Downstream Bioprocess Development for a Scalable Production of Pharmaceutical-grade Plasmid DNA

by

Luyang Zhong

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Applied Science
in
Chemical Engineering

Waterloo, Ontario, Canada, 2011

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

The potential application of a hydrogel-based strong anion-exchange (Q) membrane to purify plasmid DNAs was evaluated. The maximum binding capacity of plasmid DNA was estimated to be 12.4 mg/ml of membrane volume with a plasmid DNA recovery of ~ 90%, which is superior to other commercially available anion-exchange resins and membranes. The membrane was able to retain its structural integrity and performance after multiple cycles of usage (> 30 cycles). The inherent properties of plasmid DNA, membrane adsorbent, and the ionic environment on membrane performance were identified as the factors affecting membrane performance and their effects were systematically investigated. Plasmid DNAs with smaller tertiary structure have shorter dynamic radius and/or lowersurface charge densities, which tended to have a better adsorption and recovery than those with larger tertiary structure. Environmental Scanning Electron Microscopy (ESEM) revealed that the hydrogel structure is more porous on one side of membrane than the other, and higher plasmid DNA adsorption and recovery capacities were observed if the more porous side of the membrane was installed upward of flow in the chromatographic unit. ESEM also revealed improved pore distribution and increased membrane porosity if membrane was pre-equilibrated in the buffer solution for 16 hours. The development of better flow through channel in the hydrogel membrane upon extensive soaking further improved plasmid DNA adsorption and recovery capacities. The ionic environment affects the tertiary size of plasmid DNA; and the optimal operating pH of membrane chromatography was different for the plasmid DNAs investigated in this study. The relative contribution of these factors to improve membrane chromatography of plasmid DNAs was analyzed using statistical

modeling. It was found that the adsorption of plasmid DNA was mainly affected by the available adsorptive area associated with membrane porosity, whereas the recovery of plasmid DNAs was mainly affected by the environmental pH.

A novel, RNase-free, and potentially scalable bioprocess was synthesized using the hydrogel membrane as the technology platform for the manufacturing of pharmaceutical-grade plasmid DNA. High bioprocess recovery and product quality were primarily associated with the optimal integration of impurity removal by calcium chloride precipitation and anion-exchange membrane chromatography and the implementation of isopropanol precipitation as a coupling step between the two impurity-removing steps. Complete removal of total cellular RNA impurity was demonstrated without the use of animal-derived RNase. High-molecular-weight (HMW) RNA and genomic DNA (gDNA) were removed by selective precipitation using calcium chloride at an optimal concentration. Complete removal of the remaining low-molecular-weight (LMW) RNA was achieved by membrane chromatography using the high-capacity and high-productive hydrogel membrane. The simultaneous achievement of desalting, concentrating and buffer exchange by the coupling step of isopropanol precipitation and the high efficiency and resolution of DNA-RNA separation by anion-exchange membrane chromatography significantly reduced the operating complexity of the overall bioprocess, increased the overall recovery of plasmid DNA, and enhanced product quality by removing trace amounts of impurities of major concern for biomedical applications, such as gDNA, proteins, and endotoxin.

Key words: hydrogel membrane, plasmid DNA purification, ESEM, membrane pre-treatment, RNase-free bioprocess, selective precipitation

Acknowledgements

I am indebted to my supervisors, Dr. C. P. Chou, Dr. Jeno M. Scharer and Dr. Murray Moo-Young, for the encouragement, guidance and support they provided throughout my graduate study at University of Waterloo. I am greatly inspired by their scientific vision and keen attitude toward research work, which opened a venue to the richness of learning for me.

It is my utmost honor to have Dr. Boxin Zhao and Dr. Frank Gu as my thesis committee members. I would like to express my deepest appreciation to them for spending their precious time to provide insightful and vital comments on my thesis.

I would like to thank my lab mates, Valerie Orr, Michael Pyne, Kajan Srirangan, Karan Sukhija, Adam Westbrook, Shreyas Yedahalli, Steve George, Dr. Lin Zhang, Dr. Fred Li and Dr. Daryoush Abedi for their countless efforts to aid me in my research and making the work environment friendly and engaging.

I owe my thanks to my friends, Yifan Shi, Daoyuan Li, Dandelion Wang, Vivian Zhu, Claire Li, Candice Zhou and Ray Zhang for always being there to laugh and cry with me through these years.

I appreciated Natural Sciences and Engineering Research Council (NSERC), Canada Research Chair (CRC) program and Natrix Separations Inc. for their financial support for this project.

Finally, I would like to give special thanks to my parents, Minghui and Shuwei, for their unconditional love and inspirations throughout my life and my husband, Junkai, for his motivation and support of my academic studies.

Dedication

To my family

Table of Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Research Background

With the advancement in recombinant DNA technology, foreign genes of industrial applications can be inserted into plasmid DNA for mass production in various host cells. This has resulted in the increasing interest in using plasmid DNA as the vector for the delivery of therapeutic genes in gene therapy and DNA vaccination. This approach offers great technical advantages as compared to other conventional vectors, such as low production cost, high product stability and safety [1]. Recently, several plasmid DNA-based vaccines have progressed to clinical evaluations [2], and the ultimate scale of production is huge if they are proved to be clinically effective. Although current purification techniques exist for plasmid DNA production with end products used mostly in molecular biology work, these processes are difficult to scale up and the reagents used in the processes pose safety concerns for human applications. Therefore, an efficient downstream bioprocess for the large scale manufacturing of plasmid DNA is needed to meet the regulatory requirements for product purity, potency and safety. Ultimately, such bioprocess should only use chemicals that are generally regarded as safe (GRAS) and be free of animal-derived products (e.g. RNase, lysozyme) to produce plasmid DNAs [3].

Chromatography is considered essential for the purification of high-purity gene vectors as it offers high product resolution and uses chemicals that are GRAS [4]. It can be operated under various modes, where different physical and chemical properties of plasmid

DNA can be explored to achieve separation. Anion-exchange mode is the most commonly used amongst them by exploiting the charge density of biomolecules as the basis of separation. However, it cannot resolve plasmid DNA from other nucleic acids that are of similar charge density. This is especially the case in an RNase-free bioprocess, where HW RNA is found to co-elute with plasmid DNA if no other means of RNA reduction is done in the upstream. Beside poor resolution, many existing resin-based anion-exchangers suffer from poor binding capacity for plasmid DNA, as the small pore sizes (< 30 nm in diameter) that were once designed and optimized for protein purification, exclude plasmid DNA (with typical hydrodynamic radius around 150-250 nm) from entering the interior adsorptive surface [4]. To allow effective use of chromatographic technique for plasmid DNA purification, high-capacity membranes with convective "superpores" are developed [5, 6]. However, plasmid DNA loss due to irreversible interaction with the hydrophobic membrane supports has reduced the efficiency of membrane chromatography. Therefore, new types of high-capacity and high-productive membranes are in demand. Although several RNase-free bioprocesses have been synthesized based on anion-exchange chromatography, the bioprocesses are rather complex and time-consuming, as additional operation units are required to accommodate the resulting burden of RNA impurity [7, 8]. In this research, a high-capacity hydrogel-based anion-exchange membrane is systematically explored and integrated in a novel RNase-free downstream bioprocess to address the issues of low capacity, poor resolution from host impurities (e.g. gDNA and HMW RNA) and high complexity of the overall bioprocess.

1.2 Research Objectives

The overall objectives of this thesis are as follows:

1. Characterize hydrogel-based strong (Q) anion-exchange membrane, in terms of membrane structure, structural integrity and batch adsorption and desorption capacities for plasmid DNA.
2. Identify the factors affecting membrane performance.
3. Systematically investigate the impact of the intrinsic and extrinsic factors on plasmid DNA adsorption and recovery from the membrane.
4. Develop an efficient and scalable downstream bioprocess for the manufacturing of pharmaceutical-grade plasmid DNA using anion-exchange membrane chromatography as the final purification step.

1.3 Outline of the Thesis

This thesis consists of seven chapters. The scope of each chapter is as follows:

Chapter 1 gives an introduction to this thesis, including research background, research objectives as well as the scope of this thesis.

Chapter 2 presents a comprehensive literature review on the pharmaceutical production of plasmid DNA.

Chapter 3 investigates membrane structure, structural integrity after multiple cycles of usages and batch adsorption and desorption capacities for plasmid DNA.

Chapter 4 presents systematic investigation of the impact of the factors on membrane performance.

Chapter 5 presents bioprocess synthesis for the production of pharmaceutical-grade plasmid DNA.

Chapter 6 examines an alternative nucleic acid precipitant, PEG for its potential application in downstream bioprocess of plasmid DNA.

Chapter 7 summarizes the major achievements in this thesis and presents conclusions and recommendations for future work.

Chapter 2

Literature Review

Since the pioneering study of expressing transgenes that were inserted on a plasmid DNA as the therapeutic treatment [9], there has been a rapid advancement of plasmid DNA-based gene therapy and DNA vaccine development [10-12]. Plasmid DNA therapy is proven to be relatively inexpensive and safe to administer. In addition, they have highly stable secondary structures that are based on base-pairing hydrogen bonds, making them more stable at ambient temperature than conventional viral vaccines and protein therapeutics and this is considered as an important advantage during long-term storage [12, 13]. However, due to its inherently low infection efficacy, a relatively large dose of plasmid DNA is required [14, 15] typically in the order of a few milligrams for a full treatment of a patient [13, 16]. Currently, there are several plasmid DNA vaccines marketed or under clinical evaluation for the treatment of cancer, infectious and autoimmune diseases. The demand of pharmaceutical-grade plasmid DNAs will be soaring if they prove to be clinically effective [12]. Therefore, an efficient bioprocess that meets the required product purity, potency, and safety standards for the large-scale production of plasmid DNA is needed.

2.1 Properties of Plasmid DNA

The development of a bioprocess should always start with the thorough understanding of the properties of the target molecule and associated impurities, which will be used as the rationales for the proper selection of separation techniques. *Escherei coli (E. coli)* is a commonly used host for the production of plasmid DNA, and Table 1 summarizes the

characteristics of the components in such a host [17]. The major impurities of concern for human therapeutics are gDNA, RNA, protein and endotoxin, and Table 2 outlines FDA guidance for the acceptable levels of each impurity [3].

Table 1 Components of an *E. coli* cell and their characteristics.

Species	Amount (% w/w)	Avg. MW (kDa)
water	70	18
gDNA	0.5	2.8×10^6
tRNA	4.8	28
rRNA	0. 9	500-1000
mRNA	0. 3	660-990
pDNA	< 1	3300[a]
proteins	15	8-200
endotoxin	5	10
others	3	<1

a= for a plasmid DNA size of 6 kbp

Plasmid DNAs are double-stranded DNA molecules that carry genetic information and exist covalently closed in the bacteria cells. Due to the fact that the phosphate groups in the DNA backbone are negatively charged at pH greater than 4 [18], plasmid DNA are essentially very large polyanions, making them less physically distinct from host gDNA and HWM RNA. On the other hand, LMW RNA and protein are much smaller molecules that

can be readily separated from the plasmid DNA base on charge and/or size difference. The double helix structure of plasmid DNA coils in space and forms a higher order structure, namely the supercoiled isoform. The degree of supercoiling determines the size and charge density of plasmid DNA and is dependent on the immediate ionic environment. Other forms, such as open circular, linear, denatured or oligomeric ones can also be introduced during bioprocessing as the tertiary structure of plasmid DNA is very dynamic and sensitive to the potential shear stress encountered during the recovery processes. Studies have shown that better therapeutic outcome is associated with high percentage of supercoiled plasmid DNA in the dose [1], implying the demand of a bioprocess that favours the production of supercoiled plasmid DNA.

2.2 Bioprocess Synthesis

There are few heuristics in designing the purification scheme, such as remove the most plentiful impurities and easiest-to-remove impurities first, select separation techniques that make use of the greatest differences in the properties of the target molecule and associated impurities and make the most difficult and expensive separations last. Figure 1 illustrates a generalized block diagram of downstream bioprocess for plasmid DNA manufacturing [19], which comprises primary recovery, intermediate recovery and final purification stages. Each of the stages is reviewed separately below.

Table 2 Acceptable criteria for pharmaceutical-grade plasmid DNA and recommended assays.

Impurity	Specification	Recommended assay
Plasmid purity	>90% sc	Agarose gel electrophoresis
Plasmid identity	-	Restriction digestion Agarose gel electrophoresis
gDNA	< 0.01 μg /μg plasmid	Quantitative PCR
RNA	Undetectable (< 1%)	Agarose gel electrophoresis
Protein	< 0.01 μg/dose	BCA protein assay
Endotoxin	< 0.1 EU/μg plasmid	LAL assay

2.2.1 Primary Recovery

Plasmid DNA is an intracellular component of *E.coli*, therefore the primary recovery stage consists of cell harvesting and cell disruption to release the intracellular content. This stage involves significant reduction in bioprocess volume as well as elimination of a huge amount of impurities such as extracellular liquid, proteins and gDNA, which is in agreement with the heuristics that the most plentiful impurities are removed first. The large-scale cell harvesting is usually accomplished by centrifugation and membrane filtration with the preference in the

latter, as cell loss during centrifugation is typically 1 to 5% and membrane filtration on the other hand is demonstrated to recover essentially all cells.

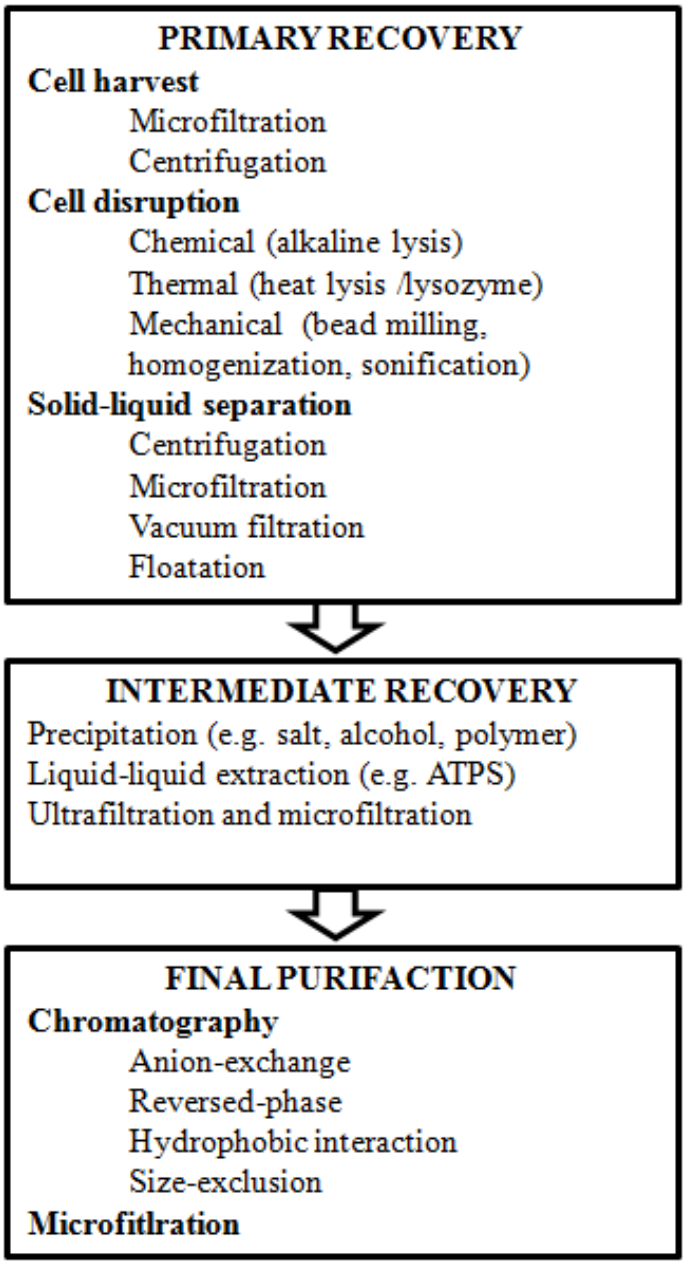


Figure 1 Generalized block diagram of downstream processing.

The most critical and troublesome step in downstream bioprocessing is cell disruption, where the cells are subjected to external force to break open and release the intercellular contents. Since plasmid DNAs and gDNA are shear stress sensitive, the method that recovers the highest amount of intact supercoiled isoform with minimum gDNA fragmentation should be used to guarantee high overall process yield. Mechanical disruption such as high pressure homogenization, bead milling and sonication usually results in significant damage to plasmid DNAs, and the usage is not recommended for supercoiled plasmid DNA recovery [18]. Enzymatic lysis using lysozyme is commonly seen in commercial kits for laboratory work, however, the use of animal-derived enzyme is restricted for the production of human therapeutics due to the potential health issues. Chemical disruption, such as alkaline lysis is a rather gentle process on the cells, is extensively practiced.

Alkaline lysis was first introduced by Bimboim and Doly [20]. It relies on the use of sodium hydroxide (NaOH) and sodium dodecyl sulphate (SDS) to disrupt cells at pH around 12.2 – 12.4, which results in reversible denaturation of plasmid DNA and irreversible denaturation of cell wall material, gDNA and protein. The rationale behind this approach is that the alkali environment disrupts the hydrogen bonds that stabilize DNA molecules, as a consequence, complementary strands separate from each other. This process is reversible for plasmid DNA only if the pH is maintained below 12.5, as the anchor base pairs are preserved which will serve as the nuclei for the renaturation of plasmid DNA in the subsequent neutralization step. At pH above 12.5, the anchor base pairs may be lost, thus making plasmid DNA denaturation irreversible. Therefore, pH should be controlled and local pH

extremes (pH > 12.5) should be avoided by sufficient mixing [21]. Also, gentle mixing should be applied to avoid excessive shear stress on nucleic acids, which would otherwise result in loss of intact supercoiled plasmid DNA and fragmentation of gDNA and HMW RNA. Fragmented gDNA and HMW RNA have smaller molecular weight, which are rather difficult to precipitate and remove in the subsequent steps, thus co-purified with plasmid DNA. Therefore, the efficiency of cell lysis affects subsequent purification processes. On large-scale production of plasmid DNA, batch mixing and continuous flow through devices have been used to mix resuspended cell paste with lysis buffer. It is claimed that the continuous flow through devices introduces lower shear than batch mixing, therefore complete, but gentle mixing of large lysis volume can be achieved, where the reaction time can be controlled by the residence time in the tubing and pipes [22]. Following alkaline lysis, the bioprocess stream is neutralized with potassium acetate for the renaturation of plasmid DNAs and the precipitation of denatured gDNA, protein and cell debris together with SDS. The precipitated biomass is then removed using solid-liquid separation techniques. Centrifuge and microfiltration are two most commonly used techniques, and some other options including depth filtration, extraction and expanded-bed adsorption (EBA) chromatography are also used [18]. At the end of primary recovery, the fermentation broth is eliminated while some purification of plasmid DNA achieved.

2.2.2 Intermediate Recovery

After primary recovery, plasmid DNA is presenting as a diluted form in the bioprocess stream, therefore volume reduction by concentration becomes the next step in the bioprocess to concentrate and further purify the plasmid DNA. Common concentration techniques

include selective precipitation, extraction, ultrafiltration and microfiltration. Depending on the technique, outcomes such as impurity removal, desalting, plasmid DNA concentration, volume reduction and buffer exchange can be achieved.

Precipitation exploits the changing physical properties of the target molecule upon interaction with the precipitants to separate it from the rest. It is usually performed by the addition of salts, solvents and polymers, each with their own advantages and disadvantages. Salts such as ammonium acetate, ammonium sulphate, sodium sulfate, calcium chloride and lithium chloride are used in many purification schemes to selectively precipitate the impurities (gDNA, RNA, protein and endotoxin) by reducing their solubility in solution while leaving plasmid DNA soluble in the solution. These impurities either have smaller molecular size or flexible structure that are more vulnerable to the access of cations into their structures as compared to the rigid double-stranded supercoiled plasmid DNA, thus making selective precipitation possible [23]. Calcium chloride was experimentally determined to be the most potent precipitant for impurity clearance, with impurity reduction of 94%, 96%, 98% and 91% for RNA, protein, gDNA and endotoxin, respectively [24]. The use of high salt precipitation is not without drawbacks, as the presence of salts in the bioprocess stream would interfere with many downstream operation units, thus an additional step of desalting is often required. Ethanol and isopropanol are well adapted solvents that are used in the selective precipitation of plasmid DNA. The addition of these alcohols would reduce the dielectric constant of the salt-rich alkaline lysates, which would result in stronger electrostatic interaction between cations in the lysates (e.g. K+, Na+) and the negatively charged phosphate groups of nucleic acids [25]. As a result, the repulsion between phosphate

groups is effectively shielded, resulting in the precipitation of plasmid DNA. Although high process yield and product purity were demonstrated with the use of these alcohols [21], the limitation is that it necessities the need of explosion proof tanks [18].

Liquid-liquid extraction using aqueous two-phase systems (ATPS) is also commonly performed to isolate plasmid DNA by partitioning biomolecules into different phases [22]. Partitioning can be done either in a polymer-polymer or polymer-salt system. There is an increasing interest in using polymer-salt system to partition nucleic acids, as such system has lower phase viscosities than polymer-polymer system, which makes handling on a large scale easier [26]. The most commonly used is the non-toxic PEG-salt system, where PEG accumulates in the top phase and salt accumulates in the bottom phase. PEG is a polymer with structure of $HO[CH_2CH_2O]_nH$ [27], which sterically excludes itself and other biomolecules, this excluded volume effect will have a considerable impact on the solubility of nucleic acids [26]. Also, the hydrophilic nature of nucleic acids will be favoured in the salt phase [28]. The factors affecting the partition and purification of biomolecules in this system include molecular weight and concentration of polymer, type and concentration of salts, ionic strength, pH values, tie line length and top to bottom phase volume ratio [29]. Therefore, the designing of the system involves systematic variation of the above mentioned factors for the desired partitioning behaviour. The main drawback of using PEG-salt system to partition nucleic acids is that they are less selective than polymer-polymer systems [26].

Ultrafiltration and microfiltration exploit the difference in molecular size between plasmid DNA and cellular impurities to achieve concentration and separation. The commonly used membranes for intermediate recovery of plasmid DNA have molecular weight cut off

(MWCO) of 30, 50, 100 and 300 kDa. Although membrane with MWCO of 100-300 kDa possess pore sizes that are an order of magnitude smaller than most plasmid DNA, studies have shown that the tertiary structure of plasmid DNA can change easily depending on the immediate ionic environment and stretch upon hydrodynamic stress and result in passing through the membrane [30]. Therefore, the operating condition should be carefully selected to ensure minimum loss of plasmid DNA. The selection of smaller MWCO should be carefully made in consideration with upstream pretreatments of the clarified lysate, as high levels of impurities would result in membrane fouling [31]. In general, the process yield can be optimized (80-100%) by carefully selecting the membrane pore size and operating conditions for the size of plasmid DNA to be recovered [31, 32].

Impurity clearance is a major technical advantage of integrating intermediate recovery step in the bioprocess. As the viscosity of cell lysate can be greatly reduced, which would otherwise cause high-pressure drops, and consequently limit the linear flow rate and process throughput for many downstream chromatographic steps [33].

2.2.3 Final Purification

Chromatography is commonly used as the final purification step in the large scale manufacturing of plasmid DNA. It can be operated in various modes, such as anion-exchange, reverse-phase, hydrophobic interaction and size-exclusion, which can be used singly or combined in many purification schemes [18, 33] to further reduce host impurities. The last three modes are primarily used following other chromatographic procedure or as a polishing step, whereas anion-exchange is best suited to capture plasmid DNA from the bioprocess stream [33]. Due to the physical and chemical similarity between plasmid DNA

and host impurities, poor selectivity and co-elution are common problems seen with chromatography. If upstream operation units can greatly reduce the impurity level in the bioprocess stream, chromatography can then take the advantages of different physical properties between plasmid DNA and associated impurities, such as charge density, molecular size and hydrophobicity, to achieve final purification. And the selection of chromatographic techniques should be made in consideration to the nature and distribution of the residual impurities.

Anion-exchange chromatography takes advantage of the interaction between negatively charged nucleic acids and positively charged chromatographic media to capture and purify plasmid DNA [33]. The anion exchanger on the chromatographic media can be classified as either strong (Q) or weak (D). Strong anion-exchanger, such as quandary ammonium, contain strong base and are able to remain positively charged over a wide range of pH values. On the other hand, weak anion exchangers such as diethylaminoetheyl (DEAE) contain weak base and tend to deprotonated at high pH values, thus having a narrow operation range of pH [18]. In both cases, bound nucleic acids are recovered with a salt gradient and an elution profile of increasing charge density is generated [33]. It is reported in several studies that longer and shallow salt gradients improve the resolution of plasmid DNA [34, 35]. This mode of chromatography can readily remove LMW RNA, oligonucleotides and some proteins, all of which bear much smaller charge density than plasmid DNA. A proposed strategy to achieve separation of low charge density impurities is to load the lysate at a sufficiently high salt concentration to avoid impurity adsorption onto the anion-exchange media. This strategy comes with an additional advantage of improved capacity for plasmid

DNA adsorption [7, 33]. However, large polyanions, such as gDNA fragments, HMW RNA and endotoxin may co-purity with plasmid DNA due to their similar charge density and adsorptive behaviours. If significant reduction of impurities levels has been done in the upstream bioprocessing steps, the plasmid DNA eluted from anion-exchange chromatography may be of high enough quality for many uses [33]. One major technical disadvantage of many anion-exchange media is the poor binding capacity for plasmid DNAs. This is associated with the inadequacy of the pores (< 0.2 μm) of most media for the mass transfer of plasmid DNA (> 0.2 μm). However, with the introduction of superporosity (> 0.2 μm) in adsorptive membranes [6, 36] and monolith supports [5], convective mass transport and improved binding capacity are observed. Table 3 compares some commercially available anion-exchange media and their binding capacities for plasmid DNA.

Table 3 Binding Capacity of plasmid DNA on commercial anion-exchange media.

Media	**Bead Size (μm)**	**Pore size (μm)**	**Capacity (mg/ml)**	**Plasmid DNA (kbp)**	**Reference**
Beads					
Q-Sepharose Big Beads	200	-	0.7	4.8	[37]
Q-Sepharose Fast Flow	90	0.19	1.3	4.8	[37]
Q-Sepharose High	34	-	2.5	4.8	[37]

Performance					
Q Hyper D	20	0.3	5.4	3.5	[7]
Fractogel EMD DEAE	40 - 90	0.8	2.45	5.9	[7]
Poros 50 HQ	50	<0.8	2.12	5.9	[7]
Monoliths					
DEAE-CIM	-	0.01 - 4	10	-	[5]
Membranes					
Mustang Q	-	0.8	10	6.1	[6]
Natrix hydrogel Q	-	0.45	13	6.4	[36]

Reverse-phase liquid chromatography (RPLC) employs non-polar chromatographic media to reversibly interact hydrophobic, non-polar regions of the biomolecules [33]. Bound molecules are eluted with decreasing polarity gradients and an elution profile of decreasing polarity or increasing hydrophobicity is generated [35]. The selectivity of this chromatography can be altered by adding amphiphilic organic ions to chromatographic buffer, which forms hydrophobic non-polar ion pairs with polar molecules, such as nucleic acids, thus making bounding of polar molecules possible. This form of RPLC is called reversed-phase ion-pair chromatography (RPIPC). The major drawback of this technique is the use of organic solvents to elute plasmid DNA, which can be toxic, mutagenic and even

explosive [38]. As a result, reverse-phase chromatography is rarely used in plasmid DNA purification on a large scale for safety concerns.

Hydrophobic interaction chromatography also exploits surface hydrophobicity of biomolecules to achieve separation [33]. Hydrophobicity of nucleic acids varies with size and structure, where higher hydrophobicity is expected for nucleic acids with higher content of exposed aromatic bases. Intact supercoiled plasmid DNAs are double-stranded nucleic acids that are covalently closed, meaning the hydrophobic bases are shielded within the helix; whereas RNAs are single-stranded nucleic acids with higher exposure of their hydrophobic bases, thus a higher interaction with the hydrophobic media. Endotoxins interact even more strongly with the hydrophobic media via lipid A moiety [39]. The binding is promoted by salt, thus HIC can be readily performed for salt-enriched cell lysate [22]. One drawback of HIC is its low binding capacity, therefore it is usually used as a polishing step operated in a condition that favours the retention of impurities and supercoiled plasmid DNA is collected in the flow through.

Size-exclusion chromatography (SEC) separates biomolecules base on molecular size [33]. The commonly used media for SEC are Sephacryl S-1000 and Superose 6B (Amersham Biosciences), with exclusion limit of 20,000 bp and 450 bp, respectively. Sephacryl S-1000 can efficiently fractionate plasmid DNA isoforms for plasmid DNA sizes above 10 kbp, however, incomplete fractionation occurs for plasmid DNA sizes smaller than 4.4 kbp [40]. Operation of Sephacryl S-1000 requires low pressure (< 20 bar) [40], thus it is not suitable for cell lysate with high contents of impurity. Otherwise, the process is very lengthy with low process throughput. Superose 6B resins, on the other hand, are more resistant to pressure,

where better process yields can be obtained with higher flow rates [40]. Nevertheless, it is less tolerant to high impurity levels in the bioprocess stream, and significant reduction in impurities in the upstream bioprocess is essential to avoid column overloading. Also, resolution of supercoiled plasmid DNA from other isoforms and gDNAs is rather poor as compared to Sephacryl S-1000. However, both media do an excellent job as the final polishing step following other chromatographic procedures, as the impurities are greatly reduced in the upstream and the plasmid DNA is more concentrated [33].

2.3 Current Bioprocesses for the Manufacturing of Therapeutic Plasmid DNA

Ideally, an efficient downstream bioprocess should involve the minimum number of operation units with the highest possible yield of plasmid DNA and clearance of other host impurities per unit of mass of host. In this section, different purification schemes representative of current downstream bioprocess (Table 4) for the manufacturing of pharmaceutical-grade plasmid DNA are discussed.

Bioprocess I represents commonly used purification schemes for the production of plasmid DNA, which relies on the use of RNase to completely remove RNA impurity in the bioprocess stream [41]. RNA is the most abundant host impurity of all, and early elimination in the bioprocess stream is desirable to greatly reduce the impurity burden to subsequent downstream bioprocess and to simplify the overall bioprocess. However, RNase is an animal derived enzyme that is a potential source of mammalian pathogens, and the use of it in human therapeutics production is restricted since the outbreak of new variant Creuzfeld-Jacob disease in the UK [42].

Bioprocess II demonstrates an RNase-free bioprocess by integrating high salt precipitation, microfiltration and anion-exchange chromatography. Complete resolution of plasmid DNA from RNA impurity was achieved by high salt precipitation of HMW RNA and TFF and anion-exchange chromatography clearance of LMW RNA. However, the use of resin-based chromatography has its disadvantage in poor binding capacity and diffusive mass transport, which limit process yield and throughput.

Bioprocess III is an RNase-free bioprocess, that is designed to purify pNGVL4a-sig/E7(detox)/HSP70 plasmid DNA vaccine for the treatment of cervical and head & neck cancers [43]. It is one of the *Developmental Therapeutics Programs* taking place at the National Cancer Institute (NCI). The bioprocess employs PEG_{8000} at 8% to selectively precipitate plasmid DNA from the alkaline lysate, followed by volume reduction using TFF. Plasmid DNA is then captured by anion-exchange chromatography using mustang Q membrane and finally polished with SEC using Sephacryl S-1000 for the reduction of gDNA and non-supercoiled isoforms of plasmid DNA. One technical advantage of using membrane chromatography is that the large pore size of the membrane can accommodate the size of plasmid DNA and allows convective mass transport, thus overcoming the limitation of diffusive transport as seen with the use of traditional resin-based chromatography [33]. Despite high product purity at the end of the process (> 95% supercoiled plasmid DNA), significant amount of plasmid DNA (> 60%) was lost during anion-exchange chromatography. Thorough investigation of mustang Q membrane was done in another study, which also reported low recovery of plasmid DNA [6]. It is suggested that the hydrophobic nature of the membrane support caused irreversible adsorption of plasmid DNA.

Thus, it is proposed that the process yield can be greatly improved by employing membrane material of hydrophilic nature.

Bioprocess IV was synthesized to purify pIDKE2 plasmid DNA, which encodes the hepatitis C virus (HCV) core, E1, and E2 structural proteins [8]. It completely avoided the use of RNase as well as precipitation, and successfully achieved criteria for purity, robustness and reproducibility required for the manufacturing of pharmaceutical-grade plasmid DNA by integrating microfiltration in TFF mode with several chromatographic steps in anion-exchange and size-exclusion modes. Despite demonstrated purity (95% supercoiled plasmid DNA) and potency of the final products, the complexity of the bioprocess is impractical and labour-intensive for on large scale production.

Table 4 Existing bioprocess for the manufacturing of pharmaceutical-grade plasmid DNA.

Bioprocess	**Description**	**References**
I	RNase digestion Vacuum filtration Depth filtration Anion-exchange resin-based chromatography IPA pp Sterile filtration	[41]

II	$CaCl_2$ pp	[7]
	TFF	
	Diafiltration	
	Dialysis	
	Anion-exchange chromatography (resin-based)	
	TFF	
III	PEG_{8000} pp	[43]
	TFF and detergent wash	
	Dissolution and filtration	
	Anion-exchange membrane chromatography (mustang Q)	
	IPA pp	
	Dissolution and filtration	
	Sephacryl S-1000 chromatography	
	IPA pp	
	Dissolution and filtration	
IV	TFF	[8]

	Sepharose CL 4B chromatography
	G25 coarse chromatography
	Anion-exchange membrane chromatography (Sartobind D)
	Sephacryl S-1000 chromatogrpahy
	TFF
	Sterile filtration

Abbreviations: IPA, isopropanol; PP, precipitation; TFF, tangential flow filtration.

Chapter 3

Characterization of Membrane Structure and Performance

The work in this chapter was published in *Journal of Chromatography B* 879 (2011) 564-572

Authors: **Luyang Zhong**[a], Jeno Scharer[a], Murray Moo-Young[a], Drew Fenner[b], Lisa Crossley[b], C. Howie Honeyman[b], Shing-Yi Suen[c] and C. Perry Chou[a,*1]

Declaration: I initiated and conducted all experiments presented in this chapter under the supervision of Dr. C. Perry Chou, Dr. Jeno Scharer and Dr. Murray Moo-Young. I am very grateful to Dr. Yuquan Ding (Department of Mechanical Engineering, University of Waterloo) for his technical assistance in ESEM.

3.1 Introduction

Amongst the numerous available methods for plasmid DNA purification, chromatographic techniques are widely adopted because they provide high resolution, use only chemicals that are GRAS, and are easily scalable [12, 13, 15]. The most commonly used method is anion-

[a] Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada, N2L 3G1

[b] Natrix Separations, Inc., 5295 John Lucas Drive, Unit 6, Burlington, Ontario, Canada, L7L 6A8

[c] Department of Chemical Engineering, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 402, Taiwan

* Corresponding author: C. Perry Chou, Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1. Telephone: 1-519-888-4567 ext. 33310, email: cpchou@uwaterloo.ca

exchange chromatography which is based on the reversible interaction between negatively charged plasmid DNA and the positively charged chromatographic media [13, 44, 45]. The application of conventional resin-based anion-exchange chromatography, however, results in low adsorption because the small pores in the resin restrict access to near-micron-sized plasmid DNAs (> 0.2 μm). Resins were originally optimized for the purification of nano-sized biomolecules such as proteins (2-10 nm) [4, 15, 46, 47]. It was shown by confocal microscopy that plasmid DNA adsorption only occurred on the outer surface of the resin and this greatly reduced the binding capacity of the resin beads [48]. Of the 10 to 100 grams of plasmid DNA loaded per litre of resin, only 0.2 to 2 grams could bind [13]. Thus, a substantial amount of chromatographic resin is needed to purify milligrams of plasmid DNA. In addition, it is time-consuming and labour-intensive to pack, clean, and regenerate the chromatographic column [13, 49]. Studies have also shown low recovery or irreversible binding of plasmid DNA to some chromatographic resins [4], either due to the small pore size or strong interaction with the resin material.

Anion-exchange membrane chromatography offers a promising alternative for large-scale purification of plasmid DNA. It does not involve packing and cleaning procedures associated with conventional resin-based chromatography. Most importantly, it allows a rapid convective transport of biomolecules through the large pores (~2 μm), in contrast to the diffusive transport through the small pores of the resins (~0.2 μm) [50]. Therefore, a higher rate of mass transfer is possible even at high flow rates [4, 50-52]. The large pore size of the membrane also allows better accessibility and greater surface area utilization, thus resulting in a higher binding capacity. Anion-exchange membranes were shown to surpass at least ten-

fold the counterpart resins in binding capacity on a per volume basis [6]. The scale-up of membrane chromatography is straightforward because the binding capacity is directly proportional to the available membrane surface area, while the scale-up of resin-based chromatography is more challenging.

Several studies reported the use of membranes to produce therapeutic plasmid DNAs with a high purity but at relatively low yield [8, 51, 53]. For economic reasons, both recovery and yield are important aspects that should be maximized to fully realize the advantage of membrane chromatography. Employing membrane chromatography, a higher yield of plasmid DNA can be achieved with fewer number of processing steps. It is generally unacceptable to have a recovery lower than 70% in any single step of a large-scale production process [44]. Some studies of plasmid DNA purification using anion-exchange membrane addressed these problems. Teeters et al. [6] used different salts and compaction agents to reduce the charge density and the size of plasmid DNA in an attempt to improve recovery, however, optimal recovery was in the range of 63-76% only. Tseng et al. [54] tried to improve plasmid DNA recovery by reducing the binding strength between plasmid DNA and the ion-exchange membrane with various alcohols and chaotropic salts in the washing buffer. They concluded that recovery was hampered by the irreversible binding of plasmid DNA to the membrane support. It is noteworthy that the membrane support in these two studies was polyethersulfone polymer, which makes the membrane partially hydrophobic. Plasmid DNAs are known to adsorb strongly onto hydrophobic chromatography resins [39]. Thus irreversible binding of plasmid DNA to the membrane support was implicated for these polyethersulfone-based membranes. Irreversible binding was also observed in another study

employing the polyethersulfone-based ion-exchange membrane [54]. It was therefore suggested that efforts to improve plasmid DNA recovery should focus on the design of membranes employing hydrophilic surfaces [6, 54].

The objective of this chapter is to explore the characteristics of a hydrogel-based strong anion-exchange (Q) membrane that are of important aspects for plasmid DNA purification, which include visualization of membrane porous structure at its working condition, analysis of structural integrity after multiple cycles of usage and determination of maximum adsorption and desorption capacity.

3.2 Materials and Methods

3.2.1 Membrane Material

The membrane explored in this study was a hydrogel-based strong anion-exchange (Q) membrane with disk diameter of 25 mm (Natrix Separations Inc., Burlington Ontario, Canada). The membrane is made by incorporating 3-acrylamidopropyl-trimethylammonium chloride (ATPAC) functionalized macroporous hydrogel polymer onto the polypropylene membrane support. The nominal pore size of the membrane is in the range of 0.3 to 0.8 μm. The crosslinking percentage ranged from 9% to 12%. The 25 mm cut disk has a membrane volume of 0.09 ml, and typical mean dynamic BSA binding capacity as determined by the manufacturer is 200 mg/ml of membrane volume at a 10% break through value. The two sides of the membrane differ in their surface texture, with one side being rougher than the other. We thereafter refer to it as the rough side or the smooth side, respectively.

3.2.2 Structural Characterization of Membrane Material

The structural analysis of the membrane was performed using Environmental Scanning Electron Microscopy (ESEM, ElectroScan Model E-3, FEI^{TM}, Hillsboro, USA). Structural visualization was carried out with Oxford INCA 350 Energy dispersive X-ray microanalysis system (Oxford Instruments, Oxfordshire, UK). Adsorbent pre-equilibration with chromatographic buffer is required for all adsorbent materials used in liquid chromatography, and 0.5 h pre-equilibration is recommended by the manufacturer for the hydrogel membrane used in this study. To ensure that the hydrogel within the membrane support would reach its full swelling potential, 16 h pre-equilibration was also explored and the resulting membrane structure was compared to that of conventional 0.5 h pre-equilibration. The two sides of the membrane were visualized before and after pre-treatments.

3.2.3 Structural Integrity Analysis

Structural integrity of the membrane can be implied by comparing the flux across the membrane after multiple cycles of usage. To perform the analysis, a single layer of membrane cut disk (25 mm) was installed in a laboratory-scale (10 ml) stirred cell (Millipore, Billerica, USA) that was pressurized by nitrogen to 172 kPa (25 psi) to drive the flow of process solution across the membrane. The flux was measured every half hour by filtering 10 ml buffer solution containing 50 mM Tris-HCl (pH 8) across the membrane. Reversibility of plasmid DNA adsorption (~100 μg) onto membranes that were subjected to multiple cycles of buffer filtration was compared to that of freshly pre-equilibrated membrane.

3.2.4 Batch Adsorption and Desorption

3.2.4.1 Plasmid DNA Preparation

E. coli cells harbouring pFlag-PalB (6.4 kb) [55] were grown overnight in Luria-Bertani (LB) media supplemented with 50 μg/ml ampicillin at 37°C. Cells were harvested by centrifuging the overnight culture at 4000 × *g* in Hettich Universal 320 R centrifuge (Hettich Instruments, Beverly, MA, USA) for 10 minutes. MaxiPreps kit (Bio Basic, Inc., Markham, Ontario, Canada) was used to purify plasmid DNA from the harvested cells. Plasmid DNA solutions used in the batch experiments were prepared by diluting purified pFlag-PalB stock solution with buffer solution containing 50 mM Tris-HCl (pH 8) to various concentrations up to 180 μg/ml.

3.2.4.2 Batch Adsorption and Desorption

Small membrane pieces with a cross-sectional area of 0.4 cm^2 were used in the batch experiments. The membrane sheets were added to the plasmid DNA solutions in 1.5-ml microcentrifuge tubes and the mixtures were allowed to equilibrate at ambient temperature in a shaker incubator (250 rpm) for 16 h. After incubation, the plasmid DNA concentration of the liquid phase was measured. The amount of plasmid DNA adsorbed onto the membrane was determined by an overall mass balance, and it was expressed as micrograms (mg) per cross-sectional membrane sheet area (cm^2). The batch experiments were conducted in triplicate. Using the Metropolis–Hastings algorithm [56], the adsorption data were analyzed to evaluate the parameters of the Langmuir isotherm (Equation (1)).

$$q = \frac{q_{\max} Kc}{1 + Kc} \quad (1)$$

where q is the amount of plasmid DNA adsorbed onto the membrane (μg/cm^2), c is aqueous concentration in equilibrium with the solid phase (μg/ml), q_{max} is the maximum adsorbed plasmid DNA (μg/cm^2) , and K is the Langmuir equilibrium constant (ml/μg). The unbound plasmid DNA was washed off by buffer solution containing 50 mM Tris-HCl (pH 8), and the elution of the adsorbed plasmid DNAs was carried out by placing the plasmid DNA saturated membrane pieces into 1 ml buffer solution containing 50 mM Tris-HCl and 0.8 M NaCl (pH 8). Desorption was conducted at ambient temperature in a shaker incubator at 250 rpm for 16 h after which no further desorption was observed.

3.2.5 Plasmid DNA Quantification

Plasmid DNA was quantified using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA) at 260 nm. The absorbance was converted to concentration (ng/μl) using the Beer-Lambert equation, $A = E \times b \times c$, where A is the absorbance, E is the extinction coefficient, b is the path length, and c is the concentration.

3.3 Results

3.3.1 Membrane Structure

The two sides of the membrane were visualized under ESEM before and after the pre-treatment methods, and the resulting membrane structures are shown in Figure 2. The rough side of the dry membrane had more fibrous membrane support exposed whereas the smooth side had uneven distribution of hydrogel polymer. Also, there were more noticeable pores on

the rough side of the dry membrane. The structure of 0.5 h pre-equilibrated membrane was similar to that of dry membrane, with the exception of improved distribution of hydrogel polymer on the smooth side. However, significant changes in membrane structure were observed upon 16 h of pre-equilibration. The hydrogel completely encased the fibrous membrane support and formed deeper, wider and more uniformly distributed pores on both sides. The porous structure became more complex on the rough side, where interconnected macropores have developed within supermacropores (up to 200 μm).

3.3.2 Membrane Integrity Analysis

Structural integrity of the membrane after multiple cycles of usage was assessed by measuring flux of 10 ml buffer solution across a single layer of membrane cut disk (25 mm) at a 0.5 h interval upon contact with aqueous solution and a final measurement was taken at 16 h. The results are summarized in Figure 3. The filtration flux of 10 ml buffer solution upon first contact with aqueous solution was 33.3 ml/min. Despite a slight decrease in flux after 2 cycles of usage, the flux was rather consistent at 25 ml/min thereafter. With continuous soaking of membrane in the buffer solution, the flux measured at 16 h was 33.3 ml/min. Figure 4 shows the percentage of plasmid DNA recovery from the membranes that were subjected to either 16 h pre-equilibration or multiple cycles of buffer filtration, which were 76% and 78%, respectively.

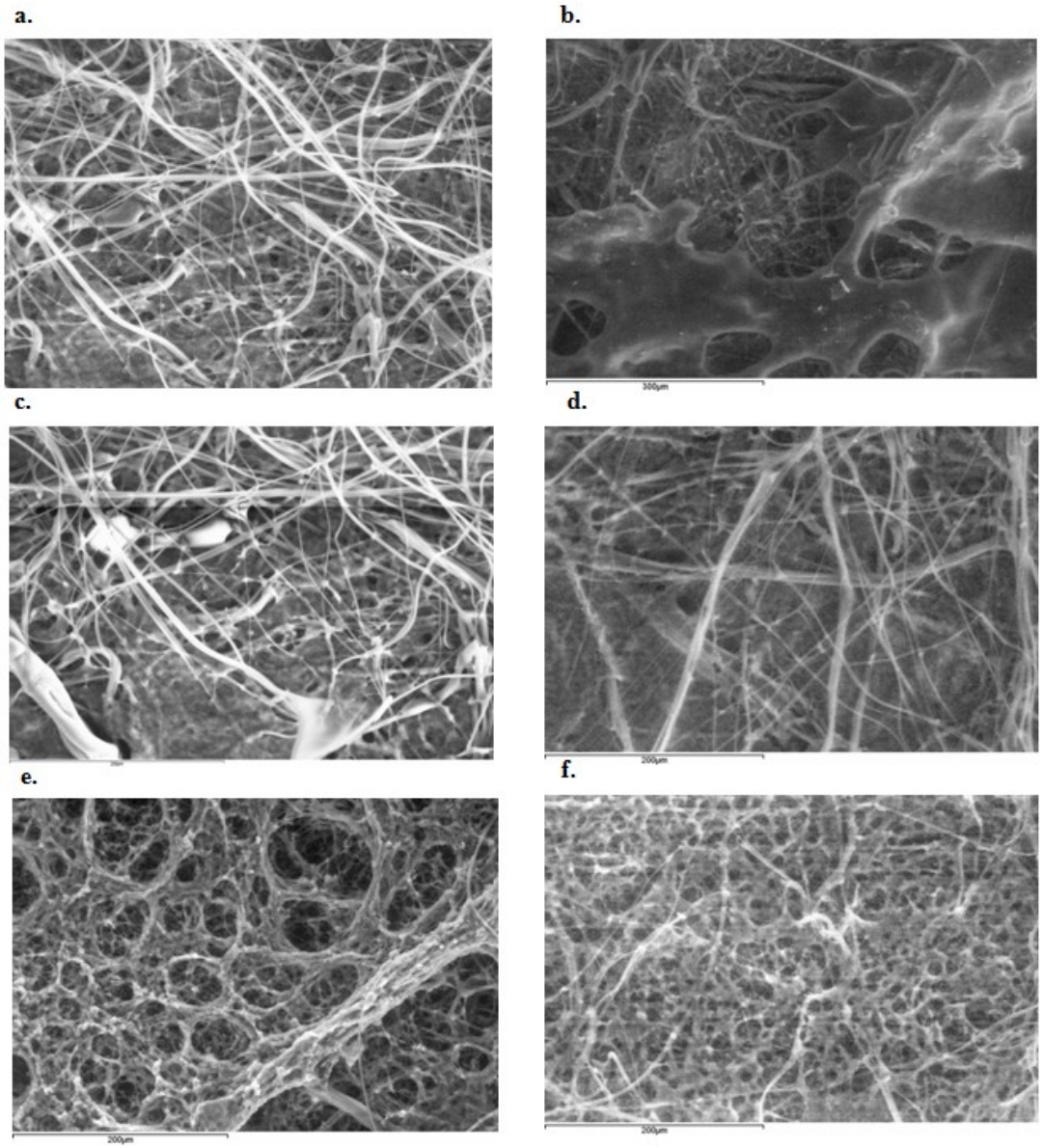


Figure 2 a) and b) are ESEM images of the rough and smooth side of the dry membrane, respectively; c) and d) are ESEM images of the rough and smooth side of 0.5 h pre-equilibrated membrane, respectively; and e) and f) are the ESEM images of the rough and smooth side of 16 h pre-equilibrated membrane, respectively. All images are at 300 X magnification.

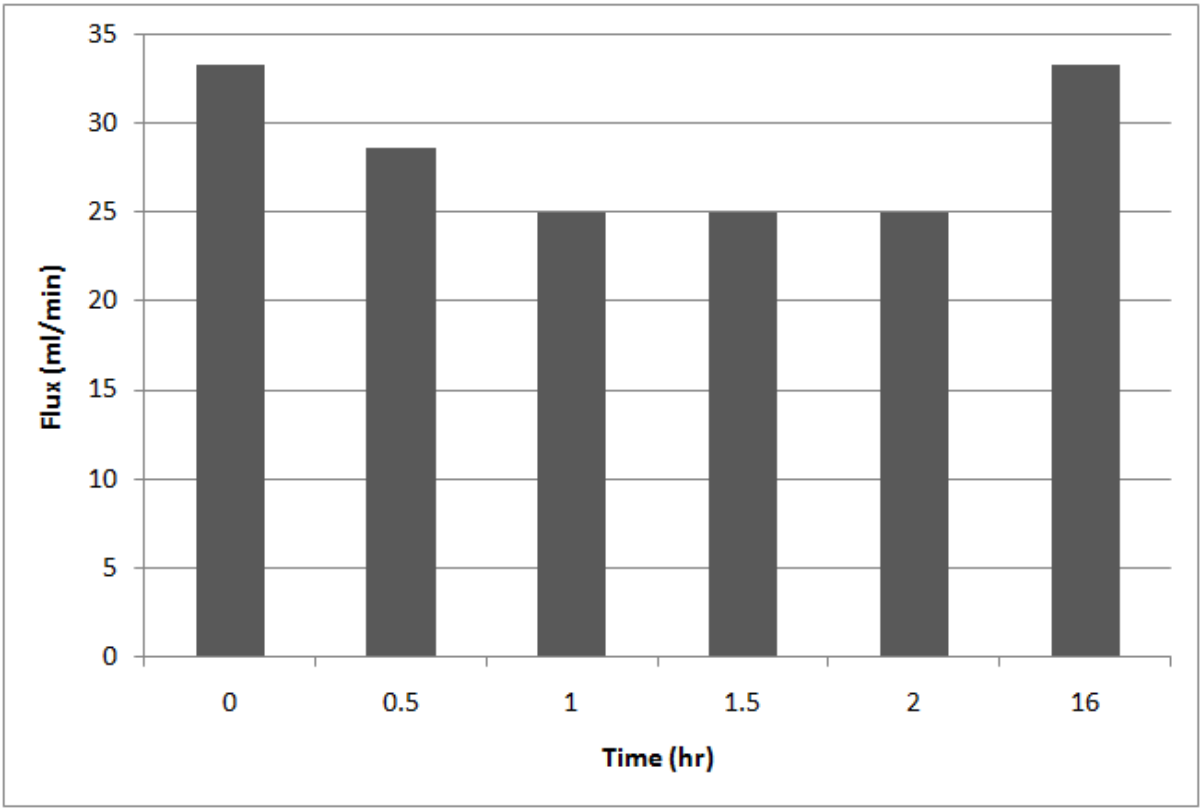


Figure 3 Flux (ml/min) of 10 ml buffer solution across a single layer of membrane cut disk (25 mm in diameter).

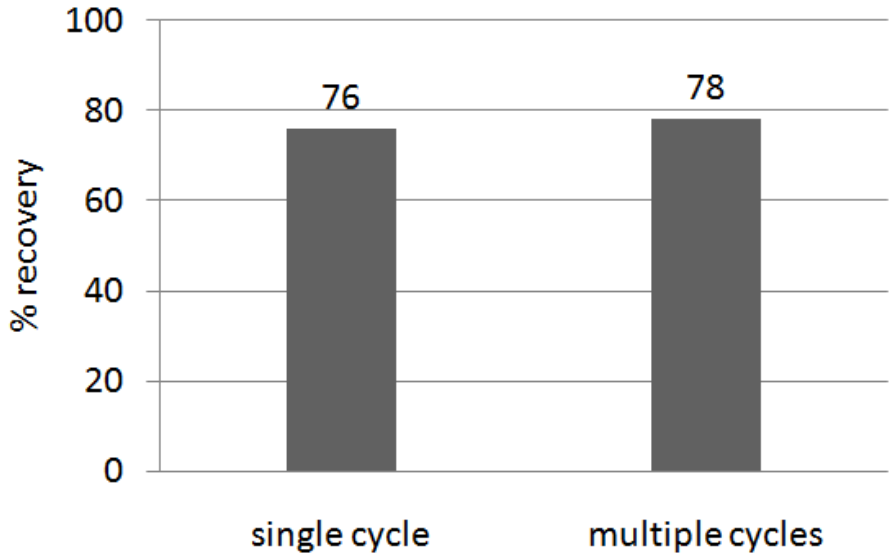


Figure 4 Percentage of recovery of pFlag-PalB from 16 h pre-equilibrated membrane and membrane subjected to multiple cycles of buffer filtration.

3.3.3 Batch Adsorption and Desorption

The static adsorption capacity of the membrane was determined by analyzing the parameters (q_{max} and K) of Langmuir adsorption isotherm using Metropolis-Hastings algorithm. The adsorption isotherm of pFlag-PalB is shown in Figure 5. The maximum amount of plasmid DNA adsorbed onto the membrane (q_{max}) and the Langmuir equilibrium constant (K) were found to be 227 μg/cm^2 (12.4 mg/ml membrane volume) and 7.4 10^{-2} ml/μg, respectively. Reversibility in plasmid DNA adsorption was assessed by eluting the adsorbed plasmid DNA from the membrane in a batch mode. Results from previous chromatographic experiments performed with a gradient elution using buffer containing 0 M to 2 M NaCl have shown that an elution buffer containing at least 0.6 M NaCl was needed to elute plasmid DNA from the membrane. Experiments were also performed with a step-wise elution scheme, where an

elution buffer containing 0.8 M and 2 M NaCl was used stepwise to see if further elution is possible beyond 0.8 M NaCl. It appears that the majority of the plasmid DNA was eluted with a buffer containing 0.8 M NaCl and further elution with 2 M NaCl did not improve recovery. Therefore, an elution buffer containing 0.8 M NaCl was chosen to desorb plasmid DNA from the membrane. Desorption process was carried out overnight until no further change in concentration was observed, and the average recovery was ~ 90%.

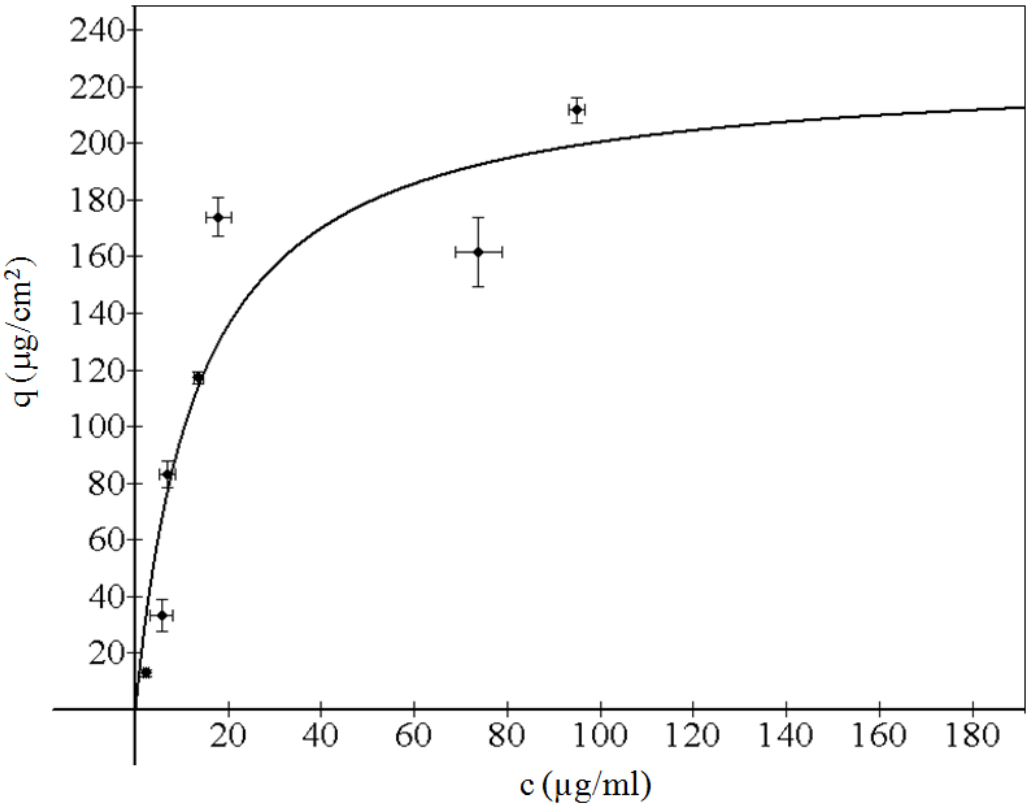


Figure 5 Batch adsorption isotherm of pFlag-PalB (6.4 kb) onto hydrogel Q membrane in buffer solution containing 50 mM Tris-HCl (pH 8).

3.4 Discussion

The characterization of membrane structure and performance is an important step in assessing the suitability of a particular membrane for the separation of biomolecules of interest, therefore membrane structure, structural integrity after multiple cycles of usage as well as the adsorption and desorption capacities were characterized for the hydrogel membrane used in this study.

ESEM was chosen to visualize membrane structure in this study for its ability to characterize sample in wet state, therefore the image is more reflective to the membrane structure at its working condition. In comparison to some other microscopic methods (e.g. Scanning Electron Microscopy), sample pre-treatment is not required for ESEM, therefore potential artifacts that would be introduced during sample preparation was avoided. Chromatographic membranes are usually preserved and shipped in a dehydrated state. According to the manufacturer's recommendation, the membrane should be pre-equilibrated in the chromatographic buffer for at least 0.5 h prior to use. However, it was noted that the typical porous structure of hydrogel-based membranes was under-developed after 0.5 h pre-equilibration and further swelling and continual pore development were observed upon a longer contact with the aqueous environment (16 h). The resulting membrane showed improved distribution of hydrogel as well as the resulting porous structure; therefore membrane pre-equilibrated in buffer solution for 16 h will be providing a better flow through channel for biomolecule separation.

The consistent filtration flux as well as the preservation of reversible adsorption of plasmid DNA after multiple cycles of buffer filtration suggested that the hydrogel membrane

used in this study possess good structural integrity, which is suitable for usage in downstream chromatography where a multiple-cycle mode is required. The improvement in filtration flux with continuous soaking of the membrane in the buffer solution (16 h) confirmed the advantage of 16 h membrane pre-equilibration for the development of better flow through channel.

The selection of the chromatographic material for the economic production of pharmaceutical-grade plasmid DNA is generally based on the adsorption capacity and reversibility. Therefore, the maximum plasmid DNA adsorption and desorption capacity of the membrane were investigated in a series of batch experiments. The maximum adsorption capacity is 227 $\mu g/cm^2$ cross-sectional membrane area or 12.4 mg/ml membrane volume. The capacity is 6 times higher than that of commercially available resin [13]. It is also notable that the adsorption capacity for plasmid DNA of similar linear size (~ 6 kb) was much higher than that of other commercially available Q membranes [57]. The average recovery was ~ 90%. The unrecovered plasmid DNA might be physically entrapped within the polymeric support as the hydrogel shrinks in solution of high ionic strength that is used to elute plasmid DNAs, or irreversibly adsorbed onto the membrane due to non-specific interaction caused by the high ionic strength of the elution buffer. Despite an incomplete desorption, the average recovery from the hydrogel membrane was substantially higher than the previously reported recoveries at 63-76% using various elution buffers containing salts and compaction agents [57]. It is postulated that the high adsorption and desorption capacities are the inherent property of the present hydrogel-based membrane adsorbent. The batch adsorption experiments were carried out by incubating membrane and plasmid DNA in the loading

buffer for 16 h, which was long enough for the hydrogel-based membrane to completely encase the hydrophobic membrane support and develop a more porous structure with a greater accessible adsorptive area (Figure 2 e and f). A comparison between hydrogel Q membrane used in study and some other chromatographic adsorbent is summarized in Table 5.

Table 5 Adsorption and desorption capacity of some anion-exchange chromatographic adsorbent.

Adsorbent	**q_{max} (mg/ml)**	**% recovery**	**References**
Hydrogel Q	12.7	90%	[36]
Mustang Q	10	60-70%	[6]
Q-Sepharose High performance	2.5	-	[7]
Q-Sepharose Fast Flow	1.3	-	[7]

Chapter 4

Investigation of Factors Affecting Membrane Performance

The work in this chapter was published in *Journal of Chromatography B* 879 (2011) 564-572

Authors: **Luyang Zhong**[a], Jeno Scharer[a], Murray Moo-Young[a], Drew Fenner[b], Lisa Crossley[b], C. Howie Honeyman[b], Shing-Yi Suen[c] and C. Perry Chou[a,*2]

Declaration: I initiated and conducted all experiments presented in this chapter under the supervision of Dr. C. Perry Chou, Dr. Jeno Scharer and Dr. Murray Moo-Young.

4.1 Introduction

In light of many technical advantages associated with the use of membrane chromatography for plasmid DNA purification, thorough understanding of the factors affecting membrane performance is the objective of this chapter for the determination of the operating conditions that favour an economic manufacturing of plasmid DNA.

---

[a] Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada, N2L 3G1

[b] Natrix Separations, Inc., 5295 John Lucas Drive, Unit 6, Burlington, Ontario, Canada, L7L 6A8

[c] Department of Chemical Engineering, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 402, Taiwan

* Corresponding author: C. Perry Chou, Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1. Telephone: 1-519-888-4567 ext. 33310, email: cpchou@uwaterloo.ca

It is known that the binding capacity of chromatographic media is in linear relationship with the available adsorptive area [44], which is a function of membrane surface porosity. As characterized in the previous chapter (Figure 2), the hydrogel membrane used in this study has different porosity between the two sides and the porosity is greatly improved with extensive membrane soaking (16 h). Therefore, membrane performance as a result of different combination of membrane orientation in the chromatographic unit and membrane pre-equilibration time was investigated. Due to the fact that separation using anion-exchange chromatography is based on the surface charge density of biomolecules, it is expected that small plasmid DNAs bearing less surface charge density would have minimal interaction with the membrane and better transport thru the porous structure, thus a higher process recovery. It is also known that the degree of supercoiling is affected by the immediate ionic environment [58]. Therefore, membrane performance was also compared for plasmid DNAs with different tertiary sizes at various pH values. And the contribution of each factor to membrane performance was statistically evaluated.

And this is the first time ever that systematic investigation of both intrinsic and extrinsic factors was done for the hydrogel membrane used in this study.

4.2 Materials and Methods

4.2.1 Preparation of Plasmid DNA

Plasmid DNA of pUC19 (2.7 kb) [59], pET20b(+) (3.7 kb) [60] and pFlag-PalB (6.4 kb) [55] were prepared similarly as described in section 3.2.4.1. The plasmid DNA feed for membrane chromatography experiments was prepared by diluting the purified plasmid DNA

to 50 μg/ml in the loading buffer (as described in section 4.2.2) at a desired pH. Same amount of plasmid DNA (~ 110 μg) was loaded onto the membrane for all experiments.

4.2.2 Chromatographic Buffers

The loading and washing buffers were 50 mM Tris-HCl, and the elution buffers contained either 0.8 M or 2 M NaCl in addition to 50 mM Tris-HCl. All buffers were prepared in 18 MΩ deionized water and adjusted to pH 7, pH 8, or pH 9.

4.2.3 Membrane Chromatography

Membrane chromatography in this study was conducted using a laboratory-scale (10 ml) stirred cell (Millipore, Billerica, USA), which was pressurized by nitrogen to 172 kPa (25 psi) to drive the flow of the plasmid DNA solution across the membrane. For each experiment, a single layer of the pre-treated membrane was overlaid in the stirred cell. Plasmid DNA solution at 2 ml was loaded onto the membrane and was pressure-driven through the stirred cell. As shown in Figure 6, plasmid DNA is subject to four possible outcomes upon loading to the membrane, i.e. (1) flow through the membrane, (2) adsorb onto the membrane reversibly, (3) adsorb onto the membrane irreversibly, and (4) be rejected by the membrane so that the plasmid DNA remains at the frontal side of the membrane.

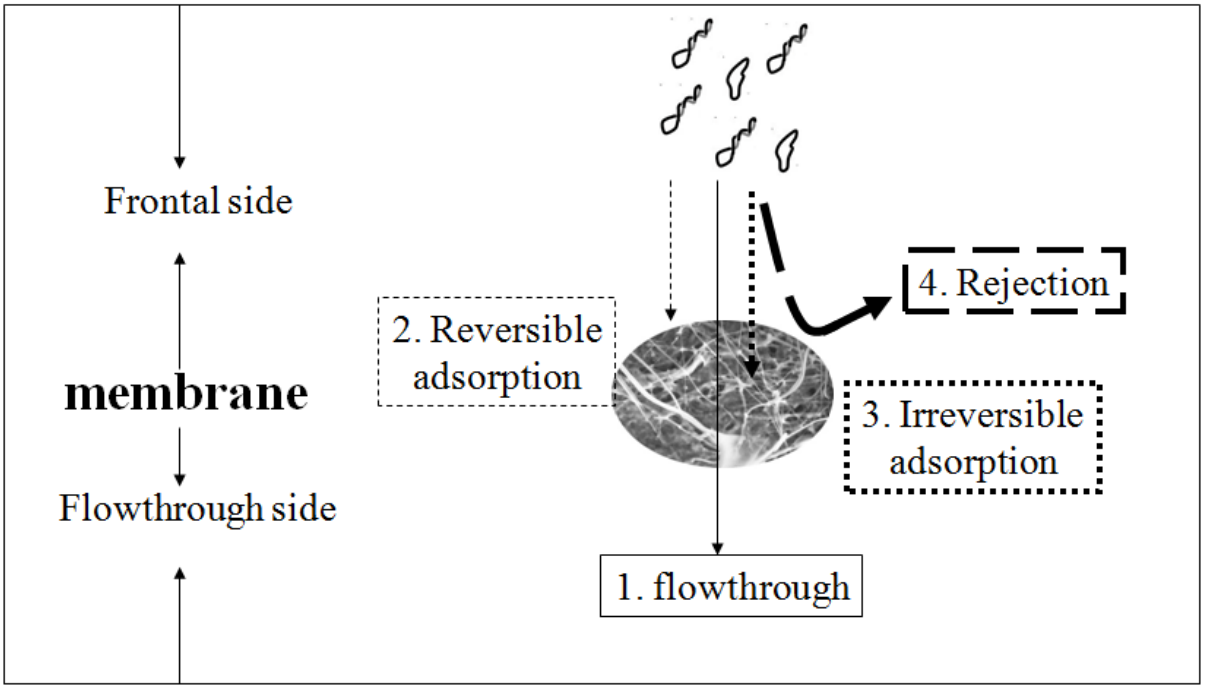


Figure 6 Potential outcomes of plasmid DNA upon loading to the membrane

The amount of plasmid DNA adsorbed onto the membrane was determined by an overall mass balance using Equation (2):

$$\% \, Ads = \frac{P_L - P_R - P_{FT}}{P_L} \times 100\% \qquad (2)$$

where P_L is the amount of plasmid DNA loaded to the membrane, P_R is the amount of plasmid DNA rejected by the membrane, P_{FT} is the amount of plasmid DNA flowing through un-adsorbed, and (P_L - P_R - P_{FT}) is the amount of plasmid DNA adsorbed onto the membrane.

After loading the plasmid DNA, the membrane was washed with 2 ml of washing buffer, followed by step-wise elution scheme with elution buffers containing 0.8 M and 2 M NaCl.

The percentage of plasmid DNA being recovered from the membrane was calculated using Equation (3) as follows:

$$\% \, Rc = \frac{\sum P_{Ei}}{P_L} \times 100\% \tag{3}$$

where P_{Ei} is the amount of plasmid DNA eluted in the *i*th elution fraction, and $\sum P_{Ei}$ is the total amount of plasmid DNA recovered from the membrane during the elution. The irreversibly adsorbed plasmid DNA onto the membrane was determined by the difference between the total amount of adsorbed plasmid DNA and the eluted amount using Equation (4):

$$\% \, Ads_I = \frac{P_{Ads} - \sum P_{Ei}}{P_L} \times 100\% \tag{4}$$

where P_{Ads} (= P_L - P_R - P_{FT}) is the total amount of plasmid DNA adsorbed onto the membrane, P_{Ads} - $\sum P_{Ei}$ is the amount of plasmid DNA irreversibly adsorbed onto the membrane, and the rest of the variables are the same as those in Equations (2) and (3).

4.2.4 Experimental Design

The intrinsic and extrinsic factors that would affect membrane performance were identified, which are plasmid DNA size, membrane orientation in the filtration unit, membrane pretreatment methods and pH of chromatographic buffers. The tertiary structure of three plasmid DNAs with different molecular sizes (2.7 kb to 6.4 kb) was compared using agarose gel electrophoresis (as described in section 4.2.5). Then, the effect of tertiary structure was related to membrane chromatography performance. To determine the optimal operating

condition of membrane chromatography, these factors were examined using fractional factorial design and their investigated levels are summarized in Table 6. The experiments were replicated for each combination of conditions.

Table 6 Fractional factorial design.

	Membrane Orientation	**Membrane Pretreatment**	**Buffer pH**
Level 1	Rough	0.5 h	7
Level 2	Smooth	16 h	8
Level 3	-	-	9

4.2.5 Analytical Methods

The concentration of plasmid DNA was quantified using NanoDrop spectrophotometer as described in section 3.2.5. In addition to the spectroscopic analysis, some plasmid DNA samples were also analyzed by agarose gel electrophoresis for comparison. To perform the analysis, samples were loaded to a 1% agarose gel for electrophoresis at 100 V for 60 min. Then, the agarose gel was stained with ethidium bromide and visualized using a UV transilluminator. The image of the agarose gel was taken by a digital camera, and the intensity of each band was scanned and quantified using an image processor (Image J software from National Institutes of Health, http://rsbweb.nih.gov/ij/). Using these quantified data, the percentages of the rejected and recovered plasmid DNAs were estimated.

4.3 Results

4.3.1 Plasmid DNA Size

The tertiary structure of three plasmid DNAs with different molecular sizes, namely pET20b(+) (3.7 kb), pFlag-palB (6.4 kb) and pUC19 (2.7 kb), was compared by agarose gel electrophoresis at pH 8 and the results are shown in Figure 7. Despite length of primary sequence, pET20b(+) had the smallest tertiary structure, followed by pFlag-PalB, and pUC19 has the largest tertiary structure (Figure 7 a). The average recovery of pET20b(+), pFlag-PalB, and pUC19 at pH 8 from the 16-h pre-equilibrated membrane was 89%, 75%, and 65%, respectively (Figure 7 b), which follows the order of their tertiary sizes from the smallest to the largest.

4.3.2 Surface Texture and Membrane Orientation

Orientation is another important consideration as the hydrogel membrane used in this study differed in its surface porosity on the two sides. To assess the effect of surface porosity, plasmid DNA adsorption and desorption of pET20b(+) were compared for membrane orientation with either the “rough” or “smooth” side upward in the chromatographic unit. As determined previously, membrane performance is dependent on the tertiary size of plasmid DNA, therefore, pET20b(+), a relatively small plasmid DNA in comparison to the other plasmid DNAs was chosen for the purpose of this experiment as it is expected to minimize the effect of plasmid DNA size on membrane performance. Therefore, any difference seen in membrane performance will be more reflective of membrane orientation.

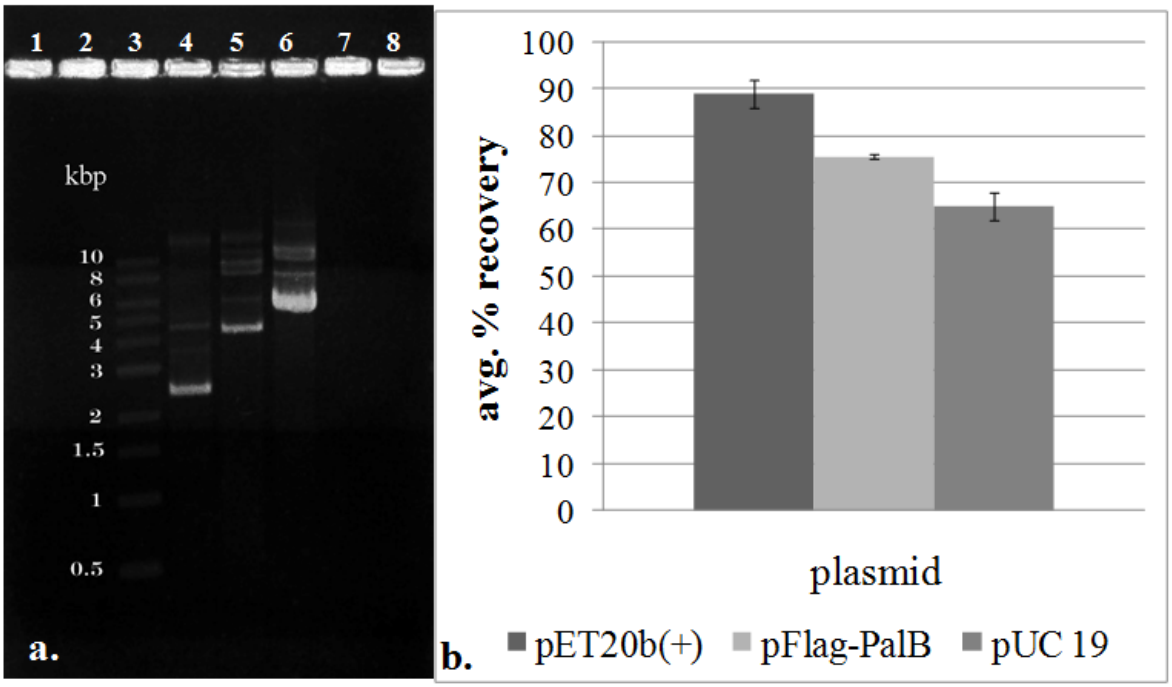


Figure 7 a) Agarose gel electrophoresis of pET20b(+) (lane 4), pFlag-PalB (lane 5) and pUC19 (lane 6), and b) averaged percentage recovery of pET20b(+), pFlag-PalB and pUC 19 at pH 8 with 16 h pre-equilibrated membrane.

The amount of rejected and recovered plasmid DNA was quantified using NanoDrop spectrophotometer, and the results are summarized in Table 7. There was significantly more plasmid DNAs being adsorbed and recovered if the membrane was oriented with the rough side upward in the chromatographic unit. The average rejection of pET20b(+) from the rough and smooth side was 20.5% and 68.0%, respectively. This significant difference in plasmid DNA rejection was also confirmed by agarose gel electrophoresis analysis. As shown in Figure 8 a, the band intensity of the rejected plasmid DNA was very faint as compared to that of plasmid DNA feed, suggesting only a small fraction of the feed was rejected if the rough side was installed upward. In the case where the smooth side was installed upward (Figure 8

b), the band intensity of the rejected plasmid DNA was as bright as the feed, suggesting a majority of the feed was rejected. Densitometric analysis of the agarose gel image was performed using Image J for the quantification of plasmid DNA recovery and rejection. The results were consistent with those obtained by spectrophotometric analysis (Table 7). The flux of plasmid DNA solution across the membrane was also higher during each step of chromatography if the membrane was installed with rough side upward, the difference was significant during loading and elution steps.

Table 7 Comparison of averaged percentage adsorption, averaged percentage recovery and flux of loading, washing and elution step between experiments using the rough side and the smooth side of the membrane as the plasmid DNA loading surface for pET20b(+) at pH 8 with 0.5-h soaking of the membrane.

Membrane Orientation	**Avg. % Adsorption**	**Avg. % Recovery**	**Loading flux (ml/m^2s)**	**Washing flux (ml/m^2s)**	**Elution flux (ml/m^2s)**
Rough	79.5 ± 1.3	77.0 ± 1.6	8.4 ± 0.3	5.0 ± 0.2	67.9 ± 0.0
Smooth	32.0 ± 1.4	67.5 ± 0.7	4.1 ± 0.0	4.1 ± 0.0	5.1± 1.0

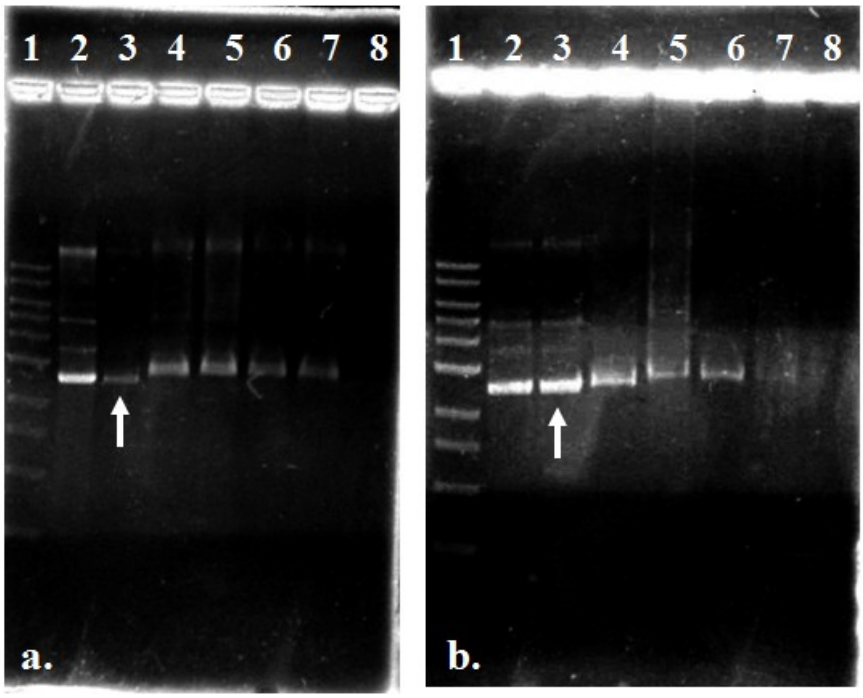


Figure 8 Agarose gel electrophoresis of samples taken from various steps of chromatographic experiments a) using rough side of the membrane as the plasmid DNA loading surface, and b) using smooth side of the membrane as the plasmid DNA loading surface. In both gels, lane 1 is DNA marker, lane 2 is plasmid DNA feed, lane 3 is the frontal side of membrane after loading and before washing, lane 4 is the frontal side of membrane before first elution with elution buffer containing 0.8 M NaCl , lane 5 is the first elution flowthrough, lane 6 is the frontal side of the membrane before second elution, lane 7 is the second elution flowthrough and lane 8 is the third elution flowthrough. Arrow in both gels is pointed at the band corresponding to rejected plasmid DNA.

4.3.3 Membrane Pre-treatment and Buffer pH

Previous experiments have demonstrated advanced porous structure formation with extensive soaking in the buffer solution (Figure 2), therefore membrane pre-treatment involving conventional 0.5 h and 16 h pre-equilibration in the loading buffer were compared for their effectiveness in plasmid DNA adsorption and desorption of pET20b(+), pFlag-PalB and pUC19. The results are summarized in Figure 9.

The average adsorption of pET20b(+), pFlag-PalB, and pUC19 by the conventional membrane pretreatment (0.5 h pre-equilibration) was 73%, 68%, and 50%, respectively. The adsorption of pET20b(+) and pFlag-PalB was improved to 96% if the membrane was pre-equilibrated for 16 h. There was no difference in the adsorption of pUC19 between 0.5 h and 16 h pre-equilibrated membrane if membrane chromatography was performed at pH 7; however, the adsorption was improved to 97% upon 16 h pre-equilibration at both pH 8 and pH 9. The application of 16 h pre-equilibration resulted in an almost complete adsorption (> 96%) of all the plasmid DNAs tested in this study, whereas the adsorption onto the 0.5 h pre-equilibrated membrane was dependent on the tertiary size of plasmid DNA.

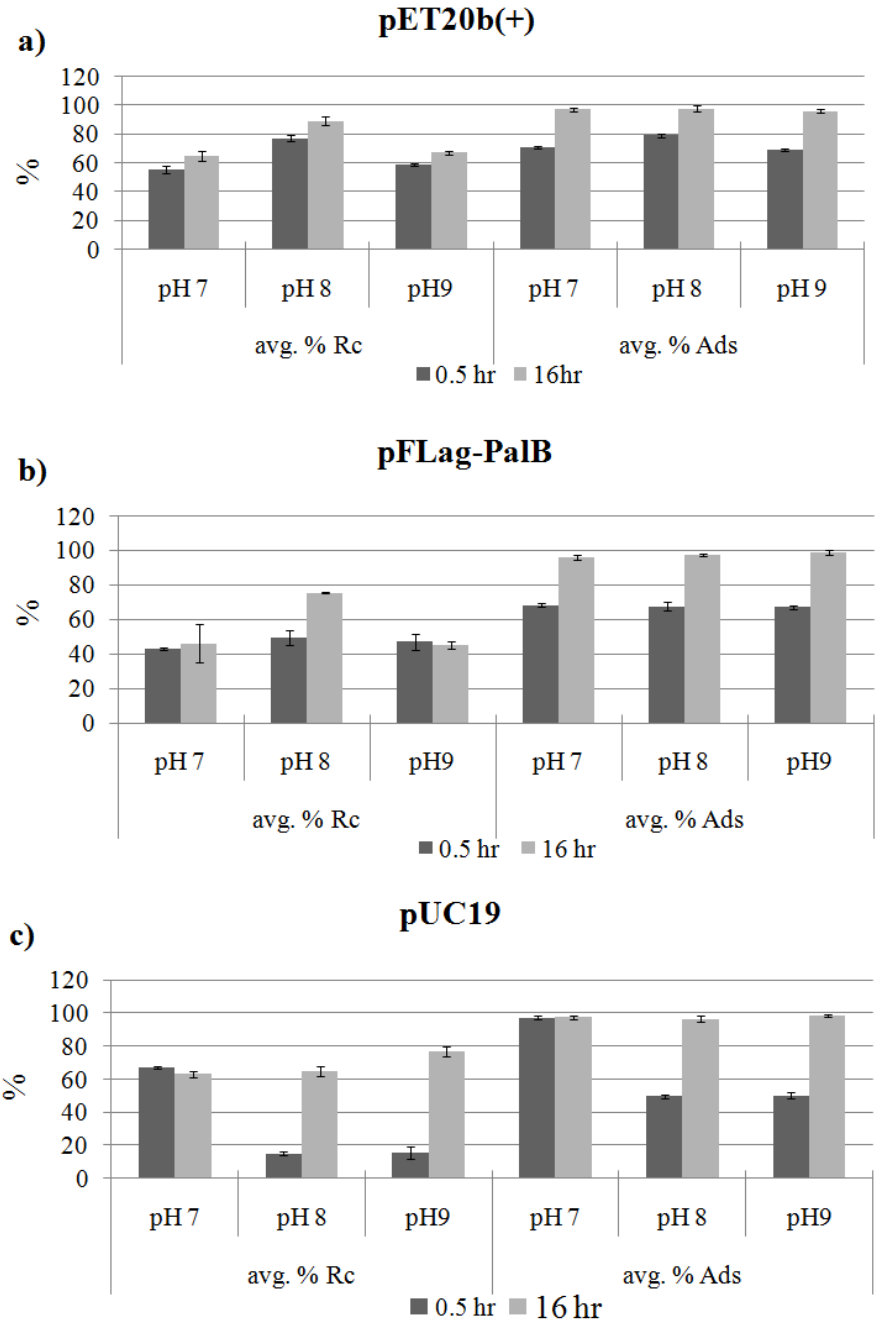


Figure 9 Comparison of the averaged percentage adsorption and recovery of a) pET20b(+), b) pFlag-PalB and c) pUC19 between experiments using 0.5 h and 16 h pre-equilibrated membranes at pH 7, pH 8 and pH 9.

Reversibility in plasmid DNA adsorption was also assessed. The highest recovery of all plasmid DNAs occurred if 16 h pre-equilibrated membrane was used (Figure 9). However, the highest recovery of pET20b(+) and pFlag-PalB occurred if membrane chromatography was performed at pH 8, whereas the highest recovery of pUC19 occurred at pH 9. For an economic production of plasmid DNA, irreversible adsorption should be kept at a minimum. It is noteworthy that the pH at which the least amount of irreversible adsorption occurred was the pH where the highest recovery was observed (Figure 10). Also, plasmid DNAs with smaller tertiary structure had lower levels of irreversible adsorption. Hence, factors affecting membrane porosity and plasmid DNA charge density should be considered to minimize irreversible adsorption for an economic production of pharmaceutical-grade plasmid DNA using anion-exchange chromatography.

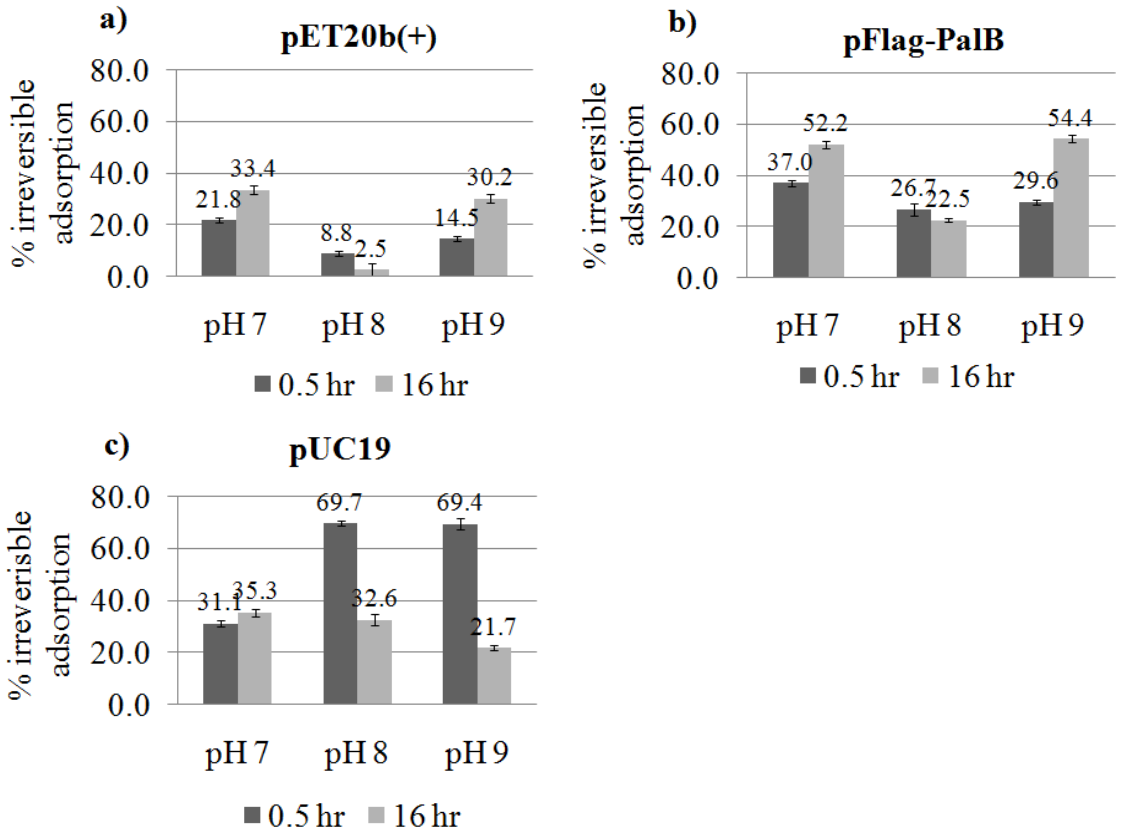


Figure 10 Percentage of irreversible adsorption of a) pET20b(+), b) pFlag-PalB and c) pUC19 to the membranes that were either soaked for 0.5 h or 16 h at pH 7, pH 8 and pH 9.

4.3.4 Factorial Contribution to Membrane Performance

Since all factors investigated herein appeared to be relevant to membrane performance, it would be valuable to obtain the contributing level of each factor for any future improvement of membrane design. Therefore, the relative contribution of various factors, including membrane orientation, membrane soaking time, and chromatographic buffer pH, to the performance of membrane chromatography was determined using fractional factorial analysis. The results are summarized in Figure 11. Note that the contribution of each factor to

the recovery was evaluated for all three plasmid DNAs (Figure 11 a, b and c). However, a similar evaluation associated with the adsorption behavior was evaluated for pET20b(+) only (Figure 11 d) since the experiments were performed at more levels of the membrane orientation factor only for this plasmid DNA. Factorial design allows the determination of the effect of a given factor at several levels of the other factors so that the conclusions are valid over a range of experimental conditions [61]. The fractional factorial experiments comprised a 2×2×3 level (orientation × soaking time × buffer pH) design (Table 6). The three-factor interactions were confounded; consequently the main effects and the two-factor interactions were evaluated. Based on the analysis, the interactions between the factors were insignificant. The main effects "explained" 80% or more of the total variability. Evidently, the response at the "best" buffer pH was not dependent on either the orientation of the membrane or the length of membrane pre-equilibration. In a similar vein, membrane orientation and pre-equilibration time appeared to be independent variables with minimal interaction. For both pET20b(+) and pFlag-PalB, the effect of the buffer pH on the recovery was significantly greater than that of the membrane pre-equilibration time and membrane orientation. However, the effect of the buffer pH and membrane orientation on the recovery of pUC19 was minor, as compared to the effect of the membrane soaking time. One can conclude that the recovery of plasmid DNAs with a small tertiary structure is mostly influenced by the buffer pH, whereas the recovery of plasmid DNAs with a large tertiary structure is mainly dependent on the membrane pore size. For the adsorption of plasmid DNAs (Figure 11 d), the effect of membrane orientation was the greatest, followed by the membrane pre-equilibration time, while the buffer pH has the smallest effect. It is

noteworthy that the combination of membrane orientation and pre-equilibration time determines the available adsorptive area of the membrane; therefore, the adsorption of plasmid DNAs was primarily dependent on the available adsorptive area of the membrane. The result is consistent to previous observations [4, 53].

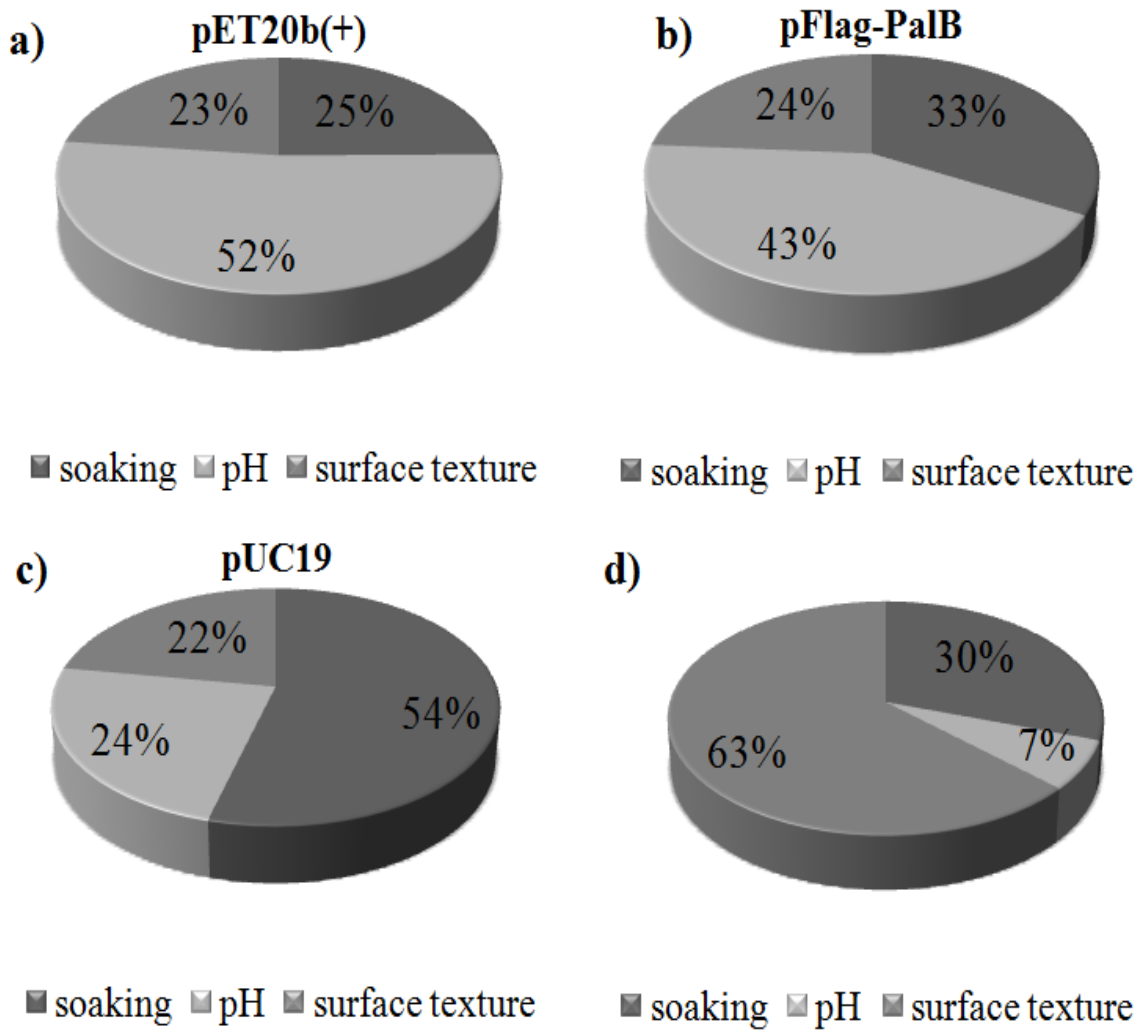


Figure 11 The relative contribution of membrane soaking time, pH of the buffer and the orientation of the membrane to plasmid recovery of a) pET20b(+), b) pFlag-PalB, c) pUC19,

and d) the relative contribution of the above mentioned factors to plasmid adsorption of pET20b(+) to the membrane.

4.4 Discussion

Membrane chromatography is promising to overcome several major challenges associated with the large-scale production of plasmid DNAs. In this study, the high-capacity hydrogel-based Q membrane was used to demonstrate its potential applicability for plasmid DNA purification. While the desired property in membrane chromatography is reversible adsorption, rejection and irreversible adsorption of plasmid DNAs can be frequently observed. Using the hydrogel-based membrane, the extent to which these undesirable events occurred was found to be dependent on various factors, such as tertiary structure of plasmid DNA, membrane porosity, membrane pre-equilibration time, and pH of chromatographic buffers.

The tertiary size of plasmid DNA, as determined by the primary sequence and the degree of supercoiling had an effect on membrane performance, where a higher adsorption and recovery were seen for plasmid DNA of smaller tertiary structure. Plasmid DNAs with a small tertiary structure have a short hydrodynamic radius so that they will be less restricted by the pores of the membranes. Also, they tend to have a lower surface charge density than plasmid DNA with a large tertiary structure, thus forming a fewer number of interactions upon loading to the anion-exchange chromatography. The experimental results of this study and other studies [23, 30, 53, 62, 63] were consistent in terms of the observation of higher recovery associated with small plasmid DNA size using anion-exchange membrane

chromatography. From a bioprocessing viewpoint, it will be desirable to use plasmid DNA of a smaller tertiary structure as the vector for gene therapy and DNA vaccinations.

Porosity appears to have a major impact on membrane performance. In this study, porosity is determined by membrane orientation and membrane pre-equilibration time. Higher adsorption, recovery and flux of plasmid DNA associated with installing the more porous side (rough side) of the membrane upward in the chromatographic unit is explained by the fact that the more porous side would provide a larger accessible area for plasmid DNA adsorption, thus the rejection due to the restrictive membrane pore size and the repulsion by the previously bound plasmid DNA could be minimized. It was shown previously that the porosity can be greatly increased by pre-equilibrate the hydrogel membrane in buffer solution for 16 h. With the pre-treatment, pores were enlarged, well-structured, and evenly distributed so that the adsorption capacity for plasmid DNA could be substantially enhanced. In addition, the hydrophilic supermacroporous hydrogel could completely encase the membrane support and the irreversible adsorption of plasmid DNA to the hydrophobic membrane support was reduced. The approach greatly reduced plasmid DNA loss associated with the irreversible adsorption to the membrane support that is commonly observed for many commercially available anion-exchange membranes. The enlargement in pores would further increase the accessible adsorptive area, thus resulting in less intermolecular competition of the plasmid DNA for the membrane binding sites. Also, the electrostatic repulsion of incoming plasmid DNA by the previously captured material on the membrane was expected to be minimized by the well developed porous structure, resulting in further reduction in the amount of plasmid DNA being rejected. This could prevent, in turn, the possible formation of

a filter cake layer at the frontal side of the membrane, which would otherwise lower the binding capacity and reduce the operational throughput. There was a greater improvement in recovery of pUC19 with the use of 16 h pre-equilibrated membrane as compared to the other two smaller plasmid DNAs, suggesting a greater impact of membrane porosity on the recovery of plasmid DNAs with a larger tertiary structure.

Unlike adsorption where the capacity was mainly influenced by the porosity of the membrane, desorption was also affected by the electric interaction between the strong anion-exchange hydrogel and plasmid DNA, which can be manipulated by the ionic environement. Therefore, reversibility was also compared at various pH values (i.e. pH 7, pH 8, and pH 9). The pH of chromatographic buffer had a more pronounced impact on desorption behaviour of plasmid DNA than other factors because it could potentially affect the size of the tertiary structure of plasmid DNA and/or surface charge density. Through a careful selection of the operating pH, the recovery can be further improved. The optimal pH appeared to be plasmid DNA-dependent. The use of a chromatographic buffer at pH 8 resulted in the highest recovery for pET20b(+) and pFlag-PalB, whereas the highest recovery of pUC19 was observed using a chromatographic buffer at pH 9. It is proposed that plasmid DNAs may have a smaller tertiary structure (due to supercoiling) and/or a lower overall surface charge under these pH conditions, which would result in less interaction with the membrane, thus in a higher recovery as compared to other (less favourable) pH values. It appears that the most favourable pH for reversibility varies with plasmid DNA, therefore the operating pH should be carefully determined for each individual plasmid.

The flux was also improved for all three plasmid DNAs if 16 h pre-equilibrated membranes were used (data not shown). The high convective flow through the supermacroporous structure of the hydrogel membrane, as compared to the diffusive transport through the interior of the resin beads, would be an important processing benefit for large-scale production. It is important to have a high throughput in addition to a high recovery and yield, and using the rough side of 16 h pre-equilibrated membrane to purify plasmid DNAs would fulfill these requirements.

Chapter 5

Bioprocess Development

The work in this chapter was included in "Developing an RNase-free bioprocess to produce pharmaceutical-grade plasmid DNA using selective precipitation and membrane chromatography", and was recently submitted to *Journal of Separation and Purification Technology*.

Authors: **Luyang Zhong**[a], Kajan Srirangan[a], Jeno Scharer[a], Murray Moo-Young[a], Drew Fenner[b,#], Lisa Crossley[b,#], C. Howie Honeyman[b], Shing-Yi Suen[c], C. Perry Chou[a,*3]

Declaration: I initiated and conducted all experiments presented in this chapter under the supervision of Dr. C. Perry Chou, Dr. Jeno Scharer and Dr. Murray Moo-Young.

[a] Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada, N2L 3G1

[b] Natrix Separations, Inc., 5295 John Lucas Drive, Unit 6, Burlington, Ontario, Canada, L7L 6A8

[c] Department of Chemical Engineering, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 402, Taiwan

Current address: BioVentures.ca, 82 Olivia Place, Ancaster, Ontario, L9K 1R4.

* Corresponding author: C. Perry Chou, Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1. Telephone: 1-519-888-4567 ext. 33310, email: cpchou@uwaterloo.ca

5.1 Introduction

Prior to the recent high demand of plasmid DNA for medical applications, technologies for plasmid DNA purification were well established only for small-scale applications, particularly in molecular biology. In addition to the limitation of scalability, these available technologies are unsuitable for the production of pharmaceutical-grade plasmid DNA as they often involve the use of toxic chemicals and animal derived products [64]. In particular, RNase, derived from bovine pancreas, is generally used to degrade RNA that is otherwise difficult to separate from plasmid DNA, but its usage raises safety concerns in view of the new variant Creuzfeld-Jacob disease outbreak in the UK [42]. The restricted use of RNase for the production of pharmaceutical-grade plasmid DNA places a great challenge on downstream bioprocessing, therefore, effective RNA removal prior to chromatography becomes necessary. Several studies reported promising clearance of RNA impurities without the use of RNase [7, 8, 65]. While the use of tangential flow filtration (TFF) in combination with resin-based column chromatography demonstrated complete clearance of RNA [8], this bioprocess was rather time-consuming and labour-intensive, particularly for large-scale production. Selective precipitations using salts are rather simple and inexpensive operations for impurity removal and are often implemented as an intermediate recovery step [21]. They are promising for RNA removal, especially HMW RNA that is otherwise difficult to separate from plasmid DNA by anion-exchange chromatography [66, 67]. As shown in a comparative study for evaluating the potential of five antichaotropic salts to precipitate RNA, calcium chloride was found to be the most potent precipitant [24]. It was suggested that, by properly coupling selective precipitations and anion-exchange chromatography, the polishing step

might not be required, since the derived plasmid already has a quality level suitable for many applications [21]. This was demonstrated in a study where the purification bioprocess involved the use of calcium chloride precipitation, TFF, dialysis, and anion-exchange column chromatography [7]. Nevertheless, these potentially tedious purification steps would be a hindrance for large–scale and economic production.

Existing methods for the purification of plasmid DNA rely mainly on chromatography, employing size-exclusion, reverse-phase, ion-exchange and hydrophobic interaction resins [33]. Of these, anion-exchange chromatography remains the most common method as it employs reversible interaction between negatively charged biomolecules and positively charged chromatographic media. It offers rapid separation and uses chemicals that are generally considered safe [21, 33]. With the development of membrane chromatography, the anion-exchange mode of separation has received greater attention as the relatively large pores (~2 μm) of the membrane allow convective mass transfer and provide a higher capacity for plasmid capture [33, 36]. Most importantly, membrane chromatography is scalable as the binding capacity is directly proportional to the available membrane surface area. However, most chromatographic techniques, including anion-exchange membrane chromatography, suffer from low resolution between plasmid DNA and undegraded RNA [68]. Although high purity of plasmid DNA can be obtained with size-exclusion chromatography (SEC) [69], the bioprocess scale-up is limited by the slow linear flow rate required for an optimal resolution [21]. Also, the small loading volume and the requirement of RNA-reduction steps (using isopropanol, ethanol, ammonium acetate, polyethylene glycol or calcium chloride) make

SEC suitable only as a polishing step following other high-capacity chromatographic steps [8, 18, 69].

In the previous chapter, we reported various technical advantages associated with a hydrogel membrane for the purification of plasmid DNA [36]. In this chapter, we demonstrate its potential application by developing a simple and yet effective RNase-free bioprocess for the production of pharmaceutical-grade plasmid DNA with a high bioprocess yield. The combined use of selective precipitation by calcium chloride and membrane chromatography by hydrogel membrane is proposed for the first time to remove total cellular RNA. In addition, we explore the use of isopropanol as a promising coupling step for simultaneous desalting, concentrating, and buffer exchange of the process stream. The clearance of impurities of major concern for biomedical applications, such as gDNA, proteins and endotoxin, is also demonstrated.

5.2 Materials and Methods

5.2.1 Bacteria Growth and Lysis

E.coli DH5α cells harbouring pET20b(+) (3.7 kb) [60] were grown and harvested as described in section 3.2.4.1. The cell pellet was resuspended in 3 ml of 50 mM Tris-HCl and 10 mM EDTA solution (pH 8.0), followed by cell lysis using a modified alkaline lysis method as described by Birnboim and Doly [20]. Alkaline lysis was gently performed by adding 3 ml of 200 mM NaOH and 1% (w/v) sodium dodecyl sulphate solution, then 6 ml of pre-chilled 3 M potassium acetate (pH 5.5) was used to neutralize the lysate for the

precipitation of cellular debris, genomic DNA and proteins. The precipitate was then removed by centrifuging at 16,000 × *g* for 10 minutes at room temperature.

5.2.2 Optimization of Calcium Chloride Precipitation for the Clearance of HMW RNA

The main purpose of this intermediate recovery step is to remove HMW RNA that is difficult to resolve from plasmid DNA using anion-exchange chromatography. To determine the concentration range of calcium chloride for the optimal removal of HMW RNA with minimal loss of plasmid, the clarified lysate was divided into 5 aliquots of equal volume, then calcium chloride stock solution (4 M) was added accordingly to reach a final concentration of 0.5, 1, 1.5, 2 and 3 M, respectively. The precipitated material including RNA, gDNA, protein and endotoxin was removed by centrifugation at 16,000 × *g* for 10 minutes after incubation at room temperature for 10 minutes. The supernatant from each precipitating concentration was collected and desalted using 60% (w/v) isopropanol, then analyzed by agarose gel electrophoresis followed by Image J densitometric measurement (as described in section 2.3.1) to evaluate plasmid DNA recovery and RNA clearance.

5.2.3 Optimization of Isopropanol Precipitation as the Desalting Step

Isopropanol is often used as a nucleic acid precipitant in the downstream bioprocess [70]. This feature can be used to couple the two major RNA clearance steps of calcium chloride precipitation and membrane chromatography by removing interfering salt content from the clarified lysate. The operating concentration of isopropanol was determined based on the precipitation profiles [65]. After adding the precipitant to cell lysate, the mixture was immediately centrifuged at 16,000 × *g* for 15 minutes at room temperature, followed by

washing with 70% (v/v) ethanol and centrifugation at 16,000 × *g* for 10 minutes at room temperature. The pellet was then resuspended in a loading buffer (as described in section 4.2.2). The same amount of cell lysate from a batch culture was used for all precipitation experiments. Plasmid DNA recovery and RNA clearance were evaluated by agarose gel electrophoresis and Image J densitometric analysis (as described in section 4.2.5).

5.2.4 Optimization of Membrane Chromatography for the Clearance of LMW RNA

Membrane chromatography was performed using BioLogic LP system with BioFrac fraction collector (Bio-Rad Laboratories Ltd.). Absorbance (280 nm) and conductivity were recorded using LP data view V1.03 software (Bio-Rad Laboratories Ltd.). Anion-exchange membrane was installed in a 25 mm stainless steel membrane disc holder (Natrix Separations, Inc.) that was integrated into BioLogic LP system. A single layer of membrane was used for each purification experiment, and it was installed with rough surface upward in the filtration unit for a better membrane performance and process yield [36]. The BioLogic LP system was programmed to pre-equilibrate the membrane with 15 ml loading buffer, followed by sample loading. The membrane was then washed with washing buffer, and the adsorbed plasmid DNA was recovered using gradient salt (NaCl) concentration. The ionic strength of the washing buffer was selected at the salt concentration which resulted in an optimal elution of impurities such as RNA, protein and endotoxin while the adsorbed plasmid DNA remained on the membrane. Gradient salt concentration for plasmid elution was established by programming the mixing ratio between loading and elution buffers employing the Bio-Logic LP system. The flow rate was programmed to be 1 ml/min. The elution fractions were

collected using BioFrac fraction collector, and subsequently analyzed for plasmid DNA recovery.

5.2.5 Analytical Methods

5.2.5.1 Quantification of Plasmid DNA

The concentration of plasmid DNA was quantified using NanoDrop spectrophotometer as described in section 3.2.5. The identity of nucleic acids (plasmid DNA, RNA, gDNA) was analyzed using agarose gel electrophoresis as described in section 4.2.5.

5.2.5.2 Quantification of Genomic DNA

Polymerase chain reaction (PCR) was performed to quantify gDNA in the process stream. Oligonucleotide primer pair of 5'-GAA TTC AAA AAT TGT GTC ATC GTC AGT GCG G -3' (sense) and 5'- CTG CAG TTA ATT CAA CCG TTC AAT CAC CAT C -3'(antisense) were used to amplify a 1194-bp region of the *atoB* gene of *E. coli*. DNA amplification was performed in GeneAmp® PCR System 2700 (Applied Biosystem, Life Technologies, California, USA) with the following temperature profile: 94°C for 4 min (initial denaturation); 20 cycles of 94°C for 1.5 min, 50°C for 45 s and 68°C for 3 min; and 68°C for 10 min (final extension). Purified *E. coli* DH5α gDNA (by Qiagen DNeasy® Blood & Tissue Kit) was used as the template. Various gDNA standards in the range of 0.05 to 1 μg/ml were used for calibration of PCR-amplified signals. The number of amplification cycle of 20 was experimentally determined to ensure that the DNA amplification was in an exponential stage with detectable signals, which were then quantified by Image J. The gDNA concentration of each sample was determined based on the calibration data using gDNA standards.

5.2.5.3 Protein assay

Protein concentration was determined by the micro-BCA protein assay (Pierce Biotechnology, Rockford, IL, USA) using the microplate method. Absorbance was measured at 562 nm with Thermo Labsystems Multiskan Ascent photometric plate reader (Thermo Scientific, Wilmington, USA), and the concentration is determined by comparing to the standard curve constructed using BSA as the protein standard.

5.2.5.4 Endotoxin assay

Endotoxins were quantitatively determined by QCL-1000® endpoint chromatogenic LAL assay (Lonza, Walkersville, MD, USA) using microplate method. Absorbance was measured at 405 nm with Thermo Labsystems Multiskan Ascent photometric plate reader, and the concentration was determined based on the calibration data using endotoxin standards with known concentrations.

5.3 Results

5.3.1 Calcium Chloride Precipitation

To determine the operating concentration for the maximal RNA clearance with minimal loss of plasmid DNA, a calcium chloride concentration range of 0.5 ~ 3 M was used and the results are summarized in Figure 12 and Table 8. Although RNA could be effectively removed from the bioprocess stream by increasing calcium chloride concentration, as seen with the decreasing band intensity of RNA and increasing ratio between plasmid DNA and RNA, the recovery of plasmid DNA was also reduced. In particular, calcium chloride

concentrations greater than 1.5 M resulted in a poor plasmid DNA recovery of less than 50%. It is generally perceived that a recovery lower than 70% for a single downstream processing step is unacceptable [44]. Therefore, calcium chloride concentrations between 1 M and 1.5 M were further analyzed for their effectiveness in HMW RNA clearance.

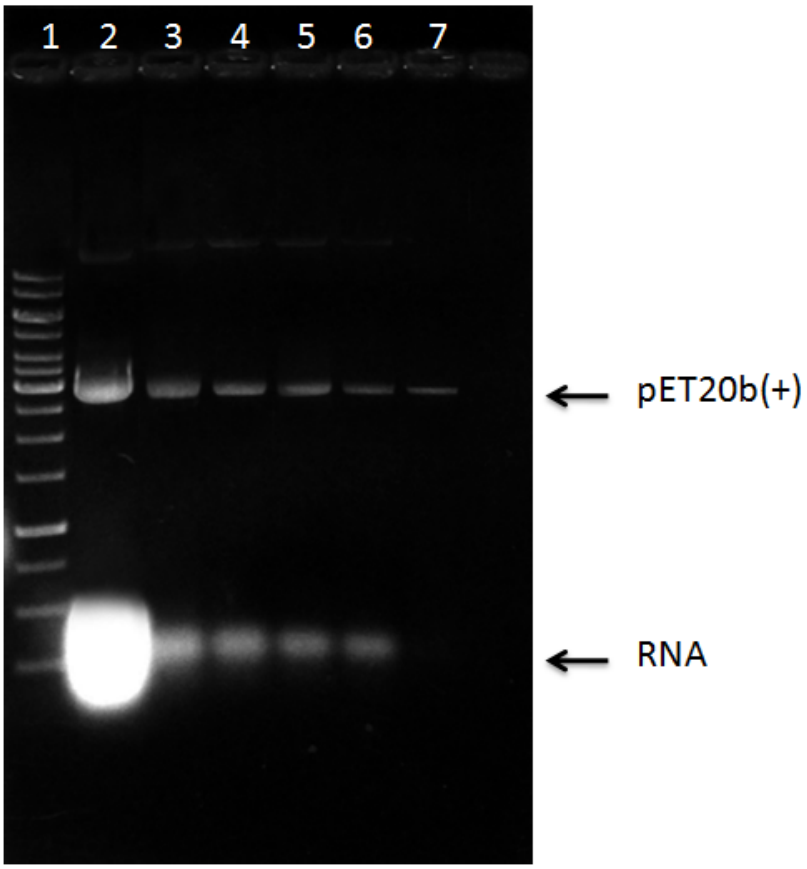


Figure 12 Agarose gel electrophoresis analysis of the supernatant after calcium chloride precipitations. Lane 1 is 1 kb DNA ladder, lane 2 is clarified lysate, lane 3 to 7 are cell lysate precipitated with 0.5, 1, 1.5, 2 and 3 M calcium chloride, respectively.

Table 8 Densitometric analysis of samples treated with calcium chloride precipitation in a concentration range of 0.5 to 3 M by Image J, and the corresponding plasmid DNA to RNA ratio and plasmid DNA recovery.

Sample	Adjusted intensity for plasmid DNA	Adjusted intensity for RNA	Plasmid DNA/ RNA	Plasmid DNA recovery (%)
0 M*	29527	889166	0.033	100%
0.5 M	26279	102680	0.256	89%
1 M	21065	61314	0.344	71%
1.5 M	17644	40086	0.440	60%
2 M	9391	30244	0.311	32%
3 M	5865	2626	2.233	20%

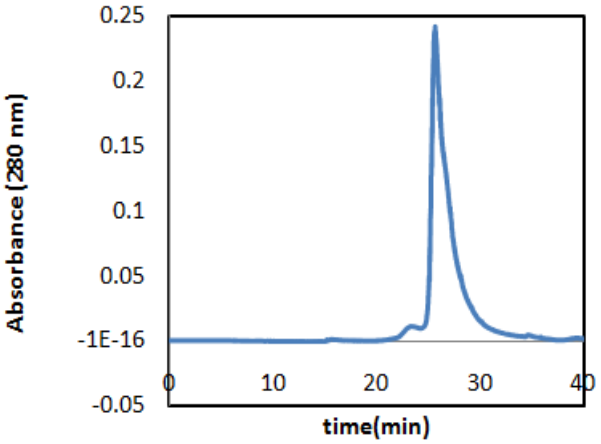
* 0 M = clarified lysate

5.3.2 Isopropanol Precipitation

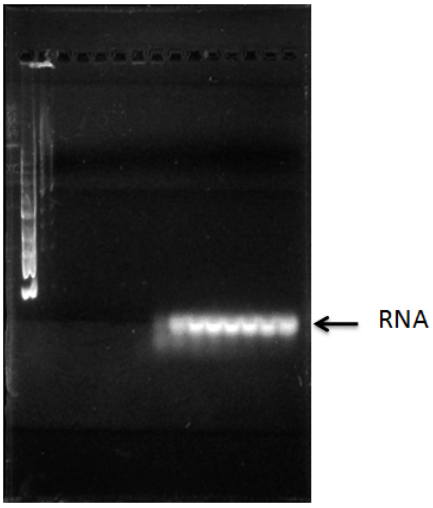
The resulting plasmid DNA concentration after calcium chloride precipitation was ~ 40 μg/ml. Based on the precipitation profiles [65], isopropanol concentration of 60% (v/v) was used to maximize plasmid DNA recovery.

5.3.3 Membrane Chromatography

The optimal operational ionic strength of the washing buffer was determined by increasing the salt concentration of NaCl in a gradient (from 0 M to 0.8 M) to a point where complete removal of the adsorbed RNA was achieved. Various fractions corresponding to the elution peak were analyzed for nucleic acid content using agarose gel electrophoresis. The results suggested that a salt concentration of 0.55 M was sufficient to remove almost all adsorbed RNA prior to plasmid elution (Figure 13). Based on the chromatogram (Figure 13 a), a washing step of 110 membrane volume was sufficient for RNA removal, which was further confirmed by agarose gel electrophoresis of various elution fractions (Figure 13 b). Therefore, in the subsequent purification experiments, LMW RNA was removed by washing buffer containing 0.55 M NaCl and plasmid DNA was then recovered in a gradient salt elution profile starting at 0.56 M.



a)



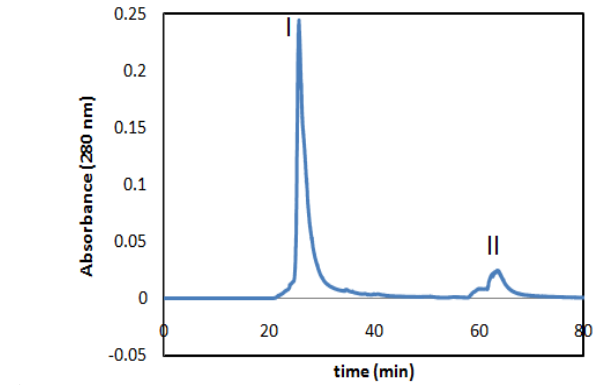
b)

Figure 13 a) Chromatogram of membrane chromatography where washing buffer containing 0.55 M NaCl was applied following 15 minutes of membrane pre-equilibration and 4 minute

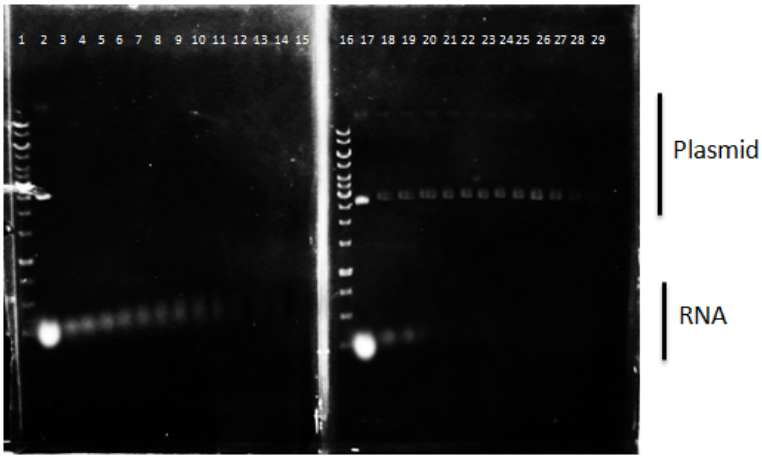
of sample loading, and b) gel electrophoresis of fractions collected from elution peak in a), lane 1 is 1 kb DNA ladder, lane 3 to lane 15 are fractions corresponding to 20 to 31 min on chromatogram.

In a reported purification strategy using only 0.2 M calcium chloride to precipitate RNA [71], the incomplete removal of HMW RNA necessitated additional chromatographic steps to purify plasmid DNA. Apparently, the concentration of the precipitating agent can possibly influence the strategy and even the performance of the subsequent chromatographic step(s). To further evaluate the validity of combining the above proposed procedure (i.e. calcium chloride precipitation, isopropanol desalting, and anion-exchange membrane chromatography), two calcium chloride concentrations lower than 1.5 M (i.e. 1 M and 1.4 M) were used in the HMW RNA removing step followed by the other processing steps. Purification performance with the two lower calcium chloride concentrations are shown in Figure 14 (1 M) and 15 (1.4 M). In the chromatograms for both cases (Figures 14 a and 15 a), the first peak corresponded to the elution of RNA during the washing step, whereas the second peak corresponded to the elution of plasmid DNA during the gradient elution step (Figures 14 b and 15 b). Note the shoulder associated with the second peak (Figure 14 a) and the trace amount of HMW RNA detected in the first few plasmid elution fractions for the lysate pre-treated with 1 M calcium chloride (Figure 14 b), they all imply that 1 M calcium chloride was insufficient for removing total HMW RNA. On the other hand, the eluted plasmid DNA was not contaminated by HMW RNA when the lysate was pre-treated with 1.4 M calcium chloride (Figure 15 b). This seemingly optimal concentration does not appear to be plasmid DNA-specific when comparing our results with other studies [7, 24], suggesting

1.4 M calcium chloride concentration can be used as a general guideline to precipitate HMW RNA from the bioprocess stream.

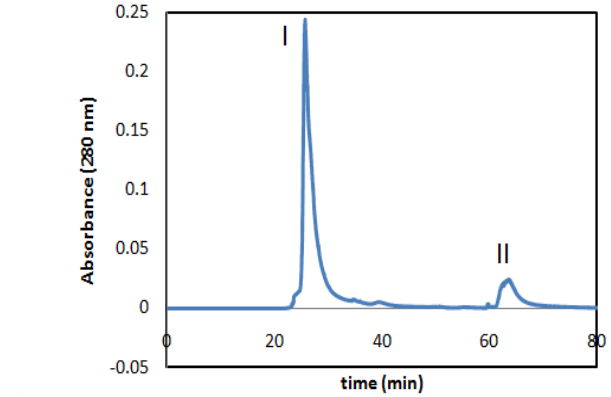


a)

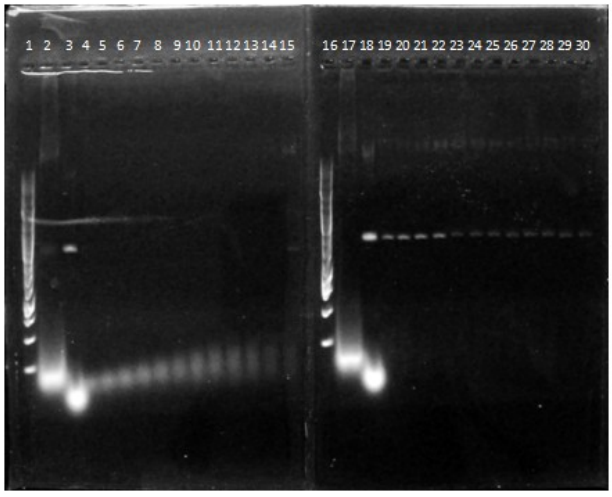


b)

Figure 14 a) Chromatogram of membrane chromatography where 1 M calcium chloride was used to remove HMW RNA from the cell lysate, and b) gel electrophoresis of elution fractions from elution peaks in a), lane 1 and 15 are 1 kb DNA ladder, lane 3 is loading lysate, lane 4 to 14 corresponds to first elution peak (I), lane 16 to 29 corresponds to second elution peak (II).



a)



b)

Figure 15 a) Chromatogram of membrane chromatography where 1.4 M calcium chloride was used to remove HMW RNA from the cell lysate, and b) gel electrophoresis of elution fractions from elution peaks, lane 1 and 16 are 1 kb DNA ladder, lane 3 to lane 15 correspond to first elution peak (I), lane 17 is loading lysate, and lane 19 to 30 correspond to second elution peak (II).

5.3.4 Bioprocess Synthesis

Figure 16 summarizes the proposed RNase-free bioprocess for the production of pharmaceutical-grade plasmid DNA. In particular, three major steps, namely selective

precipitation with calcium chloride, isopropanol desalting, and anion-exchange membrane chromatography are integrated in a simple and inexpensive manner to purify plasmid DNA effectively. As a therapeutic product, the quality of the purified plasmid DNA should meet the various criteria for impurities (e.g. gDNA, proteins, and endotoxin) as defined by regulatory agencies [3]. In this study, protein and endotoxin were analyzed using standard assay kits, whereas gDNA was quantified using PCR with an appropriate cycle number of 20 since PCR with lower (15) cycles produced weak bands and PCR with higher (30) cycles gave saturated bands (data not shown). The results of gDNA quantification are summarized in Figure 17 and Table 9. The level of gDNA was greatly reduced after calcium chloride precipitation and was undetectable in the bioprocess stream after isopropanol precipitation. Table 10 summarizes the performance of all processing steps. Apparently, desalting with isopropanol had an additional advantage in removing impurities as there was a 13-fold decrease in the protein concentration with the resulting endotoxin level as low as 4.5 EU/μg of plasmid DNA after the treatment. The subsequent use of a high-capacity anion-exchange membrane further reduced impurity levels of proteins and endotoxin, and the resulting endotoxin level is well below to those specified by the regulatory agencies (<0.01 EU/μg plasmid DNA) [38]. In addition to impurity clearance, plasmid DNA recovery is another critical factor determining the bioprocess performance. The recoveries of plasmid DNA for isopropanol precipitation and anion-exchange membrane chromatography were 83% and 82%, respectively. In fact, the current recovery for membrane chromatography is higher than other reported values [6, 8, 54]. Based on our previous characterization [36], the membrane framework becomes completely encased with functionalized hydrogel, which provides a

hydrophilic surface for reversible interaction with negatively charged biomolecules. As a result, plasmid loss due to irreversible interaction which is often associated with other types of membranes was minimized.

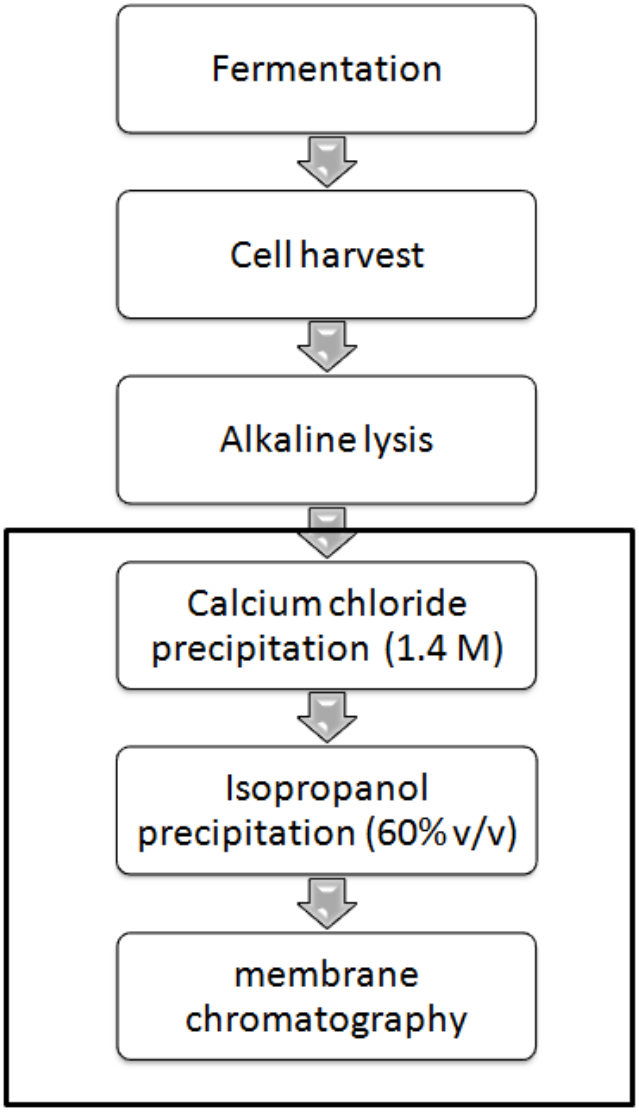


Figure 16 Overview of plasmid purification process where purification steps optimized in this study are circled.

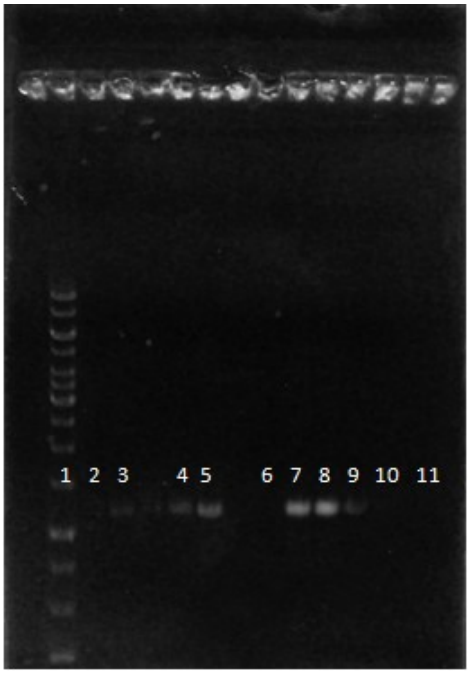


Figure 17 Agarose gel electrophoresis analysis of PCR products of gDNA fragments amplified with 20 PCR cycles. Lane 1 is 1 kb DNA ladder; lane 2 to 5 are gDNA standards prepared at 0, 0.05, 0.1 and 1 μg/ml, respectively; lane 6 and 7 are negative and positive controls, respectively; and lane 8 to 11 are samples taken from clarified lysate, post-calcium chloride precipitation, post-isopropanol precipitation and post-membrane chromatography, respectively.

Table 9 Densitometric analysis of gDNA fragments amplified by 20 PCR cycles and the corresponding concentrations calculated from the gDNA standard curve.

Sample	Adjusted intensity	gDNA (μg/ml)
0	0	0
0.05*	6630	0.05
0.1*	9141	0.1
1*	42301	1
Positive	75297	1.751
CL[#]	79235	1.842
$CaCl_2$	15983	0.372
Isopropanol	0	Undetectable
Membrane chromatography	0	Undetectable

*gDNA standards

[#]CL= clarified lysate

Table 10 Plasmid DNA and impurity levels of protein, endotoxin and gDNA after each processing step.

Processing step	Plasmid DNA (μg)	Protein (μg/μg plasmid DNA)	Endotoxin (EU/μg plasmid DNA)	gDNA (μg/μg plasmid DNA)
Clarified lysate	118	7364	190	0.12
Calcium chloride	105	-*	-*	0.04
Isopropanol	87	123.9	4.5	Undetectable
Membrane chromatography	73	0.05	0.006	Undetectable

5.4 Discussion

In the present study, a novel RNase-free downstream bioprocess was developed for the purification of pharmaceutical-grade plasmid DNA by optimally integrating calcium chloride precipitation, isopropanol precipitation and anion-exchange membrane chromatography for their demonstrated ability in HMW RNA removal, desalting and LMW RNA removal, respectively.

The use of calcium chloride at a properly determined concentration (1.4 M) has demonstrated the feasibility of complete clearance of HMW RNA with minimum loss of

plasmid DNA from the bioprocess stream prior to anion-exchange chromatography. Although no explanation has been given to the fact that calcium chloride selectively precipitates RNA while leaving plasmid DNA in solution, it is suggested that the single-stranded nature of RNAs makes it rather vulnerable for the access of divalent cations to the binding sites within their structures as compared to the rigid double-stranded structure of supercoiled plasmid DNAs [24, 72], and the precipitation is more effective for RNAs with HMW. This offers a great technical advantage, as the complete separation of HMW RNA from plasmid DNA is the major challenge in an RNase-free downstream bioprocess, especially if anion-exchange chromatography is used for the subsequent separation of biomolecules.

The high salt level remaining in the cell lysate after selective precipitation using calcium chloride interfered with anion-exchange membrane chromatography and resulted in immediate breakthrough of plasmid DNA. It is therefore essential to desalt the cell lysate prior to membrane chromatography. Also, plasmid DNA typically comprises less than 1% of total cellular mass [17], and consequently it exists in cell lysate in a dilute form. Although the majority of gDNA can be easily removed during the initial stages of the purification process, a large amount of RNA (representing ~ 80% of total cellular mass) and other impurities (proteins and endotoxin) still remain in the cell lysate. Complete separation of these impurities in excess amounts from plasmid DNA is particularly difficult. Therefore, subsequent operation units capable of desalting, concentrating and impurity reduction are needed. In consideration that isopropanol is capable of simultaneously desalt bioprocess stream, concentrate plasmid DNA and partially remove impurities from the bioprocess

stream [21], it is employed in this study as a single step to fulfill the requirements of three steps. The reduction in processing volume through resuspension of the concentrated nucleic acid pellet in buffer solution of a much smaller volume is another technical advantage associated with the use of isopropanol precipitation. Most desalting operations [7, 8] are rather time-consuming and the product in the bioprocess stream tends to be diluted or even degraded during a prolonged desalting process. Also, additional operation units are often required to concentrate plasmid DNA and reduce impurity levels [7], which rather adds operational complexity to the bioprocess. Therefore, isopropanol precipitation appears to be a promising approach by which the above technical concerns can be alleviated. Hence, it was explored as a coupling step between calcium chloride precipitation and anion-exchange membrane chromatography in this study.

The selective precipitation with calcium chloride and isopropanol not only significantly removed a large fraction of impurities but also concentrated plasmid DNA in an appropriate buffer condition for subsequent anion-exchange membrane chromatography. A strong anion-exchange membrane was selected as the chromatographic medium based on its demonstrated high capacity for plasmid DNA capture and reversibility, as reported in the previous study [36]. Using this membrane chromatographic process, the remaining impurities (LMW RNA, proteins, endotoxin) were largely eliminated. With a lower overall charge density, LMW RNA and other proteinous impurities were often eluted prior to plasmid DNA when a gradient elution operation was employed. However, due to similar chemical properties of plasmid DNA and RNA, a complete separation of these two types of biomolecules has been considered rather difficult [66, 67]. To further improve the

purification resolution, the washing buffer was supplemented with a marginally low concentration of salt (0.55 M) in order to extensively remove the adsorbed RNA and other impurities (e.g. protein) from the anion-exchange membrane without co-elution of plasmid DNA during the washing step. Besides being economic in plasmid DNA production, the determination of the proper salt concentration used in RNA elution has additional benefit in preventing the charged hydrogel from shrinking which would otherwise result in plasmid loss due to physical entrapment in the hydrogel matrix.

The bioprocess developed in this study is simple and yet effective in plasmid yield and impurity clearance, which is readily scalable for the production of pharmaceutical-grade plasmid DNA.

Chapter 6

Alternative Nucleic Acid Precipitant

The work in this chapter was partially included in "Developing an RNase-free bioprocess to produce pharmaceutical-grade plasmid DNA using selective precipitation and membrane chromatography", and was recently submitted to *Journal of Separation and Purification Technology*.

Authors: **Luyang Zhong**[a], Kajan Srirangan[a], Jeno Scharer[a], Murray Moo-Young[a], Drew Fenner[b,#], Lisa Crossley[b,#], C. Howie Honeyman[b], Shing-Yi Suen[c], C. Perry Chou[a,*4]

Declaration: I initiated and conducted all experiments presented in this chapter under the supervision of Dr. C. Perry Chou, Dr. Jeno Scharer and Dr. Murray Moo-Young.

---

[a] Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada, N2L 3G1

[b] Natrix Separations, Inc., 5295 John Lucas Drive, Unit 6, Burlington, Ontario, Canada, L7L 6A8

[c] Department of Chemical Engineering, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 402, Taiwan

Current address: BioVentures.ca, 82 Olivia Place, Ancaster, Ontario, L9K 1R4.

* Corresponding author: C. Perry Chou, Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1. Telephone: 1-519-888-4567 ext. 33310, email: cpchou@uwaterloo.ca

6.1 Introduction

Although isopropanol is a widely used nucleic acid precipitant in laboratory-scale purification of plasmid DNA, the implementation of this operation on a large scale can result in high production cost, which is associated with the capital cost of explosion-proof tank and the disposal cost of the waste. Therefore, alternative nucleic acid precipitant is in demand for a cost-effective bioprocess on the manufacturing scale. PEG is a promising alternative for its biocompatible nature, as it is often used in the formulation of bio-therapeutics. Many studies have shown promising results in plasmid DNA isolation from the cell lysate using high molecular weight PEG [73, 74]. However, most studies lack the information on the yield of plasmid DNA from this operation procedure, which is very important in determining the efficiency of the bioprocess as well as decision making for the choice of the operation unit. Therefore, the objective of this chapter is to determine the fractionation behavior of PEG8000 for nucleic acids as well as its efficiency in resolving plasmid DNA from the rest nucleic acid impurities (e.g. gDNA, RNA, other isoforms of plasmid DNA) as compared to the previously optimized isopropanol precipitation.

6.2 Materials and Methods

6.2.1 Preparation of Cell Lysate

The clarified lysate was prepared as described in section 5.2.1, and calcium chloride precipitation was performed as described in section 5.2.2, where appropriate amount of 4 M calcium chloride stock solution was added to the process stream to reach a final concentration of 1.4 M.

6.2.2 Nucleic Acid Precipitant

PEG with molecular weight of 8000 was used, and final concentrations of 3%, 6% and 10% (w/v) were compared to 60% (v/v) isopropanol for its precipitating efficiency by adding appropriate amount of freshly prepared 20% PEG stock solution to cell lysates. After adding the precipitant into cell lysate, the mixture was immediately centrifuged at 16,000 × *g* for 15 minutes at room temperature, followed by washing with 70% (v/v) ethanol and centrifugation at 16,000 × *g* for 10 minutes at room temperature. The pellet was then resuspended in a loading buffer. The same amount of cell lysate from a batch culture was used for all precipitation experiments. Figure 18 summarizes the different precipitating strategies studied for the intermediate recovery of plasmid DNA from the bioprocess stream. Plasmid DNA recovery and RNA clearance were evaluated by agarose gel electrophoresis and densitometric analysis (as described in section 4.2.5)

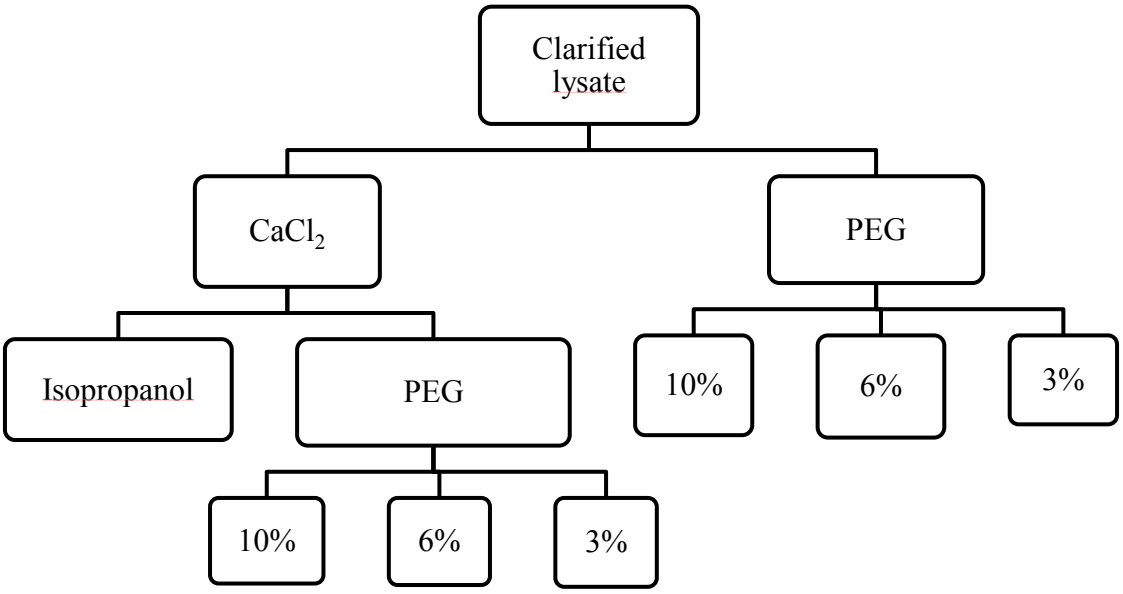


Figure 18 Overview of precipitating strategies studied for the intermediate recovery of plasmid DNA from the bioprocess stream.

6.3 Results

Isopropanol at a final concentration of 60% (v/v) was previously determined to be optimal for concentrating plasmid DNA at a concentration of ~ 40 μg/ml. Alternatively, PEG precipitation at final concentrations of 3%, 6%, and 10% were also explored following calcium chloride precipitation. The results are compared in Figure 19 (lane 2 to 5). Due to the potential interest for therapeutic applications, only plasmid DNA bands corresponding to the supercoiled form were quantified and the results are summarized in Table 11. The plasmid DNA band intensity is the highest if isopropanol was used to precipitate plasmid DNA, suggesting 60% (v/v) isopropanol is more efficient in precipitating supercoiled plasmid DNA at a concentration of ~ 40 μg/ml than PEG at all tested concentrations. Among PEG precipitations, PEG at 6% precipitated more supercoiled plasmid DNA, followed by 10% and 3% didn't precipitate any nucleic acid. The relative recovery efficiency of PEG at 10% and 6% as compared to 60% (v/v) isopropanol was 39% and 48%, respectively. RNA was undetectable in the bioprocess stream after PEG precipitation, whereas a small amount of it was precipitated by isopropanol.

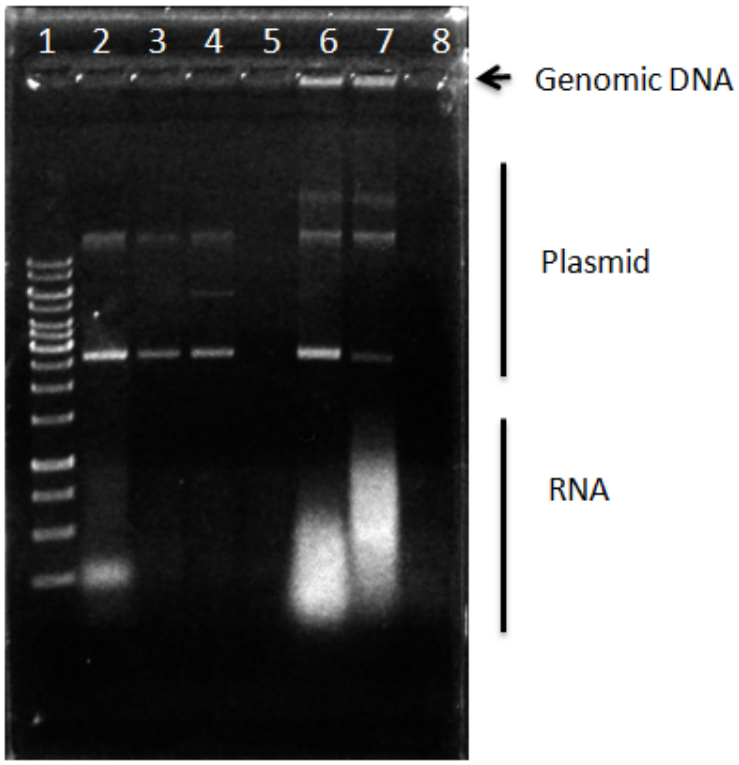


Figure 19 Agarose gel electrophoresis analysis of the nucleic acid pellets precipitated by isopropanol and PEG. Lane 2 to 5 reveal the nucleic acids precipitated with 60% (v/v) isopropanol, 10% PEG, 6% PEG and 3% PEG, respectively from the supernatant collected after calcium chloride precipitation (1.4 M); and lane 6 to 8 reveals the nucleic acids precipitated with PEG concentrations at 10%, 6% and 3%, respectively from the clarified lysate.

Table 11 Densitometric analysis of DNA bands of supercoiled plasmid DNA and the corresponding relative recovery efficiency as compared to "calcium chloride and isopropanol" precipitation strategy.

Precipitation strategy	Adjusted intensity	Relative recovery efficiency (%)
$CaCl_2$ + isopropanol	210866	100
$CaCl_2$ + 10% PEG	81476	39
$CaCl_2$ + 6% PEG	102222	48
$CaCl_2$ + 3% PEG	0	0
10% PEG	178257	85
6% PEG	38681	18
3% PEG	0	0

To investigate whether low plasmid DNA yields were associated with the combined usage of calcium chloride and PEG, PEG alone was explored to precipitate plasmid DNA directly from clarified lysate and the results are shown in Figure 19 (lane 6 to 8). In addition to various isoforms of plasmid DNA, PEG at 10% and 6% also precipitated gDNA and a huge amount of RNA. The relative recovery efficiencies as compared to isopropanol are 85% and 18% for PEG concentrations at 10% and 6%, respectively.

6.4 Discussion

While it appears that PEG in combination with calcium chloride was more effective in RNA clearance, isopropanol precipitation gave the highest yield of supercoiled plasmid DNA with decent RNA clearance. It was suspected that the combined usage of calcium chloride and PEG caused major loss of plasmid DNA, therefore PEG precipitation was performed directly following primary recovery steps. Apparently, PEG alone failed to separate gDNA and RNA from plasmid DNA effectively. The concentration of these impurities in the process stream would cause a great burden to subsequent downstream bioprocessing. In addition, the plasmid DNA yields based on the use of PEG as the precipitant remained low even without the use of calcium chloride, implying isopropanol is a more effective precipitant for recovering supercoiled plasmid DNA. The results also suggest that it is crucial to incorporate calcium chloride precipitation in the early stage of a bioprocess for gDNA clearance since the separation of gDNA from plasmid DNA can be a major technical challenge in many downstream processing operations[70].

Chapter 7
Conclusions and Recommendations

7.1 Conclusions

Membrane chromatography is promising to overcome several major challenges associated with the large-scale production of plasmid DNAs. In this study, a hydrogel-based strong anion-exchange membrane with a high binding capacity was used to demonstrate its potential applicability for plasmid DNA purification. While the desired property in membrane chromatography is reversible adsorption, rejection and irreversible adsorption of plasmid DNAs can be frequently observed. Using the hydrogel membrane, the extent to which these undesirable events occurred was found to be dependent on various factors, including the membrane porosity, the soaking time, the buffer pH and the size of plasmid DNA tertiary structure. Porosity appears to have a major impact on performance. The more porous side of the membrane would provide a larger accessible area for plasmid DNA adsorption, thus the rejection due to the restrictive membrane pore size and the repulsion by the previously bound plasmid DNA could be minimized. In this study it was shown that the porosity can be greatly increased by properly pre-treating the hydrogel membrane. With the pre-treatment, pores were enlarged, well-structured, and evenly distributed so that the binding capacity for plasmid DNAs could be substantially enhanced. In addition, the hydrophilic supermacroporous hydrogel could completely encase the membrane support and the irreversible adsorption of plasmid DNAs to the hydrophobic membrane support was reduced. The approach greatly reduced plasmid DNA loss associated with the irreversible adsorption to the membrane support that is commonly observed for many commercially available anion-

exchange membranes. The tertiary structure of plasmid DNA, as determined by the degree of supercoiling also had an effect. While the size effect on the adsorption was negated by the enlargement of pores by proper pre-treatment, but the size effect persisted for desorption. In general, a higher desorption was observed for small plasmid DNAs. Buffer pH had a more pronounced impact on desorption behaviour of plasmid DNA than other factors because it could potentially affect the size of the tertiary structure of plasmid DNA and/or surface charge density. Through a careful selection of the operating pH, the recovery can be further improved. The optimal pH appeared to be plasmid DNA-dependent. The high convective flow through the supermacroporous structure of the hydrogel membrane, as compared to the diffusive transport through the interior of the resin beads, would be an important processing benefit.

The presently developed RNase-free bioprocess based on the combined use of selective precipitations and anion-exchange membrane chromatography. IT is simple, easy to operate, economical, and effective in producing pharmaceutical-grade plasmid DNA as it involves far less purification steps in comparison to other existing technologies [7, 8, 18]. The optimal determination of the operating condition for calcium chloride precipitation serves as an important prerequisite not only to remove a significant amount of gDNA and RNA impurities in the bioprocess stream but also to enhance the performance of subsequent purification steps. The implementation of isopropanol precipitation offers several technical advantages, including desalting the bioprocess stream, concentrating plasmid DNA, and further removing various impurities in the bioprocess stream. The present study also demonstrates the technical feasibility of applying anion-exchange chromatography with

single-use high-capacity hydrogel membranes for effective purification of plasmid DNA, which saves time of labour intensive procedures (i.e. washing, cleaning, and regenerating) associated with the conventional resin-based chromatography, and at the same time provides much better membrane performance in binding capacity and biomass flow. For the chromatographic operation, the optimal determination of operating condition for the washing step ensures a complete RNA separation from plasmid DNA, which is a major technical concern for most RNase-free bioprocesses for the production of plasmid DNA. These purification steps were optimally integrated to ensure that the final product of plasmid DNA meets the regulatory standards for therapeutic applications.

7.2 Recommendations

Given the successful demonstration in the proposed bioprocess development, the plasmid product contained both supercoiled and open circular isoforms. The biological activity of plasmid DNA is often associated with its topological structure [75, 76] and the supercoiled isoform is more suitable for therapeutic applications [77]. It is believed that, by careful implementation of bioprocessing steps with optimal operating conditions, supercoiling can be enhanced. As such, following is recommended for future work to improve plasmid DNA purity.

1. A gentle and yet effective lysis technique is required to release the highest possible amount of supercoiled plasmid DNA. As it is the first step in plasmid DNA recovery from the host cells, the efficiency is critical for the overall yield.

2. Compare centrifugation with microfiltration for biomass removal, as it is proposed that microfiltration is gentle on the shear-stress sensitive plasmid DNA [18].
3. Include an additional chromatography following anion-exchange membrane chromatography, such as SEC or HIC, to selectively isolate supercoiled plasmid DNA with the consideration that such step is simple to operate with high process yield.

References

1. Manoj S, Babiuk LA, den Hurk S: **Approaches to Enhance the Efficacy of DNA Vaccines**. *Cr Rev Cl Lab Sc* 2004, **41**:1-39.
2. Han Y, Liu S, Ho J, Danquah MK, Forde GM: **Using DNA as a drug--Bioprocessing and delivery strategies**. *Chem Eng Res Des* 2009, **87**:343-348.
3. FDA: **Guidance for Industry: Considerations for plasmid DNA vaccines for infectious disease indications**. 2007.
4. Tiainen P, Gustavsson P-E, Ljunglöf A, Larsson P-O: **Superporous agarose anion exchangers for plasmid isolation**. *Journal of Chromatography A* 2007, **1138**(1-2):84-94.
5. Branovic K, Forcic D, Ivancic J, Strancar A, Barut M, Kosutic Gulija T, Zgorelec R, Mazuran R: **Application of short monolithic columns for fast purification of plasmid DNA**. *Journal of Chromatography B* 2004, **801**(2):331-337.
6. Teeters MA, Root TW, Lightfoot EN: **Adsorption and desorption behavior of plasmid DNA on ion-exchange membranes: Effect of salt valence and compaction agents**. *J Chromatogr A* 2004, **1036**:73-78.
7. Eon-Duval A, Burke G: **Purification of pharmaceutical-grade plasmid DNA by anion-exchange chromatography in an RNase-free process**. *J Chromatogr B* 2004, **804**:327-335.
8. Miladys Limonta GM, Martha Pupo, Odalys Ruiz: **The Purification of Plasmid DNA for Clinical Trials Using Membrane Chromatography**. *BioPharm* 2010, **23**.
9. Wolff J, Malone R, Williams P, Chong W, Acsadi G, Jani A, Felgner P: **Direct gene transfer into mouse muscle in vivo**. *Science* 1990, **247**(4949):1465-1468.
10. Shiver JW, Emini EA: **Recent Advances in the Development of HIV-1 Vaccines Using Replication-Incompetent Adenovirus Vectors**. *Annu Rev Med* 2004, **55**(1):355-372.

11. Gottesman MM: **Cancer gene therapy: an awkward adolescence**. *Cancer Gene Ther* 2003, **10**(7):501-508.

12. Han Y, Liu S, Ho J, Danquah MK, Forde GM: **Using DNA as a drug--Bioprocessing and delivery strategies**. *Chem Eng Res Design* 2009, **87**(3):343-348.

13. Prather KJ, Sagar S, Murphy J, Chartrain M: **Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification**. *Enzyme Microb Technol* 2003, **33**(7):865-883.

14. Tiainen P, Galaev I, Larsson P-O: **Plasmid adsorption to anion-exchange matrices: Comments on plasmid recovery**. *Biotechnol J* 2007, **2**(6):726-735.

15. Ferreira GNM, Monteiro GA, Prazeres DMF, Cabral JMS: **Downstream processing of plasmid DNA for gene therapy and DNA vaccine applications**. *Trends Biotechnol* 2000, **18**(9):380-388.

16. Leitner WW, Ying H, Restifo NP: **DNA and RNA-based vaccines: principles, progress and prospects**. *Vaccine* 1999, **18**:765-777.

17. Atkinso B, Mavituna F: **Biochemical engineering and biotechnology handbook (2nd ed.)**. *Macmillan, New York, USA* 1991.

18. Prazeres DMF, Ferreira GNM: **Design of flowsheets for the recovery and purification of plasmids for gene therapy and DNA vaccination**. *Chem Eng Process* 2004, **43**:609-624.

19. Harrison RG, Todd P, Rudge SR, Petrides DP: **Bioseparations science and engineering**. New York: Oxford Unversity Press; 2003.

20. Bimboim HC, Doly J: **A rapid alkaline extraction procedure for screening recombinant plasmid DNA**. *Nucleic Acids Res* 1979, **7**:1513-1523.

21. Ballantyne J: **Practical Methods for Supercoiled pDNA Production**. *Meth Mol Med* 2006, **127**:311-337.

22. Carnes AE, Williams JA: **Plasmid DNA manufacturing technology**. *Recent patents on biotechnology* 2007, **1**(2):151-166.

23. Eon-Duval A, MacDuff RH, Fisher CA, Harris MJ, Brook C: **Removal of RNA impurities by tangential flow filtration in an RNase-free plasmid DNA purification process**. *Anal Biochem* 2003, **316**(1):66-73.

24. Eon-Duval A, Gumbs K, Ellett C: **Precipitation of RNA impurities with high salt in a plasmid DNA purification process: Use of experimental design to determine reaction conditions**. *Biotechnol Bioeng* 2003, **83**:544-553.

25. Stephane Flock, Rudi Labarbe, Houssier C: **Dielectric Constant and Ionic Strength Effects on DNA Precipitation**. *Biophys J* 1996, **70**:1456-1465.

26. Luechau F, Ling TC, Lyddiatt A: **Partition of plasmid DNA in polymer-salt aqueous two-phase systems**. *Separation and Purification Technology* 2009, **66**(2):397-404.

27. Luechau F, Ling TC, Lyddiatt A: **Interfacial partition of plasmid DNA in aqueous two-phase systems**. *Separation and Purification Technology* 2009, **67**(1):64-70.

28. Kula M-R, Kroner K, Hustedt H: **Purification of enzymes by liquid-liquid extraction**. In: *Reaction Engineering.* vol. 24: Springer Berlin / Heidelberg; 1982: 73-118.

29. Peters TJ: **Partition of cell particles and macromolecules: Separation and purification of biomolecules, cell organelles, membranes and cells in aqueous polymer two phase systems and their use in biochemical analysis and biotechnology.** , vol. 5, 3rd edn. Chichester: John Wiley & Sons, Ltd.; 1987.

30. Arkhangelsky E, Steubing B, Ben-Dov E, Kushmaro A, Gitis V: **Influence of pH and ionic strength on transmission of plasmid DNA through ultrafiltration membranes**. *Desalination* 2008, **227**(1-3):111-119.

31. Eon-Duval A, MacDuff RH, Fisher CA, Harris MJ, Brook C: **Removal of RNA impurities by tangential flow filtration in an RNase-free plasmid DNA purification process**. *Analytical Biochemistry* 2003, **316**(1):66-73.

32. Kahn DW, Butler MD, Cohen DL, Gordon M, Kahn JW, Winkler ME: **Purification of plasmid DNA by tangential flow filtration**. *Biotechnology and Bioengineering* 2000, **69**(1):101-106.

33. Diogo MM, Queiroz JA, Prazeres DMF: **Chromatography of plasmid DNA**. *J Chromatogr A* 2005, **1069**:3-22.

34. Yamakawa H, Higashino K-i, Ohara O: **Sequence-Dependent DNA Separation by Anion-Exchange High-Performance Liquid Chromatography**. *Analytical Biochemistry* 1996, **240**(2):242-250.

35. Sofer G, Hagel L: **Handbook of Process Chromatograpy: A guide to Optimization, Scale-Up and Validation**: Academic Press; 1997.

36. Zhong L, Scharer J, Moo-Young M, Fenner D, Crossley L, Honeyman CH, Suen S-Y, Chou CP: **Potential application of hydrogel-based strong anion-exchange membrane for plasmid DNA purification**. *J Chromatogr B* 2011, **879**:564-572.

37. Ferreira GNM, Cabral JMS, Prazeres DMF: **Studies on the Batch Adsorption of Plasmid DNA onto Anion-Exchange Chromatographic Supports**. *Biotechnology Progress* 2000, **16**(3):416-424.

38. Ferreira GNM, Monteiro GA, Prazeres DMF, Cabral JMS: **Downstream processing of plasmid DNA for gene therapy and DNA vaccine applications**. *Trends Biotechnol* 2000, **18**:380-388.

39. Diogo MM, Queiroz JA, Monteiro GA, Martins SAM, Ferreira GNM, Prazeres DMF: **Purification of a cystic fibrosis plasmid vector for gene therapy using hydrophobic interaction chromatography**. *Biotechnol Bioeng* 2000, **68**(5):576-583.

40. Vo-Quang T, Malpiece Y, Buffard D, Kaminski PA, Vidal D, Strosberg AD: **Rapid large-scale purification of plasmid DNA by medium or low pressure gel filtration.** . *Biosci Rep* 1985, **5**(2):101-111.

41. Przybylowski M, Bartido S, Borquez-Ojeda O, Sadelain M, Rivière I: **Production of clinical-grade plasmid DNA for human Phase I clinical trials and large animal clinical studies**. *Vaccine* 2007, **25**(27):5013-5024.

42. Cooke GD, Cranenburgh RM, Hanak JAJ, Dunnill P, Thatcher DR, Ward JM: **Purification of essentially RNA free plasmid DNA using a modified Escherichia coli host strain expressing ribonuclease A**. *J Biotechnol* 2001, **85**:297-304.

43. Pardoll D: **Plasmid DNA Expressing E7 for the Treatment of HPV-Associated Cervical Cancer**. In.: NCI; 2004.

44. Stadler J, Lemmens R, Nyhammar T: **Plasmid DNA purification**. *J Gene Med* 2004, **6**(S1):S54-S66.

45. Eon-Duval A, Burke G: **Purification of pharmaceutical-grade plasmid DNA by anion-exchange chromatography in an RNase-free process**. *J Chromatogr B* 2004, **804**(2):327-335.

46. Diogo MM, Queiroz JA, Prazeres DMF: **Chromatography of plasmid DNA**. *J Chromatogr A* 2005, **1069**(1):3-22.

47. Unsal E, Bahar T, Tuncel M, Tuncel A: **DNA adsorption onto polyethylenimine attached poly(p-chloromethylstyrene) beads**. *J Chromatogr A* 2000, **898**(2):167-177.

48. Ljunglof A, Bergvall P, Bhikhabhai R, Hjorth R: **Direct visualisation of plasmid DNA in individual chromatography adsorbent particles by confocal scanning laser microscopy**. *J Chromatogr A* 1999, **844**(1-2):129-135.

49. Prazeres DMF, Ferreira GNM, Monteiro GA, Cooney CL, Cabral JMS: **Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy: problems and bottlenecks**. *Trends Biotechnol* 1999, **17**(4):169-174.

50. Haber C, Skupsky J, Lee A, Lander R: **Membrane chromatography of DNA: Conformation-induced capacity and selectivity**. *Biotechnol Bioeng* 2004, **88**(1):26-34.

51. Teeters MA, Conrardy SE, Thomas BL, Root TW, Lightfoot EN: **Adsorptive membrane chromatography for purification of plasmid DNA**. *J Chromatogr A* 2003, **989**(1):165-173.

52. Guerrero-German P, Prazeres DMF, Guzman R, Montesinos-Cisneros RM, Tejeda-Mansir A: **Purification of plasmid DNA using tangential flow filtration and tandem anion-exchange membrane chromatography**. *Bioprocess Biosys Eng* 2009, **32**(5):615-623.

53. Grunwald AG, Shields MS: **Plasmid Purification Using Membrane-Based Anion-Exchange Chromatography**. *Anal Biochem* 2001, **296**(1):138-141.

54. Tseng W-C, Ho F-L, Fang T-Y, Suen S-Y: **Effect of alcohol on purification of plasmid DNA using ion-exchange membrane**. *J Membr Sci* 2004, **233**:161-167.

55. Xu Y, Yasin A, Wucherpfennig T, Chou CP: **Enhancing functional expression of heterologous lipase in the periplasm of Escherichia coli**. *World J Microb Biot* 2008, **24**:2827-2835.

56. Gamerman D, Lopez HF: **Markov Chain Monte Carlo: Stochastic Simulation for Baysian Inference**, 2nd Ed. edn: Chapman-Hall/CRC UK; 2006.

57. Teeters MA, Root TW, Lightfoot EN: **Adsorption and desorption behavior of plasmid DNA on ion-exchange membranes: Effect of salt valence and compaction agents**. *J Chromatogr A* 2004, **1036**(1):73-78.

58. Lyubchenko Y, L., Shlyakhtenko L, S.: **Visualization of supercoiled DNA with atomic force microscopy in situ**. *Proc Nat Acad Sci USA* 1997, **94**(2):496-501.

59. Yanisch-Perron C, Vieira J, Messing J: **Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors**. *Gene* 1985, **33**(1):103-119.

60. Gunasekera D, Kemp RG: **Cloning, Sequencing, Expression, and Purification of the C Isozyme of Mouse Phosphofructokinase**. *Protein Express Purif* 1999, **16**(3):448-453.

61. Montgomery DC: **Design and analysis of experiments.** *John Wiley & sons, IncUSA* 2001.

62. Latulippe DR, Zydney AL: **Salt-induced changes in plasmid DNA transmission through ultrafiltration membranes**. *Biotechnol Bioeng* 2008, **99**(2):390-398.

63. Murphy JC, Fox GE, Willson RC: **Enhancement of anion-exchange chromatography of DNA using compaction agents**. *J Chromatogr A* 2003, **984**(2):215-221.

64. Prazeres DMF, Ferreira GNM, Monteiro GA, Cooney CL, Cabral JMS: **Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy: problems and bottlenecks**. *Trends Biotechnol* 1999, **17**:169-174.

65. Freitas SS, Santos JAL, Prazeres DMF: **Optimization of Isopropanol and Ammonium Sulfate Precipitation Steps in the Purification of Plasmid DNA**. *Biotechnol Progr* 2006, **22**:1179-1186.

66. Ferreira GNM, Cabral JMS, Prazeres DMF: **Anion exchange purification of plasmid DNA using expanded bed adsorption**. *Bioseparation* 2000, **9**:1-6.

67. Chandra G, Patel P, Kost TA, Gray JG: **Large-scale purification of plasmid DNA by fast protein liquid chromatography using a Hi-Load Q Sepharose column**. *Anal Biochem* 1992, **203**:169-172.

68. Prazeres DMF, Schluep T, Cooney C: **Preparative purification of supercoiled plasmid DNA using anion-exchange chromatography**. *J Chromatogr A* 1998, **806**(1):31-45.

69. Li L-Z, Liu Y, Sun M-S, Shao Y-M: **Effect of salt on purification of plasmid DNA using size-exclusion chromatography**. *JChromatogr A* 2007, **1139**:228-235.

70. Ballantyne J: **Practical Methods for Supercoiled pDNA Production**. In., vol. 127; 2006: 311-337.

71. Bhikhabhai R: **Plasmid DNA purification using divalent alkaline earth metal ions and two anion exchangers**. *US Patent 6410274 B1* 2002.

72. Semancik JS, Szychowski J: **Enhanced detection of viroid-RNA after selective divalent cation fractionation**. *Analytical Biochemistry* 1983, **135**(2):275-279.

73. Sauer M-L, Kollars B, Geraets R, Sutton F: **Sequential CaCl2, polyethylene glycol precipitation for RNase-free plasmid DNA isolation**. *Analytical Biochemistry* 2008, **380**(2):310-314.

74. Gomes GA, Azevedo AM, Aires-Barros MR, Prazeres DMF: **Purification of plasmid DNA with aqueous two phase systems of PEG 600 and sodium citrate/ammonium sulfate**. *Separation and Purification Technology* 2009, **65**(1):22-30.

75. Molloy MJ, Hall VS, Bailey SI, Griffin KJ, Faulkner J, Uden M: **Effective and robust plasmid topology analysis and the subsequent characterization of the plasmid isoforms thereby observed**. *Nucleic Acids Res* 2004, **32**:e129.

76. Cupillard L, Juillard V, Latour S, Colombet G, Cachet N, Richard S, Blanchard S, Fischer L: **Impact of plasmid supercoiling on the efficacy of a rabies DNA vaccine to protect cats**. *Vaccine* 2005(16):1910-1916.

77. Urthaler J, Buchinger W, Necina R: **Industrial Scale cGMP Purification of Pharmaceutical Grade Plasmid-DNA**. *Chem Eng Technol* 2005, **28**:1408-1420.