, the left image shows 10/90 PVP/mangiferin blend whereas the right image shows that of the neat mangiferin crystals. Keys for labels were: I = isotropic (unfilled circles), C = crystal (completely filled squares), (I + C)PVP = coexistence of isotropic and crystals for PVP rich compositions (semi-filled square), (I + C)PES = coexistence of isotropic and crystal for PES rich compositions (semi-filled circles) and (L + L) = liquid + liquid (hour glass-filled circles).

right upper pictures). In the PES-rich compositions and lower mangiferin concentrations, the lower solubility of mangiferin with PES has led to a larger (I + C)PES coexistence gap as compared to the (I + C)PVP of the PVP/mangiferin blends. At very high concentrations of mangiferin (>85 wt %), the entire field of the optical microscope view was filled with truncated spherulites of mangiferin crystals (see the right bottom pictures in Figure 5.5). It appears that the increase in mangiferin concentration has resulted in a higher nucleation
density, leading to the development of multiple spherulites, which subsequently impinge on each other.

In comparison with results of PA/PVP/mangiferin, the PES/PVP/mangiferin system exhibited a large single phase region with relatively very small liquid + liquid and solid + liquid coexistence regions, thereby providing useful guidance to controlling the membrane formation step. That is to say, a wide range of ternary composition is available in order to prepare an initially homogeneous casting solution. Moreover, the mangiferin loading can be increased without affecting the isotropic state of the initial casting solution, suggesting that PES/PVP/mangiferin blends are preferred to PA/PVP/mangiferin in the membrane formation process. Although the ternary phase diagram involving the crystalline phase is seemingly complex, the crystallinity of mangiferin can retain its phytochemical properties longer relative to the isotropic amorphous phase. Thus, the presence of mangiferin crystals may render an added advantage for prolonged time release in drug delivery.

5.4. Conclusions

In summary, the miscibility behavior of PES/PVP blend without and with mangiferin has been demonstrated. The binary PES/PVP blends were completely miscible. However, no evidence of hydrogen bonding interactions for PES/PVP blends was observed from the FTIR experiments. The PES/mangiferin and PVP/mangiferin blends were miscible at low mangiferin loading, but large spherulites developed at higher mangiferin concentrations signifying the solid-liquid coexistence phase. The FTIR results revealed varying extents of hydrogen bonding in the binary blends of PES/mangiferin and
PVP/mangiferin, which are responsible for miscibility of these binary systems. The addition of mangiferin to PES lowered the glass transition of the blends, whereas it raised glass transition of PVP/mangiferin blends. It appears that mangiferin has preferentially stronger hydrogen bonding to PVP relative to PES. The addition of mangiferin to a completely miscible PES/PVP blends led to liquid-liquid and liquid-solid phase separations in a composition dependent manner as demonstrated in their ternary morphology phase diagram. It should be emphasized that the PES/PVP/mangiferin system exhibited a larger isotropic region with smaller liquid + liquid and solid + liquid coexistence gaps as compared to those of the PA/PVP/mangiferin.
CHAPTER VI
MEMBRANE FORMATION VIA NON-SOLVENT INDUCED PHASE SEPARATION
IN RELATION TO PHASE DIAGRAMS OF POLY(AMIDE)/POLY(VINYL
PYRROLIDONE) BLENDS CONTAINING MANGIFERIN

6.1. Introduction

Synthetic polymer membranes have occupied a great prominence in numerous industrial separation processes, which include reverse osmosis, ultrafiltration, microfiltration, and gas separation. By virtue of its industrial importance, it has been the subject of extensive investigation for the past several decades, specifically on mechanism of membrane formation, mass transfer modeling and characterization. Recently, these membranes have attracted interest owing to their utility as drug delivery vehicles especially based on biodegradable polymers. A rich variety of morphologies (such as porous or dense skin layer, sponge, or finger type cross-section with asymmetric or symmetric morphology, and gradient pore cross-section) that can be realized during membrane formation has stimulated researchers to take advantage of such morphologies towards controlling the rate of drug delivery. Non-solvent induced phase separation is one of the most commonly used techniques in the preparation of synthetic membranes. In this process, a homogeneous polymer solution is cast in form of film or fiber and then immersed into a non-solvent coagulation bath. Solvent/non-solvent exchange occurs
across the interface where solvent leaves the system and non-solvent enters the system, thus making the solution thermodynamically unstable. Upon reaching the immiscibility gap, the system begins to phase separate into polymer-rich (matrix) and polymer-lean (pores) regions [42].

One of important beneficiaries of membrane technology is hemodialysis or blood purification process. Recently, hemodialysis membranes have been perceived as an active biomaterial rather than a passive filter. Such a shift in perception arose due to certain long term complications associated with hemodialysis such as dialysis induced oxidative stress (DIOS) and membrane induced inflammation in hemodialysis patients which have been primarily attributed to the bio-incompatibility of the blood-polymer interface. Hence, modification of blood-polymer interface would serve as an effective approach to reduce the ill-effects of hemodialysis. In the preceding chapters, the miscibility characteristics of PA/PVP/mangiferin blends were presented and it was demonstrated that there exist cross-hydrogen bonding interactions between the constituents that can be exploited in order to incorporate mangiferin in the polymer matrix. This chapter focuses on various aspects of membrane formation such as construction of phase diagrams, morphology evolution mechanisms and its relation to phase diagrams, mangiferin modification, and morphology analysis pertaining to unmodified and modified membranes.

Some terminologies are defined here in order to better understand the descriptions provided in this chapter. Unmodified PA and PA/PVP membranes refer to membranes that contain only PA or PA/PVP blends respectively. Modified membranes refer to samples that contain PA/mangiferin or PA/PVP/mangiferin. For simplicity, the discussion in this chapter begins with the details related to the formation of unmodified
PA membranes followed by PA/PVP membranes. For the case of modified membranes, PA/mangiferin membranes are discussed first followed by PA/PVP/mangiferin membranes.

6.2. Experimental Section

Results related to membrane formation of unmodified and mangiferin modified membranes are discussed in the following section.

6.2.1. Ternary Phase Diagrams for Polymer/solvent/non-solvent System

Non-solvent induced phase separation process was the chosen method to prepare the membranes in this work. Consequently, it is necessary to construct ternary phase diagrams before preparing the membranes. A ternary phase diagram for PA/DMSO/water system was experimentally constructed by cloud point measurement and the details of the experiment are described in Chapter III. Briefly, the amount of water that is required to turn a clear PA solution (single phase) to turbid (two phase) was regarded as the cloud point. The experiments were repeated with different concentrations of PA solutions in order to form the immiscibility envelope. Experiments were also performed with PA/PVP blends. All the experiments were duplicated in order to ensure consistency.

6.2.2. Ternary Phase Diagrams for Phytochemical/solvent/non-solvent System

As water is a non-solvent for mangiferin, it is necessary to understand the miscibility behavior of mangiferin/DMSO/water system. Details of the experiments are described in Chapter III. Briefly, DMSO and water were mixed at various compositions
followed by incremental addition mangiferin until saturation point was reached where the solution can no longer dissolve mangiferin. All the experiments were duplicated in order to ensure consistency. Optical microscopic observation was conducted after the saturation point to confirm the presence of mangiferin crystals.

6.2.3. Ternary Phase Diagrams for (Polymer + phytochemical)/solvent/non-solvent System

For simplicity, phase diagrams were constructed for (PA+ mangiferin)/DMSO/water system. The procedure is similar to that explained in section 6.2.1 except that each solution now contains PA + mangiferin instead of pure PA.

6.2.4. Membrane Casting

All membranes were cast as described in Chapter III. Unless otherwise mentioned, DMSO was used as solvent and water as non-solvent.

6.2.5. Optical Microscopy and SEM analysis

During the construction of phase diagram with cloud point measurements, it was difficult to identify the source of cloudiness. In other words, cloudiness can result from liquid-liquid phase separation or from crystallization. In order to distinguish the exact type of phase transition, samples were observed using optical microscopy technique. An optical microscope (BX60, Olympus) equipped with a 35 mm digital camera (EOS 400D, Canon) was used to observe the morphology of solutions immediately after cloud point or
saturation point was reached. For optical microscope experiments, samples were smeared on pre-cleaned optical microscope slides and viewed at a magnification of 200X.

For SEM analysis of membrane morphology, dried membrane samples were fractured in liquid nitrogen and then sputtered with silver using a sputter coater (Emitech, Model K575X) and analyzed with JEOL-JSM-7401F field emission scanning electron microscope. The sample surfaces were cleaned gently with compressed air before sputtering. In order to get consistent results, SEM data were collected at three different spots that were representatives of the whole sample.

6.3. Results and Discussion

The experimental ternary phase diagram for PA/DMSO/water system that was determined by cloud point measurements is shown in Figure 6.1. By virtue of its amorphous nature, the homogeneous solutions of PA in DMSO underwent liquid-liquid phase separation upon addition of specific amount of water. Figure 6.1 suggest that PA solutions tend to phase separate into two liquid phases by adding approximately 5 wt% of water. Owing to higher viscosity of polymer solutions (>30 wt% polymer), homogenization of the system after water addition became difficult. Hence the immiscibility gap was extrapolated to the PA/water axis at 5 wt% as per the water absorption measurements conducted in previous studies [67] with this particular type of PA. It should be emphasized that this ternary system only undergoes liquid-liquid phase separation as demonstrated in the optical microscope images shown besides the phase diagram. Under crossed polarizers, both the images were dark signifying the absence of crystalline phase. As mentioned earlier, it is important to have both hydrophobic and
hydrophilic components in hemodialysis membranes. In this regard, it is imperative to construct phase diagram consisting of PA/PVP blends in a mixture of DMSO and water, which is shown in Figure 6.2.

![Phase Diagram](image)

Figure 6.1: Ternary phase diagram for PA/DMSO/Water obtained by cloud point measurements (●) exhibiting liquid-liquid immiscibility gap. The ▲ on water/PA axis is the data reported in literature [67] based on water absorption studies. Optical microscope pictures obtained with parallel polarizers shown on either side of the phase diagram reveal the corresponding isotropic and liquid-liquid phase separated morphology.

PVP is highly soluble in both DMSO and water. As a result, there exists no immiscibility gap for PVP/DMSO/water ternary system. In other words, PVP forms homogeneous solutions in a mixture of DMSO/water at all compositions. By virtue of such solubility behavior of PVP, only the following PA/PVP blend ratios were investigated viz., 75/25, 50/50 and 25/75 as shown in Figure 6.2. Since PVP is highly hydrophilic compared to PA, a reduction in the area of immiscibility gap can be expected with increasing PVP concentration of the blends. However the observed difference in the
immiscibility gap was inappreciable, which suggest that phase separation primarily occurs due to PA while PVP dissolves in water. Membrane formation using blends of PES/PVP[13,14] and poly(acrylonitrile)/PVP[15] have been investigated in the literature. In these studies the blend system were considered to be a single homogeneous phase during initial stages of phase separation where the mobility of the polymer relative to each other is slower than the solvent/non-solvent exchange across the interface. Hence pseudo ternary phase diagrams were constructed in which polymer blends were lumped as single homogeneous phase. Given the results of miscibility characteristics of PA/PVP blends (Chapter IV) it is a fair assumption that the PA/PVP blends can be considered as

Figure 6.2: Pseudo ternary phase diagram for unmodified membranes, in which the polymer is made up of various ratios of PA/PVP. The solid line represents the immiscibility gap for PA. The results indicate that there is virtually no change in the area of the immiscibility gap due to the addition of PVP, which can be interpreted in view of high solubility of PVP in both DMSO and water.
single homogeneous phase. Therefore the quaternary system of PA/PVP/DMSO/Water can now be considered as pseudo ternary. In order to quantify the amount of PVP that was present in the final membranes, membranes were cast from feed solutions that contain various PA/PVP ratios. The non-solvent bath containing PVP/DMSO/water is evaporated at 150 °C in order to remove DMSO and water. The remaining residue was weighed which corresponds to the weight of PVP lost in non-solvent. By doing a simple mass balance, the wt of PVP in final membrane was calculated. In figure 6.3, the amount of PVP in the final membrane is plotted as a function of PVP in feed. It is evident from this plot that only a fraction of the PVP in feed can be found in the final membrane.

![Figure 6.3: Quantification of weight percentage of PVP in the final membrane. The dashed line represent the ideal case had all the PVP in the feed were present in the final membrane. Here, wt % of PVP = wt PVP*100 / (wt PA + wt PVP).](image)

6.3.1. Morphology of Unmodified Membranes

In order to study the morphology of unmodified membranes, the samples were fractured in liquid nitrogen, sputtered with silver and observed using SEM. Typical morphology of unmodified PA membrane and the effect of addition of PVP is
demonstrated in Figure 6.4. The unmodified PA membrane (Figure 6.4(a)) has a dense skin layer and the cross sectional morphology is primarily composed of finger-like channels, the size of which gradually increases along the thickness direction. The

Figure 6.4: SEM micrographs representing surface (left column) and cross sectional (right column) morphology of unmodified membranes with PA/PVP ratio (a) 100/0 (b) 75/25 and (c) 50/50. Insets represent higher magnification images. Unmodified PA membranes typically exhibit a thick and dense skin layer. Addition of PVP seems to increase thickness of the skin layer and relatively porous surface. Membranes made with 25/75 PA/PVP were too fragile to handle.
channels arose due to the hydrodynamics involved in the membrane preparation process. A thick dense skin can be clearly seen in the insets displayed in surface and cross section morphology.

Two of the important characteristics that determine the membrane performance are water permeability and diffusive properties. If the finger-like channels shown in Figure 6.4(a) are assumed to be cylinders that are perpendicularly oriented to flow, the water permeability of the membranes can be expressed as

\[ Q = \frac{\Delta P n \pi r^4}{8 \eta L} \]  \hspace{1cm} (6.1)

where \( Q \) is the volumetric flow rate/unit area, \( \Delta P \) is the pressure drop, \( n \) is the number of pores per unit area (i.e., pore density), \( r \) is the mean pore radius, \( \eta \) is the viscosity of water and \( L \) is the length of the channel across the membrane thickness. It can be seen from Eq. 6.1 that the mean pore radius of the membrane is the important parameter that is greatly influences the water permeability through the membrane due to its fourth power dependence. In this regard, the water permeability of the unmodified PA membranes would be significantly low due to the dense nature of the skin layer. From the cross-sectional morphologies (Figure 6.4), the ratio of the pore diameters of the surface and the bottom, \( r_{\text{top}}/r_{\text{bottom}} \) can be estimated to be approximately 1/5. Such a gradient in pore size (i.e., the asymmetric nature) ensures that a significant pressure gradient exists across the thickness of the membranes resulting in removal of toxins from blood to the dialysate. Here \( r_{\text{top}} \) and \( r_{\text{bottom}} \) represent the radius of pore at top and bottom of the membrane.
The second important characteristics are the diffusive permeability of the solute (urea and uric acid in case of hemodialysis). Based on the straight cylindrical pore assumptions, the solute permeability can be expressed as

\[ J_s = \lambda D \varepsilon \frac{\partial C}{\partial x} \]  \hspace{1cm} (6.2)

where \( J_s \) is the solute flux (mass of solute/unit area unit time), \( \lambda \) is the partition coefficient of solute, \( \varepsilon \) is membrane porosity, \( D \) is the solute diffusivity, \( \partial C/\partial x \) is the concentration gradient across the membrane. Membrane porosity \((\varepsilon = n\pi r^2)\) is a function of both pore size and number whereas the partition coefficient is inversely proportional to the degree to which a solute is excluded from entering a pore. Hence, in order for the membrane to provide higher solute permeability (urea removal rate in case of hemodialysis) lower wall thickness and higher porosity are highly desired. From this point of view, the thicker skin layer and smaller pores present on the skin layer of unmodified PA membranes appear to be less ideal. Unmodified PA membranes were also prepared by varying polymer concentration (10 wt% - 20 wt%) and solvent (dimethyl acetamide and dimethyl formamide) (data not shown). The surface and cross sectional morphological characteristics of all those membranes were similar to that illustrated in Figure 6.4(a). For unmodified PA membranes, the phase separation occurs between PA and solvent/non-solvent mixture.

It is interesting to note the effect of PVP addition on membrane morphology, which appears to be more pronounced in surface morphology. The skin layer at higher PVP concentration (50/50 PA/PVP) consists of relatively large pore compared to that of
dense PA membranes. The cross sectional morphology seems to be unchanged consisting of progressively increasing channels size. In case of PA/PVP membranes, it is possible that phase separation occurs between polymer and solvent/non-solvent mixture and also between the two polymers. Even though, the presence of PVP improves the biocompatibility of the membranes, there seems to be a very marginal change in surface porosity of the membranes. Upon careful observation, it can be realized morphological changes due to PVP addition are dictated by thermodynamics and kinetics of phase separation process. In order to explain the changes that occurred due to PVP addition, it is important to understand the mechanism of morphology evolution, which is explained in the next section.

6.3.2. Mechanism of Membrane Formation in Relation to Phase Diagram

Upon contact with the non-solvent bath, if the system reaches the metastable region, polymer-poor phase begins to nucleate in the skin layer. These sites serve as the point of entry of non-solvent into the film and coagulate the polymer. Due to strong affinity between solvent/non-solvent, the coagulation front propagates through the thickness of the film. As solvent/non-solvent exchange occurs, the structures that are formed later stage become progressively larger. Finally, the non-solvent escapes from the bottom of the film. This force is sufficient enough to push the film off the glass substrate. The skin layer that was formed during the initial stages of membrane formation crosses the glass transition temperature during solvent/non-solvent exchange and forms a skin layer at the surface, thereby retarding the entry of non-solvent. As solvent/non-solvent
exchange occurs, further nucleation of polymer poor phase occurs along the walls of the already formed channels. The composition path traversed by the system is represented by dash-dotted line in Figure 6.5 and the schematic of morphology evolution is represented in Figure 6.6. Therefore, the formation of finger-like structure is possibly due to the combined effect of thermodynamics and hydrodynamics involved during the membrane formation process. Further, nucleation and growth mechanism is a slower process owing to the metastable nature of the system, which needs to overcome an energy barrier in order to grow.

Figure 6.5: Hypothetical composition pathway (−−−) inscribed inside a ternary phase diagram when the system traverses metastable region.
So far, details pertaining to unmodified PA and PA/PVP membranes have been discussed. In the following section, mangiferin modification of membranes and its effect on membrane morphology is discussed. Since mangiferin is directly mixed with the feed solution for membrane formation, it is important to understand the miscibility characteristics of mangiferin/solvent/non-solvent system. Hence mangiferin/DMSO/water system will be discussed first followed by PA/mangiferin membranes and PA/PVP/mangiferin membranes.

![Diagram](image)

Figure 6.6: Schematic representing the nucleation and growth of polymer-poor phase at skin layer and growth of finger-like structures along with a SEM micrograph of finger-like structures. The figure represents the time evolution of one such finger as solvent/non-solvent exchange occurs.

6.3.3. Phase Diagrams for Mangiferin/DMSO/water

Intrinsically, mangiferin is a small crystalline molecule. Before attempting to fabricate mangiferin modified PA membranes, it is imperative that the miscibility behavior of mangiferin in a solvent/non-solvent mixture be understood. Figure 6.7 illustrates the liquid-solid phase transition boundary for mangiferin/DMSO/water ternary
system. It should be emphasized that the only isotropic-crystal phase transition (i.e., crystallization) was observed in this system as confirmed by the optical microscope images shown on either side of phase diagram in Figure 6.7. Under crossed polarizers, the needle-like crystals of mangiferin can be clearly seen. No liquid-liquid phase separation was observed in this system.

Figure 6.7: Ternary phase diagram for mangiferin/DMSO/Water demonstrating the liquid-solid phase transition region. This ternary system showed only liquid-solid transition, no liquid-liquid phase separation was observed. Optical microscope picture to the right of the phase diagram (crossed polarizer) shows the needle-like crystals of mangiferin dispersed in an isotropic matrix while the micrograph to the left (parallel polarizer) reveals no morphology signifying the isotropic state.

6.3.4. Ternary Phase Diagrams for Mangiferin Modified Membranes

Figure 6.8 shows a comparison between the immiscibility gaps experimentally obtained for unmodified PA and mangiferin modified PA systems. The immiscibility gap
for the modified system in this figure corresponds to 70/30 PA/mangiferin ratio. A series of such mangiferin modified solutions were prepared and water was added to each of the solutions to identify the cloud point as explained in experimental section. As illustrated in Figure 6.8, the addition of mangiferin has lead to an increase in the immiscibility gap of the modified system. Cloud point experiments were also conducted with other PA/mangiferin ratios such as 90/10 and 80/20 but the change in immiscibility area compared to unmodified PA was too small and was within the experimental errors (data not shown).

**Figure 6.8: Overlay of cloud point curves for unmodified PA (filled circles) and mangiferin modified PA (filled triangles) in a mixture of DMSO and water. Each solution of modified system was prepared with PA/M ratio of 70/30. The above figure indicates a marginal increase in the liquid-liquid immiscibility area due to mangiferin addition.**
6.3.5. Effects of Mangiferin Modification

In order to understand the effect of mangiferin modification, membranes were cast from feed solutions in which the PA/PVP/mangiferin ratios were varied as shown in Table 6.1. All the compositions tabulated in this table are the feed compositions that were employed to cast various membranes. Experiment 1 corresponds to unmodified PA and PA/PVP membranes. Experiment 2 corresponds to modified PA/mangiferin membranes. Experiment 3 corresponds to PA/PVP/mangiferin membranes that were prepared with fixed mangiferin composition while varying PA/PVP ratio.

The simple case of PA/mangiferin membranes (experiment 2) were formed first. The membrane casting procedures are as discussed in the experimental section. The feed solution contained 17.5 wt % total solids. Here total solids refer to weight of PA and mangiferin. The employed PA/mangiferin ratios were 90/10, 80/20, 70/30 and 65/35. During the membrane formation process, some of the mangiferin from the cast film was lost in the non-solvent bath. In order to quantify the amount of mangiferin lost (or mangiferin that was retained in the membrane), the non-solvent bath containing water, mangiferin and DMSO were cooled at 15 °C for 96 hours. During the cooling process, the mangiferin crystallized out of the mixture, which were washed, dried, and weighed. From a simple mass balance calculation, the amount of mangiferin that was present in the membrane was calculated. As illustrated in Figure 6.10, the higher the mangiferin loading in the feed solution, the higher was the loss during membrane formation. However, the mangiferin loading in the final membranes saturates ~ 26 wt%. A visual comparison between unmodified and mangiferin modified PA membranes is given in Figure 6.11.
The yellowish tinge of modified membranes can be clearly discerned. During SEM observation, the modified membranes containing PA/mangiferin ratio of 90/10 and 80/20 exhibited similar surface and morphological features (data not shown) to that of the unmodified PA membranes. Despite containing mangiferin, these membranes did not exhibit morphological features that would make them appealing in terms of water and solute permeability. The modified membrane with 70/30 PA/mangiferin ratio is shown in Figure 6.12 (b). The surface of these membranes contained pores of the ~ 100 nm (inset). Unlike the unmodified membranes, majority of the cross section is made up of network

<table>
<thead>
<tr>
<th>Experiment</th>
<th>PA</th>
<th>PVP</th>
<th>Mangiferin</th>
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structure, which might have possibility evolved due to the spinodal decomposition process of phase separation. It is also interesting to notice that the size of the features in the cross section progressively increases in the thickness direction. By virtue of higher

Figure 6.9: A plot of actual wt % of mangiferin in the final membrane as a function of wt% of mangiferin in feed. The plot shows that the concentration of mangiferin of the final membrane saturates out approximately around 26 wt%. The dashed line represents the ideal case, had there been no loss of mangiferin in the non-solvent bath. Here wt % of mangiferin = wt of mangiferin*100/ (wt of PA + mangiferin)

Figure 6.10: Photograph of unmodified and mangiferin modified PA membranes clearly demonstrating the change in color due to mangiferin modification.
surface porosity and absence of a thick skin layer, these membranes can be expected to
possess higher water and solute permeability. Also the pore size range of these
membranes is ideally suited for ultrafiltration process such as hemodialysis. Any further

Figure 6.11: SEM micrographs representing surface (left column) and cross sectional
(right column) morphology of membranes with PA/M ratio (a) 100/0 (b) 70/30; (c) 65/35.
Micrographs in the insets represent higher magnification image of the surface and cross
section. Significant difference in membrane morphology was observed by addition of 30
wt % mangiferin. The membranes become highly micro porous at 65/35 PA/M ratio. The
aggregates shown in (c) might be the crystals of mangiferin. The feed ratio of
PA/mangiferin corresponds to experiment 2 in Table 6.1.
increase in the PA/mangiferin ratio (65/35) results in the formation of membranes with high surface porosity (Figure 6.12 (c)). Even though these membranes appear to have even highest water and solute permeability, the surface pore size range is too large to retain useful proteins from the blood stream. The bicontinuous network structure can be clearly seen in the cross sectional morphology. Even though the morphology appears to have the signature of spinodal decomposition, by no means it is a proof for the same. In addition, this membrane also exhibited highly rough surface which might be injurious to the red blood cells. PA/mangiferin ratio cannot be increased beyond 65/35 in the feed solution. Attempts to increase this ratio sabotaged the homogeneity of the feed solution resulting in liquid-liquid phase separation, which rendered the feed solutions useless for membrane forming purposes. In order to optimize the total solids concentration needed to fabricate the optimum membrane, feed solutions were prepared with increasing total solids concentration at a fixed PA/mangiferin ratio of 70/30. The total solids concentration was varied from 10 wt % to 20 wt % with increments of 2.5 wt %. This particular concentration window was chosen because the membranes at lower total solids concentration (< 10 wt %) were too fragile to handle and at higher concentration (>20 wt %) were too viscous to cast.

6.3.6. Effect of PVP Addition on Mangiferin Modified Membranes

As mentioned in the introduction, one of the frequently employed additives for fabrication of hemodialysis membranes is PVP, which is often considered to improve the biocompatibility of the membranes. Addition of PVP also optimizes the hydrophilic/hydrophobic balance of the hemodialysis membranes that in turn avoids the
unfavorable interactions between blood proteins and the membranes. From the previous sections, it became clear that membranes that were prepared with 17.5 wt% of total solids containing PA/mangiferin ratio of 70/30 were optimal in terms of morphology and mangiferin loading. In order to examine the effect of PVP addition on mangiferin

![SEM micrographs](image)

Figure 6.12: SEM micrographs representing surface (left column) and cross sectional (right column) morphology of membranes with fixed mangiferin composition while varying the PA/PVP ratio. The data corresponds to experiment 3 of Table 6.1. Micrographs in the insets represent higher magnification image of the surface and cross section. It is evident from the figure that increase in PVP concentration has lead to the bicontinuous structure evolution and also an increase in pore size.
modified membranes experiment 3 (Table 6.1) was devised. In experiment 3, for a fixed mangiferin composition, the PA/PVP ratio was varied. Membranes were cast and analyzed for morphology.

The effect of PVP addition on mangiferin modified membranes is displayed in Figure 6.12. It can be clearly seen that the increase in PVP concentration makes the surface of the membrane to be highly porous due to loss of both PVP and mangiferin and resulting in formation of bicontinuous structure. However, by no means this is a proof of occurrence of spinodal decomposition. It should be pointed out here, that the results of miscibility characteristics of PVP/mangiferin blends showed that mangiferin is highly miscible with PVP than with PA. In the hindsight, this was appealing because PVP

![Figure 6.13: Amount of mangiferin present in the final membrane as a function of mangiferin in feed when the polymer consists only of PA and 75/25 PA/PVP.](image)

Figure 6.13: Amount of mangiferin present in the final membrane as a function of mangiferin in feed when the polymer consists only of PA and 75/25 PA/PVP. It is evident that by virtue of water affinity of PVP combined with mangiferin’s affinity for PVP results in decreased concentration of mangiferin in the final membrane compared to the case when PVP absent in the feed. Here wt % of mangiferin = wt of mangiferin*100/(wt of PA + PVP + mangiferin)
could have been used to increase the mangiferin loading in the membranes. However, the water solubility of PVP nullified that advantage. Hence it is necessary to optimize the amount of PVP that is needed to optimize mangiferin loading in PA/PVP/mangiferin membranes.

Figure 6.13 represent the amount of mangiferin present in the final membrane when PVP was added to the feed solution. From Figure 6.13, it can be clearly inferred that the presence of PVP in feed results in reduction of mangiferin concentration of the final membrane compared to its absence in the feed. The dashed line represents the ideal condition had all mangiferin of feed were retained in the membrane. It is obvious from the figure that addition of PVP to the feed solution causes an increased loss of mangiferin compared to the feed solution containing no PVP. This effect is more pronounced at higher PVP concentrations. From mangiferin loading point of view, it is possible to prepare modified membranes with PA/PVP ratio as high as 75/25, which could provide a larger contact area between blood and the membrane due to the presence of network morphology. In these calculations, the wt % was calculated with respect to total solids (i.e., wt PA + wt PVP + wt mangiferin).

The morphological changes that are induced by virtue of PVP addition to mangiferin modified membranes can be explained in relation to the phase diagram. For example, if the system reaches the unstable gap, small compositions fluctuations begin to grow and results in the formation of a bicontinuous structure which is the signature of spinodal decomposition. Further, spinodal decomposition is a rapid process owing to the thermodynamically unstable nature of the system. In this case, the non-solvent can enter the film through a larger surface area (of the polymer-poor phase) available, thus
preventing the formation of the finger-like structure. However, depending upon the forces that exist locally, a combination of the structures is also possible. The composition pathway that the system might have traversed in order for morphology evolution via spinodal decomposition is depicted in Figure 6.14.

Figure 6.14: Hypothetical composition pathway (---) inscribed in a ternary phase diagram that the system traverses during membrane formation when the system traverses unstable region. The network structure of PA/PVP/mangiferin suggests the likelihood of morphology evolution via spinodal decomposition, however by no means it is a proof.

6.4. Conclusions

In summary, PA and PA/PVP membranes with and without mangiferin were formed via non-solvent induced coagulation process at isothermal conditions. Effects of parameters such as mangiferin loading and PVP addition on membrane morphology were
exemplified using SEM micrographs. Since a fraction of mangiferin was lost into the non-solvent bath during the membrane formation process, the same was quantified as a function of the processing variables. Mangiferin loss was enhanced when PVP was added to the feed solution. Membranes were optimized for mangiferin loading and microscopic morphology by systematically varying the processing variables. By virtue of the inherent yellow color of mangiferin, the modified membranes exhibited a pale yellow appearance compared to unmodified membranes. For PA and PA/PVP membranes, the morphology evolution was dominated by the hydrodynamics involved during the membrane formation process. The skin layer of these membranes was typically dense and without pores. However, the skin layer of PA/PVP/mangiferin possessed network morphology suggesting a possibility of spinodal decomposition mechanism of morphology evolution.
CHAPTER VII
MISCIBILITY STUDIES AND MEMBRANE FORMATION OF POLY(AMIDE)/POLY(VINYL PYRROLIDONE) BLENDS CONTAINING GENISTEIN

7.1. Introduction

In the preceding chapters, miscibility studies and membrane formation pertaining to PA/PVP/mangiferin system have been described in detail. During the material selection process, it was thought that the presence of glucose molecule in mangiferin might alter the blood glucose levels of hemodialysis patients (false positive). As majority of hemodialysis population is diabetic in nature, a phytochemical without glucose molecule was sought which would also possess good anti-oxidant properties. Hence, genistein was chosen as the second phytochemical in this dissertation. This first half of this chapter is focused on miscibility of PA and PVP with genistein followed by their ternary blends. The second half focuses on aspects of membrane formation such as construction of phase diagrams, membrane casting, genistein modification, and morphology analysis of membranes with and without genistein.
7.2. Experimental Section

The miscibility behavior of PA/genistein, PVP/genistein, and PA/PVP/genistein blends was established using DSC, optical microscope, and FTIR studies. The experimental details pertaining to sample preparation and instrumental procedure has been described in section 4.2. Similar procedures were employed for PA/PVP/genistein blends, unless otherwise mentioned.

7.2.1. Ternary Phase Diagrams for Polymer/solvent/non-solvent System

Ternary phase diagram for PA/DMSO/water was constructed as explained in section 6.2.1.

7.2.2. Ternary Phase Diagrams for Phytochemical/solvent/non-solvent System

Briefly, DMSO and water were mixed at various compositions followed by incremental addition genistein until saturation point was reached where the solution can no longer dissolve genistein. All the experiments were duplicated in order to ensure consistency and optical microscopy was employed to confirm the presence of crystals.

7.2.3. Ternary Phase Diagrams for (Polymer + phytochemical)/solvent/non-solvent System

Phase diagrams were constructed for (PA + genistein)/DMSO/water system as explained in section 6.2.3 except that each solution contains (PA + genistein) blends instead of pure PA.
7.2.4. Membrane Casting and Morphology Analysis

All membranes were cast and analyzed for morphology using SEM as described in section 6.2.5. Unless otherwise mentioned, DMSO was used a solvent and water as non-solvent.

7.3. Results and Discussion

Figure 7.1 exhibits the overlaid plots of DSC and TGA thermograms of pure genistein along with its chemical structure. It can be clearly noticed that there is a significant weight loss of genistein that coincided with the crystal melting peak. The thermal stability of genistein resembled that of mangiferin in this respect. The solvent cast PA/genistein samples were transparent to naked eyes, but tinted golden color owing to the inherent color of genistein.

The DSC thermograms of PA/genistein blends are displayed in Figure 7.2. A single Tg can be clearly discerned upon addition of genistein and the Tg of blends shifts to lower temperature. The decrease in the Tg of the PA/genistein blends may be attributed to the lower Tg of genistein, if any exists. The presence of crystal melting peak at higher genistein concentrations (> 40 wt % of genistein) signifies the occurrence of liquid-solid phase transition and also underscores the solubility limit of genistein in PA. Compared to the invariant nature of crystal melting peak PA/mangiferin blends, significant movement can be observed for PA/genistein blends, which also suggest that genistein acts as a diluent molecule and depresses the melting point.
Figure 7.1: Overlay of DSC and TGA thermograms for genistein illustrating the decomposition of genistein occurring at its melting point. The inset shows the chemical structure of genistein.

Figure 7.2: Second run DSC thermograms for PA/genistein showing single $T_g$ and its systematic movement to lower temperature at lower genistein concentrations whereas an endothermic crystal melting peak at higher genistein concentrations. Appreciable melting point depression can also been seen in blends.
Figure 7.3 (a) exhibits the FTIR spectra of PA/genistein blends in the 1500-1800 cm\(^{-1}\) range. It should be emphasized that both PA and genistein are self-associating components, capable of forming intra-molecular hydrogen bonding. In general, the shift in band due to hydrogen bonding can occur under the following scenarios. A band shifts towards higher frequencies when hydrogen bonds are released. On the other hand, shift towards lower frequency occurs due to formation of newer hydrogen bonds. The second aspect contributing to the movement of bands is the strength of hydrogen that is released compared to the strength of hydrogen bonds that are newly formed. So, the competition between intra-molecular hydrogen bonding and intermolecular hydrogen bonding combined with the strength of various possible interactions results in peak shift that is observe in typical IR spectrum. Unlike PA/mangiferin blends, the addition of genistein, the amide I band of PA exhibits a relatively appreciable shift to lower frequencies due hydrogen bonding with the hydroxyl groups of genistein. In contrast, the aromatic C=C band does not show any movement since it is not involved in any specific interaction. The assignments \(\nu_{8a}-\nu_{8b}\) represent the C=C ring stretching vibrations that is characteristic of six-membered heterocyclic ring (for example benzene). However, unlike benzene, these pairs are not degenerate in phenyl compounds. The addition of genistein to PA also causes the amide I band to split into two bands. The band at higher frequency might correspond to the release of intra-molecular hydrogen bonds existing between the amide groups whereas the frequency at lower frequency may be due to the formation of cross hydrogen bonding interactions occurring between amide groups of PA and hydroxyl and carbonyl groups of genistein.
Figure 7.3: FTIR spectra recorded at 100 °C for PA/genistein blends (a) in the range of 1500-1750 cm\(^{-1}\) demonstrates hydrogen bonding formation as shown by the systematic movement of amide I band of PA and carbonyl band of genistein to lower frequencies and (b) in the 2800-3600 cm\(^{-1}\) range. Explicit C-H stretching bands corresponding to genistein and PA and a sharp O-H stretching band for genistein can be observed in (b).
It is more difficult to analyze band movements in the N-H stretching region due to the overlap of O-H and N-H stretching bands. It is of interest to note the sharpness of the O-H stretching peak in case of genistein (Figure 7.3 (b)) and compare it with that of mangiferin (Figure 4.5). In mangiferin, there exists significant intra-molecular hydrogen bonding between the hydroxyl groups due to the number as well as the position of the hydroxyl groups as a result a broad O-H stretching peak was observed. However, in genistein, the degree of intra-molecular hydrogen bonding less significant as the hydroxyl groups are sterically hindered. This results in a sharp band for hydroxyl stretching. Another spectral difference between PA/mangiferin and PA/genistein system is the presence of separate C-H stretching bands corresponding to aliphatic and aromatic frequencies. In mangiferin, the broadness of the O-H stretching dominates this region thereby suppressing the distinction of C-H stretching peaks. Upon mixing, the carbonyl bands of both genistein as well as PA shift to lower frequency suggesting the occurrence of cross hydrogen bonding interactions between the two entities. The possible interactions in these blends include N-H…C=O (self), N-H…C=O (cross), C=O…O-H (cross) and N-H…O-H (cross).

7.3.1. Thermal and Miscibility Characteristics of PVP/genistein Binary Blends

There were some similarities and differences between PVP/mangiferin and PVP/genistein blends. Similar to that of PVP/mangiferin blends, the DSC thermograms of PVP/genistein samples showed single $T_g$. However, the movement of $T_g$ was in the opposite direction to that of the PVP/mangiferin blends, i.e., $T_g$ of PVP/genistein blends consistently shifted to lower temperatures with increasing genistein concentration (Figure 7.3 (c)).
7.4). No liquid-liquid phase separation was observed in the entire composition range, but large spherulitic structures developed at higher genistein concentrations signifying the isotropic liquid + crystal coexistence regions. It can be clearly seen that the depression of melting point of PVP/genistein blends is similar to that of PVP/mangiferin blends and in addition, the isotropic + crystal transition occurs at comparable composition of genistein and mangiferin (70 wt %). This observation combined with the larger isotropic composition range for PVP/genistein blends suggests that genistein is also seemingly more miscible with PVP than with PA. The suppression of $T_g$ of the blends upon genistein may be due to the lower glass transition of genistein (if any exists) compared to that of PVP.

Figure 7.4: Second run DSC thermograms of PVP/genistein blends showing appreciable melting point depression and also low temperature shift of $T_g$ of the blends.
Figure 7.5: FTIR spectra recorded at 100 °C for PVP/genistein blends (a) in the range 1500–1800 cm\(^{-1}\) demonstrates hydrogen bonding formation illustrated by the systematic movement of C=O band of the blends to lower frequencies. The aromatic C=C band of genistein doesn’t show any shift and (b) in the 2800–3600 cm\(^{-1}\) range illustrating the broadening of the O-H band upon mixing.
PVP does not self-associate, but contains functional groups (C=O) that are capable of forming hydrogen bonds with mangiferin via electron donor-acceptor type of interaction. Figures 7.5 (a) and (b) illustrate the FTIR spectrum of PVP/genistein blends in the 1500-1800 and 2800-3600 cm\(^{-1}\) range, respectively. These spectra were acquired at 100 °C to minimize moisture absorption. The FTIR analysis of the present PVP/genistein system is relatively straightforward. As the concentration of PVP is increased the hydroxyl band becomes broader and shifts to lower frequencies while reducing its magnitude. However, the sharp O-H stretching band of genistein disappears by adding 30 wt % PVP. The corresponding effect can be explicitly seen in the carbonyl spectral region in which the carbonyl peak of PVP shifts to lower frequencies due to hydroxyl-carbonyl interactions. However, the interactions seem to be weaker compared to that of PVP/mangiferin blends, which is probably due to the presence of only three hydroxyl groups of genistein as compared to eight hydroxyl groups of mangiferin.

7.3.2. Miscibility Characteristics of PA/PVP/genistein Ternary Blends

The miscibility behavior of PA/PVP/genistein blends was determined via DSC and POM experiments. The weaker interaction between PVP and genistein is also manifested in ternary blends. Upon addition of genistein to the PVP rich-blends (e.g., 25/75 PA/PVP, Figure 7.6 (a)), a single glass transition is evident up to 30 wt% of genistein. However, beyond 30 wt % dual glass transition temperatures were observed between 40-60 wt% of genistein suggesting phase separation. Further, the T\(_g\) of both the phases shifted to lower temperature which was different from the mangiferin system. However, in case of 50/50 PA/PVP blends (Figure 7.5 (b)), liquid-liquid phase separation
Figure 7.6: Second run DSC thermograms for PA/PVP/genistein ternary blends with (a) 25/75 PA/PVP ratio exhibiting a single $T_g$ at low genistein composition and two $T_g$s at higher genistein compositions, which shift consistently to lower temperature with increase in genistein composition. (b) 50/50 PA/PVP ratio showing similar trend as 25/75 PA/PVP blends expect that liquid-liquid phase separation occurs at even lower genistein compositions.
occurred at even lower concentration (20 wt %) and this phase separated trend continues with further increase of genistein concentration in which the dual glass transitions shifting to lower temperatures. Similar trend was observed for when genistein was added to 75/25 PA/PVP system (data not shown). The phenomena of liquid-liquid phase separation and solid-liquid phase transition can be confirmed by an independent approach such as POM. The PA/genistein and PVP/genistein blends were transparent to naked eyes.

Figure 7.7: Ternary morphology phase diagram, mapped out with the DSC and optical microscope data for the PA/PVP/genistein blends, showing various coexistence regions. Polarized and unpolarized of optical microscope images representing typical morphology of each region are shown in relation to the ternary phase diagram. Keys for labels are: I = isotropic (unfilled circles), C = crystal (completely filled squares), (I + C)_{PVP} = coexistence of isotropic and crystals for PVP rich compositions (left-half-filled squares), (I + C)_{PA} = coexistence of isotropic and crystal for PA rich compositions (right-half-filled squares), L + L = liquid + liquid (left-half-filled circles), L + L + C = liquid + liquid + crystal (hour glass-filled circles).
showing no indication of liquid-liquid phase separation at low genistein loading. Increase in genistein concentration resulted in crystallization of genistein showing the large spherulitic structures in the continuum of isotropic liquid signifying the isotropic-crystal (liquid-solid) coexistence phase. At high concentrations of genistein, the entire field of optical microscope view was filled with solid crystals. In the case of PA/PVP blends, the addition of genistein revealed liquid-liquid phase separated domains in addition to isotropic single phase and crystal + liquid coexistence phase. Finally, all the DSC and POM data were utilized in order to construct a ternary phase diagram of PA/PVP/genistein system as shown in Figure 7.7. A quick comparison between PA/PVP/mangiferin (Figure 4.9) and PA/PVP/genistein ternary phase diagrams, suggest qualitatively similarity between the two systems. However, the difference lies in the areas of different coexistence regions and the glass transition behavior of the blends.

7.3.3. Membrane Formation with PA/PVP/genistein System

The phase diagram of PA/DMSO/water system was described in section 6.3.1 and the detailed morphological analysis of the unmodified membrane was illustrated in section 6.3.2. All the unmodified membranes (i.e., PA as well as PA/PVP membranes) exhibited finger-like cross sectional morphology with gradient in channel size across the cross section, which ensures that during hemodialysis process the uremic toxins are transported from the blood side to the dialysate side. Finger formation was probably initiated by nucleation of polymer-poor phase in polymer-rich matrix. However, hydrodynamics associated during the immersion of polymer solution into water bath drives the formation of progressive larger channels. The system vitrified upon reaching a
certain non-solvent concentration due to glass transition effects and the final membrane structure gets frozen.

7.3.4. Phase Diagrams for Genistein/DMSO/water System

Genistein is a small crystalline molecule that has a pale golden color. The miscibility behavior of pure genistein in a solvent/non-solvent mixture needs to be understood before blending with the polymers. Figure 7.8 illustrates the liquid-solid

Figure 7.8: Ternary phase diagram for genistein/DMSO/Water demonstrating the liquid-solid phase transition region. This ternary system showed only liquid-solid transition, no liquid-liquid phase separation was observed. Optical microscope picture to the right of the phase diagram (crossed polarizer) shows the needle-like crystals of genistein dispersed in an isotropic matrix while the micrograph to the left (parallel polarizer) reveals no morphology signifying the isotropic state.
phase transition boundary for genistein/DMSO/water ternary system. It should be pointed out that the only liquid-solid phase transition (i.e., crystallization) was observed in this system as confirmed by the optical microscope images shown on either side of phase diagram in Figure 7.8. Under crossed polarizers, the needle-like crystals of genistein can be clearly seen. No liquid-liquid phase separation was observed in this system. Qualitatively, the phase diagram of genistein/DMSO/water resembles that of mangiferin/DMSO/water system (Figure 6.7) consisting of isotropic and isotropic + crystal coexistence regions. However genistein solution tends to crystallize at a higher water composition compared to that of mangiferin solution.

7.3.5. Ternary Phase Diagrams for (PA + genistein)/DMSO/water System

Figure 7.9 shows a comparison between the immiscibility gaps experimentally obtained for pure PA and genistein modified PA systems. The immiscibility gap for the modified system in this figure corresponds to 70/30 PA/genistein ratio. A series of genistein modified solutions were prepared, and water was added to each of the solutions to identify the cloud point as explained in experimental section. As illustrated in Figure 7.9, the addition of genistein has lead to an increase in the immiscibility gap. Once cloud point was reached, samples were immediately observed under optical microscope to identify the source of cloudiness (liquid-liquid phase separation vs. liquid-solid phase transition). In all the samples, the system showed only liquid-liquid phase separation upon reaching cloud point. Nevertheless, the boundary delineates single phase region from two phase region.
Figure 7.9: Overlay of cloud point curves for unmodified PA and genistein modified PA in a mixture of DMSO and water. Each solution of modified system was prepared with PA/genistein ratio of 70/30. The above figure indicates a marginal increase in the liquid-liquid immiscibility area due to genistein addition.

7.3.6. Morphology of Genistein Modified Membranes

In order to understand the effect of genistein modification, membranes were cast from feed solutions in which the PA/PVP/genistein ratios were varied as shown in Table 7.1. All the compositions tabulated in the table represent the feed composition that was used to cast the membranes. The simple case of PA/genistein membranes (experiment 2) were cast first. The membrane casting procedures were described in the experimental section. The feed solution contained 17.5 wt % total solids. Here total solids refer to weight of PA + genistein. The following PA/genistein ratios were used viz., 90/10, 80/20,
and 70/30. As with mangiferin modification (during the membrane formation process), some of the genistein from the cast film was lost in the non-solvent bath. In order to quantify the amount of genistein lost, the non-solvent bath containing water, genistein, and DMSO was cooled to 15 °C and maintained for 96 hours. During the cooling process, the genistein crystallized out of the mixture, which were washed, dried, and weighed. From a simple mass balance calculation, the amount of genistein that was present in the membrane was calculated. As illustrated in Figure 7.10, up to 30 wt% genistein in feed, almost no loss of genistein was observed. However, beyond 30 wt %, appreciable amount was genistein was found in the non-solvent bath. The genistein loading in the final membranes saturated at approximately 35 wt%. By virtue of the pale golden color of

Table 7.1: Feed composition ration of PA/PVP/Genistein used in casting various membranes

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genistein, a visual comparison between unmodified and genistein modified PA membrane rendered no appreciable color change. The amount of genistein present in the final membranes was relatively larger when comparing with mangiferin modified membranes (Figure 6.10).

Figure 7.10: A plot of actual wt % of genistein in the final membrane as a function of wt% of genistein in feed. The plot shows that the concentration of genistein of the final membrane saturates approximately around 35 wt%. The dashed line represents the ideal case, had there been no loss of genistein in the non-solvent bath. The data corresponds to experiment 2 shown in Table 7.1. Here wt % of genistein = wt of genistein *100/ (wt of PA + genistein).

SEM micrographs displayed in Figure 7.11 illustrates the morphological changes that occur in membranes due to genistein modification. It is evident from the micrographs that even 10 wt% of genistein resulted in formation of genistein crystals on the membrane surface as well as in the cross section (Figure 7.11 (b)). However, these crystals were found to be embedded in the surface. The embedded crystals were also found at higher genistein loading such as 80/20 and 70/30 (Figures 7.11 (c) and (d) respectively). At higher genistein concentration, the walls of the finger-like channels in the cross sections
Figure 7.11: SEM micrographs representing surface (first column), low resolution cross sectional (second column) morphology, and high resolution cross sectional (third column) morphology of membranes with PA/genistein (PA/G) ratio (a) 100/0, (b) 90/10, (c) 80/20, and (d) 70/30. Micrographs in the insets represent higher magnification image of the surface. Even at very low genistein concentration embedded crystals can be seen on the surface of modified membranes. As the concentration genistein increases, seaweed type crystals can be observed on the surface. The feed ratio of PA/genistein corresponds to experiment 2 shown in Table 7.1.
are also filled with genistein crystals (inset of Figure 7.11 (d)). The appearance of embedded crystals on the membrane surface was a distinguishing feature between mangiferin modified and genistein modified membranes. Even though, both genistein and mangiferin are crystalline molecules, the final membrane morphologies of the modified membranes were very different. Although, it was possible to make genistein modified membranes with a feed composition as high as 50/50 PA/genistein, only up to 70/30 ratio was employed due to genistein loss at higher composition range. The difference between the crystallization kinetics as well as the glass transition effects between PA/mangiferin and PA/genistein blends might be the contributing factors which could explain the observed difference in morphology. Experiments were also carried out to identify the optimum total solids concentration. Similar to mangiferin modification, membranes that were formed from solution that contained < 10 wt% total solids were too fragile to handle and > 20 wt % were too viscous to cast. A total solid concentration of 17.5 wt% was

![Figure 7.12: Low resolution (left) and high resolution (right) surface micrographs of a 50/50 PA/genistien membrane clearly indicating that at higher genistein concentration (> 30 wt%), large spherulitic structures grows on the surface which contributes to elevated loss of genistein at this concentration range as seen in Figure 7.10. In the vicinity of the spherulitic structures embedded crystals can also be seen.](image)
employed for membrane casting which would enable a direct comparison between mangiferin and genistein modification.

Figure 7.12 illustrates the morphology of membranes that were formed with very high genistein concentration (50/50 PA/genistein). It can be inferred from the micrographs that at higher genistein concentration, large spherulitic structures tend to grow on the membrane surface that might be easily removed during the membrane formation process. This scenario also correlates well with the trend observed in Figure 7.10. Clearly, genistein modified membranes are good examples of amorphous/crystal system. Extensive work pertaining to binary system consisting of an amorphous and a crystal component has been carried out by Matkar et al. [133] and Rathi et al. [134].

7.3.7. Effect of PVP Addition on Genistein Modified Membranes

In order to examine the effect of PVP addition on genistein modified membranes experiment 3 (Table 7.1) was devised, in which, for a fixed genistein composition, the PA/PVP ratio was varied. Membranes were cast and analyzed for morphology and genistein loading. The effect of PVP addition on genistein modified membranes is displayed in Figure 7.13. It can be clearly seen that the increase in PVP concentration leads to the suppression of crystal formation. This trend can be explained in view of the miscibility behavior of PA/PVP/genistein blends. The results of miscibility characteristics of PA/PVP/genistein blends suggested that genistein is highly miscible with PVP than PA (as observed by the larger single phase region of PVP/genistein compared to PA/genistein blends). As a result, the increase in PVP concentration results in the formation of
modified membrane in which genistein is dispersed in amorphous state in the polymer matrix. Even though, PVP suppresses crystal formation, it is still necessary to quantify the amount of genistein that is present in the final membrane. Figure 7.14 represent the amount of genistein that is present in the final membrane as a function of

![Figure 7.13: SEM micrographs representing surface (first column), low resolution cross sectional (second column) morphology, and high resolution cross sectional (third column) morphology of membranes with PA/PVP/genistein ratio (a) 66.5/3.5/30, (b) 63/7/30, and (c) 52.5/17.5/30. The feed composition of PA/PVP/genistein corresponds to experiment 3 of Table 7.1. Micrographs in the insets represent higher magnification image of the surface. It is evident from the figure that increase in PVP concentration has lead to the suppression of crystal formation in the modified membrane.](image)
feed composition. It is obvious from Figure 7.14 that addition of PVP to the feed solution causes an increased loss of genistein compared to the feed solution without PVP. This effect can be extrapolated to higher PVP concentrations at which even higher loss of genistein could be encountered. In these calculations, the wt % was calculated with respect to total solids (i.e., wt PA + wt PVP + wt genistein). This observation is qualitatively similar to the mangiferin modified membranes. However, the saturation range of genistein modified membranes is actually higher than that of the mangiferin modified membranes, which is probably due to the crystallization of genistein during membrane formation.

Figure 7.14: Amount of genistein present in the final membrane as a function of genistein in feed for two cases where the polymer consists only of PA and 75/25 PA/PVP. It is evident that the presence of PVP in feed reduces the actual amount of genistein in the final membranes which can be attributed to the water affinity of PVP. The dashed line represents the ideal condition had all the genistein in feed were present in the final membrane. Here wt % of genistein = wt of genistein *100/ (wt of PA + PVP + genistein).
7.4. Conclusions

In summary, this chapter focused on the results obtained from PA and PA/PVP membranes with and without genistein. Similarities and differences between PA/PVP/mangiferin and PA/PVP/genistein systems were highlighted and discussed. Effects of genistein and PVP addition on membrane morphology were exemplified using SEM micrographs. Unlike the mangiferin modified membranes, genistein modified membranes showed the presence of crystals even at very low genistein concentration. The suppression of glass transition temperature by genistein as well as the crystallization kinetics of genistein might be responsible for the appearance of crystals during morphology evolution. As some loss of genistein was encountered in the non-solvent bath during the membrane formation process, the amount of genistein in the final membrane was quantified. As opposed to embedded crystals at low genistein concentration, large spherulitic structures were observed on the membrane surface at very high genistein concentration which agrees well with the quantification studies that concentration of genistein present in the final membrane saturates around 35 wt %. Similar to mangiferin system, loss of genistein was enhanced when PVP was added to the feed solution. Addition of PVP to the feed also suppressed the crystallization of genistein in the modified membrane surface. Membranes were optimized for genistein loading and microscopic morphology by systematically varying the processing conditions.
CHAPTER VIII
DETERMINATION OF IN VITRO CYTOTOXICITY, ANTI-OXIDANT AND ANT-INFLAMMATORY PROPERTIES OF UNMODIFIED AND MODIFIED MEMBRANES

8.1. Introduction

In the preceding chapters, detailed miscibility characteristics of polymer/phytochemical blends have been described, subsequently followed by illustration of membrane formation, morphology description, and effects of phytochemical modification on membrane morphology. This chapter deals with important in vitro blood compatibility experiments that are needed to be evaluated to ensure the safe and effective performance of the membranes. The success of any biomaterial depends on carefully designed biocompatibility experiments. The most important consideration of any biomaterial selection is the cytotoxicity induced by the biomaterial in intended application. Hence, cytotoxicity forms the initial criterion for biomaterial selection. Cytotoxicity provides an estimate of the harmfulness of the biomaterial and is usually designed to detect cell death using variety of techniques. The lack of cytotoxicity is necessary but not sufficient prerequisite for biocompatibility. Numerous other aspects need to be considered when evaluating biocompatibility. Understanding other possible interactions between cells and a biomaterial is critical for
the successful development of a biomedical device. These interactions are composed of various biological processes and may be manifested in the form of adhesion and proliferation of cells onto a biomaterial surface, non-specific inflammation, specific immunological reactions, blood-material interactions, and infection.

8.2. Experimental Section

Experimental methods pertaining to the in vitro blood compatibility studies are explained in detail in the following section.

8.2.1. In vitro Cytotoxicity Studies

In vitro cytotoxicity studies were conducted by measuring cell viability upon exposure to unmodified membranes, pure phytochemicals as well as phytochemically modified membranes. A nucleic acid dye, 7-Amino-actinomycin D (7-AAD), (BD-Via-Probe™ from BD Bioscience, CA, US) was used as a viability probe for dead cell exclusion, based on uptake of 7-AAD. Fresh blood mixed with the dye is used as negative control, whereas blood incubated with membrane and/or phytochemicals served as the experimental samples. The assay protocol is as follows. 100 µl of fresh blood was pipetted in to separate polypropylene tubes and mixed with 2 ml of ammonium chloride lysis buffer solution (8.26 g ammonium chloride, 1 g potassium bicarbonate, and 0.037 g ethylenediamine-tetraacetic acid dissolved in 1 liter deionized water). The tubes were allowed to stand at room temperature for 10 minutes during which most of the red blood cells are lysed. The tubes were centrifuged at room temperature for 5 minutes. The supernatant was discarded and the cells were twice washed and centrifuged with 2 ml of
phosphate buffer saline (PBS, Baxter, IL, US. pH = 7.4) solution. The supernatant was discarded. 20 µl of 7-AAD was added to the cells and were stored under dark conditions for 10 minutes to permit cell uptake. The cells were resuspended with 500 µl of PBS and subjected to flow cytometry. 7-AAD is a nucleic acid dye and is excluded from live cells. As a result, during flow cytometry, the dead cells fluoresce while the live cells do not fluoresce. The number of cells in each sample was adjusted between 10,000 to 20,000 cells/ml with PBS.

8.2.2. In vitro Anti-oxidant Properties by Dihydrorhodamine (DHR) Assay

Dihydro rhodamine (DHR) is a non-fluorescent molecule that is readily taken up by neutrophils. In presence of reactive oxygen species (ROS), DHR 123 is converted to rhodamine which fluoresces green and can be detected using a flow cytometer. Venous blood was obtained from adult volunteers. Ten milliliter specimens were collected in lithium heparin Vacutainer® tubes (Becton Dickinson, Rutherford, NJ) and used within 12 h of sampling. All reagents for the oxidative burst assays were obtained from Sigma Aldrich (MO, US). The stock solution of dihydrorhodamine 123 was 10 mg DHR in 2.0 ml dimethyl sulphoxide (DMSO), stored in 50 µl aliquots at −70 °C. For the assay, 20 µl of stock was dissolved in 980 µl PBS to give a working solution of 100 µg/ml. Phorbol myrisate acetate (PMA) was used to activate neutrophils to undergo oxidative burst. PMA stock solution was made by dissolving 1.0 mg PMA in 1.0 ml DMSO. This was also stored in 50 µl aliquots at −70 °C. For the assay, 10 µl of stock solution was diluted with 90 µl of PBS to give a working concentration of 5 µg/ml.
One hundred micro liters of whole blood samples were pipetted into three separate polypropylene tubes. These were used as stimulated, resting and reagent blank tests. Twenty-five microliters of 5 μM PMA solution were added to the stimulated tubes and 25 ml PBS was added to the resting and reagent blank tubes. All tubes were incubated at 37 °C for 15 min. Then 25 μl working DHR solution were added to the stimulated and resting samples and 25 μl PBS was added to the reagent blank followed by 15 min incubation at 37 °C. The tubes were centrifuged at 1800 rpm for 5 min. The supernatant was discarded and the cells were resuspended in PBS and centrifuged again. After the second centrifugation, the supernatant was discarded and replaced with 1.0 ml of immunoprep® (Beckman Coulter, CA, US) solution. Immunoprep reagent is a rapid whole blood lysing solution. The immunoprep reagent’s gentle lysing action reduces cell stress and minimizes cell shrinkage while maintaining cellular integrity and morphology. The solution is comprised of 3 ready-to-use reagents: reagent A lyses the red blood cells, reagent B buffers the solution and stops the lysing process and reagent C fixes the cells. The solutions were filtered through 50 μm filter and analyzed with a flow cytometer. The composition of the solutions is proprietary.

Before acquiring data the instrument was set up using a reagent blank sample. The forward and side light scatter profiles were adjusted to ensure that the neutrophil population was clearly displayed. Fluorescence was measured on the FL1 green channel (wavelength 530 nm). Data were then collected from the reagent blanks and all resting and stimulated tubes. A total of 10,000 events were collected for each sample. After collecting the data, the plot of forward scatter vs. side scatter was used to select the neutrophil population by its typical location (as shown in Figure 3.3). A histogram of
rhodamine fluorescence from this population was then obtained which directly corresponds to the ROS level. Each experiment was duplicated. Before subjecting the unmodified and modified membranes to DHR assay, the anti-oxidant properties of pure phytochemicals in solution were measured. An example of assay conditions for this purpose is tabulated in Table 8.1.

Table 8.1. An example of DHR assay experiment using mangiferin solution

<table>
<thead>
<tr>
<th>Sample number</th>
<th>DHR</th>
<th>PMA</th>
<th>Mangiferin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3,4</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5,6</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>7,8</td>
<td>−</td>
<td>−</td>
<td>+ (200 µg/ml)</td>
</tr>
<tr>
<td>9,10</td>
<td>+</td>
<td>−</td>
<td>+ (200 µg/ml)</td>
</tr>
<tr>
<td>11,12</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>13,14</td>
<td>+</td>
<td>+</td>
<td>+ (200 µg/ml)</td>
</tr>
<tr>
<td>15,16</td>
<td>+</td>
<td>+</td>
<td>+ (100 µg/ml)</td>
</tr>
<tr>
<td>17,18</td>
<td>+</td>
<td>+</td>
<td>+ (50 µg/ml)</td>
</tr>
<tr>
<td>19,20</td>
<td>+</td>
<td>+</td>
<td>+ (25 µg/ml)</td>
</tr>
<tr>
<td>21,22</td>
<td>+</td>
<td>+</td>
<td>+ (10 µg/ml)</td>
</tr>
</tbody>
</table>

In the above table, ‘+’ and ‘−’ represent the presence or absence of a particular component respectively. For all the assays, 100 µl of blood was used and after mixing all the ingredients the final volume was made up to 1000 µl with PBS. The concentration of
mangiferin represented in parenthesis represents the final concentration in the whole 1000 µl of final solution. PMA and DHR were used at 10 µg/ml and 100 µg/ml respectively in all the samples. Samples 1,2 contains fresh blood with PBS. Tubes 3-10 test whether DHR, PMA, and/or mangiferin contribute to oxidative burst which leads to an increase in the fluorescence. Samples 11,12 serve as positive controls which contain stimulated neutrophils. Samples 13-22 are the experimental samples in which mangiferin is added at a decreasing concentration.

8.2.3. Determination of Cytokine Profiles

The necessity to accelerate commercialization of drugs to the marketplace, together with the increasing cost of research and development, has led to emphasis on biomarkers in Phase I clinical trials. In this context, biomarkers show considerable promise for improving the efficiency and progression of drug development and regulatory decision making early in clinical development. Biomarkers serve as a basis for the selection of lead drugs for clinical trials and for characterization of a disease. Accordingly, the major hurdle is the identification and evaluation of appropriate biomarkers than can gain widespread acceptance. From a clinical point of view, the levels of biomarker should be influenced in response to drug treatment and finally to the clinical end point. It should be kept in mind that biomarkers alone may not necessarily represent a clinical end point. However, the evaluation of biomarkers in phase-I clinical trials offers short-term, but important implications to the pharmaceutical and biomedical industry.

Surrogate biomarkers help understand the mechanism of action of compounds and dose selection that facilitates progression of compounds into clinical development. No
single biomarker can provide all of the required clinical information. Therefore, it is necessary to incorporate multiple biomarkers that can adequately explain the full spectrum of relevant information on the efficacy of a compound. Given the cost associated with each of the assays it would be ideal to have a sensitive and cost-effective assay that will simultaneously quantify multiple biomarkers in a small sample volume.

For the case of hemodialysis membranes, cytokines have been identified as good surrogate biomarkers that can be correlated to clinical complications and therefore the progression of the disease. Cytokines (cyto = cell; and kinos = movement) are a family of proteins that are involved in numerous immunological functions including the regulation and production of other cytokines. They play an important role in the regulation of hematopoiesis, mediating the differentiation, migration, activation, and proliferation of phenotypically diverse cells. Thus, it is commonly accepted that analysis of multiple cytokines in biological fluids will have useful implication in research and clinical studies. Tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) are some of the important cytokines that exert various physiological effects in hemodialysis. For example, it has been revealed that endotoxins from dialysate and complement fragments of C5a induce secretion of interleukin-1β (IL-1β) from neutrophils. These factors have been suggested to cause hyperthermia during hemodialysis and muscular atrophy, osteoporosis, and articular fibrosis in patients on long-term hemodialysis. Thus, the cytokines are attracting close attention as inflammation-associated factors that have systemic effects.

Whole blood was collected from the volunteer in EDTA containing Vacutainer® tubes. The blood was stimulated ex vivo with 100 ng/ml lipopolysaccharide (LPS) for 24
hours at room temperature. Blood was centrifuged at 1800 rpm for 10 min and the plasma was separated. An additional centrifugation step of the plasma at 5000 rpm for 10 min at 4 °C was included to completely remove platelets. Cytokine levels in LPS-stimulated plasma samples were determined using a Luminex® cytometer.

For cytokine measurements, standard matched pairs of antibodies for each cytokine (TNF-α, IL-6 and IL-1β) were obtained and each capture antibody was coupled to carboxylate-modified 5.6 µm polystyrene microspheres by the manufacturer. The primary antibodies specific to a particular cytokine was prefixed on a particular family of microspheres (please refer to Figure 3.5 for details). The primary antibodies coupled microspheres were purchased from the manufacturer (Bio-rad Laboratories, Hercules, CA). Microspheres, calibrators, and detection reagent were diluted in phosphate-buffered saline containing 10 mg/ml bovine serum albumin and 500 mg/ml sodium azide. Microspheres (2000/well of each specificity; 50 µl) were incubated with different concentrations of each cytokine (50 µl) for 30 min at room temperature. Subsequently, without washing, biotin labeled secondary antibodies were added (50 µl), and the mixture was incubated for an additional 30 min at room temperature. Fifty micro liters of streptavidin-phycoerythrin (16 µg/ml in assay buffer) was added to the wells and incubated at 37 °C for 15 min. Unbound analytes were removed using a vacuum manifold and the analyte bound microspheres washed three times with 100 ml/wash of assay buffer. Following the last wash step, 100 ml of assay buffer was added to each well. Fifty micro liters of sample was analyzed on the Luminex 200® system. As mentioned in Chapter 3, Luminex® flow cytometer combines the principles of flow cytometry and immunofluorescence where the surface of the polystyrene microspheres is used to build
the assay. An average of 100 microspheres was acquired for each population of analyte-specific microspheres, and the mean fluorescence intensities were reported. Duplicate samples for each cytokine concentration were averaged, and the zero analyte background was subtracted from each value.

8.2.4. Statistical Analysis

Data obtained from DHR assay was subjected to unpaired two-tailed Student’s t-tests and the results are expressed in form of mean ± standard deviation. \( p \)-value less than 0.05 were considered statistically significant.

8.3. Results and Discussions

Results of the \textit{in vitro} blood compatibility studies are explained in great detail in the following section.

8.3.1. An Example of DHR Assay Data

Before beginning the analysis of the data, an example is shown in the following paragraph describing the details of the actual data and the method to interpret the data. The image shown in Figure 8.1(a) is the data obtained from the flow cytometer. This figure shows the plot of forward scatter vs. side scatter that is used to discriminate the cells between monocytes, lymphocytes, and neutrophils. The boundary that is drawn towards top of the figure which is labeled ‘A’ is called the gate which is used to select the neutrophil population. The image shown in Figure 8.1 (b) is the fluorescence signal obtained from the selected neutrophil population and the signal value is shown as ‘X-
mean’. Figure 8.1 (b) shows an image corresponding to the sample containing only blood and PBS. Figure 8.1 (c) shows the image of fluorescence signal data obtained from a sample containing blood and PBS mixed with DHR and PMA. This sample contained

Figure 8.1: (a) Example of an actual forward scatter vs side scatter plot obtained from the flow cytometer showing the neutrophil population selected by using a gate and labeled as ‘A’. Fluorescence signal obtained from the neutrophils present in (b) unstimulated blood (i.e., blood + PBS) and (c) stimulated blood (blood + PBS + DHR + PMA) showing a shift in signal to higher values as indicated by X-mean. Lines B and E shown in (b) and (c) are used to gauge the movement of the signal to higher or lower values.
detection agent. Here blood with PBS served as negative control (−ve control) where as blood with PBS containing DHR and PMA served as positive control (+ve control). From Figure 8.1, it is evident that there is some background fluorescence signal associated with whole blood. The raw data obtained from the X-mean values of various samples are plotted in Figure 8.2.

8.3.2. Determination of ROS Levels Using DHR assay

As mentioned previously, in the DHR assay, a non-fluorescent DHR molecule gets oxidized into a fluorescent rhodamine molecule in presence of reactive oxygen.

![Figure 8.2: Raw data obtained from the DHR assay showing mean fluorescence signal of the neutrophil population for different samples. From the figure it is clear that the addition of DMSO, PMA, or DHR to blood do not lead to any significant increase in fluorescence signal. However, the addition of both DHR and PMA results in a significant increase in the fluorescence signal which is the expected trend and also confirms that the correctness of the assay. Here blood with PBS serves as the control. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate *p* < 0.05 imply statistical significance as compared to the corresponding control.](image-url)
species (ROS). The fluorescence signal measured from the assay is directly proportional to the ROS levels, in which PMA is used as stimulating agent and DHR is used as detection agent. Here blood with PBS served as negative control (−ve control) where as blood with PBS containing DHR and PMA served as positive control (+ve control). From Figure 8.1, it is evident that there is some background fluorescence signal associated with whole blood. The raw data obtained from the X-mean values of various samples are plotted in Figure 8.2.

![Figure 8.2: Raw data obtained from the X-mean values of various samples.](image)

Figure 8.3: Reduced data for the DHR assay obtained by applying equation 8.1 to the raw data. Here the y-axis represents ROS levels in percentage. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate p < 0.05 imply statistical significance as compared to the corresponding control (i.e., B+P).

Since there is some background fluorescence associated with fresh blood, it is necessary to reduce the data. The following formula was used to reduce the data (Figure
8.3) in such a way that the stimulated neutrophils (blood + PBS + DHR + PMA) has 100% ROS levels and unstimulated neutrophils has 0% ROS levels (blood + PBS).

\[
\text{% ROS level} = 100 - \left( \frac{x - z}{x - y} \right) \times 100
\]  

(8.1)

Where \( x \) = mean fluorescence obtained from \(+\)ve control (blood + PBS + DHR + PMA)

\( y \) = mean fluorescence obtained from \(-\)ve control (blood + PBS) and

\( z \) = mean fluorescence obtained from the samples under consideration.

Figure 8.4: An example of data obtained from viability test. (a) Forward scatter vs. side scatter data. (b) Percentage cells (in this example 99%) that was viable among 10,000 cells that were analyzed and (c) Percentage cells that was viable (in this example 99.4) from the gated region A of figure (a). The viability data reported in this chapter were that corresponding to figure (b) i.e., representation of viability for the entire system of cells.
Figure 8.5: Effect of chemicals used in the DHR assay on cell viability. The results indicate that the chemicals used for the DHR assay did not cause any appreciable cell death compared to the control.

It is also essential to confirm that the chemicals that were used in the DHR assay are not toxic to the cells. An example data from viability test is illustrated in Figure 8.4. Therefore, cell viability studies were conducted with DHR, PMA and DMSO (used to dissolve DHR and PMA) as described in section 8.2.1 and the results are shown in Figure 8.5, which indicate that the chemicals employed in DHR assay are not cytotoxic as the cell viability were as good as fresh blood.

8.3.3. DHR Assay with Unmodified Membranes

One of the objectives of this work is to demonstrate that unmodified membranes causes an increase in oxidative stress, which eventually leads to dialysis induced oxidative stress. In order to determine the change in levels of ROS that might be caused
Figure 8.6: (a) DHR assay performed with unmodified membranes fabricated with 100/0, 95/5, 90/10 and 75/25 PA/PVP ratios. ROS levels of the membrane samples (blood + PBS + membrane + DHR + PMA) were compared with that of the positive control (blood + PBS + DHR + PMA) and the results indicate that the contact of blood with unmodified membranes has led to an increased ROS levels. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate p < 0.05 imply statistical significance as compared to the positive control. (b) Cell viability studies with unmodified membrane suggesting no appreciable cell death.
by the unmodified membrane, the assay needs to be modified with additional samples. For these experiments, unmodified membranes were cast as described in Chapter 3. The membranes were cast and dried under sterile conditions inside a biosafety laminar flow hood. For the assay, two small circular samples (2 x 0.4 cm$^2$) were punched out from the cast films and placed inside the test tubes. Blood and PBS were added to each of the test tubes and the samples were incubated at 37 °C for 3 hours (to simulate hemodialysis conditions). After three hours, the membrane samples were removed from blood and agitated with 1 ml of PBS in order to dislodge the cells that were in contact with the membrane. The rest of the assay was similar to the standard assay consisting of incubation with DHR and PMA followed by lysis of red blood cells. Finally all the samples were analyzed by flow cytometry. The unmodified membrane samples consisting of the following PA/PVP ratios were used in this experiment: 100/0; 95/5, 90/10 and 75/25. As with the standard assay, fresh blood with PBS served as the negative control and blood with DHR and PMA served as positive control which consists of stimulated cells. The mean fluorescence values of the membrane samples were compared with that of the positive control. The results of the DHR assay are displayed in Figure 8.6 (a) and cell viability results are shown in Figure 8.6(b). The results clearly indicate that the blood contact with unmodified membranes has lead to an increase in ROS level. For example, with a 90/10 PA/PVP membrane the increase in ROS level was as high as 30% compared to that of the positive control.

This outcome is very significant in the context of hemodialysis. Typical hemodialysis patients’ blood comes in contact with the membrane approximately 3 hours/session. It can be anticipated that the oxidative stress builds up in the patient’s
body with each session. However, the natural anti-oxidant mechanisms present in the body acts quickly to suppress the increase in ROS levels. For end stage renal disease (ESRD) patients, hemodialysis is a life sustaining treatment and they are prescribed to this treatment for the rest of their lives or until kidney transplantation. Hence, over a period of time, the excessive production of ROS overpowers the natural anti-oxidant defense mechanisms eventually leading to dialysis induced oxidative stress (DIOS). DIOS becomes a serious issue about 2-5 years after the beginning of hemodialysis treatment.

8.3.4. DHR Assay with Pure Mangiferin

The main objective of this dissertation was to fabricate phytochemically modified hemodialysis membranes and demonstrate the ability of such membranes to suppress ROS levels. However, before measuring ROS levels with mangiferin modified membrane, it is essential to know the effect of pure mangiferin. Since water is a non-solvent for mangiferin it is difficult to form a homogeneous solution of mangiferin with PBS. Hence mangiferin was dissolved in DMSO and added to the test tubes for DHR assay. For these experiments, the standard DHR assay was set up with additional test tubes corresponding to different concentrations of mangiferin. The concentration range of mangiferin was chosen between 25–1000 µg/ml. Here the concentration represents the amount of mangiferin present in the final solution (i.e, blood + PBS + DHR + PMA + mangiferin + DMSO). For each assay 900 µl of PBS was used as a result the concentration of DMSO in the final mixture was negligible to cause any effect on the DHR assay or on the cell viability. Also, it has been shown in Figures 8.3 and 8.5 that
DMSO did not cause any significant change in ROS levels or in cell viability. The data for DMSO (in Figure 8.3 and 8.5) were obtained with 10 times the concentration of the current assay. So any contribution of DMSO towards ROS levels can be safely ignored.

The results obtained for the DHR assay with mangiferin solution is demonstrated in Figure 8.7 (a). The data indicate that at lower concentrations (up to 500 µg/ml) mangiferin increases ROS levels in blood compared to the positive control and suppression of ROS levels can be seen only above 500 µg/ml. The ROS levels were decreased by 20% at 500-1000 µg/ml concentration range. However, the cell viability data (Figure 8.7 (b)) indicate that at 1000 µg/ml concentration, the viability drops to 95% compared to 99% and above for fresh blood. Combined, these data suggest that although it is possible to realize the anti-oxidant properties of mangiferin, a price had to be paid in terms of cell viability. In addition, 500-1000 µg/ml of mangiferin seems to be too high in comparison to the therapeutic levels of drugs employed by pharmaceutical industry.

A significant difference was observed between the measurements of Garcia et al. [135] and data shown in Figure 8.7 both in terms of % ROS suppression as well as in the concentration range over which the suppression was observed. According to literature, the average suppression capability of mangiferin was about 80% in the concentration range of 1–100 µg/ml. The data in Figure 8.7 suggest approximately 20% suppression in the concentration range of 500–1000 µg/ml. There are four possible important reasons that might contribute to the observed difference. The first reason lays the difference in techniques that are being employed in these two studies. Garcia et al. measured extracellular ROS levels as opposed to intracellular ROS levels in this study. The second source of difference comes from the detection molecules that were employed. Current
Figure 8.7: (a) DHR assay performed with mangiferin in solution in the concentration range of 25-1000 µg/ml. ROS levels of samples containing mangiferin (blood + PBS + mangiferin + DHR + PMA) were compared with that of the positive control (blood + PBS + DHR + PMA). An increase in ROS levels was observed at low mangiferin concentration and 20% suppression was observed at high concentration. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate p < 0.05 which imply statistical significance as compared to the positive control. (b) Cell viability studies indicate no appreciable cell death at lower concentration. However, viability drops to 95% at 1000 µg/ml concentration indicating appreciable cell death.
study employed DHR, which is a non-fluorescent dye molecule that freely enters the cells and gets localized inside the mitochondria (the power generating unit of the cell). On the other hand, the literature evidence had employed the oxyBURST® reagent which is based on dichlorodihydrofluorescein diacetate (DCF-DA), which was one of the earliest technologies that was employed to detect oxygen radical concentrations. DCF-DA is a non-fluorescent molecule which gets converted to DCF and fluoresces when attacked by oxygen radicals [136]. However, the major limitations associated with DCF-DA include photo-oxidation of DCF-DA to DCF and leaking from the cells rather than getting concentrated within the cells. Another important limitation of DCF-DA is the susceptibility to attack from any radicals rather than only by oxygen radicals. As a result, the direct proportionality between concentration of ROS and fluorescence signal emitted by DCF needs to be evaluated carefully.

In order to understand the third probable reason, it is important to look into the mechanism of formation of reactive oxygen species. Upon activation of the cells, the membrane-bound NADPH (nicotinamide adenine dinucleotide phosphate) and cytosolic components of the enzyme assemble in the membrane to form the active enzyme. NADPH oxidase catalyzes the reduction of O$_2$ to superoxide anion (O$_2^-_{}$), which then rapidly dismutates to hydrogen peroxide (H$_2$O$_2$) (electron transport chain). Subsequently, H$_2$O$_2$ may be converted by the enzyme myeloperoxidase into highly reactive compounds such as hypochlorous acid (HOC$\cdot$) [28]. The importance of functionally intact NADPH oxidase is demonstrated clinically in patients suffering from chronic granulomatous disease (CGD). The impaired function of the NADPH oxidase system in CGD results in recurring life-threatening infections. The DHR assay is routinely employed for clinical
evaluation of CGD patients. Hence DHR assay probes the changes occurring during the early stage of electron transport chain as opposed to the extracellular ROS levels measured which provides information at some later stages. The last difference lies in the cells used for the study. In literature, the experiments were conducted using peritoneal macrophages derived from mouse whereas current study had utilized neutrophils derived from human blood.

8.3.5 ROS Levels in Mangiferin Modified Membranes

After the effect of pure mangiferin on ROS levels has been established, experiments were designed to determine the effect of mangiferin in membranes i.e., PA/M membranes. Mangiferin modified membranes (PA/M) were cast as described in the previous section and the experimental protocol employed for the DHR assay was exactly same as that of unmodified membranes i.e., the membrane area that contacts blood was fixed for each type of membrane (2 x 0.4 cm$^2$). The results of the DHR assay are shown in Figure 8.8 (a) and viability results are shown in Figure 8.8 (b). Experiments were conducted with the following PA/M ratio: 90/10, 80/20 and 70/30. Given the effect of unmodified membranes and pure mangiferin on ROS levels, mangiferin modified membranes can be anticipated to increase the ROS levels more than the unmodified membranes and the experimental data agrees with the hypothesis. From the information available on the amount of mangiferin present in the membranes (as quantified in the chapter 6 and 7), the concentration range of mangiferin present in membranes was estimated to be the range of 100-200 µg/cm$^2$. Experiments were also conducted with
Figure 8.8: (a) DHR assay performed with mangiferin modified membranes containing the following PA/M ratio: 90/10, 80/20 and 70/30. ROS levels of mangiferin modified samples were compared with that of unmodified membranes (\textdagger) control). An increase in ROS levels was observed for all the mangiferin modified membranes. Values shown are mean ± standard deviation obtained from three experiments (\(n = 3\)). Asterisks indicate \(p < 0.05\) imply statistical significance as compared to the positive control. (b) Cell viability studies indicate no appreciable cell death due to mangiferin modified membranes.
Figure 8.9: (a) Comparison between ROS levels of PA/PVP and PA/PVP/M membranes. In this experiment, each of the PA/PVP/M membrane contained 30% mangiferin while the polymer fraction (70%) has the same PA/PVP ratio as that of the unmodified membranes. ROS levels in PA/PVP/M membranes were found to be even larger than that of PA/PVP membranes in all the cases. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate p<0.05 imply statistical significance as compared to the positive control. (b) Cell viability studies indicate no appreciable increase in viability due to mangiferin addition.
PA/PVP/M membranes and the results that are displayed in Figure 8.9(a) and (b) also show an increase in ROS levels which was similar to that of PA/M membranes.

8.3.6. DHR Assay with Pure Genistein

During the material selection process, it was realized that the presence of glucose in mangiferin might lead to unwanted increase in blood glucose levels (false alarm) as diabetes is a primary cause of kidney failure. Hence genistein was chosen as an alternative phytochemical [137,138,139,140] which does not contain glucose. Before testing genistein modified membranes, ROS levels in blood were tested with pure genistein. The experimental protocol for DHR assay and cell viability was exactly same as that of mangiferin solutions and the outcomes are plotted in Figure 8.10 (a) and (b).

Two important conclusions can be made from Figure 8.10 (a) viz., genistein was able to suppress ROS levels significantly even at concentrations as low as 25 µg/ml and the suppression of ROS occurs in a dose dependent manner i.e., higher concentration of genistein results in higher suppression of ROS levels. Genistein has been reported to inhibit the priming events necessary for high level ROS production. These antioxidant effects of genistein have been demonstrated in a number of experiments: in HL-60 (human promyelocytic leukemia cells) cancer cells and human polymorphonuclear cells cultured in vitro. The reported levels of ROS inhibition by genistein (approximately 50% in the concentration range 15–100 µg/ml) [99] agrees well with the present studies. In addition, a dose-dependent behavior of ROS suppression was also observed by Polkowiski et al.[141].
Figure 8.10: (a) DHR assay with genistein solution in the concentration range of 25-200 µg/ml. ROS levels of samples containing genistein (blood + PBS + genistein + DHR + PMA) were compared with that of the positive control (blood + PBS + DHR + PMA). Dose dependent suppression of ROS levels was observed in the whole concentration range. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate p < 0.05 imply statistical significance as compared to the positive control. (b) Cell viability studies indicate no detrimental effect on cells due to the presence of genistein.
As pointed out in section 8.3.3, the priming events that lead to the formation of ROS (electron transport chain) needs to be kept in mind during the interpretation of antioxidant properties of compounds. In this case, genistein is successfully able to interfere with the expression of NADPH, which is the first step of the electron transport chain to form superoxide anion and subsequent dismutation to H$_2$O$_2$. Due to the ability of genistein to prevent NADPH expression, long term oral administration of genistein has been recommended in order to improve the health of endothelial cells [142]. The cell viability results displayed in Figure 8.10(b) suggest no detrimental effect due to the presence genistein in the concentration range employed.

Since genistein seems to possess the intended anti-oxidant effect, experiments were designed with genistein modified membranes i.e., PA/G membranes. The experimental protocol was same as that employed for PA/M membranes. The outcome of the DHR assay and the cell viability are shown in Figure 8.11 (a) and (b) respectively. It can be inferred from Figure 8.11 (a) that genistein modified membranes were able to emulate the inhibition characteristics of pure genistein and also in a dose dependent manner. From the information available on the amount of genistein present in the membranes (Figure 7.4), the dose dependent suppression of ROS levels can be explained i.e., 70/30 PA/G membrane has higher genistein concentration in the membrane compared to 80/20 or 90/10 PA/G membranes. Similar results were obtained with PA/PVP/G membranes and a comparison between PA/PVP membranes and PA/PVP/G membranes is presented in Figure 8.12 (a) and (b). In summary, the results obtained with PA/G and PA/PVP/G membranes were very encouraging from a hemodialysis
Figure 8.11: (a) Effect of genistein modified (PA/G) membranes on ROS levels. ROS levels of PA/G membranes were compared with that of the positive control. Dose dependent suppression of ROS levels was observed which correlated with the amount of genistein present in the membranes. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate p < 0.05 imply statistical significance as compared to the positive control. (b) Cell viability studies indicate no appreciable cell death due to genistein modified membranes.
Figure 8.12: (a) Comparison between ROS levels of PA/PVP and PA/PVP/G membranes. In this experiment, each of the PA/PVP/G membrane contained 30% genistein while the polymer fraction (70%) has the same PA/PVP ratio as that of the unmodified membranes. ROS levels in PA/PVP/G membranes were found to be approximately 50% less than that of PA/PVP membranes in all the cases. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate p < 0.05 imply statistical significance as compared to the positive control. (b) Cell viability studies indicate no appreciable change in viability between the PA/PVP and PA/PVP/G membranes.

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perspective as the modification of currently employed membranes with genistein might be able to suppress DIOS in HD patients in the long run.

8.3.7. Effect of Glucose on Generation of Oxygen Radicals

During the material selection process, it was thought that the presence of glucose molecule in mangiferin might alter the blood glucose levels of hemodialysis patients (false alarm), leading to potential pitfall during the actual application. As a majority of hemodialysis population is diabetic in nature, a phytochemical without glucose molecule was sought which would also possess good anti-oxidant properties. Hence, genistein was chosen as the second phytochemical in this dissertation. The comparison of results obtained with pure mangiferin and genistein confirmed the initial hypothesis that the presence of glucose in mangiferin might be detrimental in terms of anti-oxidant properties. In order to gather some evidence for this hypothesis, the DHR assay and cell viability experiments were performed with pure glucose. In this experiment, glucose was dissolved in PBS and a concentration range of 25-1000 µg/ml was used. The rest of the protocol was similar to that employed for genistein and mangiferin solutions. The results are shown in Figure 8.13 (a) and (b), which clearly indicate that glucose increases intracellular oxygen radical generation up to 20% higher than that of the positive control. Literature evidence supports this idea of increased ROS generation by leucocytes after oral ingestion of glucose [143].

The basic unit of mangiferin and genistein, which are xanthone and isoflavone respectively, are almost similar, if not exactly the same. Since genistein had shown a positive anti-oxidant effect and glucose had shown a negative anti-oxidant effect, an
Figure 8.13: (a) DHR assay with glucose solution in the concentration range of 25-1000 µg/ml. ROS levels of samples containing glucose (blood + PBS + glucose + DHR + PMA) were compared with that of the positive control (blood + PBS + DHR + PMA). ROS levels were found to increase due to the addition of glucose as high as 20% more than the positive control in the whole concentration range. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate p < 0.05 imply statistical significance as compared to the positive control. (b) Cell viability studies indicate no detrimental effect on cells due to the presence of glucose.
Figure 8.14: DHR assay demonstrating the effect of glucose addition on genistein. The addition of glucose to genistein (i.e., genistein/glucose mixtures) seems to increase the ROS levels compared to pure genistein. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate p < 0.05 imply statistical significance as compared to the positive control.

Figure 8.15: DHR assay comparison between vitamin E, mangiferin, and genistein. Compared to vitamin E, genistein seems to be a better anti-oxidant in the employed concentration range. Values shown are mean ± standard deviation obtained from three experiments (n = 3). Asterisks indicate p < 0.05 imply statistical significance as compared to the positive control.
experiment was devised in which DHR assay was performed with a 75/25 and 50/50 mixture of genistein/glucose and the results were compared with that of pure genistein and glucose in solution. The results described in Figure 8.14 convincingly illustrate that the presence of glucose can indeed reduce the anti-oxidant capability of genistein. In other words, the observed ROS levels of genistein increased after the addition of glucose.

8.3.8. Comparison between Anti-oxidant Properties of Vitamin E, Mangiferin, and Genistein

The currently available commercial technology for hemodialysis consists of vitamin E coated membranes. Vitamin E is a lipid soluble anti-oxidant molecule which forms one of the natural anti-oxidant defenses in human body. The DHR assay was performed with vitamin E solution and the results were compared with genistein and mangiferin solution data. The results are shown in Figure 8.15, which suggest that genistein might be a better anti-oxidant than vitamin E in the range of concentration that was used in the assay.

8.3.9. Effect of mangiferin modified membranes on cytokine levels

Cytokine levels of the unmodified and phytochemically modified membranes were measured using principles of ELISA. The results of cytokine profiles for PA/M and PA/PVP/M membranes are shown in Figure 8.16 (a) and (b) respectively. Interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured simultaneously using the Luminex® flow cytometer. In general, the results indicate that both unmodified as well as mangiferin modified membranes increased the cytokine levels
Figure 8.16: Blood plasma levels of IL-1β, IL-6, and TNF-α simultaneously measured (a) by incubating (blood + LPS) with unmodified and PA/M membranes and (b) by incubating (blood + LPS) with unmodified and PA/PVP/M membranes. Here (blood + LPS) serves as positive control and fresh blood serves as negative control.
compared to that of the positive control. However, some discrepancies that were observed with IL-1β levels may be due to experimental errors. This result confirms the fact that blood contact with hemodialysis membranes results in increased production of inflammatory cytokines. PA/M membranes significantly increased the production of TNF-α compared to that of the positive control but lesser than unmodified membranes. However, at lower mangiferin concentration, a slight suppression was observed. Levels of IL-1β also followed a similar trend as that of TNF-α whereas IL-6 increased in a dose dependent manner consistently.

On the other hand, PA/PVP/M membranes showed a slight suppression of TNF-α compared to that of the unmodified membranes. However the levels were significantly higher compared to that of positive control. Levels of IL-6 were marginally less compared to unmodified membranes and positive control while IL-1β levels were consistently higher than both unmodified membranes as well as positive control. Experiments conducted by Leiro et al. [97] suggest a slight decrease in TNF-α mRNA synthesis. However, no suppression of in vitro synthesis of TNF-α was observed when LPS was used as inducing agent. However, a dose dependent suppression of TNF-α was observed both LPS and interferon-γ were used together to stimulate cytokine secretion. Other examples showing an increase cytokine levels with unmodified membranes were also observed by Sasaki et al.[40].

8.3.10. Effect of genistein modified membranes on cytokine levels

Cytokine levels were also measured with genistein modified membranes. All the experimental details were similar to that of the mangiferin modified system. The results
Figure 8.17: Blood plasma levels of IL-1β, IL-6, and TNF-α simultaneously measured (a) by incubating (blood + LPS) with unmodified and PA/G membranes and (b) by incubating (blood + LPS) with unmodified and PA/PVP/G membranes. Here (blood + LPS) serves as positive control and fresh blood serves as negative control.
obtained with PA/G and PA/PVP/G membranes are displayed in Figure 8.17 (a) and (b) respectively. It can be inferred from Figure 8.17 (a) that PA/G membranes effectively suppressed the *in vitro* secretion of IL-1β and TNF-α in a dose dependent manner whereas the levels of IL-6 were slightly suppressed only at higher genistein concentrations (for example 70/30). On the other hand PA/PVP/G membranes were able to suppress all of the three cytokines however to different extents. Genistein has been known to be a very effective inhibitor of protein tyrosine kinases, which mechanistically play an important role in the secretion of IL-1β and TNF-α [144]. As a result, the *in vitro* levels of both these cytokines were highly reduced. Based on the cytokine measurements genistein modified membranes seem to possess excellent anti-inflammatory properties compared to that of the mangiferin modified membranes.

8.4. Conclusions

In summary, two *in vitro* blood compatibility studies were conducted viz., DHR assay and measurement of cytokine profiles. Unmodified membranes were subjected to DHR assay and it was shown that both PA as well as PA/PVP membranes induce increased production of oxygen radicals, which supported the idea that prolonged contact of blood with synthetic polymer surface is the primary source of dialysis induced oxidative stress. Pure phytochemicals were subjected to both assays in solution. It was found out that at low concentration range (25-200 µg/ml) mangiferin caused an increase in oxidative stress whereas it showed about 20% suppression in ROS levels at 500-1000 µg/ml range. On the other hand, genistein showed a dose dependent suppression of oxidative stress in the entire concentration range (25-1000 µg/ml). Mangiferin modified
membranes (i.e., PA/mangiferin and PA/PVP/mangiferin) were cast and subjected to DHR assay. Given the solution behavior of mangiferin towards oxygen radical generation and similar effect unmodified membranes, it was not surprising to observe a higher oxidative stress in mangiferin membranes compared to that of unmodified membranes. However, when the experiments were executed with genistein modified membranes, dose dependent reduction in ROS levels was observed.

It was hypothesized that the presence of glucose moiety in mangiferin might be responsible for the unexpected behavior of mangiferin which lead to increased generation of oxygen radicals. Experiments were conducted with pure glucose in solution and it was shown that glucose induces an increased oxidative burst. Considering the close similarity of flavonoid structure between genistein and mangiferin, DHR assay was performed with a physical mixture of genistein and glucose in an attempt to imitate mangiferin’s structure. The addition of glucose to pure genistein caused an increase production of oxygen radicals. Hence it was concluded that it might be possible to realize the full antioxidant potential of mangiferin by cleaving the glucose moiety from mangiferin.

Measurement of cytokine profiles revealed that genistein modified membranes were effectively able to suppress the levels of IL-1β, IL-6, and TNF-α more effectively than mangiferin modified membranes. Protein tyrosine kinases play an important role in the process of cytokine secretion induced by LPS. The well known inhibition of protein tyrosine kinases by genistein might be responsible for the observed suppression of cytokines.
CHAPTER IX

SUMMARY AND RECOMMENDATIONS

9.1. Summary

In this dissertation, an attempt was made to fabricate functional hemodialysis membranes that could suppress two of the important long term complications associated with hemodialysis namely dialysis induced oxidative stress and membrane induced inflammation. PA/PVP and PES/PVP blends, which are currently used as hemodialysis membranes were chosen. Mangiferin and genistein were chosen as the phytochemicals. The phytochemicals were decided to be incorporated into the membrane by physically mixing with the polymer feed solution that is used to fabricate the membranes.

In Chapter 4, the miscibility studies of PA/PVP/mangiferin blends were discussed. PA/PVP blends were shown to be completely miscibility owing to cross hydrogen bonding between the two polymers. Occurrence of cross hydrogen bonding interactions in PA/mangiferin as well as PVP/mangiferin blends was demonstrated. Further, mangiferin was shown to have preferential affinity towards PVP compared to PA. Ternary phase diagram for PA/PVP/mangiferin system was established and various co-existence regions such as isotropic, liquid-liquid, isotropic + crystal and crystal regions were identified.

Miscibility studies of PES/PVP/mangiferin blends were demonstrated in Chapter 5. PES/PVP blends were completely miscible in entire composition range although FTIR
studies indicated no specific interactions between the two species. Addition of mangiferin to PES reduced the glass transition temperature of PES/mangiferin blends. Ternary phase diagrams for PES/PVP/mangiferin blends indicated the presence of a larger single phase region compared to that of PA/PVP/mangiferin blends.

Membrane formation with PA/PVP blends and mangiferin modification were illustrated in Chapter 6. Membranes were cast as film via non-solvent induced phase separation process. Morphology of unmodified membranes revealed a dense skin layer. However, the cross section showed finger-like channel which progressively increased in size along the cross section. Mangiferin modification of PA membranes led to an increase in surface porosity of the membranes whereas PA/PVP/mangiferin membranes not only showed microporous surface but also network structure which might have been the result of spinodal decomposition. Due to preferential affinity of DMSO with water, some amount of PVP as well as mangiferin was lost during membrane formation. The loss of mangiferin and PVP were quantified.

Miscibility characteristics of PA/PVP/genistein blends were shown in Chapter 7 followed by formation of genistein modified membranes. SEM micrographs revealed that even at very low genistein concentration (in feed) embedded crystals were observed on the membrane surface. As the concentration of genistein was increased (in feed) large spherulitic structures evolved on the membrane surface. Similar to mangiferin, some amount of genistein that was also lost during the membrane formation was quantified. The addition of PVP to the feed solutions seemed to have suppressed the formation of genistein crystals in PA/PVP/genistein membranes.
Finally, the outcomes of *in vitro* blood compatibility experiments performed with human blood neutrophils were presented in Chapter 8. Unmodified membranes were shown to increase the concentration of oxygen radicals, which lent support to the idea of DIOS. Mangiferin increased the ROS levels of neutrophils at low concentration (25–200 µg/ml) whereas approximately 20% reduction in ROS levels was found at higher concentration (500–1000 µg/ml). By virtue of aforementioned behavior of pure mangiferin membranes, PA/mangiferin as well as PA/PVP/mangiferin membranes also increased the generation of oxygen radicals in neutrophils. On the other hand, pure genistein as well as genistein modified membranes was illustrated to suppress ROS levels in a dose dependent manner. In order to understand if the presence of glucose in mangiferin led to the observed increase in oxygen radical concentration, experiments were performed with genistein/glucose mixture (genistein does not contain glucose). The addition of glucose to genistein resulted in increased production of oxygen radicals by neutrophils. Measurement of cytokine profiles revealed that genistein modified membranes were effectively able to suppress the levels of IL-1β, IL-6, and TNF-α more effectively than mangiferin modified membranes. The well known inhibition of protein tyrosine kinases by genistein might be responsible for the observed suppression of cytokines.

9.2. Recommendations

Even though the anti-oxidant and anti-inflammatory properties were demonstrated, one of the important characteristics of hemodialysis membrane is thrombosis i.e., formation of clot. Therefore, an immediate extension of the results
presented in this dissertation will be to conduct hematology experiments with unmodified and phytochemical modified membranes especially estimation of time needed for clot formation and changes in platelet characteristics due to membrane contact.

The improvement in quality of life of patients is one of the critically important objectives in design, development, and modification of any biomedical device. The most serious limitation of hemodialysis patients is the inability to work which is caused due to extreme fatigue caused by dialysis. Any attempt that could cut down the dialysis time will provide a huge sigh of relief to these patients. One of the methods that can be considered towards reducing the dialysis time is to enable the hemodialysis membrane to remove toxins by both filtration as well as absorption. In this regard, it is necessary to understand the interactions of the membranes and phytochemicals with urea. The second method that could reduce the time is to provide maximum contact area between blood and membrane surface which can be achieved by reducing the fiber diameter. Development of portable hemodialyzer units will be one the most challenging as well as the most rewarding opportunities, which could benefit the dialysis population immensely. With the expanding application of electrospinning process, the days of portable dialyzers are not far away.

The research presented in this dissertation not only helps to develop novel materials and devices but also to utilize environmentally friendly and renewable resources for such ventures thereby enabling value addition to agricultural products.
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