UNIVERSITI PUTRA MALAYSIA

PARTITIONING BEHAVIOURS AND SELECTIVE RECOVERY OF THERAPEUTIC PROTEIN IN AQUEOUS TWO-PHASE SYSTEM

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FK 2016 21

PARTITIONING BEHAVIOURS AND SELECTIVE RECOVERY OF THERAPEUTIC PROTEIN IN AQUEOUS TWO-PHASE SYSTEM

By

CHOW YIN HUI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

February 2016

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PARTITIONING BEHAVIOURS AND SELECTIVE RECOVERY OF THERAPEUTIC PROTEIN IN AQUEOUS TWO-PHASE SYSTEM

By

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February 2016

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Immunoglobulin G (IgG) is a type of high value therapeutic protein widely applied to the treatment of various chronic diseases such as cancer, immune and inflammatory disorders. The conventional expensive and rate-limiting chromatography-based downstream processing of IgG has been considered as the bottleneck in producing commercially viable therapeutic products. This thesis focused on the development and application of aqueous two-phase system (ATPS) as an effective and economical approach to recover the IgG from crude feedstock. The partition of pure IgG and the extraction of IgG from an artificial mixture of proteins, which contained IgG and bovine serum albumin (BSA) at a concentration that simulates the common IgG/impurities ratio, were significantly affected by the polyethylene glycol (PEG) molecular weight, phase compositions, and the addition of sodium chloride (NaCl). The monoclonal human IgG1 was successfully recovered from the Chinese Hamster Ovary (CHO) cell supernatant by using an ATPS composed of 14.0% (w/w) PEG 1450, 12.5% (w/w) phosphate (pH 7.5), and 5.0% (w/w) NaCl in the first forward extraction. A total yield of 81.38%, high IgG purity of 95.06% and *PF* of 8.91 were achieved after the back extraction step. Also, relationship which describes the effect of the difference in composition of the phase-forming component between the top and bottom phases on the interfacial partitioning of protein as well as relationship which linearly correlates the protein partitioning behaviour to phase compositions and system pH were proposed and verified by studying the partitioning behaviour of a model protein, BSA, in the PEG-phosphate ATPS. The results of goodness of fit test showed that the former relationship and an extended form of the latter relationship, which incorporated with the influence of NaCl concentration, were both applicable to the correlation of the partitioning behaviour of IgG in the ATPS which contained complex protein solutions. The molecular dynamics (MD) simulation of the partitioning of BSA in an optimised ATPS confirmed that the ATPS is a biocompatible separation technique. Therefore, these results open a promising prospect for the application of ATPS as an effective alternative purification tool in the downstream processing of IgG.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

SIFAT-SIFAT PENGASINGAN DAN PEMULIHAN KHUSUS PROTEIN TERAPEUTIK DALAM SISTEM DUA FASA AKUEUS

Oleh

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Imunoglobulin G (IgG) adalah sejenis protein terapeutik yang diguna secara meluas untuk rawatan pelbagai penyakit kronik seperti kanser, keradangan dan gangguan imun. Untuk mengatasi cabaran dari pemprosesan hiliran konvensional IgG yang memakan masa dan tinggi kosnya, pembentukan kaedah alternatif untuk menulenkan IgG menjadi semakin terdesak. Dengan itu, penyelidikan ini mengkaji pembangunan dan aplikasi sistem akueus dua fasa (ATPS) sebagai kaedah alternatif yang ekonomi dan berkesan bagi penulenan IgG dari supernatan kultur sel. Pengekstrakan IgG tulen dan IgG daripada campuran protein tiruan yang mengandungi IgG dan albumin serum lembu (BSA) pada kepekatan yang menyerupai nisbah IgG/bendasing yang biasa, telah terjejas dengan ketara oleh berat molekul polietilena glikol (PEG), komposisi fasa dan penambahan natrium klorida (NaCl). IgG1 manusia monoklonal telah berjaya dipulihkan daripada supernatan kultur sel ovari hamster China (Chinese Hamster Ovary (CHO)) dengan mengguna ATPS yang mengandungi 14.0% (w/w) PEG 1450, 12.5% (w/w) fosfat (pH 7.5) dan 5.0% (w/w) NaCl pada pengekstrakan peringkat pertama. Hasil pemulihan keseluruhan, ketulenan dan faktor penulenan yang masing-masing sebanyak 81.38%, 95.06% dan 8.91 telah dicapai selepas pengekstrakan balik IgG ke fasa fosfat yang baru. Selain itu, model yang menerangkan kesan perbezaan kepekatan polietilena glikol (PEG) antara dua fasa pada pengasingan interfasa protein dan model yang menghubung kaitkan pengasingan protein dengan komposisi fasa dan sistem pH telah dibentuk dan disahkan dengan kajian sifat-sifat pengasingan protein model, BSA, dalam ATPS PEG-fosfat. Keputusan analisis statistik menunjukkan bahawa model pengasingan interfasa protein dan model sifat-sifat pengasingan protein yang telah diperkembangkan untuk merangkumkan pengaruh kepekatan NaCl boleh digunakan untuk ramalan sifat-sifat pengasingan protein dalam ATPS yang mengandungi larutan protein yang kompleks. Simulasi dinamik molekul (MD) bagi pengasingan BSA dalam ATPS mengesahkan bahawa ATPS adalah teknik penulenan protein yang bioserasi. Oleh itu, kajian ini menunjukkan bahawa aplikasi ATPS mempunyai prospek yang cerah sebagai kaedah penulenan alternatif dalam pemprosesan hiliran IgG.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Mohd Shamsul Anuar for his unfailing support, invaluable guidance, and encouragement throughout the course of this research.

I would like to thank Professor Dr. Ling Tau Chuan for giving me the opportunity to pursue my postgraduate study in UPM before his transfer to the University of Malaya and for his kind assistance throughout this study. Thank you to my co-supervisors, Dr. Bimo Ario Tejo for his invaluable suggestions as well as continuous support though he had transferred to Surya University, Indonesia, and to Professor Dr. Arbakariya Bin Ariff for his professional advice and support.

My profound gratitude also goes to Dr. Yap Yee Jiun from University of Nottingham Malaysia Campus for his great assistance, insightful ideas and generous knowledge sharing throughout this research.

Special thanks to the staffs of Laboratory of Immunotherapeutic and Vaccines (LIVES) and Department of Process and Food Engineering for their kind assistance. I would also like to thank my fellow friends who had been working together in the laboratories for their kind help and motivation (Dr. Tan Joo Shun, Dr. Show Pau Loke, Dr. Grrace Ng Hui Suan, Dr. How Chee Wun, Naimah Haron, and Tam Yew Joon).

The financial supports provided by the Graduate Research Fellowship of UPM and the MyBrain15 of the Ministry of Education Malaysia are gratefully acknowledged.

Lastly, I wish to thank my parents and my sister for their unwavering support, understanding and love.

I certify that a Thesis Examination Committee has met on 2 February 2016 to conduct the final examination of Chow Yin Hui on her thesis entitled "Partitioning Behaviours and Selective Recovery of Therapeutic Protein in Aqueous Two-Phase System" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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a	Constant which depends on the polymer type, molecular weight, concentration, temperature and salt type
a_{H^+}	Hydrogen ion activity
A	Constant which relates the ln G to $\Delta[PEG]$
AEX	Anion exchange chromatography
ATPS	Aqueous two-phase system
b	Constant which depends on the polymer type, molecular weight, concentration, temperature and salt type
B	Constant of the interfacial partitioning relationship
BCA	Bicinchoninic acid
BHK	Baby hamster kidney cells
BSA	Bovine serum albumin
C	Concentration of the dissolved solid
C_0	Liquid equilibrium concentration in contact with the flat solid
C_B	Concentration of solute partitioned to the bottom phase
C_i	Charge of the impurity
C_T	Concentration of solute partitioned to the top phase
CEX	Cation exchange chromatography
CH	Constant domain on the immunoglobulin G heavy chain
CHO	Chinese Hamster Ovary
CL	Constant domain on the immunoglobulin G light chain
d_p	Diameter of particle
DBC	Dynamic binding capacity
DHFR	Double dihydrofolate reductase
E	Measured potential

E^o	Standard electrode potential
$\boldsymbol{F}$	Force
F	Faraday constant
Fab	Antigen binding fragment
Fc	Crystallisable fragment
FDA	Food and drug administration
G	Interfacial partition coefficient
H	Heavy chain of immunoglobulin G
HAMA	Human Anti-Mouse Antibodies
HEK	Human embryonic kidney
HIC	Hydrophobic interaction chromatography
IGF-I	Insulin-like growth factor I
IgG	Immunoglobulin G
k	Boltzmann constant
K	Partition coefficient
K_0	Partition coefficient of protein at the pH which corresponds to the protein pI
K_2HPO_4	Dipotassium hydrogen orthophosphate
KH_2PO_4	Potassium dihydrogen orthophosphate
L	Light chain of immunoglobulin G
L	Length
m	The number of fitted parameters
mAb	Monoclonal antibody
MD	Molecular dynamics
MMC	Mixed-mode chromatography
n	Number of moles

N	The number of measurements
N_i	The amount of solute in the phase *i*
NaCl	Sodium chloride
NRTL	Non-Random Two-Liquid
PEG	Polyethylene glycol
PF	Purification factor
pI	Isoelectric point
QbD	Quality by Design
R	Gas constant
R_g	Radius of gyration
R_h	Hydrodynamic radius
R_i	Radius
R^2	Coefficient of determination
RMSD	Root mean square deviation
SASA	Solvent accessible surface area
SEC	Size exclusion chromatography
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STL	Slope of the tie-line
T	Temperature (K)
TEMED	Tetramethylethylenediamine
TLL	Tie-line length
TPP	Three phase partitioning
UNIQUAC	Universal Quasi-Chemical
v	The number of degrees of freedom
V_R	Volume ratio
VH	Variable domain on the immunoglobulin G heavy chain

VL	Variable domain on the immunoglobulin G light chain
VRF	Virus retentive filtration
Y_{BE}	Yield of the back-extraction
Y_T	Yield at the top phase of the ATPS
$y_{exp,i}$	The i^{th} experimentally measured data
$y_{pred,i}$	The i^{th} predicted data
Z	Net protein charge
$[i]$	Concentration of solute or ion i
σ	Interfacial tension
σ_i^2	Variance of the measurement
τ	Solid-liquid interfacial energy
Ω	Molecular volume of the solid
θ	Contact angle
γ	Constant which relates $\Delta\Phi$ to E
$\Delta\gamma$	Difference in cell surface free energy between the phases
$\Delta\psi$	Difference in electrical potential between phases
$\Delta\Phi$	Interfacial potential difference
ΔE	Energy which varies from phase to phase
Δp	Change of pressure
ΔP_i	Difference in the concentration of phase-forming component i between the top and bottom phases
$\Delta[PEG]$	PEG concentration difference between the top and bottom phases of the aqueous two-phase system
$\mathbb{R}$	Real number
χ_v^2	Reduced chi-squared
α	Constant which relates ln K to TLL^2

α'	Constant which relates ln K to pH
α''	Constant which relates ln K to ln[Cl^-]
β	Constant of the relationship between ln K and TLL^2
β'	Constant of the relationship between ln K and pH
β''	Constant of the relationship between ln K and ln[Cl^-]

CHAPTER 1

INTRODUCTION

1.1 Background

Antibodies, also known as immunoglobulins, are divided into five different classes (i.e. IgG, IgM, IgA, IgD and IgE) which differ in size, carbohydrate composition, and the sequence of amino acid in the heavy chain. Among various immunoglobulins, immunoglobulins of the IgG class are widely applied to diagnose and to treat various diseases and disorders, such as cancer, transplant rejection, immune and inflammatory disorders (Elvin et al., 2013). Since the first commercialization of therapeutic antibody in 1986, this class of biotechnology derived drugs have been hailed as a magic bullet in various medical applications due to their inherent high specificity (Ecker et al., 2014). Following the approval of the humanised and fully human antibodies (i.e. antibodies of non-human origin whose protein sequences have been genetically modified to enhance their resemblance to antibodies produced naturally in the human body) such as Xolair and Arzerra, the global sales revenue reached $75 billion in 2013, constituting nearly half of the biopharmaceutical products market (Ecker et al., 2014; Walsh, 2014). In view of the increasing market demand, market introduction of newly approved antibody products and the prevalence of cancer and diseases, the global sales are projected to increase at 8% per year, reaching $125 billion in 2020. Meanwhile, the growing market needs, the call for less costly products, the growing competition between the biopharmaceutical companies, and the economic constraints of healthcare systems have adversely increased the pressure for the establishment of a cost-effective and faster pharmaceutical production (Gagnon, 2012; Sommerfeld and Strube, 2005).

In response to these needs, the upstream process development has advanced remarkably in the last few decades. While earlier *in vivo* production (Sommerfeld and Strube, 2005) yielded milligram to gram quantities of IgGs, the continuous improvements in the upstream processing of IgG have allowed their volumetric productivities to increase by 20-fold over the past two decades (Kuczewski et al., 2011). However, the economies of scale brought by this improvement have failed to translate to the downstream processing of antibody which constitutes up to 50-80% of the total manufacturing costs (Walsh, 2010).

In general, most of the purification schemes of the antibody depend on the application of Protein A affinity chromatography, which needs prior centrifugation and filtration steps, as the primary capture and purification step (Shukla and Thömmes, 2010). This step is then followed by two chromatographic polishing steps and a filtration step. The high specific binding affinity of protein A ligand towards the Fc domain of antibody provides high selectivity and more than 95% product-related purity from a clarified cell culture supernatant (Hahn et al., 2003). However, this major rate-limiting process accounts for more than 70% of the downstream costs and an industrial scale protein A column could cost up to $1.5 million (Azevedo et al., 2009b; Walsh, 2010). Moreover, its purification

performance is overshadowed by several drawbacks such as the low capacity, long processing time, complex scale-up, high pressure drop within column and low proteolytic and chemical stability which may contaminate the end product (Low et al., 2007). In addition to this, the polishing steps also suffer from the similar limitations aforementioned and the use of viral filters could cost $25,000 per production run (Gronemeyer et al., 2014; Walsh, 2010). As a whole, the conventional recovery processes are laborious, time-consuming, expensive and finite, which in turn make the therapeutic antibody product extremely expensive. Versatile and economical alternative purification methods are therefore required to be developed in order to improve the process throughput, scalability, and the processing time of the IgG purification processes (Thömmes and Etzel, 2007). Alternatively, they can reduce the number of purification stages, make excellent complements to the conventional operations and enhance the cost effectiveness of the recovery operations.

In recent years, the aqueous two-phase system (ATPS), a bioseparation process that exploits the differential partitioning of solutes in the immiscible two-aqueous solutions, has been recognised as a versatile and superior alternative recovery step that can overcome several technical limitations of the conventional chromatographic purification processes for therapeutic antibodies. This purification method has not only shown good purification performance in the recovery of various proteins and enzymes, but also a number of industrial applications (Asenjo and Andrews, 2011). Judging from the product selectivity, recovery and purity, the use of inexpensive phase-forming chemicals has made this emerging technique more attractive and competitive. Its merits also include simplicity, high capacity, biocompatible separation environment, ease of upscale and continuous operation (Goja et al., 2013). Most importantly, the integration of the clarification, concentration and purification of proteins into a single ATPS extraction step can reduce the processing time, the number of unit operations, running costs and capital expenditure of the manufacturing process as well as providing favourable product yield and purity (Igarashi et al., 2004b; Rosa et al., 2010). These process advantages over the conventional primary chromatographic purification technique thus make the ATPS an excellent recovery method feasible for the large-scale purification of high value therapeutic IgG from complex feedstock.

1.2 Research Problems

The pressures for alternative low-cost and rapid pharmaceutical production are on the rise from various aspects. The economic constraints of healthcare systems, growing market demand and competition between companies inevitably place crucial challenges to the typically expensive and time-consuming conventional downstream processing that has failed to keep up with the advancement in the upstream processes (Rosa et al., 2011). The high production cost of $100-1000 per gram of therapeutic proteins not only makes the products not commercially viable, but also causes the antibody-based pharmacotherapy extremely expensive and may not be affordable for everyone (Sommerfeld and Strube, 2005). Thus, the development of alternative antibody recovery processes that could produce comparable or improved yield and decrease the purification cost and time are indispensable prerequisites to expand the market for the therapeutic antibodies. One of such alternative IgG purification methods that could circumvent these bottlenecks is the ATPS. However, despite the considerable academic research efforts as well as the favourable process economics and advantages offered, the industrial scale

application of the ATPS is still limited (Oelmeier et al., 2012b; Rosa et al., 2010). The limited application is caused by the poor understanding of the complex solute partitioning mechanism in the ATPS that makes the ATPS method development rather time, labour, and material intensive (Benavides and Rito-Palomares, 2008). Under such circumstances, a clear molecular picture of the underlying protein partitioning mechanism needs to be devised and detailed models in the form of equations which could predict and elucidate the protein partitioning behaviour have to be derived to achieve process optimisation in a rapid and inexpensive way. Unfortunately, most of the available models either demand excessive experimentally determined parameters or only valid over a limited set of condition. Besides this, the accumulation of product at the interface of the ATPS, a common phenomenon that has been reported in many literatures, has however been neglected by many researchers. This phenomenon is detrimental to the protein recovery process as it results in product loss and may cause subsequent process complication. This interfacial partitioning behaviour ought to be thoroughly investigated and accounted in order to fully exploit the industrial capabilities of the ATPS for the recovery of IgG.

In light of these constraints, besides determining the best conditions for the recovery of IgG in the polymer-salt ATPS that promote rapid and cost effective recovery process, this work also sought to identify and formulate the relationships between the system variables and each of the "true" partitioning (i.e. the partition of protein between two bulk aqueous phases and with no accumulation of protein at the interface) and the interfacial partitioning of proteins (Figure 1.1). This approach could enhance the understanding of the ATPS and pave the way for the process development of industrial applications. Additionally, the molecular dynamics (MD) simulation of a small replica of the protein containing ATPS was demonstrated for the first time in the literature to give a clearer molecular insight into the underlying protein partitioning mechanism of the ATPS. Therefore, this work focused on the understanding, characterisation, and correlations of the "true" and interfacial partitioning behaviour of proteins with the ATPS parameters, as well as the selective recovery of IgG in the ATPS to guide the process development of ATPS as a rapid and economical IgG recovery process.

1.3 Objectives

In summary, the objectives of this research were:

1. To identify and formulate the factors which contribute to the interfacial partitioning of a model protein, BSA, in the polymer-salt ATPS.
2. To characterise the partitioning behaviour of a model protein, BSA, in the polymer-salt ATPS by performing MD simulation and developing relationships between the partitioning behaviour of protein and ATPS parameters.
3. To characterise the partitioning behaviour of pure IgG in the polymer-salt ATPS by studying and correlating the effect of ATPS parameters to the partitioning behaviour of pure IgG.
4. To recover the IgG with high yield and purity from crude feedstock by using the polymer-salt ATPS.

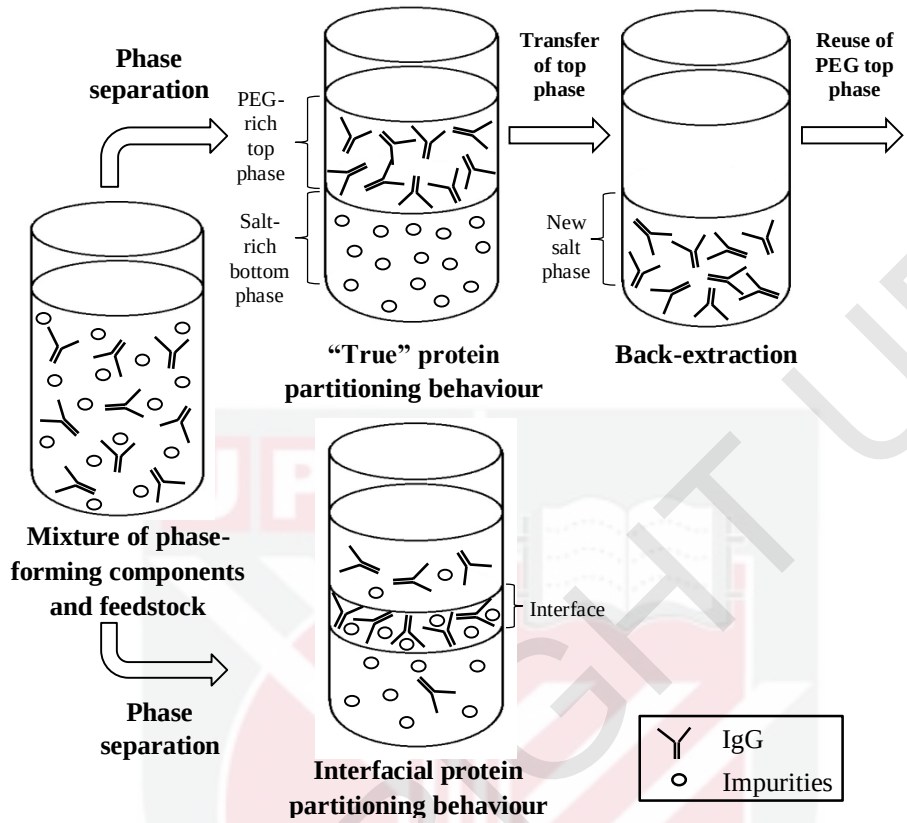

Figure 1.1: Protein partitioning in the ATPS

1.4 Summary of the research

In this study, the ATPS was adopted for the recovery of IgG. The initial investigation focused on assessing the factors which affect the partitioning behaviour of a model protein and the pure IgG in the polyethylene glycol (PEG)-phosphate ATPS in order to develop relationships which could provide a better understanding of the interfacial protein partitioning and "true" protein partitioning behaviour. The best ATPS conditions for the selective recovery of monoclonal human IgG1 from the Chinese Hamster Ovary (CHO) cell culture supernatant was then determined. Also, the proposed relationships were applied to provide a framework for elucidating, correlating and ultimately predicting the IgG partitioning in the ATPS. The scope of this work is depicted in Figure 1.2.

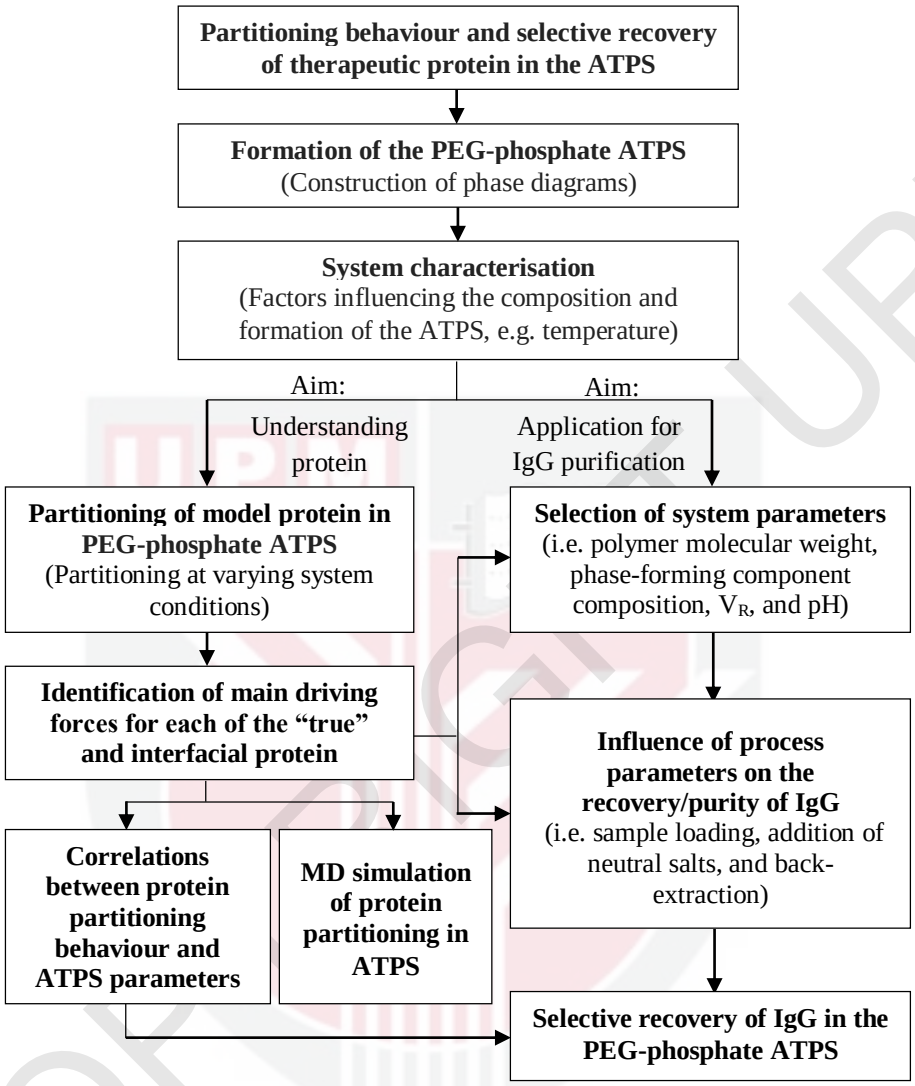

Figure 1.2: Schematic overview on the scope of the research.

The subject matter of this thesis is organised as follows:

Chapter 2 is a literature review of the current market of the therapeutic antibody products, its application and the advancement of the upstream process. The limitations of the conventional downstream processes, potential alternatives and the practical strategies of the ATPS process development for product recovery are critically discussed in terms of efficiency and economic viability.

Chapter 3 details the factors which contribute to the interfacial partitioning of a model protein (BSA) in the ATPS. An alternative method to evaluate the interfacial partition coefficient of protein in the ATPS was formulated.

Chapter 4 describes the protein partitioning behaviour of BSA in the PEG-phosphate ATPS. The BSA serves as both the model protein for the evaluation of purification performance and as model impurity for the impurities present in an IgG containing feedstock. Relationships that correlate the protein partitioning with the ATPS parameters are developed and MD simulation of the partitioning of protein in an optimised ATPS is performed. The findings provide a new valuable insight into the underlying protein partitioning mechanism.

With reference to the partitioning behaviour of the BSA studied in Chapter 4, Chapter 5 details the influence of the ATPS parameters on the partitioning behaviour of pure IgG. The relationship proposed in Chapter 4 is further extended to correlate the partitioning behaviour of IgG with the ATPS parameters. The findings can serve as a guide for the recovery of various monoclonal antibodies (mAbs) from crude feedstock.

Chapter 6 demonstrates the selective recovery of IgG from simulated protein mixture and crude feedstock, as well as the applicability of the proposed relationships in correlating the ATPS extraction of IgG from complex protein solution.

Chapter 7 outlines the main conclusions of the studies in this work. Overall, the ATPS can effectively recover high value therapeutic protein, IgG with high product yield and purity from complex feedstock.

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