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DEVELOPMENT OF FORCED-FLOW ELECTROPHORESIS FOR PROTEINS CONCENTRATION

HAIRUL NAZIRAH BINTI ABDUL HALIM

UNIVERSITI SAINS MALAYSIA
2007

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by

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science

OCTOBER 2007

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LIST OF SYMBOLS

		Unit
A	Membrane area	(m^2)
C_o	Initial protein concentration	(g/L)
C_{bulk}	Concentration of solute in bulk	(g/L)
C_f	Final protein concentration	(g/L)
C_p	Cumulative protein concentration in permeate	(g/L)
C_{wall}	Concentration of solute near membrane wall	(g/L)
E	Electric field strength	(V/cm)
E_o	Standard electrode potential at 298 K	(V)
h	Channel height (distance between anode and cathode)	(cm)
I	Electric current	(A)
J	Permeate flux	($L/m^2.h$) ($m^3/m^2.s$)
M_R	Mass of protein in retentate	(g)
r	Specific electric resistance	-
R	Electric resistance	(Ω)
R_a	Hydraulic resistance due to protein adsorption	(m^{-1})
R_b	Hydraulic resistance due to pore blocking	(m^{-1})
R_c	Hydraulic resistance due to the cake layer	(m^{-1})
R_{cc}	Hydraulic resistance due to compression of the cake layer	(m^{-1})
R_{cp}	Hydraulic resistance due to the concentration polarization	(m^{-1})
R_g	Global hydraulic resistance	(m^{-1})
R_m	hydraulic resistance due to intrinsic membrane	(m^{-1})
R_p	Percentage of protein rejection	(%)
P_R	Retentate pressure	(Pa)
P_f	Filtrate pressure	(Pa)
P_F	Feed pressure	(Pa)
V	Electric potential (voltage)	(V)
V_o	Initial volume of feed solution	(L)
V_p	Cumulative volume of permeate	(L)

Greek letters

k	Conductivity	(μS/cm)
μ	Dynamic viscosity	(Pa.s)
ΔP	Transmembrane pressure	Pa
Δt	Time difference	(h)
ΔV	Cumulative volume difference	(L)

LIST OF ABBREVIATIONS

AEM	Anion exchange membrane
BSA	Bovine Serum Albumin
BGG	Bovine γ-globulin
CEM	Cation exchange membrane
EMF	Electro-membrane filtration
FFE	Forced-Flow Electrophoresis
HSA	Human Serum Albumin
IEF	Isoelectric focusing
IEP	Isoelectric point
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IVIg	Intravenous immunoglobulin
M	Electrode material
MF	Microfiltration
MWCO	Molecular weight cut off
NWP	Normal water permeability
PBS	Phosphate buffer solution
PES	Polyethersulfone
SPE	Soy protein extract
TMP	Transmembrane pressure
UF	Ultrafiltration

PEMBANGUNAN ELEKTROFORESIS ALIRAN PAKSA UNTUK PEMEKATAN PROTEIN

ABSTRAK

Dewasa ini, aplikasi proses penurasan ultra (UF) dalam bidang bioteknologi telah diterima dengan baik untuk memekat dan menulen produk-produk protein plasma seperti albumin dan immunoglobulin. Walaubagaimanapun, penurunan hasil telapan yang ketara dengan masa kerana pengutuban kepekatan dan membran tersumbat merupakan satu pembatasan ke atas kecekapan penggunaan proses penurasan ultra.

Dalam kajian ini, penggunaan medan elektrik luar dalam UF yang dikenali sebagai Elektroforesis Aliran Paksa (FFE) telah dibangunkan sebagai kaedah alternatif untuk memperbaiki prestasi penurasan. FFE telah digunakan untuk memekatkan dua jenis protein iaitu Albumin Serum Lembu (BSA) dan γ-globulin Lembu (BGG). Modul FFE telah dikaji menggunakan elektrod keluli tanpa karat sebagai katod manakala campuran oksida titanium iridium sebagai anod. Dua jenis membran telah digunakan dalam projek ini iaitu membran polietersulfon (PES) dan membran penukar kation (CEM). Di bawah penggunaan medan elektrik, protein yang bercas bergerak kearah elektrod yang bercas berlawanan dan bergerak menjauhi permukaan membran UF. Fenomena ini menghalang partikel-partikel daripada termendap ke atas permukaan membran UF dan mengurangkan pengutuban kepekatan berhampiran permukaan membran UF dengan menggunakan daya elektroforesis.

Perbandingan di antara penurasan ultra konvensional dan proses FFE membuktikan bahawa prestasi proses di bawah pengaruh medan elektrik telah memberi keputusan yang lebih baik. Pada keadaan kekuatan medan elektrik (E) 3.0 V/cm, tekanan menerusi membran (TMP) 100 kPa, kepekatan awal protein (C_o) 0.50

g/L, pH 8.0 dan 20 mM larutan penimbal fosfat (PBS), kadar hasil telapan FFE bagi penurasan BSA dan BGG, telah bertambah baik sebanyak 116.67% dan 46.56%, masing-masing berbanding penurasan ultra konvensional. Rintangan hidraulik global bagi BSA dan BGG berkurang sebanyak 53.94% dan 31.87%, masing-masing di bawah keadaan yang sama.

Prestasi bagi proses FFE dipengaruhi oleh potongan berat molekul (MWCO) bagi membran PES, kekuatan medan elektrik (E), tekanan menerusi membran (TMP), kepekatan awal protein (C_o), pH awal penimbal dan kepekatan awal ionik bagi larutan penimbal fosfat (PBS). Didapati bahawa 30 kDa membran polietersulfon (PES) adalah sesuai bagi penurasan BSA manakala 50 kDa membran polietersulfon (PES) pula sesuai bagi penurasan BGG. Pada E = 3.0 V/cm, keadaan optimum operasi bagi pemekatan kedua-dua BSA dan BGG dalam sistem FFE ialah pH 8.0, 20 mM PBS, C_o 0.50 g/L dan TMP 100 kPa. Pada keadaan ini, kadar hasil telapan bagi BSA ialah 33.78 L/m^2.h manakala bagi BGG ialah 14.29 L/m^2.h. Rintangan hidraulik global bagi BSA dan BGG ialah1.17 x 10^{13} m^{-1} and 2.77 x 10^{13} m^{-1}, masing-masing. Pada akhir eksperimen, BSA dan BGG telah dipekatkan daripada 0.50 g/L kepada 1.45 g/L dan 0.76 g/L, masing-masing. Daripada kajian ini, telah didapati bahawa penggunaan medan elektrik dalam UF telah memperbaiki prestasi penurasan untuk pemekatan larutan protein.

DEVELOPMENT OF FORCED-FLOW ELECTROPHORESIS FOR PROTEINS CONCENTRATION

ABSTRACT

Nowadays, the application of ultrafiltration (UF) process in the field of biotechnology has been well accepted to concentrate and purify plasma protein products such as albumin and immunoglobulin. However, the significant flux declines with time due to concentration polarization and membrane fouling is a limitation to the efficient use of ultrafiltration process.

In this work, the application of external electric field in UF which is known as Forced-Flow Electrophoresis (FFE) was developed as an alternative technique to improve the filtration performance. The FFE was used to concentrate two types of proteins, Bovine Serum Albumin (BSA) and Bovine γ-globulin (BGG). The FFE module was studied using a stainless steel electrode as a cathode while titanium iridium mixed oxide electrode as an anode. Two types of membrane were used in this project which include polyethersulfone (PES) membrane and cation exchange membrane (CEM). Under the application of electric field, the charged protein moves towards the opposite charged electrode and migrates away from the UF membrane surface. This phenomenon prevents particles from depositing onto the UF membrane surface and minimized the concentration polarization near the UF membrane by the imposed electrophoretic force.

The comparison between conventional ultrafiltration and FFE process has proven that the process performance under the influence of electric field gave better results. At the condition of electric field strength (E) of 3.0 V/cm, transmembrane pressure (TMP) of 100 kPa, initial protein concentration (C_o) of 0.50 g/L, pH 8.0 and 20

mM of PBS, the permeate flux of FFE for BSA and BGG filtration improved for about 116.67% and 46.56%, respectively as compared to the conventional UF. The global hydraulic resistance of BSA and BGG were minimized by 53.94% and 31.87%, respectively under the same conditions.

The performance of FFE process was affected by molecular weight cut off (MWCO) of PES membrane, electric field strength (*E)*, transmembrane pressure (TMP), initial protein concentration (C_o), initial pH buffer and initial ionic strength of phosphate buffer solution (PBS). It was found that the 30 kDa of PES membrane was suitable for BSA filtration while the 50 kDa of PES membrane was suitable for BGG filtration. At E = 3.0 V/cm, the optimum operating conditions for concentrating both BSA and BGG in FFE system were pH 8.0, 20 mM of PBS, C_o of 0.50 g/L proteins and TMP of 100 kPa. At this condition, the permeate flux of BSA was 33.78 L/m^2.h while for BGG was 14.29 L/m^2.h. The global hydraulic resistance for BSA and BGG was 1.17 x 10^{13} m^{-1} and 2.77 x 10^{13} m^{-1}, respectively. At the end of experiment, the BSA and BGG was concentrated from 0.50 g/L to 1.45 g/L and 0.76 g/L, respectively. From this research, it was found that the application of electric field in UF has improved the filtration performance for concentrating proteins solution.

CHAPTER ONE

INTRODUCTION

1.1 The Demand of Plasma Protein Products

In recent years, the demand of plasma protein products especially albumin and immunoglobulin has highly increased especially for biotechnological and biomedical applications. Almost 50 years, albumin has been used clinically as a volume expender for hypovolumia, thermal injury, hypoalbumineria, shock and burn (Albumin therapy, 2005). On the other hand, immunoglobulin is a type of antibody which could be reacted with specific antigens to fight infections and to boost the body's natural defence system. Most of the immunoglobulins have significant contribution to the therapy options in curing autoimmune and immunodeficiency, antihemophilic, hepatitis and cancer diseases.

One of the major biopharmaceutical companies in the world which manufactures and market plasma products such as albumin, intravenous immunoglobulin (IVIg) and clotting factor is CSL Limited, Melbourne, Australia. Figure 1.1 shows the total revenue for the year of 1999 to 2006 which has increased from $US 504.3 million to $US 2903.5 million. It was reported in CSL Limited Financial Report 2005-2006, that the total revenue in 2006 has increased by 9.55% to $US 2903.5 million as compared to 2005. The market of plasma products, especially albumin and immunoglobulin continues to grow with approximately 12-13% per annum.

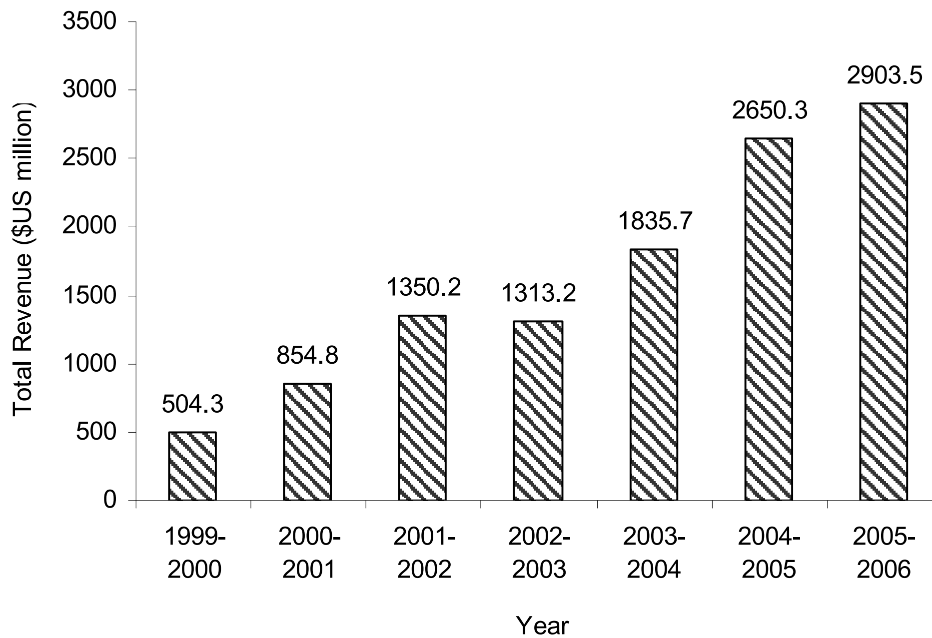


Figure 1.1 Total Revenue (CSL Limited, 2006)

Figure 1.2 and Figure 1.3 show the market demands for both albumin and intravenous immunoglobulin (IVIg) which were reported by Robert (2006) in Market statistics and trends of plasma protein products. The actual global consumption of both proteins has been slightly increased since 1990 until 2006. A projected trend extrapolated until 2016, with respect to the global demand for intravenous immunoglobulin and albumin is represented in both figures. The trend is based on historical demand figures dating from 2000 onwards and suggests an increase in demand of 7.0 tonnes of albumin and 5.1 tonnes of intravenous immunoglobulin annually for the period of 2006 – 2016. The high demand of plasma products should be in lines with the production capacity of the plasma products and will turn to reduce gradually without the appropriate plant facilities and process development. Thus, the maintenance and process improvement are considered as one of the important requirements in the production process.

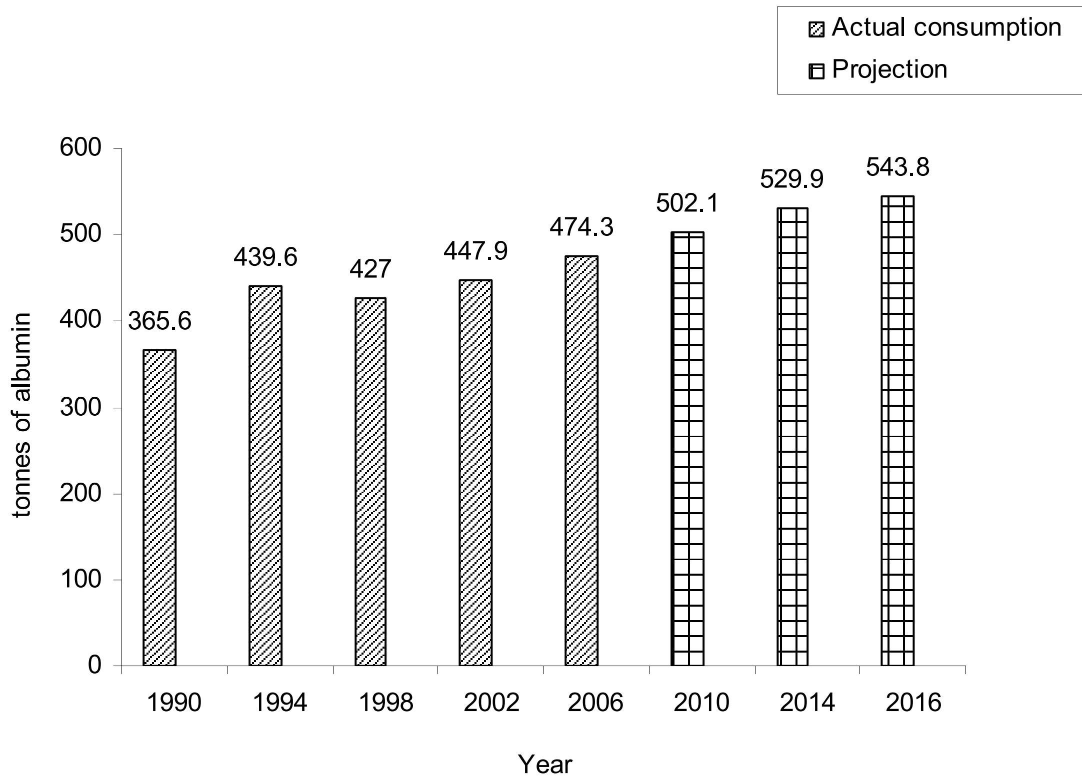


Figure 1.2 Albumin actual global consumption and projection (Robert, 2006)

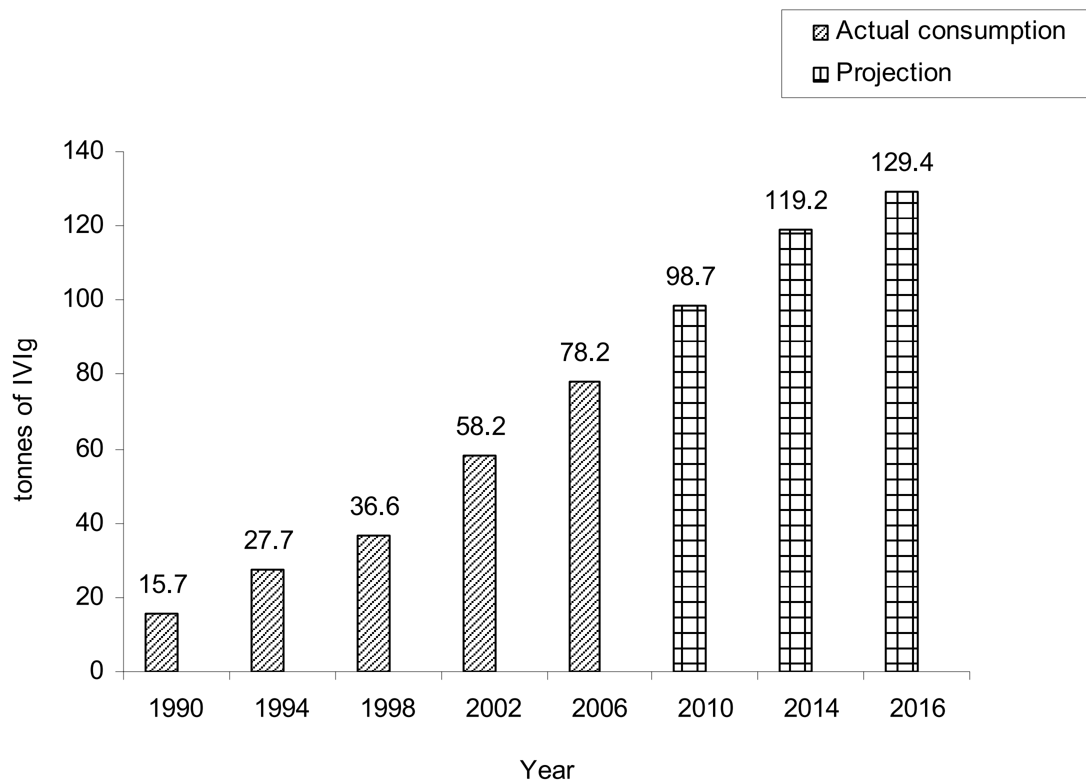


Figure 1.3 Intravenous immunoglobulin (IVIg) actual global consumption and projection (Robert, 2006)

1.2 Membrane Application in Pharmaceutical Industry

The applications of membrane processes in the field of biotechnology are widely used both in the laboratory and in industry to clarify, concentrate and purify macromolecular products. The developed membrane operations have played an important role in the production of high value added products, such as antibiotics, enzymes, albumin, antibody and other bio-products (Tarazaga *et al.*, 2006).

In downstream processing, membranes are suitable to be used for separation of desired material from unwanted components and concentrating the diluted biological substances to the desired final concentration. Membrane processes such as ultrafiltration, microfiltration, reverse osmosis, pervaporation and electrodialysis are finding niches in downstream processing of recombinant proteins and proteins produced from fused cell cultures (Crespo and Boddeker, 1994). Combining membrane processes with other unit processes such as precipitation, affinity ligand adsorption and coupling several chromatographic techniques one after the other such as ion exchange, gel filtration and affinity chromatography also becoming economically attractive which has been applied in commercial plasma fractionation process.

Although membrane technology has not played a critical role in the discovery process of the biological revolution, it is an important component in the production of valuable pharmaceuticals and other biological molecules. The development of novel approaches to improve the efficiency of downstream processes is desirable to simplify processing or improve product yield and purity. Since the growing demand for the production of protein biopharmaceuticals products has been largely increased, there is consequently an increasing requirement to improve the efficiency of bioprocess involved. One way of achieving this could be to develop tools and techniques for the rapid, smaller energy consumption and operation flexibility.

1.3 Problem Statement

The development of an alternative method for the industrial protein concentration is desirable since it is gaining great importance in the biopharmaceutical processes. Nowadays, membrane technology has been well accepted in commercial application as a unit process in the downstream part of biopharmaceutical processes to concentrate most valuable components such as albumin and immunoglobulin. Ultrafiltration is preferred for protein concentration, ethanol removal and desalting because there is less denaturation and more efficient than vacuum freeze-drying (lyophilization) or thin film evaporation (Porter, 1990). A major advantage of concentration by ultrafiltration over conventional evaporation or lyophilization is the reduction of energy cost due to no latent heat required to evaporate the diluted protein solution while maintaining product purity under ambient conditions (Porter, 1990).

The application of ultrafiltration process in the field of biotechnology is being increasingly integrated into the recovery schemes for the production of valuable biological molecules. The problems facing with the ultrafiltration process are flux declination during the operation time. The limitation is attributed to several factors, namely, broad membrane pore size distribution, concentration polarization, membrane fouling, solute-solute and solute-membrane interactions (Nakatsuka and Micheals, 1992). The accumulation of solute particles on the membrane surface which known as concentration polarization increases the viscosity and osmotic pressure during a process. On the other hand, the deposition of retained colloidal and macromolecular material on the membrane surface creates membrane fouling which closely related with the interaction between protein charged and the membrane surface charge during the process. These phenomena offer to some external extra resistance to the solvent flow through the membrane. These limitations consequently contribute to the cost of the process as it requires frequent cleaning and replacement of the membrane.

There are four types of membrane modules in the ultrafiltration which is tubular, hollow fiber, spiral wound and plate-and-frame modules. Among these, the plate-and-frame module found to be high resistant to fouling. In downstream processing of plasma fractionation process, spiral wound ultrafiltration has been used as a unit operation to concentrate the albumin and immunoglobulin. One of the disadvantages of the module is if only a part of the spiral wound ultrafiltration membrane fails (due to fouling); the entire module must be discarded (Schafer *et al*., 2005). However, in plate-and-frame module, if the membrane in one of the plates fails, it can be individually replaced and the entire module does not have to be discarded (Schafer *et al*., 2005).

Intensive researches on alternative anti-fouling strategy for plate-and-frame module have been carried out by some researchers. A challenge faced in the development of the new techniques which can improve the flux performance as compared to the conventional ultrafiltration. Several techniques have been developed including promoting turbulence at or near a membrane surface (Oussedik *et al.*, 2000), gas sparged membrane filtration (Cheng and Li, 2007; Cheng 2002) and electro-membrane filtration (Park, 2006; Tarazaga *et al*., 2006; Weber and Stahl, 2002; Karthik *et al*., 2002; Oussedik *et al*., 2000; Iritani *et al*., 2000; Mameri *et al*., 1999; Weigert *et al*., 1999 and Zumbusch *et al.*, 1998). Among these techniques, the electro-membrane filtration (EMF) seems to be the most effective methods to minimize the existing problems. In EMF process, the application of electric field at low operating pressure will minimize the accumulation of the solutes on the membrane surface by imposing the electrophoretic force.

There are a few configurations of electro-membrane filtration modules. The simplest configuration is to insert the electrodes into the suspension and permeate channels which have been investigated by Weber and Stahl (2002), Karthik *et al*. (2002), Oussedik *et al*. (2000), Iritani *et al*. (2000), Mameri *et al*. (1999) and Zumbusch

et al. (1998). However, the major disadvantage of this configuration is that the electrolysis products enter the filtration cycle and significantly change the suspension pH value (Weigert *et al*., 1999). Alternatively, the ion exchange membrane (cation exchange membrane and anion exchange membrane) were used to separate the filtration and rinsing cycle which at the same time helps to prevent the direct contact between feed and electrolysis products (Pribyl *et al*., 2003; Bargeman *et al*., 2002; Weigert *et al*., 1999; O'Connor *et al.,* 1996).

It was reported by Hwang and Kammermeyer (1975) that most of the synthetic polymeric anion exchange membranes (AEM) are not stable as compared to the cation exchange membrane (CEM). When the feed streams contain high molecular weight anions, they usually adsorb on the AEM and thus foul the surface. At a sufficient electric current, the transport depletion occurs where the total ionic concentration near the AEM interface becomes severely depleted due to the concentration polarization effect. The ionic concentration is often polarized to such an extent which resulted in the changes of the pH value near the interface (Hwang and Kammermeyer, 1975). In this case, the AEM which is unstable at a high pH value generally deteriorates and the effective membrane life is shortened. In order to eliminate such a membrane deterioration, the array of cation exchange membrane (CEM) and ultrafiltration membrane will be investigated in this project rather than the usual anion-cation membrane combination. The process is known as Forced-Flow Electrophoresis (FFE).

In the Forced-Flow Electrophoresis, the employment of two types of membranes; polyethersulfone ultrafiltration and cation exchange membranes in the plate-and-frame module are expected to minimize the concentration polarization effect in the membrane processes under the influence of external electric field. The movement of protein charged away from the membrane surface under the influence of

electric field has a potential to improve the filtration performance as compared to the conventional ultrafiltration in terms of flux decay and concentration polarization effect.

1.4 Research Objectives

The present research study has the following objectives:

1. To develop a Forced-Flow Electrophoresis (FFE) method to improve the ultrafiltration process for concentrating proteins solution, Bovine Serum Albumin (BSA) and Bovine γ-globulin (BGG) based on the difference in terms of charge and molecular size.
2. To compare the performance of Forced-Flow Electrophoresis (FFE) and conventional ultrafiltration (UF) in terms of the permeate flux and global hydraulic resistance for BSA and BGG.
3. To study the process parameters covering molecular weight cut off (MWCO) of membrane, electric field strength, transmembrane pressure, initial protein concentration, initial pH buffer and initial ionic strength of buffer in terms of flux profile, global hydraulic resistance and final protein concentration.

1.5 Scope of Study

Two types of proteins, Bovine Serum Albumin (BSA) and Bovine γ-globulin (BGG) purchased from Sigma Aldrich (USA) were used to study the performance of FFE process. The albumin and γ-globulin were selected because they are found in major fraction in the human protein plasma and have valuable potential to be used clinically as a therapeutic agent. The technique of Rotofor System was used to determine the isoelectric point (IEP) of protein. The ultrafiltration membrane of Biomax polyethersulfone (PES) membranes made by Millipore Corporation (USA), with different molecular weight cut off (MWCO), 30 kDa and 50 kDa were studied to find the highest rejection of the protein. The experiment was conducted using fabricated FFE module.

The performance of conventional ultrafiltration and FFE process were compared in term of the permeate flux and global hydraulic resistances. The pertinent process parameters were identified to gauge the process performance covering transmembrane pressure (100 to 250 kPa), initial protein concentration (0.50 to 7.50 g/L), initial pH (pH 5.0 to 8.0) and initial ionic strength of buffer (20 to 150 mM). The effect of electric field strength (E) was studied in the range of 1.0 to 20.0 V/cm for each process parameter to study the filtration behavior on each process parameter. The performance of the Forced-Flow Electrophoresis (FFE) process was studied in terms of flux profile, global hydraulic resistance and final protein concentration.

1.6 Organization of Thesis

Chapter 1: Introduction describes the general application of protein plasma products, albumin and gamma-globulin and the current biopharmaceutical demand in the world. Then, the application of membrane processes in the pharmaceutical industry is described in general. The problem statement of the research is stated to give the clear objectives of the present study. The scope of the study covers the research work done to meet these objectives.

Chapter 2: Literature Review is divided into four main sections. The first section describes the structures and the characteristics of protein plasma, albumin and gamma-globulin. The second section elaborates the industrial method for protein concentration such as thin film evaporation, vacuum freeze-drying and membrane filtration. The third section describes the ultrafiltration process covering ultrafiltration membrane, concentration polarization and fouling problem, the interaction between protein charge and membrane charge on fouling and the various filtration techniques to enhance ultrafiltration. Section four describes the proposed Forced-Flow Electrophoresis (FFE) to improve the performance of ultrafiltration of protein solution.

This section covers the description of the process, the driving force involved, force interaction and electrokinetic effect in FFE process and various configuration of electro-membrane filtration (EMF) module.

Chapter 3: This chapter is divided into seven main sections. The first section is about the materials and chemicals used in the present work. The second section presents the flowchart of the overall process study. The third section describes the set up of FFE module and experimental rig used in the experiment. Section four describes the experimental procedures in this work followed by parameters study of FFE process in section five. The analytical techniques used in this experiment were described in section six while the calculation of experimental data is given in section seven.

Chapter 4: Results and Discussion chapter presents the experimental data and results along with the useful observations and findings. It covers the discussion about isoelectric point of BSA and BGG, selection of membrane pore size, comparison between ultrafiltration and Forced-Flow Electrophoresis (FFE) and parameter study of Forced-Flow Electrophoresis (FFE). The parameters studied include electric field strength, transmembrane pressure, initial protein concentration, initial pH buffer and initial ionic strength of buffer. It is discussed in term of flux profile, global hydraulic resistance and final protein concentration.

Chapter 5: Conclusions and Recommendations chapter presents the concluding remarks on the potential of Forced-Flow Electrophoresis to improve the ultrafiltration of protein solutions. Some recommendations for the future studies are also given in this chapter.

CHAPTER TWO

LITERATURE REVIEW

2.0 Introduction

This chapter presents the literature review which relates to this work. It can be divided into four main sections which include the description of plasma proteins, industrial method for protein concentration, ultrafiltration and Forced-Flow Electrophoresis (FFE) system.

2.1 Plasma Proteins

Plasma is a liquid presents in blood, in which the erythrocytes, leukocytes and platelets are suspended. It is mainly composed of water, proteins and inorganic electrolytes. The plasma protein constitutes about 7% of blood plasma containing albumin, globulins and fibrinogen. The plasma protein excluding its clotting factors (such as fibrinogen) is known as blood serum that falls into two main protein groups which are albumins and globulins. Table 2.1 shows the summary of the blood components.

Table 2.1 Summary of Blood Component (Cambrian College, 2005)

Component of the Blood					
Plasma (55%)			**Formed Element (45%)**		
Protein (7%)	Water (91%)	Other Solutes (2%)	Platelets	Leukocytes	Erythrocytes
Albumins (58%) Globulins (38%) Fibrinogen (4%)		Ions Nutriens Waste Products Gases Regulatory Substances		Neutrophils (60-70%) Lymphocytes (20-25%) Monocytes (3-8%) Eosinophils (2-4%) Barophils (0.5-1%)	

2.1.1 Human Serum Albumin

Human Serum Albumin is a highly soluble protein with a molecular weight of 67,000 Daltons. Human serum albumin is constructed of a single chain of 585 amino

acids, crosslinked by 17 disulfide bridges. It has been used as a therapeutic agent for over 50 years (Matejtschuk *et al.*, 2000). The main functions of albumin include maintaining the colloid osmotic pressure and transporting hormones, enzymes, fatty acids, metal ions and drugs. At normal physiological concentrations of plasma proteins, albumin contributes 80% of the colloidal osmotic (oncotic) pressure of plasma. Clinical uses of albumin as therapeutic has been widely used to treat hypovolemic shock, thermal injury, hypoalbuminaemia, trauma, surgery and blood loss, burn management and plasma exchange (Albumin Therapy, 2005).

2.1.2 Gamma Globulin

Gamma globulin or also known as immunoglobulin is a type of protein found in serum plasma with a molecular weight of 158,000 Daltons. It is a type of antibody which could be reacted with specific antigens to fight infections and to boost the body's natural defense system (Peeters, 1975). The immunoglobulin is formed from two identical light (L) and two identical heavy (H) polypeptide chains. These chains are linked by interchain disulfide bridges from the carboxyl-terminal ends of the light chain to the heavy chain between the heavy chains in their mid-region (Painter and Dorrington, 1979). The heavy chains make it possible to divide antibodies into major immunoglobulin groups termed IgG, IgA, IgM, IgD and IgE. Small differences in the structure of the heavy chains have made it possible to subdivide immunoglobulins (IgG) into four subclasses (IgG1 to IgG4) and IgA and IgM into two subclasses. The IgG molecule is shown in the Figure 2.1. Most of the immunoglobulin have significant contribution to the therapy options in curing autoimmune and immunodeficiency, antihemophilic, hepatitis and cancer diseases. Table 2.2 shows the properties of albumin and IgG.

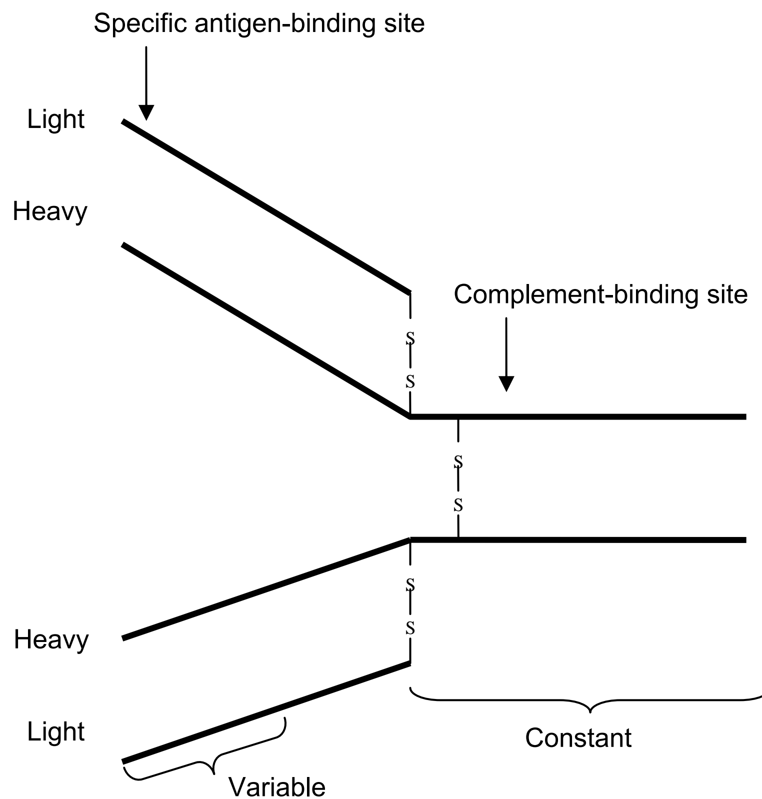


Figure 2.1 Structure of immunoglobulin molecule (Source: Chanarin *et al.*, 1984)

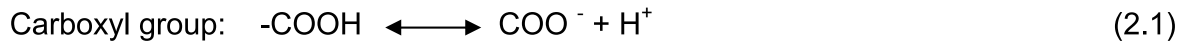
Table 2.2 Properties of albumin and IgG

	Albumin (BSA, HSA)	**IgG (γ-globulin)**
Molecular weight (Voet *et al.*, 2002)	67,000 Daltons	158,000 Daltons
Isoelectric point (Voet *et al.*, 2002)	pH 4.9	pH 6.6
Equivalent radius (Yoshikawa *et al.*, 2004; Causserand *et al.*, 2001)	3.61 nm	5.60 nm
Diffusivity at 20^0C (10^{-7} cm^2/s) (Chan and Chen, 2004)	6.1, 6.7	3.8, 4.0

2.1.3 Isoelectric Point (IEP) of Protein

Proteins are amphoteric (or zwitterionic) compounds and are therefore either positively or negatively charged because they contain both acidic and basic residues.

Most of the charges of protein come from the pH-dependent ionization of amino acid side-chain carboxyl and amino groups as shown in Equation 2.1 and Equation 2.2.



Carboxyl group: $-COOH \longleftrightarrow COO^- + H^+$ (2.1)

Amino group: $-NH_2 + H^+ \longleftrightarrow NH_3^+$ (2.2)

These proteins show different net charges at different pH media (Peng *et al*., 2004). The net charge of a protein is determined by the pH of the surrounding medium and the number and types of amino acids carrying amino or carboxyl groups.

For each protein species, the pH at which the net charge of the protein is zero is defined as the isoelectric point (IEP). At this pH, there are equal numbers of positive and negative charges on the molecule. If the protein is dissolved around its isoelectric point value, the protein tends to precipitate because the tertiary structure of protein is disturbed (Pincet *et al*., 1995). In a solution with a pH above the isoelectric point, a protein has a negative net charge while when the solution is below a protein's isoelectric point, the protein has a positive net charge (Rodgers, 1999). The IEP values of the proteins of interest are important for any means of protein separation. It is therefore possible to promote the charge effect to achieve an effective concentration of the protein by adjusting the solution's pH value. Table 2.3 shows the isoelectric points of several common proteins.

Table 2.3 Isoelectric points of several common proteins (Voet *et al*., 2002)

Protein	**IEP (pH)**
Serum Albumin (human)	4.9
γ – Globulin (human)	6.6
Fibrinogen (human)	5.8

Hemoglobin (human)	7.1
Insulin (bovine)	5.4
Ovalbumin (hen)	4.6

2.2 Industrial Method for Protein Concentration

In the industrial processes, there are quite a number of feed streams which have to be concentrated to obtain the solute in pure form. The industries involved include food, dairy, pharmaceutical, biotechnological and chemical industries. Generally, the concentration of the required products in the feed stream is very low (Crespo and Boddeker, 1994). This implies that all the solvent (in most cases water) must be removed in order to achieve the final product concentration. Several methods for protein concentration in industrial scale have established such as thin film evaporation, vacuum freeze drying (lyophilization) and membrane processes.

2.2.1 Thin Film Evaporation

Evaporation is primarily associated with the removal of water and other solvent by boiling in batch processes. Evaporation is conducted by vaporizing a portion of the solvent to produce a concentrated solution of thick liquor. Most evaporators are heated by steam condensing on metal tubes. The material to be evaporated flows inside the tubes.

Thin Film Evaporation is operated under high vacuum while the temperature of the liquid can be kept low during the process. It is once-through operation where the feed liquor passes through the tubes only once at the evaporation temperature and can be quickly cooled as soon as it leaves the evaporator. Then, it leaves the unit as a thick liquor. This process is especially useful for heat-sensitive materials such as pharmaceutical products and foods. However, the disadvantage of this process is the high energy demand during operation which leads to high energy cost.

2.2.2 Vacuum Freeze Drying

Freeze drying also known as lyophilization is a dehydration process that being used more commonly in the bioseparations, food industry and pharmaceutical industry. In pharmaceutical industries, the freeze drying method is often used to increase the shelf life of products and reserved for materials that are heat-sensitive, such as proteins, vaccines, enzymes, microorganisms, and blood plasma (Freeze drying, 2007). In bioseparations, freeze drying can also be used as a late-stage purification procedure, because it can effectively remove solvents. Furthermore, it is capable of concentrating molecules with low molecular weights that are too small to be filtered out by a filtration membrane (Freeze drying, 2007).

Generally, the rotary freeze dryers are usually used with liquid products such as pharmaceutical solutions and tissue extracts. There are three stages in the complete freeze drying process which consist of freezing, primary drying, and secondary drying. For a larger scale operation, freezing is usually done using a freeze drying machine. In this step, it is important to freeze the material at a temperature below the eutectic point of the material. Since the eutectic point occurs at the lowest temperature where the solid and liquid phase of the material can coexist, freezing the material at a temperature below this point ensures that sublimation rather than melting occurs in the following steps.

During the primary drying phase the pressure is lowered and enough heat is supplied to the material for the water to sublime. In this initial drying phase about 98% of the water in the material is sublimated. This phase may be slow, because if too much heat is added the material's structure could be altered. In this phase, pressure is controlled through the application of partial vacuum. The vacuum speeds up

sublimation process making it useful as a deliberate drying process. Furthermore, a cold condenser chamber and/or condenser plates provides a surface(s) for the water vapour to re-solidify on. Condenser temperatures are typically below −50 °C.

The secondary drying phase aims to sublime the water molecules that are adsorbed during the freezing process, since the mobile water molecules were sublimated in the primary drying phase. This part of the freeze-drying process is governed by the material's adsorption isotherms. In this phase, the temperature is raised even higher than in the primary drying phase to break any physico-chemical interactions that have formed between the water molecules and the frozen material. Usually the pressure is also lowered in this stage to encourage sublimation.

This process is relatively expensive due to high energy demands during the process. It consumes long processing time and the additional of too much heat to the material can cause melting or structural deformation (Freeze drying, 2007).

2.2.3 Membrane Processes

Pressure-driven membrane processes are being increasingly integrated into isolation and recovery schemes for the production of valuable biological molecules. Well known membrane processes such as microfiltration (MF) and ultrafiltration (UF) are known to be effective methods for removal of particulates and protein concentration. The heart of the microfiltration field is sterile filtration using microfilters with small pores which microorganisms cannot pass through the membrane. Some of the sterile microfiltration applications replace thermal sterilization which may offer a direct energy saving in the process and indirect saving through avoidance of heat exchange equipment (Baker *et al.*, 1991).

The diversity of applications for UF in pharmaceutical industry includes the concentration, purification, desalting, fractionation and sterilization of the pharmaceutical products such as viruses, hormones, vaccines, antibiotics, albumin and antibodies (Porter, 1990). Membrane processes is suitable for biological products because it is usually performed at ambient temperature which is suitable for temperature sensitive solutions to be treated without the constituents being damaged or chemically altered (Porter, 1990). In ultrafiltration process, the separation is achieved by concentrating the large molecule present in the feed on one side of the membrane, while the solvent and microsolutes are depleted as they pass through the membrane. In some applications, the product is the retentate and the objective is to concentrate or purify the retained species by passing unwanted contaminant through the membrane. In other applications, the product is the permeate, and the objective is to remove unwanted contaminant which are large enough to be retained by the membrane. However, in a few applications, both retentate and filtrate contain valuable products.

A major advantage of concentration by UF over evaporation and lyophilization is that the salts are not concentrated by the process, but freely pass through the membrane (Crespo and Boddeker, 1994). Thus, the ionic environment for the protein remains constant. Furthermore, since no latent heat is required in this process, the energy costs are reduced as compared to the evaporation and lyophilization processes. Table 2.4 shows the advantages and disadvantages of the thin film evaporation, lyophilization and membrane processes for protein concentration.

Table 2.4 Advantages and disadvantages of the thin film evaporation, lyophilization and membrane processes for protein concentration

Method	Advantage	Disadvantage
Thin Film Evaporation	▪ alternative process to concentrate heat-sensitive products	▪ temperature sensitivity lead to protein denaturation

		▪ high energy demands lead to high energy costs
Vacuum Freeze Drying (lyophilization)	▪ capable of concentrating molecules with low molecular weights that are too small to be filtered out by a filtration membrane. ▪ less damage to the substance than other dehydration methods using higher temperatures	▪ relatively expensive process ▪ high energy demands lead to high energy costs ▪ long process time. Protein sample is sensitive to degradation or denaturation, should dictate the use of heat and refrigeration in processing
Membrane Processes	▪ the salts are not concentrated by the process, but freely pass through the membrane. Thus, the ionic environment for the protein remains constant ▪ low energy cost due to no latent heat required ▪ less product denaturation because the product does not contact with air interface	▪ concentration polarization and fouling problems

2.3 Ultrafiltration

2.3.1 Ultrafiltration Membranes

Ultrafiltration is a type of separation process in which large molecules or colloidal particles are filtered from the solution by means of suitable membrane. The average pore diameter of the membrane is in the range of 10 – 1000 Ǻ (0.001 to 0.1μm). They have a finely porous surface layer on a much more open microporous substrate. The finely porous surface layer performs the separation while the microporous substrate provides mechanical strength. The pore size of the ultrafiltration membranes usually characterized by their molecular weight cut-off (MWCO). The molecular weight cut-off is defined as the molecular weight of the globular protein molecule that is 90% retained by the membrane.

Membranes are made from a number of polymers. Typical polymers include; cellulose, cellulose diacetate and triacetate, polyacrylonitrile, polysulfone, polyethersulfone (PES), polypropylene, polyethylene, and others. These polymers

have shown to provide the basic properties such as mechanical strength and ability to be cleaned. The selection of suitable membrane materials that exhibit low non-specific protein adsorption is one of the important criteria in protein separation process.

The chemical composition of the polymer can have a profound effect on the adsorbtivity of proteins. The property of the membrane material which describes its interaction with proteins is classified as the relative hydrophobicity. Usually hydrophilic surface groups such as hydroxyls, sulfonates and amines result in low protein fouling and hydrophobic such as aromatic or aliphatic hydrocarbons result in significant hydrophobic adsorption (Rodgers, 1999). However, many hydrophilic groups can contribute to extensive protein interaction due, possibly, to dipole-dipole or coulombic interaction especially for polyethersulfone and cellulose acetate (Rodgers, 1999). Figure 2.2 shows the repeat unit structure for polyethersufone (PES). It can be seen that the PES is made up of diphenylen sulfone repeating units. The oxygen molecules on the $–SO_2$ groups have two unshared electrons which can allow for significant hydrogen bonding between membrane and protein.

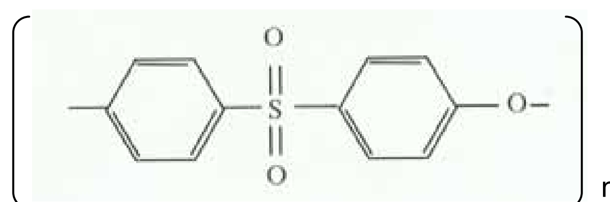


Figure 2.2 Repeating unit structure for polyethersulfone

2.3.2 Fouling and Concentration Polarization in Ultrafiltration

Membrane fouling and concentration polarization are two aspects of a same problem which is the build-up of retained species in the boundary layer on a membrane surface. Both phenomena have in common; gradually to reduce the permeation flux through the membranes and change the selectivity of the process. Concentration polarization describes the concentration profile of solutes in the liquid phase adjacent to the membrane surface. It is reversible mechanism which disappears as soon as the operating pressure has been released (Crespo and Boddeker, 1994). Concentration polarization can be represented by the concentration gradient adjacent to the membrane as shown in Figure 2.3. The magnitude of concentration polarization is determined by the balance between the convection towards the membrane, due to solvent flux and back-transport from the membrane to the bulk due to the concentration gradient. The possible back transport mechanism includes the molecular (Brownian) diffusion, interaction induced-migration (electrokinetic effects) and shear induced-diffusion (Schafer *et al.*, 2005).

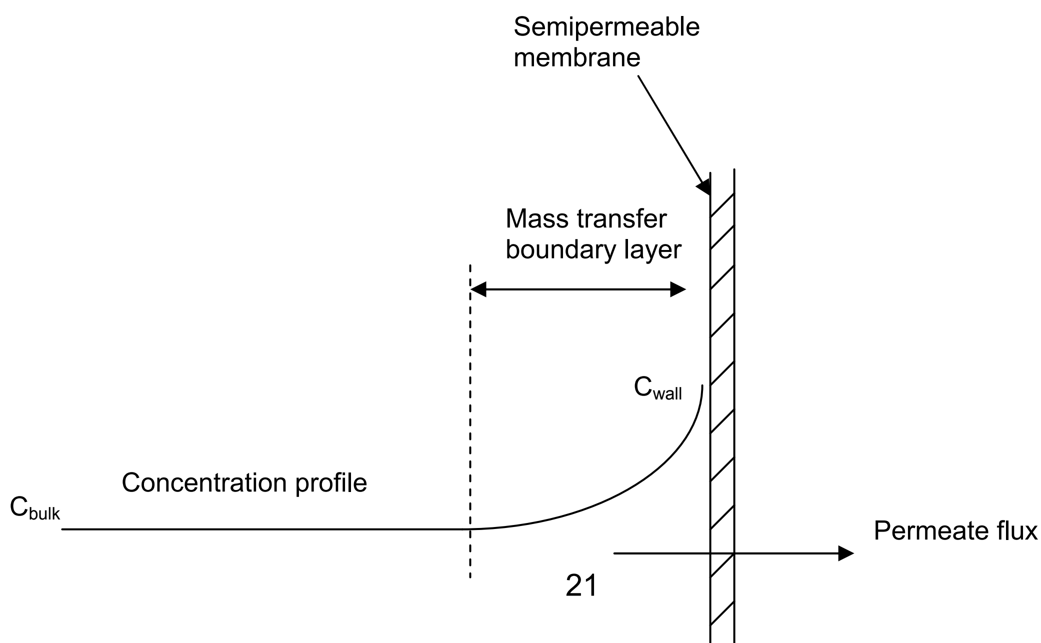


Figure 2.3 Solute concentration gradient in semipermeable membrane

According to Koros *et al*. (1996), fouling is defined as a process resulting in loss of performance of a membrane due to deposition of suspended or dissolved substances on its external surfaces, at its pore openings, or within its pores. One part of fouling may be permanent (irreversible), because a mechanical and chemical cleaning is required to restore the membrane properties. One fraction of fouling may be non-permanent (reversible) as in some cases, the deposited material is swept away by the cross flow just after the pressure difference has been released. Fouling can be subdivided in essentially five mechanisms, each monitored as a build up of global hydraulic resistance (R_g) (Rooda and van der Graaf, 2001):

- adsorption inside the membrane pores (R_a);
- blocking of the membrane pores (R_b);
- concentration of foulants near the membrane surface, also called concentration polarisation (R_{cp});
- deposition on the membrane surface forming a cake layer (R_c);
- compression of the cake layer (R_{cc}).

During filtration these mechanisms may occur simultaneously. According to Oussedik *et al.* (2000), the global hydraulic resistance, R_g (m^{-1}) through the membrane may be expressed by the sum of the hydraulic resistance due to protein adsorption onto the membrane R_a, pore blocking R_b, the concentration polarization R_{cp}, the cake layer R_c, and intrinsic membrane R_m. The average pore diameter and the porosity of the membrane mainly determine the initial resistance of the membrane (R_m). The global hydraulic resistance, R_g (m^{-1}) is expressed in Equation 2.3 as follow:

$$R_g = R_a + R_b + R_{cp} + R_c + R_m \quad (2.3)$$

The relationship between the permeate flux and the global hydraulic resistance of membrane is shown in Equation 2.4 as follow:

$$J = \frac{\Delta P}{\mu R_g} \tag{2.4}$$

Where J is the permeate flux ($m^3/m^2.s$), ΔP is the transmembrane pressure (Pa) and μ is the dynamic viscosity (Pa.s). The dynamic viscosity of aqueous protein solutions can be determined by an empirical correlation as shown in Equation 2.5 (Kozinski and Lightfoot, 1972):

$$\mu = 0.001 \exp^{0.01244\, Co} \tag{2.5}$$

Where C_o is the initial protein concentration (g/L).

2.3.3 Effect of Protein-Membrane Interactions on Membrane Fouling

The importance of protein-membrane interactions in determining the rate of protein transport during ultrafiltration have been studied by many researchers. Physicochemical properties of the protein and the hydrodynamics of the membrane system have significant effects on protein adsorption and membrane fouling (Jones and O'Melia, 2001). Due to their structure and chemical properties, proteins tend to be adsorbed on the membrane surface by the interaction mechanisms such as ionic, entropic, van der Waals interactions and hydrogen bonding (Muller *et al.*, 2003). The electrostatic repulsion exists between the membrane pore wall and the protein molecules for the same charged case, whereas electrostatic attraction is involved for the oppositely charged case.

Many problems with protein fouling in ultrafiltration is directly associated with the basic properties of proteins and the synthetic membrane's chemistry (Rodgers, 1999). The membrane fouling and protein adsorption are strongly dependent on protein-protein and protein-membrane interactions (Fane *et al.*, 1983). Protein-protein interactions affect the porosity of the cake layer on the membrane, while protein-membrane interactions affect irreversible adsorption onto the membrane (Jones and O'Melia, 2001). The interaction of charged membrane and proteins is strongly affected by pH and ionic strength of buffer solution. Most of the experimental studies of electrostatic interactions in membrane systems have been reported by obtaining data for a single protein over a range of pH or ionic strength (Pujar and Zdyney, 1994; Yang and Tong, 1997; Burns and Zydney, 1999).

According to Jones and O'Melia (2001), at higher pH values, where the protein and membrane are negatively charged, the electrostatic repulsion created by protein-membrane interactions had reduced fouling problem. Similar observation were reported by Reis *et al.* (1999) and Nakao *et al.* (1988) that the protein rejection was greatest under conditions where the membrane and protein had same charges due to the strong electrostatic repulsion. It has been reported that the flux decline is more pronounced at the IEP of the protein (Mukai *et al.*, 1997; Ricq *et al.*, 1999; Ohmori and Glatz, 1999; Jones and O'Melia, 2001). At the IEP of protein, the protein solubility tends to be lowest and the repulsive electrostatic interaction between protein molecules and membrane surface is minimized (Nakamura and Matsumoto, 2006). The interaction between BSA molecules and pore surface would be repulsive at pH above the IEP of BSA, negligible at pH of the IEP and attractive in pH range below the IEP (Nakamura and Matsumoto, 2006).