NOVEL APPROACHES TO THE PURIFICATION OF PLASMID DNA

FOR THERAPEUTIC APPLICATION

by

Diane Kendall

Thesis submitted for the degree of

Doctor of Philosophy

In

Biochemical Engineering

The Advanced Centre for Biochemical Engineering
Department of Biochemical Engineering
University College London
Torrington Place
London
WC1E 7JE
To Malcolm Lilly and Peter Dunnill,
for inventing the discipline and changing my life.
I like to think that in the not too distant future, this thesis will be read by other Students of Biochemical Engineering. I have thought long and hard about what advice I could impart, but in the end I decided to add my tuppence-worth regarding thesis writing: Set the correct page sizes and margins before you start writing up, it saves a lot of hassle in the end.

Firstly I have to thank my Mother and Father for being themselves, and always being there.

Next on the list, my Supervisor, Gary, for all his help and guidance, and correcting my awful grammar.

Special thanks also to all those who have haunted Huntley Street during my time at UCL, providing invaluable punctuation in the working week in addition to many pearls of wisdom, probably many more than I remember. Cheers guys.

Many thanks also to the Gene Team at UCL, in particular Susanna, Ronan (aka The Cell Paste Leprechaun), Fran, Pat and Leigh, to name but a few. Thanks also to Andy, for all his technical help and advice with CCC.

Also thanks to the BBSRC, for paying to let me in the lab, and for keeping me solvent.

Out of the Department, thanks to Martin, my housemate of 3 years, for introducing me to many of the better points of living in London, and to Jo from ULU Accommodation Office for keeping a roof over my head.

And last, but far from least, many thanks to Nic Murrell for pointing out the obvious, (plus an extra side helping of thanks for correcting my grammar).
Abstract

At the present time there is considerable interest in the development of scaleable and reproducible plasmid DNA purification protocols for vaccine and gene therapy. The work presented in this thesis addresses two of the most significant purification challenges namely, the initial purification of the plasmid DNA from related contaminants in high yield and the subsequent high-resolution separation of different plasmid DNA forms.

The ability of nitrocellulose to selectively bind single stranded DNA (ssDNA), both in powder and sheet membrane form, was initially investigated. In order to maximise the volume of lysate that could be processed per m² membrane area, the use of an integrated unit operation, comprising tangential-flow filtration coupled with the adsorption of contaminants onto nitrocellulose membranes as a single processing step was then examined, and indicated the utility of the operation to adsorb ssDNA and proteins from model solutions. Tangential-flow filtration-adsorption of E.coli lysates containing a plasmid product was shown to decrease the levels of chromosomal DNA contamination by 75% w/w. Total plasmid DNA concentration and supercoiled content of the permeate were virtually identical to those of the feed indicating close to 100 % yield. Results were similar for E.coli lysates containing either a 6.9 kb or a 20 kb plasmid. Significant reductions in RNA, endotoxin and protein levels were also observed.

The use of liquid-liquid countercurrent chromatography (CCC) for the preparative scale fractionation of different forms of plasmid DNA was subsequently examined. Fractionation of supercoiled and open circular plasmid DNA (6.9 kb) could be achieved using a phase system comprising 12.5 % w/w PEG 600 and 18 % w/w K₂HPO₄. Addition of isopropyl alcohol (2 % w/w) was found to be beneficial to the separation by alteration of the phase physical properties. Residual protein, RNA and chromosomal DNA did not co-purify with the plasmid DNA fractions further increasing the purity of the final product. Preparation of lysate prior to loading onto the CCC column by aqueous two-phase partitioning was found to decrease chromosomal contamination by 90 % with 25 % w/w yield loss of plasmid DNA.
CONTENTS

ACKNOWLEDGEMENTS ................................................................. 3

ABSTRACT .................................................................................. 4

CONTENTS .................................................................................. 5

TABLE OF FIGURES ................................................................. 13

TABLES ..................................................................................... 18

1. INTRODUCTION ...................................................................... 18

1.1 Significance and Conceptual Challenges ......................... 18

1.1.1 Use and formulation of pharmaceutical grade plasmid ....... 20
1.1.2 Considerations for purification strategies ....................... 22

1.2 Molecular structure and properties of Nucleic Acids and other cellular constituents. .............................................. 22

1.2.1 The molecular structure of DNA ........................................... 22
1.2.1.1 Primary and secondary structure of DNA ......................... 22
1.2.1.2 Tertiary structure, function and properties of DNA .......... 25
1.2.2 Structure and properties of other cellular contaminants .... 26
1.2.3 Typical product specifications ............................................. 28

1.3 The manufacturing process for plasmid DNA .................. 30

1.3.1 Production and primary recovery operations ................... 30
1.3.1.1 Fermentation ................................................................. 30
1.3.1.2 Alkaline Lysis ............................................................. 31
1.3.2 Downstream purification
1.3.2.1 Intermediate purification
1.3.2.2 High-resolution purification
1.3.3 Published process sequences

1.4 Background to the novel process techniques investigated
1.4.1 Theory of hydrophobic interactions and selective adsorption of contaminants
1.4.1.1 Theory of hydrophobic interactions
1.4.1.2 Selective adsorption of contaminants
1.4.2 Countercurrent chromatography
1.4.2.1 The nature of aqueous two-phase systems
1.4.2.2 Examples of separations using ATPS
1.4.2.3 Countercurrent chromatography
1.4.2.3.1 The Archemedian screw principle
1.4.2.3.2 Early countercurrent chromatography techniques
1.4.2.4 CCC designs employing planetary motion
1.4.2.4.1 Multilayer coil planet centrifuge (J-Type)
1.4.2.4.1.1 Phase distribution within J-type CCC and considerations for mode of operation
1.4.2.4.1.2 Factors affecting the retention of the stationary phase
1.4.2.4.2 Cross-axis synchronous flow through coil planet centrifuge
1.4.2.4.3 Toroidal coil centrifuge
1.4.2.4.4 Selection of countercurrent chromatography system for bio-separations
1.4.2.5 Scale up of countercurrent chromatography

1.5 Aims of the project
2. MATERIALS AND METHODS......................................................... 75

2.1 Preparation of commonly used buffers and model solutions......................................................... 75

2.2 Preparation of plasmid containing lysate................. 76

2.2.1 Plasmid and culture conditions......................................................... 76
2.2.2 Alkaline lysis of E. coli cells and lysate clarification ............. 78

2.3 Primary purification of plasmid DNA by batch adsorption using nitrocellulose powder................................. 79

2.3.1 An alternate protocol for the preparation of plasmid containing lysate......................................................... 79
2.3.2 Preparation of nitrocellulose powder................................. 79
  2.3.2.1 Room temperature grinding and washing......................... 80
  2.3.2.2 High temperature washing protocol......................... 80
2.3.3 Stirred batch adsorption experiments................................. 81
2.3.4 Regeneration of nitrocellulose powder................................. 81

2.4 Primary purification of plasmid DNA by integrated filtration-adsorption............................................. 82

2.4.1 Pre-purification of lysate by selective calcium chloride precipitation of contaminants................................. 82
2.4.2 Membrane modules and filtration conditions................................. 82
  2.4.2.1 Membrane types and equilibration................................. 82
  2.4.2.2 Dead-end filtration-adsorption................................. 83
  2.4.2.3 Tangential-flow filtration-adsorption................................. 83
2.4.3 Regeneration protocols for the nitrocellulose membrane........... 85
2.5 Plasmid purification by countercurrent chromatography

2.5.1 Concentration and preparation of feed

2.5.2 Composition and separation of aqueous two-phase systems

2.5.2.1 Construction of binodal phase diagrams

2.5.3 Preparation of feed by aqueous two-phase extraction

2.5.4 Equilibrium partitioning of plasmid DNA in ATPS

2.5.5 CCC instrumentation and operation

2.6 Analytical techniques

2.6.1 Plasmid DNA analysis by agarose gel electrophoresis

2.6.1.1 Precipitation of nucleic acids by isopropanol precipitation

2.6.1.2 Agarose gel electrophoresis conditions

2.6.2 Chromosomal DNA analysis

2.6.2.1 Preparation and labelling of the chromosomal DNA probe

2.6.2.2 Chromosomal DNA analysis following nitrocellulose filtration

2.6.2.3 Chromosomal DNA analysis following CCC

2.6.3 Quantitation of purified DNA samples by measuring absorbance at 260 nm

2.6.4 Quantitation of un-purified DNA samples using the picogreen assay

2.6.5 BioRad DC protein assay

2.6.6 Endotoxin analysis (Limulus amebocyte lysate gel clot assay)

2.6.7 Estimation of solids content by measuring absorbance at 600 nm
3. PRIMARY PURIFICATION OF PLASMID DNA BY BATCH ADSORPTION USING NITROCELLULOSE POWDER........... 101

3.1 Aims and introduction................................................................. 101

3.2 Batch adsorption of ssDNA from model solutions and plasmid containing liquors................................. 102

3.2.1 Batch adsorption of ssDNA from a model solution............... 102
3.2.2 Capacity of nitrocellulose to adsorb chromosomal DNA from partially clarified lysate.............................. 103
3.2.3 Regeneration of nitrocellulose powder................................. 105
3.2.4 Discussion.............................................................................. 108

2. PRIMARY PURIFICATION OF PLASMID DNA BY INTEGRATED MEMBRANE FILTRATION-ADSORPTION... 111

4.1 Aims and introduction................................................................. 111

4.2 Filtration-adsorption of model ssDNA solutions: determination of membrane capacity and effect of filtration operating mode..................................................... 112

4.2.1 Comparison of cellulose acetate and nitrocellulose membranes ...................................................................... 112
4.2.2 Adsorptive properties of nitrocellulose membrane at varying retentate recirculation rates............................................. 114
4.2.3 Discussion.................................................................................. 118
4.3 Tangential-flow filtration-adsorption of partially clarified *E. coli* lysates .............................................................. 120

4.3.1 Filtration-adsorption of partially clarified *E. coli* lysates containing a 6.9 kb plasmid................................................................................ 120

4.3.2 Effect of plasmid size on plasmid DNA recovery .................... 124

4.3.3 Membrane cleaning and regeneration ...................................... 125

4.3.4 Discussion................................................................................. 128

4.4 Effect of lysate pre-purification on membrane capacity .............. 130

4.4.1 Effect of RNAse addition during lysis .................................... 130

4.4.2 Effect of CaCl₂ precipitation .................................................... 133

4.4.3 Discussion................................................................................... 135

4.5 Conclusions................................................................................ 136

5. COUNTERCURRENT CHROMATOGRAPHY FOR THE HIGH RESOLUTION PURIFICATION OF PLASMID DNA............. 139

5.1 Aims and introduction................................................................. 139

5.2 Choice of phase system and hydrodynamic properties ................ 140

5.2.1 Phase separation and equilibrium plasmid distribution on various ATPS................................................................. 140

5.2.2 Stationary phase retention........................................................ 143

5.3 Initial results on CCC fractionation of plasmid DNA... 146
5.3.1 CCC fractionations carried out at 800 rpm.................................146  
5.3.2 CCC fractionations carried out at 600 rpm.................................149  
5.3.3 Discussion ....................................................................................154  

5.4 Effect of feed composition and solute loading..............156  
5.4.1 Effect of variation of plasmid concentration.......................156  
5.4.2 Effect of buffer composition.......................................................157  
5.4.3 Discussion ....................................................................................161  

5.5 Use of plasmid feed prepared by aqueous phase extraction of a crude \textit{E. coli} lysate.........................163  
5.5.1 Preparation of plasmid feed by batch aqueous phase extraction of crude \textit{E. coli} lysate.................................................................163  
5.5.2 CCC using plasmid feed prepared by aqueous phase extraction........................................................................164  
5.5.3 Discussion ....................................................................................167  

5.6 Conclusions.....................................................................................169  

6. Final discussion and conclusions .........................172  
6.1 Final discussion.................................................................................172  
6.1.1 Initial economic evaluation of process options...............173  
6.1.2 Evaluation of a novel process for the purification of plasmid DNA........................................................................177  
6.2 Future Work.....................................................................................179
6.2.1 Studies on the selective adsorption of contaminants.........179
6.2.2 Studies on countercurrent chromatography and aqueous two-
phase extraction.................................................................180

APPENDICES.............................................................................183

APPENDIX A.............................................................................184
Initial economic evaluation of the process options................184

APPENDIX B.............................................................................186
Supplementary CCC chromatograms........................................186

7. NOMENCLATURE.................................................................189

8. REFERENCES.........................................................................190
**Table of Figures**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Diagrammatic representation of the primary structure of DNA</td>
</tr>
<tr>
<td>1.2</td>
<td>Diagrammatic representation of the secondary structure of DNA</td>
</tr>
<tr>
<td>1.3</td>
<td>Overview of the steps used in whole process sequences for plasmid purification published to date</td>
</tr>
<tr>
<td>1.4</td>
<td>Demonstration of the 'bubble and bead' principle to illustrate the Archimedean screw effect</td>
</tr>
<tr>
<td>1.5</td>
<td>Simplified diagram to illustrate the construction and planetary motion of the J-type CCC</td>
</tr>
<tr>
<td>1.6</td>
<td>Illustration of the distribution of the mixing and settling zones present during rotation of CCC column on a twin bobbin machine</td>
</tr>
<tr>
<td>1.7</td>
<td>Simplified diagram of a type X cross-axis coil planet centrifuge</td>
</tr>
<tr>
<td>1.8</td>
<td>Simplified diagram of a type XL cross-axis coil planet centrifuge</td>
</tr>
<tr>
<td>2.1</td>
<td>DOT, OUR and CER profiles for a DH5α pSVβ 75 L fermentation</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic diagram of the tangential-flow filtration rig used and detail of the membrane housing</td>
</tr>
<tr>
<td>2.3</td>
<td>Typical calibration curve for Picogreen assay</td>
</tr>
<tr>
<td>2.4</td>
<td>Typical calibration curve for BioRad DC protein assay</td>
</tr>
<tr>
<td>3.1</td>
<td>Agarose gel analysis of feed and incubated lysate supernatant from batch adsorption experiments</td>
</tr>
<tr>
<td>3.2</td>
<td>Agarose gel analysis of feed and incubated lysate supernatant from batch adsorption experiments with fresh and regenerated nitrocellulose powder</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Dead-end filtration-adsorption of a solution of pure ssDNA through cellulose acetate or nitrocellulose membranes of pore size 0.45μm</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Tangential-flow filtration-adsorption of a solution of pure ssDNA through a nitrocellulose membrane</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Tangential-flow filtration-adsorption of a solution of BSA through a nitrocellulose membrane</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Agarose gel analysis of feed and permeate fractions from a tangential-flow filtration-adsorption run using a partially clarified containing a 6.9 kb plasmid</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Southern slot-blot analyses of feed and permeate fractions from a tangential-flow filtration-adsorption run using a partially clarified lysate containing a 6.9 kb plasmid</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>Permeate flux and composition during the tangential-flow filtration-adsorption of a partially clarified E. coli lysate containing a 6.9 kb plasmid</td>
</tr>
<tr>
<td>Figure 4.7</td>
<td>Agarose gel analysis of feed and permeate fractions from a tangential-flow filtration-adsorption run using a partially clarified E. coli lysate containing a 20 kb plasmid</td>
</tr>
<tr>
<td>Figure 4.8</td>
<td>Agarose gel analyses of feed and permeate fractions from three sequential tangential-flow filtration-adsorption runs, with two regeneration stages</td>
</tr>
<tr>
<td>Figure 4.9</td>
<td>Agarose gel analysis of feed and permeate fractions from a tangential-flow filtration-adsorption of a partially clarified lysate containing a 6.9 kb plasmid obtained from cell paste from the 75 L fermentation lysed in the presence or absence of RNAse</td>
</tr>
<tr>
<td>Figure 4.10</td>
<td>Agarose gel analyses of feed and permeate fractions from two consecutive tangential-flow filtration-adsorption runs using lysate containing a 6.9 kb plasmid, previously pre-purified by CaCl₂ precipitation</td>
</tr>
</tbody>
</table>
Figure 5.1 Phase diagrams for various phase systems.................................144
Figure 5.2 CCC chromatograms showing plasmid DNA elution for various ATPS at a mobile phase flow rate of 0.5 mL min$^{-1}$ and CCC operation at 800 rpm.................................................................148
Figure 5.3 Agarose gel analysis of feed and selected fractions collected during a CCC run carried out using a phase system comprised of 12.5 % w/w PEG 600 and 18 % w/w K$_2$HPO$_4$ at 600 rpm and a mobile phase flow rate of 0.5 mL min$^{-1}$.................................................................151
Figure 5.4 CCC chromatogram of plasmid DNA eluted during a CCC run carried out using a phase system comprised of 12.5 % w/w PEG 600 and 18 % w/w K$_2$HPO$_4$ at 600 rpm and a mobile phase flow rate of 0.5 mL min$^{-1}$.................................................................151
Figure 5.5 CCC chromatogram of plasmid DNA eluted during CCC carried out at a temperature of 40°C using a phase system comprised of 12.5 % w/w PEG 600 and 18 % w/w K$_2$HPO$_4$ at 600 rpm and a mobile phase flow rate of 0.5 mL min$^{-1}$.................................................................153
Figure 5.6 CCC chromatograms of plasmid DNA eluted during a selection of runs using feed prepared with different buffer compositions and concentration of plasmid.................................................................159
Figure 5.7 Southern slot-blot analysis of different plasmid DNA feed preparations for CCC..................................................................................................................161
Figure 5.8 Southern blot of analytical gel for feed and selected fractions collected from the stationary PEG phase following a CCC run carried out with feed prepared by precipitating with IPA and resuspension in TE buffer .................................................................161
Figure 5.9 CCC chromatogram of plasmid DNA eluted using a phase system comprised of 2 % w/w IPA 12.5 % w/w PEG 600 18 % w/w K$_2$HPO$_4$. CCC carried out at 600 rpm with mobile phase flow rate 0.5 mL min$^{-1}$........................................................................165
Figure 5.10 Agarose gel analysis of fractions collected from the Feed and eluted fractions collected during CCC carried out using a phase system comprised of 2 % w/w IPA 12.5 % w/w PEG 600 18 % w/w K₂HPO₄. CCC carried out at 600 rpm with mobile phase flow rate 0.5 mL min⁻¹............................................................................................166

Figure 5.11 Southern slot-blot analysis of feed and eluted fractions containing plasmid collected during CCC using a phase system comprised of 2 % w/w IPA 12.5 % w/w PEG 600 18 % w/w K₂HPO₄. CCC carried out at 600 rpm with mobile phase flow rate 0.5 mL min⁻¹.............167

Figure 6.1 Proposed novel plasmid purification process and typical conventional process.................................................................178

Appendix CCC chromatogram of plasmid DNA eluted during CCC carried out B.1 using a phase system comprised of 12.5% w/w PEG 600 (stationary phase) and 18.5% w/w K₂HPO₄, 0.15 M NaCl (mobile phase) at 600 rpm and a mobile phase flow rate of 0.5 mL min⁻¹..........................186

Appendix CCC chromatogram of plasmid DNA eluted during a CCC runs B.2 carried out with a bobbin rotational speed 600 rpm and a mobile phase flow rate of 0.5 mL min⁻¹ as described in Section 2.5.5 were used in both experiments. 1 mL feed injected at the start of the CCC run, before coil had been equilibrated.................................187

Appendix CCC chromatogram of plasmid DNA eluted using a phase system B.3 comprised of 2 % w/w IPA 12.5 % w/w PEG 600 (stationary phase) 18.5 % w/w K₂HPO₄ (mobile phase). CCC carried out at 600 rpm with mobile phase flow rate 0.5 mL min⁻¹. Feed prepared as the lower phase of a phase system comprising 12.5 % w/w PEG 600, 16 % w/w K₂HPO₄ (as 40 % w/w stock solution) 5 % w/w IPA and 37.5 % w/w E. coli lysate containing plasmid. 10 mL feed injected at the start of the CCC run, before coil had been equilibrated.........................................................188
### Tables

**Table 1.1** Release specification for Allovectin-7 plasmid DNA ........................................... 29

**Table 2.1** Composition of buffers commonly used in this work ............................................. 75

**Table 2.2** Composition of selected phase systems used for CCC experiments ........................................ 87

**Table 2.3** Summary of the experiments carried out using the countercurrent chromatograph ........................................ 91

**Table 5.1** Equilibrium plasmid partition coefficients, phase separation times and phase volume ratios for selected aqueous two-phase systems ........................................ 142

**Table 5.2** Equilibrium plasmid partition coefficient, phase separation times and stationary phase retention of selected aqueous two-phase systems ........................................ 145

**Table 6.1** Comparison of the novel plasmid DNA purification techniques investigated ........................................ 172
1. Introduction

1.1 Significance and conceptual challenges

1.1.1 Use and formulation of pharmaceutical grade plasmid DNA

At present there is considerable interest in the development of new DNA based therapeutics, many of which are currently in early stage clinical trials (Hasan et al. 1999; Mountain 2000; Mhashilkar et al. 2001). DNA vaccines against conditions such as HIV, various cancers and influenza are under development at this time (Hasan et al. 1999; Shroff et al. 1999; Koide et al. 2000; Mhashilkar et al. 2001). Other therapeutic possibilities include gene therapy products aimed at correcting at source the genetic defects involved in inherited diseases (Taylor 1998).

Consequentially there is also a need for robust process scale protocols for the production of large quantities of pure DNA products at a reasonable cost. Much is expected from this new generation of vaccines, which utilise plasmid DNA encoding antigenic proteins (expressed by the patients protein expression system), for vaccination. Such DNA vaccines will be less heat labile than conventional protein vaccines, (Hasan et al. 1999) thus eliminating the need for cold chain transportation and reducing costs. Early indications from clinical trials, following DNA vaccination with plasmid encoding antigen (Tighe et al. 1998; Hassett et al. 2000; Powderject Pharmaceutical PLC. 2001; Vical Incorporated. 2001) indicate long-lived protection equal to, or exceeding that resulting from conventional vaccination. Due to the similarity of the DNA vaccines at molecular level, which differ only in the sequence of the of the vaccine gene inserted into the vector, it should be possible to use generic growth and purification processes for different DNA vaccines (Robinson et al. 1997).
There are several delivery systems that may be used for DNA vaccine, or gene therapy applications (Mhashilkar et al. 2001). Replication deficient recombinant retrovirus, adenovirus or adeno-associated viral vectors capable of accommodating up to 9 kb of genetic information are being developed. These are efficient with regard to gene transfer and are effective at reproducing themselves, but may cause an immunogenic response within the patient which is undesirable (Crystal 1995). The recent deaths of patients involved in clinical trials using such vectors has caused concern (Fox 1999). In order to circumvent the problems associated with viral vectors other researchers are designing new methods for introducing DNA into cells. Plasmid DNA-liposome complexes are under development (Crystal 1995), and in many cases naked plasmid DNA may be used (Horn et al. 1995; Mahvi et al. 1997; Robinson et al. 1997; Hasan et al. 1999). In 2001, 63% of protocols approved by the US Recombinant Advisory Committee for the delivery of therapeutic DNA to the target tissue employed retroviral vectors. Adenoviral vectors were utilised in 16% of approved protocols, liposomes accounted for 13 %, adeno-associated vectors 2 % and the remaining 6 % of protocols employed a variety of vector systems, most of which are based around the injection of naked DNA (Mhashilkar et al. 2001). If the amount of information to be introduced increases substantially it may be necessary to move to, much larger artificially constructed chromosomes (Willard 1998; Brown et al. 2000). The final form of the DNA product will depend on the method and purpose of administration, and may affect processes used for plasmid DNA purification.

This project focuses on the downstream processing of naked plasmid DNA and is concerned with investigation and development of two novel purification concepts. The selective adsorption of single stranded chromosomal DNA and other cellular contaminants from the double stranded plasmid DNA model product was investigated as a primary purification step. Here the aim was to increase the capacity of conventional chromatographic techniques for the
subsequent high-resolution purification of plasmid DNA. In addition, a novel high-resolution technique utilising countercurrent chromatography for the separation of supercoiled plasmid DNA from contaminant open circular plasmid DNA and chromosomal DNA was established. This approach has the potential to circumvent many of the problems associated with conventional purification techniques such as high cost of matrix, low capacity for plasmid DNA, and processing difficulties associated with the loading of the high viscosity process stream onto conventional packed columns.

1.1.2. Considerations for purification strategies

Therapies based on proteins produced through recombinant DNA technology have been established for some time (Watson et al. 1992). Many methodologies for the purification of such proteins on an industrial scale are well documented and rationalised, with a wide range of chromatography matrices for high-resolution protein separations being commercially available. In contrast, although the structure of DNA is well known, and many methodologies exist for the production, manipulation and purification of DNA at the laboratory scale, most of these are not readily amenable to scale up (Sambrook et al. 1989).

Another potential problem for the purification of DNA vectors is the shear sensitivity of large nucleic acid molecules, which could result in high product losses. The shear sensitivity of plasmid DNA increases sharply with the size of the molecule and decreasing ionic strength (Levy et al. 1999a; Levy et al. 1999b). Chromosomal DNA from the cell strain used in the production of the plasmid product will also be very shear sensitive. The degradation of such molecules in the process stream may result in contaminant species of similar size as the plasmid product, which will act in a similar manner during subsequent purification steps, and therefore be difficult to remove (Levy et al. 2000c).
1. Introduction

DNA molecules with the same molecular mass as a given protein have a significantly larger dynamic volume in comparison. Typical particle diameters for a 3.8 kb plasmid (approximately 2500 kDa) in 20 mM Tris-HCl 1mM EDTA pH 7.6 buffer are in the range 0.2 – 0.3 μm as measured by visualisation with atomic force microscopy (Lyubchenko and Shlyakhtenko 1997). This is 1 – 2 orders of magnitude greater than typical for proteins, which are generally retained by ultrafiltration membranes of pore size 1 – 20 nm according to molecular weight (Moses and Cape 1994). Over a decade ago Ellegren and co-workers (Ellegren and Laas 1989) demonstrated that double stranded DNA fragments of only 800 bp (approximately 530 kDa) were excluded from 100 nm diameter pores during size exclusion chromatography on Superose 6. The limited diffusion of DNA molecules into the pores of chromatographic matrices reduces the capacity of the matrix for plasmid DNA and contaminating chromosomal DNA fragments. Traditional anion exchange matrices are reported to typically have capacities 1000 fold lower for DNA than for proteins (Collins et al.). Lyddiatt and co workers report that for 50 nm diameter nano-particulates (i.e. viral particles or small plasmids) surface binding alone can be calculated to account for the capacity of 100 nm spherical adsorbent particles (Lyddiatt and O'Sullivan 1998).

Due to the therapeutic application of plasmid DNA products highly pure plasmid DNA, intact and in the supercoiled form is required (Bonilla et al. 1991; Middaugh et al. 1998; Bergan et al. 2000). Contaminating fragments of host chromosomal DNA must be removed (Marquet et al. 1997b; Prazeres et al. 1999; Levy et al. 2000c). Ribonucleic acids (RNA) are an essential part of the cellular machinery, and are found in great quantities in cells. The profusion of RNA molecules together with their chemical, and for larger species, physical resemblance to the plasmid complicates purification (the similarities will be considered in more detail in Section 1.2.2). Endotoxins, lipopolysaccharides shed from the bacterial cell wall, although chemically quite different to the plasmid product, have often been found to co-purify with plasmid DNA (QIAGEN 07/1999). Due to the antigenic nature of endotoxin
molecules, it is particularly important to ensure pharmaceutical products are essentially free of endotoxin contamination. In addition host cell proteins along with cellular debris must also be removed. These are generally quite different chemically and in size from plasmid DNA, thus are likely to be the least problematic. The structure and properties of plasmid DNA and the major contaminants found in the process stream will be considered in more detail in Section 1.2.

1.2 Molecular structure and properties of nucleic acids and other cellular constituents

1.2.1 The molecular structure of DNA

1.2.1.1 Primary and secondary structure of DNA

Watson and Crick proposed the double helix structure of DNA in 1953 (Watson and Crick 1953). DNA can be thought of as a polymer comprised of four types of very similar monomers known as nucleotides. Nucleotides consist of a deoxyribose sugar and phosphate (which form the 'backbone' of the DNA strand) plus one of four bases (adenine, guanine, thymidine or cytosine). The order in which the bases appear along the DNA strand forms the basis of the genetic code. The native form of DNA is the double helix structure, which consists of two strands covalently through specific hydrogen bond formation between the bases placed opposite each other in the helix (Watson and Crick 1953; Matthews and van-Holde 1990). The bases are found in the centre of the double helix sandwiched between the phosphate-sugar backbone. The molecular structure of the four bases is such that hydrogen bonds can only form between specific pairs: Guanidine and cytosine will form three hydrogen bonds between them, adenine and thymidine are capable of forming two hydrogen bonds (Watson and Crick
1. Introduction

1953; Matthews and van-Holde 1990). It is this specificity which allows for the accurate replication of the DNA strands during cell division.

![Diagram of DNA structure](image)

**Figure 1.1.** Diagrammatic representation of the primary structure of DNA

The basic molecular structure of a nucleotide, and the two-dimensional orientation in the DNA polymer is illustrated in Figure 1.1. The shape, and interactions between the nucleotide monomers in the DNA strands are such that the DNA chain will form the familiar three dimensional double helix structure (Figure 1.2), with one turn of the helix completed every 10 nucleotides or 34 Å (Watson and Crick 1953).
Under some circumstances the double stranded helix may become denatured to yield two single strands of DNA (ssDNA). For example, *in vivo* local separation of the strands may be induced to facilitate transcription or translation by the appropriate enzyme complexes (Matthews and van-Holde 1990). *In vitro* DNA can be denatured through heating or by exposure to high pH (Birnboim 1983; Sambrook et al. 1989). In most cases this denaturation is reversible, the DNA strands will re-anneal to the double stranded form (dsDNA), guided by the base specificity, once suitable conditions return. At extremely high pH (pH > 12) this effect may be irreversible (Birnboim 1983).
1. Introduction

1.2.1.2 Tertiary structure, function and properties of DNA

The tertiary structure of DNA varies (Matthews and van-Holde 1990). In eukaryotes the majority of DNA occurs as non-circular chromosomes, whereas prokaryotic cells contain a large circular chromosome, and may also have smaller circular DNA molecules known as plasmids. It is these plasmids, once re-engineered in the laboratory to contain the nucleotide sequence encoding the required information, which may be transformed back into a suitable bacterial host, grown up and purified for use as gene therapy vectors or DNA vaccines.

*In vivo*, the double helix of the DNA molecule will twist around itself, so that the DNA assumes a supercoiled form. Enzymes called topoisomerases are responsible for introducing (or removing) the extra twists in the DNA strand that causes it to assume the supercoiled configuration (Matthews and van-Holde 1990). The degree of supercoiling seen also varies, chromosomes will become more tightly coiled just prior to cell division, but adapt a more relaxed configuration during normal metabolism of the cell to facilitate access of enzymes to sites on the DNA. *In vitro*, a greater degree of supercoiling has been artificially induced by the inclusion of 160 mM NaCl to the plasmid suspension buffer (Lyubchenko and Shlyakhtenko 1997).

The majority of plasmid DNA isolated from the host cell will be in the supercoiled (SC) form. Plasmids may also be present as dimers (due to the two new plasmids becoming linked following replication) and the covalently closed open circular plasmid form (Matthews and van-Holde 1990). During processing, shear and chemical degradation may cause nicks in one of the DNA strands, which will cause the plasmid to relax from the SC form to open circular (OC) plasmid form. If both DNA strands are broken in the same place, linear plasmid forms will result (Levy et al. 1999b). It is desirable to remove OC and linear forms of plasmid from the final product to ensure the potency
and stability of the final dosage form (Bonilla et al. 1991; Middaugh et al. 1998; Bergan et al. 2000).

DNA molecules are negatively charged (Matthews and van-Holde 1990). This is the basis of separation by anion exchange. As the charge carried by each nucleotide is the same, the overall charge correlates directly to the size of the DNA molecule. The backbone of the helix is hydrophilic, whereas the bases at the centre of the helix are hydrophobic. Relaxed or linear forms of dsDNA will tend to be more hydrophilic than tightly supercoiled forms, where the hydrophobic bases may become partially exposed due to the contortion of the DNA molecule. Lengths of ssDNA where the bases are completely exposed are the most hydrophobic form of DNA (Mao et al. 1998; Prazeres et al. 1998; Diogo et al. 1999). The shear sensitivity of DNA molecules increases with size, with plasmids of 29 kb in size having been found to be readily degraded by relatively moderate shear forces of a magnitude commonly found in process equipment (Levy et al. 1999b; Levy et al. 2000c). Care must be taken with the design of downstream processes to ensure the shear forces present are kept below a critical level, both to ensure the integrity of larger product molecules and to minimise shear of large contaminant chromosomal DNA molecules during early stages of the process (Levy et al. 2000c).

1.2.2 Structure and properties of other cellular contaminants

Plasmid DNA is an intracellular product of the host cell, therefore it is to be expected that a variety of cellular contaminants will be present in the process stream. The structure and properties of chromosomal DNA has been considered in Section 1.2.1. The same features will now be considered briefly for other major contaminants of plasmid containing process streams.

RNA is a major contaminant in plasmid containing process streams. The basic structure of is similar to that of DNA except that (I) the base uracil replaces thymidine, (II) the backbone of the molecule contains ribose sugar
instead of deoxyribose and, (III) RNA molecules are generally single stranded (except for the hairpin loop structures formed within the strand by the transfer RNA) (Matthews and van-Holde 1990). The chemical and structural similarities, along with the profusion of RNA within cells, make this contaminant difficult to separate from plasmid DNA.

There are three main types of RNA. Messenger RNA (mRNA) is responsible for transferring the DNA encoded message from the chromosome to the ribosome's, which use the mRNA template to synthesise the encoded polypeptide. The ribosome's themselves contain ribosomal RNA (rRNA). The third main type of RNA is transfer RNA (tRNA) which carries specific amino acids and will recognise the sequence on the mRNA in the ribosome / mRNA complex; thus the correct sequence of amino acids is constructed (Matthews and van-Holde 1990).

Endotoxins are lipopolysaccharide molecules shed from the surface of the bacterial cell wall (Petsch and Anspach 2000). They possess both hydrophobic and hydrophilic regions (Petsch and Anspach 2000) and so tend to form micelle structures, which are similar in size and density to a large DNA molecule, and will often interact in a similar way to DNA during anion exchange chromatography. This particular contaminant, which is especially undesirable due to the danger of eliciting an immunogenic response, can thus also be particularly difficult to remove (QIAGEN 07/1999; Green et al. 1997; Prazeres et al. 1998).

Proteins tend to be globular molecules, and can have widely varying chemical and physical properties. Proteins are made up of a chain of amino acids, of which there are 20 different common naturally occurring forms (Matthews and van-Holde 1990)). The structure of a protein can be thought of as the primary sequence of amino acids, which then forms a secondary structure (α helix or β sheet). Following the manufacture of the amino acid sequence upon the cells ribosome's, and formation of the secondary structure, the protein is
folded into a complex tertiary globular structure, the shape being fixed by disulphide bonds. The association of folded protein sub-units with other protein sub-units, (and often specific metal ions), results in a quaternary structure for some proteins. As discussed in Section 1.1.2 the overall size of a 'typical' protein molecule is much smaller than plasmid DNA of similar molecular weight. Also, the charge carried varies between amino acids, and given the variations possible in the arrangement of the amino acid monomers to make up a protein, the net charge a particular protein may carry is highly variable.

Other contaminants which must be removed include lipids, cellular debris and media components. However, there is no evidence from the literature to date that any of these have been found problematic to purify from plasmid DNA, and the importance is therefore considered negligible compared to the main contaminants already discussed.

1.2.3 Typical product specifications

Given the therapeutic nature of plasmid DNA products, the international regulatory authorities will set stringent standards for the maximum allowable levels of cellular contaminants, such as chromosomal DNA, RNA, proteins and endotoxins present in drug formulations. These, in turn, will be dependent on the dose to be administered. The acceptable cellular DNA contamination has been set at 100 pg per dose (Marquet et al. 1997b). Maintaining such levels will be very challenging when quantities of plasmid in the order of 100 μg are to be injected (Marquet et al. 1995). For example, the release specifications for plasmid DNA to be used in a cancer clinical trial have been stipulated for the various contaminants, RNA, protein, chromosomal DNA and endotoxin, and are shown in Table 1.1 (Marquet et al. 1997a). As a rule of thumb 1EU can be taken as equivalent to 100 pg endotoxin (Petsch et al. 1998).
Although the removal of all contaminants must be considered, the greatest challenge at this stage remains the removal of chromosomal DNA, given the similarities of the product and contaminant molecules. The SC plasmid form is also the type form required for the final product. Hence it is desirable to purify, if possible, OC and linear forms of plasmid from the SC plasmid product to ensure the potency and stability of the final dosage form (Bonilla et al. 1991; Middaugh et al. 1998; Bergan et al. 2000).

**Table 1.1.** Release specification for Allovectin-7 plasmid DNA. Compiled from Marquet et al. 1997a. See Figure 1.3 for the purification process associated with this particular product Horn et al. 1995.

<table>
<thead>
<tr>
<th>Test</th>
<th>Specification</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Clear colourless solution</td>
<td>Visual observation</td>
</tr>
<tr>
<td>Size of plasmid product</td>
<td>Approximate size predicted from plasmid map</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>Circular plasmid DNA</td>
<td>≥ 95 % (nucleic acids)</td>
<td>Agarose gel electrophoresis and anion exchange HPLC</td>
</tr>
<tr>
<td>Chromosomal DNA</td>
<td>&lt; 0.01 μg μg⁻¹ plasmid DNA</td>
<td>Southern blot</td>
</tr>
<tr>
<td>Protein</td>
<td>Undetectable</td>
<td>BCA protein assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Undetectable</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>Residual ethanol</td>
<td>≤ 250 ppm</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>&lt; 0.1 EU μg⁻¹ plasmid DNA</td>
<td>LAL assay</td>
</tr>
<tr>
<td>Sterility</td>
<td>No growth through 14 days</td>
<td>USP</td>
</tr>
<tr>
<td>Gene expression</td>
<td>Expression similar to working reference standard</td>
<td>In vitro transfection / FACS assay</td>
</tr>
<tr>
<td>General safety test</td>
<td>Per USP</td>
<td>Per USP</td>
</tr>
</tbody>
</table>
1.3 The manufacturing process for plasmid DNA

1.3.1 Production and primary recovery operations

1.3.1.1 Fermentation

*Eschericia coli* is by far the most common choice to date as host for the production of plasmid DNA. The organism is well characterised and there exist a multitude of strains with various characteristics that may be desirable. *E. coli* grows quickly and is amenable to industrial scale fermentation. Due to the large amount of information that it may be necessary to include in a gene therapy vector, it may ultimately be necessary to use a vector other than plasmid, such as Human Artificial Chromosomes (Taylor 1998; Willard 1998; Brown et al. 2000). However, plasmid DNA produced using *E. coli* as host is a good model system given products under development at this time (Mhashilkar et al. 2001).

To ensure that the maximum amount of plasmid product is produced during fermentation, various strategies have been developed. It is desirable that each cell produces the highest possible number of plasmids. As the production of plasmid DNA will place a metabolic burden on the host cells, those which have 'lost' the plasmid during growth will grow more quickly than cells containing plasmid (Watson et al. 1992). Thus it is desirable to select for those cells which contain plasmid. An antibiotic resistance marker is commonly incorporated onto the plasmid for this reason and the corresponding antibiotic introduced into the medium to select for plasmid containing cells (Sambrook et al. 1989; Watson et al. 1992). Due to concerns regarding the spread of antibiotic resistance and potential allergic reactions, care is needed to ensure antibiotics utilised in the fermentation stages are removed downstream. Alternative antibiotic free strategies, such as the ORT vector which enables de-repression of an essential chromosomal gene hence
exerting selection for cells containing the ORT vector, have also been
developed (Williams et al. 1998). Some plasmids naturally occur in greater
numbers in their host cells than do other plasmids. Such high copy number
plasmids are therefore desirable as a base vector for plasmid DNA
therapeutic products, in order to maximise yield of plasmid (Watson et al.
1992). Plasmid production may be induced near the end of the fermentation,
using various techniques, for example through the use of a heat inducible
origin of replication for the plasmid or by chloramphenicol induction
(Sambrook et al. 1989; Chen et al. 1997). By optimising the combination of
host strain, vector and growth conditions (i.e. fed batch versus batch
fermentation and the use of defined as opposed to complex culture media)
plasmid yield can be enhanced. Yields as high as 220 µg plasmid DNA mL⁻¹
fermentation broth have been reported (Lahijani et al. 1996). A high titre of
plasmid from the fermentation, in addition to increasing the profitability of the
process through increased yield, also has the effect of decreasing the relative
level of contaminants. It is therefore essential that reproducibly high plasmid
titres be achieved during fermentation for the success of the downstream
processes, both in terms of the purification achieved and for the maximum
quantity of end product.

1.3.1.2 Alkaline Lysis

As plasmid DNA is intracellular, the plasmid product must be released from
the cells following harvest from the fermenter, before purification can take
place. Due to the shear sensitivity of both the plasmid and chromosomal
DNA, mechanical disruption techniques commonly used for the release of
recombinant protein from cells are not effective as too much plasmid DNA
product is lost (Carlson et al. 1995). The alkaline lysis method of Birnboim
and Doly (Birnboim and Doly 1979; Birnboim 1983), has the advantage of
being a low shear operation, and also facilitates the purification due to the
precipitation of contaminant molecules during the neutralisation step. Alkaline
lysis is almost universally utilised in published plasmid purification processes
for those reasons (the exception being the heat lysis step developed by Merck Research Laboratories (Merck Incorporated. 1995)).

The lysis reaction is initiated by the addition of an equal volume of 0.2 M sodium hydroxide (NaOH) containing 1% w/v sodium dodecyl sulphate (SDS) to the harvested cells, suspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8 (TE) buffer. The SDS interacts with the proteins and lipids in the cell wall to render them soluble causing the cells to lyse. The pH of the solution is such that proteins and chromosomal DNA, once released from the cells are irreversibly denatured, while the denaturation of plasmid DNA is reversible (Sambrook et al. 1989). Typically, five minutes is allowed for the completion of the lysis reaction and the denaturation of DNA and protein (Ciccolini et al. 1998). The reaction mixture is then neutralised by the addition of one volume of 3.0 M chilled potassium acetate. The solubility of SDS decreases with temperature, so that SDS-protein complex precipitate at this point. RNA, cell debris, high molecular weight chromosomal DNA and other impurities also precipitate with the salt-detergent complexes to form an insoluble floe. However plasmid DNA will re-nature and remains in solution as the pH is reduced upon potassium acetate addition.

At large scale the mixing of the lysis reaction mixture after the addition of the alkaline detergent and potassium acetate presents a challenge (Doran 1995). It is important to ensure thorough mixing of the cell suspension and alkaline detergent to avoid localised extremes of pH, since irreversible plasmid denaturation will occur at pH values over 12.5 (Prazeres et al. 1998). However, shear levels must be kept to a minimum during mixing for the neutralisation step to avoid damaging the delicate floc and releasing contaminants back into the plasmid-containing liquor (Levy et al. 2000c). One approach is the use of dough mixing equipment developed for use in the food industry (Marquet et al. 1995). Ciccolini and co-workers (Ciccolini et al. 1999) have suggested two separate mixing strategies. Mixing for the lysis step utilises impinging jets, while for the neutralisation step, air assisted injection
of cold potassium acetate into the base of the reactor is used. Additional air sparged in through the base of the reactor following neutralisation will provide additional low shear mixing and aid the flotation of the floc (Ciccolini and al 1999).

For large plasmids (>100kb), there is an apparent loss of the desired plasmid product when the above alkaline lysis procedure is performed using potassium acetate for the neutralisation step (Sinnett et al. 1998). This may be because larger plasmids have insufficient time to reanneal during the reaction. The procedure can be modified to utilise sodium acetate instead of potassium acetate. Under these conditions a higher yield of plasmid is achieved, but this is offset by higher levels of contaminants such as RNA and protein in the final supernatant. In the adapted protocol suggested by Sinnett and co-workers, sodium N-lauroylsarcosine (SLS) was employed instead of SDS, and in this case no loss of large plasmids was observed when the lysis was performed.

Following lysis, the aqueous layer containing the desired plasmid product must be separated from the floc. At laboratory scale this is commonly achieved by centrifugation (QIAGEN 07/1999). For industrial scale processes it is likely that filtration will be the preferred choice of unit operation due to the high shear forces commonly encountered in industrial scale centrifuges (Levy et al. 2000c). Work carried out by Theodossiou and co-workers (1997) suggested that the best compromise between purity and loss of plasmid during filtration is achieved with a 5 μm polypropylene cloth. Filtration resulted in 99.4 % w/w removal of solids and 96.8 % w/w removal of protein, at the expense of a 33 % w/w loss of plasmid. The addition of a filter-aid was not found to be useful. Improved removal of protein and solids in this case was offset by a 70 % w/w loss of plasmid (due to plasmid DNA adsorbing onto the diatomaceous earth) and a marked reduction in the rate of filtration, (Theodossiou et al. 1997).
1.3.2 Downstream purification operations

1.3.2.1 Intermediate purification

The removal of major contaminants, following initial plasmid release (as described in Section 1.3.1.2), may be effected in various ways. As stated above, the alkaline lysis method of Birmboim and Doly (Birnboim and Doly 1979; Birnboim 1983) is almost universally used as the initial cellular disruption step and has the advantage that a large proportion of cellular debris, proteins, and large chromosomal DNA fragments are precipitated. The resulting insoluble floc produced at this stage may be readily removed by centrifugation (QIAGEN 07/1999), or flotation and filtration (Theodossiou et al. 1997; Varley et al. 1999).

The remaining contaminants at this point in the process can be removed in a variety of ways. RNA is a major contaminant, and is not completely eliminated by precipitation as a result of the alkaline lysis procedure. At laboratory scale digestion with RNase is commonly used to break the RNA polymer into small monomers, which are more readily separated (QIAGEN 07/1999; Sambrook et al. 1989). Enzymatic digestion is however likely to be expensive at industrial scale. Also, the bovine pancreas is a common source of RNase. The use of enzymes derived from such sources will not be acceptable to the regulatory authorities in the production of pharmaceutical grade products due to potential risk with regards to the presence of mammalian pathogens such as the prion which causes BSE (Durland and Eastman 1998). In order to avoid addition of exogenous RNase Monterio and co-workers (Monterio and al 1999) showed that the activity of endogenous nucleases remaining following the removal of the floc formed in the alkaline lysis step can result in up to a 40 % w/w reduction in host RNA levels when lysates are incubated at 37°C. This however resulted in 9 % w/w plasmid loss. More recently the modification of an E. coli host strain to include an expression cassette for RNase A integrated into the host genomic DNA has been reported (Cooke et
al. 2001). The expressed RNA is translocated to the periplasm of the cell from whence it is released upon lysis and acts to hydrolyse the bulk of host RNA. This methodology avoids the use of exogenous animal derived RNAse and thus should be acceptable to the regulatory authorities.

There are several alternative methods for the removal of RNA that do not rely on endogenous RNAse activity. The ability of diatomaceous earth to absorb RNA in favour of SC plasmid DNA has been the basis of a recent patent (Horn et al. 1996). The retention of plasmid DNA while low molecular weight contaminants are removed with the permeate during ultrafiltration has also received considerable investigation. Bussey and co-workers (Bussey 1998) described the use of tangential flow filtration, using membranes with typical molecular weight cut off (MWCO) between 300-500 kDa, for the purification of plasmids up to 50 kb. Contaminants such as proteins and low molecular weight RNA pass through the membrane while the plasmid is retained. However shear may be a problem, particularly with larger plasmids. Kahn and co-workers (Kahn et al. 2000) have recently combined a filtration process with extended exposure to alkaline conditions during the alkaline lysis step (with the effect that remaining RNA contaminants were reduced in both size and abundance). Tangential flow filtration was carried out with a polyethersulfone membrane with nominal MWCO between 500 and 1000 kDa (for plasmids in the range of 5.6 – 10 kb). 0.5 ft² of membrane was required per 10-15 g cells (7-20 mg plasmid) processed. Greater then 99 % w/w RNA and 95 % w/w protein was removed; however, endotoxin levels remained high (2400 ± 1700 EU mL⁻¹) and no mention is made of the reduction in the levels of chromosomal DNA contamination, which appear to be high from the analytical gels published. The initial depletion of RNA levels in the process liquor before the high-resolution stages may, however, prove useful.

Purification of plasmid DNA on the basis of differential solubility’s compared to contaminant molecules has been exploited through fractional precipitation using Isopropyl alcohol (IPA), various salts (such as lithium chloride,
ammonium acetate or ammonium sulphate), polymers such as polyethylene glycol (PEG) or combinations of these (Durland and Eastman 1998; Ferreira et al. 2000; Levy et al. 2000c). The use of the compaction agents spermidine and spermine (small cationic molecules which bind to the minor grooves of a dsDNA molecule, resulting in 4 – 6 orders of magnitude reduction of the volume occupied by the DNA molecule) has also been described (Murphy 1999). Shear, resulting in plasmid losses during resuspension of precipitated plasmid is again a danger at industrial scale. Work carried out by Collins and co-workers (Collins et al.) utilised a two step PEG precipitation process to further purify clarified lysates containing a 6.9 kb plasmid. 5% w/v PEG precipitation first removed contaminating DNA and protein, while the subsequent 8% w/v PEG step selectively precipitated the bulk of the plasmid from remaining contaminants. The method has the advantage that at this point the plasmid precipitate can be resuspended in a buffer suitable for the next unit operation. The removal of contaminants in this way was found to decrease the competition for binding sites on a chromatographic matrix during subsequent purification increasing the capacity of the matrix for plasmid by a factor of 2 (Collins et al.). More recently, McHugh and co-workers (McHugh and Hoare 2001) investigated the use of CaCl₂ for the selective precipitation of contaminants present in plasmid containing process liquors, while the plasmid remains the supernatant. Addition of 0.2M CaCl₂ to a process stream, which had previously been clarified and concentrated by IPA precipitation, resulted in a purification factor of approximately 6.5. This was mainly due to the precipitation of RNA, with some clearance of single-stranded chromosomal DNA also being observed (McHugh, personal communication. 2000; McHugh and Hoare 2001). The fractionation of DNA has also been achieved by aqueous two-phase extraction, either using systems comprised of the immiscible polymers PEG and dextran (Rudin and Albertsson 1966; Favre and Pettijohn 1967; Walter et al. 1985), or PEG-salt systems (Cole 1991; Andrews et al. 2001). These results will be further discussed in Section 1.4.2.
1. Introduction

1.3.2.2 High-resolution purification

High-resolution techniques for the separation of the plasmid product from chromosomal DNA and other contaminants, such as protein and endotoxin can be achieved through the use of chromatography. In some cases resolution of OC and SC plasmid has also been achieved. The use of anion exchange chromatography (Ferreria et al. 2000b), size exclusion chromatography (Horn et al. 1995; Ferreira et al. 1997) and hydrophobic interaction chromatography (Diogo et al. 1999), and also reverse-phase HPLC (Green et al. 1997) have been described for the purification of pharmaceutical grade plasmid DNA. Less conventional methods, such as expanded bed anion exchange chromatography or fluidised bed adsorption (Varley et al. 1999; Ferreria et al. 2000a; Theodossiou et al. 2000; Thwaites et al. 2001), membrane chromatography systems (Van-Huynh et al. 1993; Giovannini et al. 1998; Nochumson et al. 2000), magnetic beads (Levison et al. 1998), triple helix affinity chromatography (Wils et al. 1997; Simon et al. 2001), affinity chromatography utilising a zinc finger-glutathione S-transferase fusion protein as the ligand (Woodgate et al. 2002) and the combination of conventional chromatography mass transfer effects in the presence of an electric field (Cole et al. 2000; Park 2001) have also been described.

Anion exchange chromatography is efficient at separating plasmid products from other biological molecules such as proteins, due to the much higher charge typically carried by nucleic acids. Small RNA molecules are also well resolved. However this method is less efficient with regard to large RNA and chromosomal DNA fragments of similar size to the plasmid which may have a similar charge. Endotoxins may also be difficult to remove by this method (Durland and Eastman 1998). Similarly, size exclusion chromatography will not adequately resolve contaminants of similar size to the plasmid product (Durland and Eastman 1998). Reverse phase high performance liquid chromatography can be an extremely effective purification step, but utilises
reagents which are both toxic and expensive, and the requirement for high pressure will complicate scale up, and may result in shear damage to large plasmids. Hydrophobic interaction chromatography has been shown to be efficient at separating plasmid that has been denatured, for example by localised pH extremes during lysis (to the single stranded form) (Diogo et al. 1999). Diogo and co-workers attest that ongoing work indicates the utility of the operation to separate RNA and chromosomal DNA from plasmids. Triple helix affinity chromatography (Wils et al. 1997; Simon et al. 2001) is efficient at purifying plasmid product from RNA, chromosomal DNA and endotoxins, but the matrix is costly, and the technique requires that the plasmid be engineered to contain a homopurine sequence for the affinity separation. No differentiation between plasmid forms is possible using this method. The use of various chromatographic steps in whole plasmid purification processes is considered in more detail in the following section.

1.3.3 Published process sequences

Several whole process sequences have been reported for the preparation of pharmaceutical grade plasmid DNA. These are summarised in Figure 1.3. Horn and co-workers (Horn et al. 1995) describe a single chromatographic step process which utilised multiple upstream precipitation steps (IPA, ammonium acetate and PEG 8000) after lysis before the purified and concentrated feed was loaded onto a size exclusion column. However the yield of plasmid DNA over the chromatographic step was only 51 % w/w, and residual *E. coli* chromosomal DNA levels were not reduced to levels that would be acceptable to the regulatory authorities for a pharmaceutical grade product. Ferreira and co-workers (Ferreira et al. 1999) compared the yield and purity of plasmid DNA obtained through two alternate process routes both incorporating two chromatographic separation steps. Initially the process stream was clarified and concentrated through multiple precipitation steps (IPA, lithium chloride, potassium acetate or ammonium acetate, PEG 8000) prior to sequential anion exchange and size exclusion chromatography steps.
1. Introduction

Purification factors of 105 ± 10, 82 ± 8 and 102 ± 9 with corresponding yields of 91 ± 4, 88 ± 0.5 and 88 ± 5 % w/w were achieved over the multiple precipitation steps with lithium chloride, ammonium acetate or potassium acetate respectively. The overall yield was 24 ± 6 % w/w. In order to investigate the performance of the chromatographic steps alone, a second process route was investigated which excluded the multiple precipitation steps following lysis. The exclusion of the clarification and concentration prior to anion exchange chromatography reduced yield over the two chromatographic steps due to the exclusion of early fractions of the plasmid peak. Excellent purity was achieved through this process, with the overall yield higher than when the clarification and concentration steps were included in the process (37 % w/w ± 8 compared to 24 % w/w ± 6 overall). This process demonstrated that it was possible to obtain plasmid of a purity and quality required for pharmaceutical grade product in terms of RNA, chromosomal DNA and protein, but endotoxin levels were not determined. However, significant amounts of plasmid product are lost during the chromatographic stages.

Diogo and co-workers (Diogo et al. 2000) have exploited the hydrophobic nature of contaminants such as RNA, protein, endotoxin and chromosomal DNA present in plasmid DNA process streams in the use of hydrophobic interaction chromatography for the purification of pharmaceutical grade plasmid DNA. Here two precipitation steps (IPA and ammonium sulphate) were employed before a single chromatographic stage. The yield over the chromatography step was 95 % w/w, a drastic increase over that observed for size exclusion. Clearance of contaminants (RNA, protein, endotoxin and chromosomal DNA) was also to levels acceptable to the regulatory authorities, and up to 45 mg plasmid L⁻¹ of matrix was loaded onto the column.
Figure 1.3. Overview of the steps used in whole process sequences for plasmid purification published to date. Intermediate centrifugation and ultrafiltration steps have been omitted from this diagram for simplicity.
Expanded bed anion exchange chromatography is of interest as an alternative to the traditional packed bed mode of operation. The high viscosity of process streams following lysis presents problems in that it can cause high back pressures during traditional column chromatography. Varley and co-workers (Varley et al. 1999) describe the use of an optimised alkaline lysis step, followed by a bag filtration step before the lysate is adsorbed onto an expanded bed anion exchange column. Optimisation of the lysis step for plasmid recovery and contaminant precipitation is achieved through scrupulous attention to the temperature at which the reaction is carried out, mixing stages and the pH and exact composition of buffers. Elution of the plasmid is in packed bed mode and does not use toxic or flammable solvents. Finally the eluted plasmid is concentrated by ultrafiltration before a final size exclusion step. Ferreira and co-workers (Ferreria et al. 2000a) report purification factors of 36 ± 1 for expanded bed operation, with close to 100% yield when less than one settled volume of the packed bed was loaded onto the column. However increasing the loading resulted in a drastic reduction in both yield (35 ± 2 % w/w compared to 109 ± 3 % w/w for 250 mL and 50 mL unclarified lysate loaded onto 100 mL settled volume matrix) and purification factors (6.9 ± 0.7 compared to 36 ± 1 for 250 mL and 50 mL unclarified lysate loaded onto 100 mL settled volume matrix). At present the matrices commercially available are designed for the purification of proteins and again the low capacity of the expensive matrix for plasmid DNA seriously reduces the cost effectiveness of the operation.

The use of novel 20-40 μm high density pellicular expanded bed anion exchange matrices designed specifically for the capture of plasmid DNA and other large macromolecules was been shown to increase the capacity of the expanded bed capture step for a model DNA solution from ~ 50 μg mL⁻¹ to 6 mg mL⁻¹ (Theodossiou et al. 2000). Zhang and co-workers (Zhang et al. 2001) have compared the performance of various types of adsorbent solid phases (pellicular, solid and porous beads) for the
purification of nanoparticulates (i.e. virus-like particles and plasmid DNA). The most useful conformation was again found to be small (93 µm) high density particulates, coated with a thin skin of agarose to give a pellicular finish. In this way diffusional limitations are minimised by the presence of the ligand on the outer surface of the particle, while the small diameter of the beads ensures maximum surface area is available for adsorption. The density of the particle facilitates use at high linear velocities, or with high viscosity solutions in fluidised bed applications (Thwaites et al. 2001; Zhang et al. 2001). In contrast, nanoparticulates were excluded from the interstitial void of porous particles, while contaminants present in the process stream (such as RNA and proteins) were able to penetrate within the beads porous internal structure. The increased time for mass transfer resulted in extensive post binding washing of the adsorbent to displace the contaminants within the porous structure, which in turn extended the processing time.

Another approach to the purification of plasmid DNA is based around membrane chromatography. The use of DEAE-cellulose membranes for the purification of plasmid DNA from crude lysate has been reported (Van-Huynh et al. 1993). The use of a membrane stack should result in greater capacity than conventional chromatography matrices and enable higher flow rates to be used for processing (Van-Huynh et al. 1993). However yields were low (33 % w/w) and both phenol and chloroform are utilised in the method described. The separation of OC and SC plasmid forms using anion-exchange membrane chromatography has been demonstrated (Giovannini et al. 1998). No toxic solvents were used during the chromatography steps, however the procedure was carried out using a plasmid feed which had been previously pre-purified by CsCl density gradient centrifugation. Further investigation into this technology will be required to elucidate the effectiveness of this approach. More recently Nochumson and co-workers (Nochumson et al. 2000) described the utility of Mustang Q (Pall Filtration, Portsmouth, UK) anion exchange
membranes for the purification of 2.7 kb plasmid DNA. Clarified lysate, prepared using the alkaline lysis method was loaded onto the Mustang Q module. Sequential separation of RNA, and OC plasmid from SC plasmid was achieved by gradient elution. Between 2 and 4 mg plasmid was loaded onto a module of 10 mL volume. 98.4 % w/w SC plasmid, and 1.6 % w/w OC plasmid was purified from a feedstock containing 95.7 % w/w SC plasmid, and 4.3 % w/w OC plasmid. No information was given however for overall yield or purification factors for other contaminants such as RNA, protein or chromosomal DNA.

While the processes described above are sufficient for the production of the relatively small amounts of plasmid DNA required for clinical trials, significant improvements are desirable for the development of industrial scale manufacturing processes. At present a major disadvantage of the use of conventional chromatography matrices for plasmid purification is the low capacity of commercially available matrices for DNA (Prazeres et al. 1998). This can be explained by the much larger hydrodynamic volume of plasmid DNA compared to protein (Levy et al. 2000c). Ferreira and co-workers (Ferreira et al. 1999) have shown that capacities can be improved through the use of intermediate purification steps, such as precipitation, to remove impurities, but the improvement in capacity for the matrix (and subsequent reduction in costs) is be balanced against product losses and increased processing times. New matrices designed specifically for the purification of DNA and other large macromolecules, intended to increase the capacity of chromatography steps, are under development (Whitney et al. 1998; Theodossiou et al. 2000; Thwaites et al. 2001). However, as the cost of such specialist matrices is likely to be high, any increases in the total amount of plasmid, which may be loaded per cycle, through the depletion of problematic contaminants such as chromosomal DNA prior to loading, are desirable. Hence the development of an intermediate purification step with high yield of plasmid product, such as selective adsorption by nitrocellulose, is of interest. Separations based on aqueous
two-phase systems such as countercurrent chromatography, are of interest as an alternative to conventional chromatography operations, and have the advantage of potentially high capacity coupled with the low cost of the phase forming chemicals. The background to nitrocellulose adsorption, and countercurrent chromatography are discussed in more detail in the following section.

1.4 Background to the novel process techniques investigated

1.4.1 Theory of hydrophobic interactions and selective adsorption of contaminants

1.4.1.1 Theory of hydrophobic interactions

The tendency of ssDNA to adsorb to nitrocellulose has been exploited in Southern blot analysis for some time (Southern 1975; Oss et al. 1987; Sambrook et al. 1989). The binding of ssDNA is thought to be due to the hydrophobic interaction between the exposed bases of the ssDNA and the nitrocellulose. However the dsDNA and RNA present do not bind (Oss et al. 1987). A similar technique for the analysis of RNA exists (Northern blotting) which makes use of diazobenzoxymethyl cellulose for the binding of RNA (Alwine et al. 1977).

Many theories for the mechanism behind hydrophobic interactions have been proposed, but none have been universally accepted (Amersham-Pharmacia 1999). Precipitation of proteins by salting out laid the foundation for the science behind hydrophobic interactions, when Tiselius noted that 'proteins and other substances which are precipitated at high concentrations of neutral salts (salting out), often are adsorbed quite strongly already in salt solutions of lower concentrations than is required
for their precipitation, and that some adsorbents which in salt free solutions show no or only slight affinity for proteins, at moderately high salt concentrations become excellent adsorbers (Tiselius 1948; Amersham-Pharmacia 1999).

The theory proposed by Hjerten (Hjerten 1977) is based around the well known thermodynamic relationship:

$$ \Delta G = \Delta H - T \Delta S $$ \hspace{1cm} [1.1]

Where \( \Delta G \) (J) is the free energy in the system, \( \Delta H \) (J) the enthalpy in the system, \( T \) (K) is temperature and \( \Delta S \) (J K\(^{-1}\)) the entropy. The addition of neutral salts increases the polarity of the solvent and results in a gain in entropy due to structure changes in the water surrounding the hydrophobic groups (Porath et al. 1973). In turn the interaction of non-polar hydrophobic patches on the target solute with a hydrophobic support (i.e. alkyl ligands for hydrophobic chromatography) becomes energetically favourable (Amersham-Pharmacia 1999). Melander and Horvarth propose that hydrophobic interactions are due to an increase in the surface tension of water arising from the structure forming salts dissolved in it (Melander and Horvarth 1977). Srinivasan and Ruckenstein proposed that hydrophobic interactions are due to van der Waals attraction forces (Srinivasan and Ruckenstein 1980). The binding of ssDNA to nitrocellulose is through the energetically favourable association of hydrophobic bases with nitrocellulose, while the interaction of dsDNA or RNA is not energetically favourable in the conditions used in this work (Oss et al. 1987). It is not, however the aim of this work to test or expand on the theories suggested for hydrophobic interactions between nitrocellulose and nucleic acids, but rather to exploit it.
1.4.1.2 Selective adsorption of contaminants

In the 1960's and 1970's, various authors reported the use of nitrocellulose powder for the purification of nucleic acids. Cohen and Miller (Cohen and Miller 1969) described the purification of circular R-factor DNA isolated from *E. coli* through the selective adsorption of undesirable denatured plasmid forms to nitrocellulose powder in 300 mM NaCl, 30 mM sodium citrate buffer. The low ionic strength was achieved through the dilution of lysate obtained following a lysis step which did not make use of a high ionic strength neutralisation step (as used in the alkaline lysis procedure described in Section 1.3.1.2), with an equal volume of 600 mM NaCl, 60 mM sodium citrate buffer. Earlier Boezi and Armstrong (Boezi and Armstrong 1967) had reported the fractionation of nucleic acids on nitrocellulose columns. Suggested applications for nitrocellulose column chromatography were the preparation of RNA free from DNA (following the denaturation by heat of DNA species), the separation of native DNA species from denatured DNA, and the purification of mRNA. Following washing steps utilising distilled water only (to remove loose fragments of nitrocellulose) the column was equilibrated with a buffer comprised of 0.5 M KCl, 0.01 M Tris (pH 7.3), before loading of the nucleic acids to be purified in the same buffer. At concentrations of 0.5M KCl denatured DNA species, but not RNA or double stranded DNA, are retained in the column. A low salt buffer (0.01 M Tris, pH 7.3) or distilled water were employed to elute bound denatured DNA species. The capacity of nitrocellulose columns for denatured DNA was observed to be 64 \( \mu g \) mL\(^{-1}\), with a yield of 80-100 % w/w reflecting that a small amount of native DNA may also be absorbed. Popovic and co-workers (Popovic and Wintzerith 1983) describe the separation of ribosomal nucleic acids on nitrocellulose columns. In this case the small and large rRNA species from rat liver were separated, with the large rRNA species being strongly retained on the column in 0.5-0.7 M NaCl buffer. A higher ionic strength buffer (0.9-1.7 M NaCl) was required for the retention of the less
hydrophobic, small rRNA species. Popovic and co-workers also investigated the effect of varying the cation present in the buffer, and for both rRNA species the retention was found to increase for the cations investigated in the order Li⁺ > Na⁺ > K⁺ > Cs⁺. This does not follow the well known Hofmeister series, the order of salting-out (precipitation) observed for proteins (Amersham-Pharmacia 1999). The selectivity of the binding at various salt concentrations and compositions suggest that selective purification of plasmid DNA product on nitrocellulose should be possible.

Filtration through nitrocellulose membranes has also been shown to drastically reduce the levels of ssDNA contaminants (Wohlhieter et al. 1966; Levy et al. 2000a), although the capacity is limited. Wohlheiter and co-workers (Wohlhieter et al. 1966) determined that the capacity of nitrocellulose membrane to adsorb denatured *E. coli* DNA. Samples were prepared by boiling *E. coli* DNA for 10 minutes in 150 mM NaCl 15 mM sodium citrate, followed by rapid cooling on ice. The ssDNA thus prepared was mixed with native double stranded *E. coli* DNA. Samples were then purified by CsCl density gradient centrifugation and adjusted to 0.5 M KCI before filtration through nitrocellulose. The capacity of nitrocellulose to adsorb single stranded DNA from this model system was calculated to be about 20 μg cm⁻² with an efficiency of 90 % ssDNA adsorbed. A small amount of dsDNA was also retained following filtration through nitrocellulose. The pore size of the nitrocellulose membrane used has an impact on the efficiency of the purification achieved, membranes with pore sizes of 0.45 μm (up to 95 % ssDNA adsorbed) are more effective than membranes with pore sizes 5.0, 1.2 0.22 0.05 and 0.01 μm (Wohlhieter et al. 1966). Membrane pore size is not thought to be the major factor affecting the efficiency of adsorption, rather the percentage of nitrogen in the membrane as membranes both with pores larger and smaller than 0.45 μm did not perform as well (Wohlhieter et al. 1966). More recent work carried out by Levy and co-workers (Levy et al. 2000a) in the context
of pharmaceutical plasmid purification processes, investigated the potential of nitrocellulose filtration, operated in dead-end mode, to selectively adsorb chromosomal DNA (denatured following the alkaline lysis procedure) from highly clarified process liquors. In these laboratory scale experiments, an ionic strength of 1.5 M NaCl was used during the adsorption step, and levels of chromosomal DNA contamination dropped from 26% to below 1% (Levy et al. 2000a).

Membrane chromatography for the purification of proteins is gaining acceptance (Henricksen 1996; Gebauer et al. 1997). The large surface area present within the membrane pores offers the potential to both increase capacity and throughput. The ligand is directly accessible on the surface of the membrane’s pores so that diffusion is not limiting when enabling operation at high flow rates. It may also be possible to work with ‘dirty’ feedstocks, due to the potential to operate the membranes in a tangential flow mode. It may be possible to apply a similar technology to the purification of plasmid DNA through the selective retention of contaminant nucleic acids.

Recently, alternative membrane chemistries to nitrocellulose, including cationized nylon membranes and polyvinyl difluoride have been advocated for use in Southern blot techniques (Sambrook et al. 1989), the attraction for nucleic acids being due to strong electrostatic and hydrophobic interactions respectively. These membrane chemistries do not differentiate between RNA and DNA or other macromolecules such as proteins in terms of the selective binding seen with nitrocellulose, and it may be possible to exploit these, or other, novel membrane chemistries in the purification of DNA. Nucleic acids have also been observed to adsorb to silica (Theodossiou et al. 1997), and diatomaceous earth (Horn et al. 1996). As part of their research into the persistence of genetically modified plasmids in the soil, Poly and co-workers (Poly et al. 2000) noted differences in the strength of adsorption of linear chromosomal and
supercoiled plasmid DNA onto clay minerals. Such observations suggest that it may be possible to develop alternate selective adsorption strategies for plasmid DNA purification.

1.4.2 Countercurrent chromatography

1.4.2.1 The nature of aqueous two-phase systems

The immiscible nature of aqueous and organic liquids, such as water and oil, is a well-known phenomenon. Combinations of certain polymers, or a polymer and salt, in aqueous solution can also result in the formation of two immiscible phases (Walter et al. 1985). These can be used to effect separations of biological solutes through preferential partitioning of solutes between the two phases. Phase diagrams indicate the concentrations of phase forming solutes required for the formation of two immiscible phases and can be simply constructed by careful dilution of a phase forming mixture of known composition until the cloud point, (the minimum limit of concentration at which two phases are formed for that particular ratio of phase forming solutes), is reached. A binodal phase diagram is constructed by plotting cloud points for a range of compositions for the phase forming polymers or salts in question (Walter et al. 1985).

Polyethylene glycol (PEG) and dextran are two polymers that are known to form aqueous two-phase system (ATPS) when mixed in appropriate proportions, PEG will be found in the upper phase while dextran is present in the lower. The use of such systems for the purification of a diverse range of products, from proteins and nucleic acids to organelles and whole cells has been described (Walter et al. 1985). The cost of the polymers, dextran in particular, has always been prohibitive to the scale up of such separation processes and has prompted the development of new, and cheaper alternatives, such as Reppal (a hydroxypropyl starch polymer) to replace dextran (Kaul and Mattiasson 1986; Tjerneld et al.
1. Introduction

1986; Cesi and Narodoslawsky 1996; Planas et al. 1996; Petruszka et al. 2000). Alternatively a PEG-salt phase system may be used, where PEG again forms the upper phase over a salt rich lower phase. ATPS contain a high proportion of water compared to aqueous-organic systems (80-90% compared to 40-50% (Diamond and Hsu 1992)), which combined with the relatively low interfacial tension provide a gentler environment for the separation of labile biological macromolecules (Albertsson 1986; Diamond and Hsu 1992). However, for ATPS the low interfacial tension, and small differences in the density of the two phase's results in exceedingly slow settling times compared to aqueous-organic or organic-organic phase systems.

The affinity of a given macromolecule for each of the two phases is dependent on the charge, size and hydrophobicity of the molecule. The phase to which a given component will partition will be influenced by ionic composition, pH and the molecular weight of polymers present in the phase system (Walter et al. 1985). In the case of high molecular weight nucleic acids, the high net charge of the molecule means that the partitioning behaviour is much more dependent on the ionic composition of the phases than is the case for proteins. In addition, major differences have been observed between the partitioning behaviour of linear and supercoiled DNA in ATPS (Pettijohn 1967). In contrast, low molecular weight compounds, including some RNA, will partition fairly equally between the phases. The partition coefficient (K) of a molecule for a given phase system can be determined by a simple test tube procedure (Walter et al. 1985), whereby a sample is introduced into the phase system to be studied. After thorough mixing, the contents of the tube are allowed to settle and once two clear layers are formed aliquots from both phases are taken. K is obtained for each of the sample constituents from the following equation:
1. Introduction

\[ K = \frac{C_t}{C_b} \]  \hspace{1cm} [1.2]

Where \( C_t \) and \( C_b \) are the concentration of the solute of interest in the upper and lower phases respectively. Differences in the \( K \) values of solutes present in a given sample can form the basis for a separation.

1.4.2.2 Examples of separations using ATPS

In 1967, Rudin and Albertsson (Rudin and Albertsson 1966) first described a method for the purification of DNA from micro-organisms based on ATPS. Soon after an alternative method was described by Favre and Pettijohn (Favre and Pettijohn 1967). Both methods revolve around aqueous two-phase extraction using a PEG-dextran system at different ionic strengths to selectively partition solutes between the phases. The protocol of Rudin and Albertsson involves selectively partitioning proteins and low molecular weight RNA's (following chloroform extraction of the cell lysate) to the top phase by multiple extractions at 4 M NaCl, while the high molecular weight DNA of interest favours the lower dextran phase. The ionic strength of the system is then reduced by extraction against a fresh upper phase system not containing salt, resulting in the selective transfer of DNA to the upper phase, leaving high molecular weight single-stranded RNA behind. The protocol described by Favre and Pettijohn (1967) (Favre and Pettijohn 1967) uses similar principles, but the DNA is selectively moved from the upper phase to the lower phase in the final step. In this case, cell lysate was initially equilibrated in a system for which all the nucleic acids favour the upper phase. Large molecular weight RNA molecules, denatured DNA and most of the low molecular weight RNA molecules are then removed to the lower phase, while dsDNA remains in the top phase. dsDNA is then partitioned to the lower phase, while ssDNA and large molecular weight RNA molecules remain in the upper phase. dsDNA is then returned to the upper phase.
More recently the use of ATPS for the purification of circular forms DNA has been described by Ohlsson et al (Ohlsson et al. 1977). Their method results in the separation of ssDNA and RNA from double stranded circular plasmid DNA, and as such would be convenient for use after the denaturation of chromosomal DNA following alkaline lysis. 500 μl 16.8 % w/w dextran 500 and 250 μl 18.4 % w/w PEG was added to the cleared lysate to give a final volume of 1 mL, with a salt concentration of 0.01 M sodium phosphate buffer (pH 6.8). The double stranded plasmid DNA preferred the upper PEG phase at the low salt concentration used. Multiple extractions improved the purification at the expense of greater losses of the plasmid product.

The use of PEG-salt systems for the extraction of nucleic acids and various other macromolecules has been extensively described. Ribeiro and co-workers (Ribeiro et al. 2000) investigated the partitioning of an 8.5 and 7 kb plasmids in ATPS comprised of PEG 600 – K₂HPO₄ and PEG 300 - K₂HPO₄. Plasmid was found to preferentially partition to the lower phase for the phase system using the PEG 600 – K₂HPO₄ system, and to the upper phase with the PEG 300 – K₂HPO₄ system. The low yields observed (41 % w/w and 35 % w/w yield for PEG 600 and PEG 300 respectively) were due to the plasmid accumulating at the interface of the phase system. The tendency of large particulates to accumulate at the interface can be attributed to the consequential reduction in the amount of energy required for the formation of the interface, and hence the reduced interfacial tension (Walter et al. 1985). A more recent paper (Ribeiro et al. 2002) expands upon the earlier work, PEG with a range of molecular weights were investigated, as was the effect of the mass of neutralised lysate loaded into the ATPS. For plasmid purification, a system using PEG 600 and comprising 40% w/w lysate load is recommended. Under these conditions, RNA was found mainly at the interface, proteins were not
detected in the plasmid containing lower phase and genomic DNA was reduced 7.5 fold.

Huddleston and co-workers (Huddleston et al. 1996) and Kulkarni (Kulkarni et al. 1999) detail the use of PEG-phosphate systems for the partitioning of proteins for application in purification and extractive fermentation respectively. The processing of inclusion bodies using ATPS has also been described (Walker and Lyddiatt 1998). Marcos and co-workers (Marcos et al. 1998) considered the use of PEG-sodium citrate systems for the purification of antibiotic. Lin and co-workers (Lin and Chu 1995) report on the partitioning of cehalosporin C and desacetyl cephalosporin C using high speed countercurrent chromatography (more fully described in Section 1.4.2.4) using a PEG 600 ammonium sulphate aqueous two-phase system. The advantageous effect of various additives such as urea, KSCN and water-soluble organic solvents to increase the difference between the partition coefficients for the two species, and hence the separation efficiency, is noted.

The solutes present in biological suspensions have been shown to affect the behaviour of aqueous two-phase systems (Rito-Palomares and Cueto 2000). Lebreton and co-workers (Lebreton et al. 2001) reported that the addition of biomass to ATPS reduced the sensitivity of ATPS to operational conditions. For robust separations a system that will not be greatly affected by variations in the composition of the feed suspension is desirable. Although the purification of various macromolecules using ATPS has been shown to be effective at laboratory scale, the multiple extraction steps often required will prove labour intensive if carried out manually. Hence various approaches to make use of the selective partitioning of biological macromolecules in ATPS without the need to manually introduce fresh phases have been investigated.
Rito-Palomares and co-workers report the application of a two stage extraction process for the recovery of protein from yeast (Rito-Palomares and Lyddiatt 2000) and animal blood (Rito-Palomares et al. 2000). An initial extraction was carried out using a suitable PEG–phosphate system, and the cell debris and contaminants removed in the lower phosphate rich phase. A back extraction was then performed (utilising ATPS with lower PEG content). The protein rich upper phase is pumped to a mixer where the requisite volume of fresh phosphate rich solution is added before being pumped to a column. The protein rich phosphate phase is harvested, and the PEG rich phase recycled to be mixed with fresh protein rich PEG solution and the cycle is repeated again. This mode of operation, extraction and back extraction, with replenishment of the phases as required, allows the upper PEG phase to be recycled. In addition the lower phosphate rich phase can be reclaimed by ultrafiltration for reuse. The recycling of the phase forming polymer and salt was not found to significantly affect the partitioning of the protein.

Counter current distribution (CCD) is a technique developed to facilitate purification using two-phase systems through the automation of multiple extraction steps (Walter et al. 1985). The two phases containing the solute mixture to be separated are mixed and allowed to settle, before the phases are split and the apparatus rotated so that each phase is brought into contact with a fresh volume of the opposite phase. The phases are again mixed and the cycle repeated until the prerequisite number of mixing and settling stages have been carried out. He La metaphase chromosomes have been separated in this way (Walter et al. 1985), thus this method should be sensitive enough for the fractionation of plasmid DNA. The behaviour of chromosomes varied in different concentrations of the polymers, and this was used to move chromosomes selectively to the top phase, through the multiple extraction steps it was possible to perform using the automated technique. However the suitability of CCD for scale up is restricted due to the CCD equipment becoming rather bulky,
elaborate, fragile and difficult to operate for industrial volumes (Ito and Conway 1996).

Another approach to reducing the labour required to carry out multiple extractions using ATPS has been described by Muller et al. (Muller and Kutemeier 1982). Microgranular cellulose is used as a support for the dextran rich stationary phase, which forms a thin film on the stationary particles. The PEG rich upper phase is then pumped through the column and solute particles are separated according to their preference for the stationary dextran phase or the mobile PEG phase, those with the highest affinity for the PEG phase being eluted first. DNA fragments of between 20 and 30 000 base pairs were separated in this way, the resolution being limited by the size of the target DNA molecules. However the procedure is described with respect to the fractionation of a preparation of dsDNA restriction fragments. It was also noted that pBR322 plasmid DNA eluted from the column well in advance of RNA, thus the separation of ssDNA species from double-stranded plasmid DNA is also likely to be good. The resolution achievable by this method was described as comparable to that achieved by polyacrylamide or agarose gels, and has a higher capacity and greater potential for scale up. More recently silica (LiChristopher Diol 4000) and hydrophobic polyvinyl polymers (LiParGel 650) were investigated as the solid supports for the separation of proteins using ATPS (Wingren et al. 1994). However, some of the constraints common to conventional chromatography, such as the requirement for a highly pure and concentrated sample, restrictions on the flow rates possible due to the presence of tightly packed solid matrix and the restriction of the surface area available to the solutes, will still apply on scale up.

1.4.2.3 Countercurrent chromatography

Countercurrent chromatography (CCC) is an emerging low pressure chromatographic technique, which separates molecules on the basis of
different partition coefficients between two immiscible liquid phases. For detailed reviews of the technique see the books by Conway (Conway 1990) and Ito (Ito 1988; Ito and Conway 1996). Within the CCC column, one liquid phase (the stationary phase) is held in place by centrifugal forces created as a result of rotating spirally wound tubing i.e. the CCC column. A second liquid phase (the mobile phase) is then continuously pumped through the column and is subjected to multiple stages of phase mixing (during which solute mass transfer occurs) and settling with the stationary phase. Those components having a higher affinity for the mobile phase are eluted first. The alternate stages of mixing and separation are a result of the cardiodal path (Figure 1.5 B) that the coil follows which generates a variable 'g' field. In this way a large number of stages can be generated in a single machine; for example spinning the column at 800 rpm for 1 hour will generate $4.8 \times 10^4$ mixing and settling stages. There are several different designs of CCC machine available (Conway 1990; Ito et al. 1998) and these will be considered in greater detail in Section 1.4.2.4.

1.4.2.3.1 The Archimedean screw principle

More than 2000 years ago, the Greek mathematician Archimedes used a rotating helical structure, the Archimedean screw, to lift river water against the force of gravity up onto the riverbank. This principle can also be used to move two immiscible liquids past each other in a countercurrent manner by rotating a coiled tube in a gravitational field. This is also the principle by which one of two immiscible liquid phases can be retained within the rotating CCC column.

For instance in the case of a water-filled coiled tube into which a glass bead and an air bubble are introduced, the air bubble will always remain at the top of the coil and the glass bead at the bottom due to the effect of gravity. Figure 1.4 illustrates this effect. As the tube is rotated around the
axis of the coil, both the air bubble and glass bead will move toward one end of the tube. Both objects, whether lighter or heavier than water move in the same direction. The end of the tube toward which the objects move is termed the 'head' of the coil, and the opposite end the 'tail' (Ito and Conway 1996). In the case of two immiscible liquid phases being present in the coil (as opposed to a bubble and a bead). The lighter phase moves toward the top of the coil with each helical turn, and the heavier drops to the bottom. Given time the two phases will establish a hydrodynamic equilibrium, with each phase occupying roughly equal space at the head of the coil with excess of either phase being pushed back to the tail. Further rotation of the two phases simply results in the mixing of the two phases with each turn of the coil with no change in the overall volumetric distribution of the phases (Ito and Conway 1996).

Figure 1.4. Demonstration of the 'bubble and bead' principle to illustrate the Archimedean screw effect. The air bubble, being lighter than the liquid in the coil always occupies the high point of the coil and the bead, being denser, the low point. Both will progress toward the 'head' as the coil is rotated. After Ito and Conway (1996).

1.4.2.3.2 Early countercurrent chromatography techniques

Droplet CCC is a liquid-liquid partition method introduced in 1970 which takes advantage of the hydrodynamic equilibrium condition described above. The coil is first filled with the chosen stationary phase, and then rotated around its axis while the other phase is introduced from the head
end of the coil. The two phases establish a hydrodynamic equilibrium within each turn of the coil, until the mobile phase finally emerges from the tail of the coil. A solute mixture to be separated is introduced at the head of the coil and is subjected to a series of mixing and settling steps, before each solute is eluted according to their respective affinity for the two phases (solute with high affinity for the mobile phase being eluted first).

The speed of revolution of the coil has been found to have a profound effect on the phase distribution in the coil (Ito and Conway 1996). At a slow rotation (10-20 rpm) the two solvent phases are quite evenly distributed. Under these conditions the centrifugal force generated by the rotation of the coil is negligible, and the Archimedean screw effect acts evenly on the two phases, both phases moving toward the head in the upper and lower portions of the coil dependent on the respective densities. As the rotational speed increases the centrifugal force generated begins to negate the gravitational effect, thus the progression of the lighter phase toward the head is retarded as the net effect of the centrifugal force and the downwards gravitational force accelerate the progress of the heavier phase toward the head end of the coil. At a critical speed range, between 60 and 100 rpm, the two phases are completely separated, with the heavier phase at the head, and the lighter at the tail. After this critical speed further increase in the speed of rotation of the coil introduces a stronger radial force field which negates the gravitational effect and the phases become redistributed so that the heavier phase occupies the outer part of the coil, and vice versa, resulting in the phases gradually returning to an even distribution through the coil. At the critical speed (60-100 rpm) when the two phases are completely separated in the coil, the hydrodynamic equilibrium condition present enables a large degree of retention of stationary phase during CCC in the coil if the chosen mobile phase is introduced from the end that the chosen stationary phase would occupy (i.e. if mobile phase is lighter, elute head
Although the capability of droplet CCC to achieve separations has been demonstrated (Ito and Conway 1996), the method is time consuming due to the fact that retention of the stationary phase relies on a relatively weak Archimedean screw force operating under unit gravity restricting the useful flow rate of the mobile phase before depletion of stationary phase would result in loss of peak resolution. The use of a centrifugal force field, achieved by rotating the coil at relatively high velocity in planetary motion, enhances the Archimedean screw force acting on the coil, enabling higher flow rates of mobile phase to be utilised. A number of synchronous planetary motion types have been developed for seal-free flow-through centrifuge systems for CCC (Ito and Conway 1996). Three main types of countercurrent chromatograph (J-type, cross-axis and toroidal coil) will be considered in subsequent sections. The distribution of the two phases for various types of planetary motion has been studied. With both the J-type and the cross-axis systems, the planetary motion employed distributed the phases unilaterally in a closed end coil, (as has been observed for coil rotating in unit gravity as described in Section 1.4.2.3.2). It is this equilibrium state between two phases which can be applied to achieving separations by CCC.

1.4.2.4.1 Multi-layer coil planet centrifuge (J-type)

Figure 1.5 schematically shows the design of a J-type coil planet centrifuge. The design consists of two gears, the central ‘sun’ gear being interlocked with the planetary gear. This arrangement produces a particular type of planetary motion whereby the column holder is rotated around the central sun gear axis, while the planetary gear causes
simultaneous rotation of the column holder around its own axis. The column itself is fabricated by winding a single continuous tube around the column holder making multiple layers of coils. Originally a counterweight was used to balance the system. However this proved awkward, as the weight would have to be adjusted to account for different densities of the solvent systems used. This problem has been overcome by placing multiple column holders symmetrically around the rotary frame as shown in Figure 1.5.

Figure 1.5. (A) Simplified diagram to illustrate the construction and planetary motion of the J-type CCC and the position of the coils in relation to the central axis of revolution. (B) Illustration of the 2 dimensional path followed by a single point on the coil through space during one revolution ($\beta = 0.85$). S indicates the position of the central sun gear.
The parameter $\beta$ is computed by dividing the radius of the coil ($r$) by the orbital radius of revolution of the coil ($R$), and is an important factor in determining the hydrodynamic distribution of the two immiscible phases in the coil. The coil follows a two dimensional path through space during operation. The effect of the $\beta$ value on the path acceleration achieved in different stages has been analysed (Menet and Ito 1993). At low $\beta$ values ($\beta = 0.1$) the path is practically circular and the centrifugal forces present consistent with those expected from a completely circular motion. As the $\beta$ value increase, an inward loop develops (Figure 1.5 B) as the increase in the radius of the holder begins to impact on the path that the coil follows. For $\beta = 0.25$, the centrifugal force fields from the two gears cancel out around the modification in the circular path. At higher $\beta$ values ($0.7 \geq \beta \geq 0.4$) the centrifugal fields reverse during the inwards loop, the relative magnitude and direction of the centrifugal forces at the various points along the path being important for the vigorous mixing achieved with high $\beta$ values.

### 1.4.2.4.1.1 Phase distribution within J-type CCC and considerations for mode of operation

A recent paper by Sutherland and co-workers (Sutherland et al. 2000) considers the relative actions of the hydrostatic and Archimedean forces present during CCC. In Archimedean screw helical spirals operating under unit gravity, the heavy phase goes to the 'head' end of the coil and the light phase to the 'tail'. In gravitational systems like the J-type centrifuge, the distribution is reversed, with the light phase moving toward the 'head' and the heavy phase being displaced toward the 'tail' (Conway 1990; Ito and Conway 1996; Sutherland et al. 2000). When the 'tail' is at the periphery of the coil (coil is rotated in the same direction as coil has been wound) hydrostatic forces can also be expected to force the heavier phase to the 'tail'. In the case when the bobbin is rotated in the direction
opposite to the direction in which the coil has been wound onto it, placing the tail in the centre and the head at the periphery of the coil, the Archimedean and hydrostatic forces oppose one another. The direction in which the heavier phase will travel is dependent on the density difference between the two phases, the heavier phase can be expected to travel to the tail when the density difference is low, but to the head for greater differences in density as the action of hydrostatic forces wins out. During operation of the CCC machine, it is therefore desirable to pump the mobile phase against the stationary phase. Thus if the chosen stationary phase is the heavier one (for normal phase chromatography) the mobile phase should be pumped from ‘tail’ to ‘head’, when the column is rotated in the forward direction. Conversely if the chosen stationary phase is the lighter one (for reverse phase chromatography) the mobile phase should be pumped from ‘head’ to ‘tail’. In order that the Archimedean forces and the hydrostatic work together, it is generally desirable that the column be rotated in the forward direction. However this can cause problems with the columns becoming unwound (Sutherland et al. 2000) so columns are often rotated in the opposite direction to that in which they are wound. For ATPS with low density difference it is to be expected that the heavier phase will be displaced by the lighter and so move toward the tail, so the same rules apply regarding the direction of flow for the mobile phase.

The hydrodynamic motion and phase distribution within a J-type column has been observed by Conway (Conway and Ito 1984) (Illustrated in Figure 1.6) using a J-type CPC equipped with a spiral column and a transparent plastic cover. A two-phase solvent system comprising chloroform and water, each stained with a dye to facilitate stroboscopic observation was utilised in the experiment. After steady state hydrodynamic equilibrium was established two distinct zones could be observed; approximately one fourth of the area closest to the centre showed violent mixing of the two phases (the mixing zone), while in the remainder of the coil the phases were separated into two layers forming a
linear interface (settling zone). The mixing zone is always in the section of the loop of the coil nearest to the centre of rotation, while the coil itself rotates around it's own axis, thus the mixing zone is travelling through the spiral column toward the head at a rate equal to the column rotation. In any part of the column the two phases are thus subjected to repeated mixing and settling stages at a frequency of over 13 times per second for a rotational speed of 800 rpm (Ito and Conway 1996), hence a high partition efficiency can be achieved by high speed CCC.

**Figure 1.6.** Illustration of the distribution of the mixing and settling zones present during rotation of CCC column on a twin bobbin machine. Top diagram indicates location of the mixing zones (black bars) in the coil created due to the planetary motion created as it orbits the sun gear (S). The lower diagram shows straightened sections of the coil and illustrates the progression of the mixing zones (M) from the 'tail' to the 'head'.

1.4.2.4.1.2 Factors affecting the retention of the stationary phase

The amount of stationary phase retained is an important factor affecting the degree of resolution achieved by CCC techniques, higher resolutions being achieved with greater stationary phase retention. In general greater
retention of stationary phase means a greater degree of resolution. In 1999 Du and co-workers reported a linear relationship between the square root of the mobile phase flow rate (u) and the degree of stationary phase retention (S_f) for a range of aqueous-organic solvent phase systems and for a range of column diameters (Du et al. 1999). This allows prediction of S_f according to the flow rate of the mobile phase from just two retention experiments. The relationship can be represented as follows;

\[ S_f = a - bu^{0.5} \]  \[1.3\]

Where a and b are constants, S_f is the stationary phase retention, and u is linear flow rate (appropriate units). They also note that the value of a remains remarkably constant for hexane - ethyl acetate - water two-phase systems (although markedly different values are reported for other solvent systems) while the value of b declines as the relative volume of hexane to ethyl acetate increases. This lead the authors to speculate that the value of a may be related to the composition of the phase system, whereas the value of b appears to be related to the volume ratio of the solvents.

The same authors have also investigated the relationship between mobile phase flow rate and separation efficiency for a separation carried out with an aqueous-organic phase system. For conventional chromatography, utilising a solid stationary phase, the relationship between the separation efficiency, measured as height equivalent to a theoretical plate (HETP) and linear flow rate (u) can be described by the Van Deemeter Equation;

\[ \text{HETP} = A + B/u + Cu \]  \[1.4\]

where A, B and C are constants, and u is linear flow rate (appropriate units). B/u describes the decrease in HETP (indicating increased
separation efficiency) due to the reduction in longitudinal diffusion of solutes at higher flow rates, while Cu describes the increase in HETP (decreased separation efficiency) due to mass transfer limitations resulting in decreased transfer of solutes between mobile liquid phase and solid chromatographic matrix at higher flow rates.

The Van Deemeter equation can also be applied to CCC separations. In the case of high speed CCC carried out at fixed stationary phase retention using a two-phase solvent system of n-hexane - ethyl acetate - water (3:5:1 v/v/v), HETP was observed to increase steadily with increasing flow rate of the mobile phase above a critical flow rate (u>1 cm s\(^{-1}\)) (Du et al. 2000). From the Van Deemeter equation, the function B/u is hypothesised to represent the increase in mixing of the phases and interfacial area available for mass transfer at higher flow rates during CCC. The factor Cu again represents the increased resistance to mass transfer of solutes between the phases at high flow rates. A linear correlation was observed to exist between B and C, and the partition coefficient of a given solute, hence HETP could be calculated as a function of K and u;

\[
HETP = Du^K
\]  

where D is the HETP value at the minimum critical flow rate (1 cm s\(^{-1}\) for the experiment reported), K is the partition coefficient of a given solute and u is the linear velocity (appropriate units) (Du et al. 2000). It is possible that such mathematical models will eventually allow the performance of given phase systems during CCC to be predicted.

CCC conducted with J-type machines has been used with organic-organic or aqueous-organic phase systems to effect a variety of separations, including polyketide antibiotics (Booth and Lye 2001), flavonoid glycosidases and engine oil antiwear agent (Sutherland et al. 1998).
The time taken for a phase system to settle in a simple test tube experiment is a useful rule of thumb for the estimation of stationary phase retention, the phase systems with the fastest settling times achieving the highest stationary phase retention values during CCC (Ito and Conway 1996). In the main, due to the requirement for fast settling times, aqueous-organic or organic-organic phase systems have been used rather than aqueous two-phase systems, which are more suited to the separation of labile biological macromolecules such as plasmid DNA. Alternative designs, such as the toroidal coil and cross-axis have been found to be better suited to use with ATPS and will be discussed in the following sections.

1.4.2.4.2 Cross-axis synchronous flow through coil planet centrifuges

This type of CCC machine, described by Ito and co-workers (Ito and Conway 1996) differs from the J-type, as the axis of rotation of the column is perpendicular to the axis of rotation of the rotary shaft. The cross-axis machines are further classified into X and L types and various hybrid designs such as XL, XLL and XLLL according to the position of the column holder on the rotary shaft. For the type X cross-axis machine (Figure 1.7) the column holder revolves around the central axis of the centrifuge and simultaneously rotates about it's own axis at the same angular velocity. In so doing it always maintains the same distance (R) from the central axis. Again the parameter β (r/R) has a major influence on the hydrodynamics of the system. In the most recent experimental machines, it is possible to shift the column holder laterally along the rotary shaft (Figure 1.8). The degree of lateral shift of the holder has been found to be vital for retention of viscous solvent systems, such as ATPS. The lateral shift of the column holder is described by \( \delta = \frac{L}{R} \) where L is the distance from the mid portion of the rotary shaft to the column and R is the total radius of revolution around the mid shaft. For the original cross-axis model type X; \( \delta = 0 \), for type XL; \( \delta = 1 \), for type XLL; \( \delta = 2 \), for type XLLL; \( \delta \)
1. Introduction

Use of the cross-axis coil planet centrifuge with the column holder shifted laterally along the rotary shaft increases the magnitude of the laterally acting centrifugal field. This is thought to suppress emulsification and enables superior retention of ATPS such as PEG 1000: K$_2$HPO$_4$ and PEG 8000-dextran T500 (Shinomiya et al. 1993; Shinomiya et al. 2000), which has previously been found to be poor in models such as the J-type and cross-axis type X coil planet centrifuge.

**Figure 1.7.** Simplified diagram of a type X cross-axis coil planet centrifuge ($\delta = 0$) showing the position of the columns in relation to the central axis of revolution axis (vertical black rod). Note the coils are not offset in the z axis. Not to scale.
The column in a cross-axis coil planet centrifuge follows a three-dimensional path due to the coil rotating in the plane perpendicular to the rotary shaft. The influence of the $\beta$ value is not as pronounced as for the J-type machine, although the additional variable for the positioning of the column holder along the rotary shaft does influence the centrifugal forces present (Menet and Ito 1993). In the X – L positions the influence of the lateral force is greatly increased compared to the L position, where acceleration is produced mainly perpendicular to the CCC column.

Ito and co-workers have also reported on the effect of revolution speed and mobile phase flow rate on stationary phase retention using cross-axis coil planet centrifuge with ATPS. The optimum speed of revolution in
terms of stationary phase retention will vary with the design of machine and the phase system used but is likely to be in the region of 800 rpm (Goupy et al. 1995; Ito and Conway 1996; Shinomiya et al. 1998). For slower revolution speeds it was suggested that the lateral acting centrifugal force is too weak to provide optimal separation of the phases, and above this value retention again decreased presumably due to excessive mixing of the two phases.

Cross-axis machines have been used to effect many separations using ATPS, both with PEG-salt systems and PEG-dextran. Mainly protein separations have been reported (Shinomiya et al. 2000; Shibusawa et al. 2001), but the separation of plasmid DNA from RNA, utilising a 4 % w/w PEG 6000, 5 % w/w dextran system has also been described (Madava and Ito 1988). The apparatus used was a non-synchronous cross-axis design coil planet centrifuge, of the XLLL type. The columns were rotated around their own axis at 5rpm, while the speed of rotation of the column holders around the central shaft was 1000 rpm. The linear flow rate of the mobile phase (PEG) was 0.3 cm s\(^{-1}\). Plasmid DNA eluted immediately after the solvent front, with RNA eluting soon after. Some overlap between the plasmid and RNA peak was seen. No separation of the OC and SC plasmid forms was observed, and the separation of chromosomal DNA from plasmid DNA was not considered.

An alternative design of CCC machine that has been utilised to effect separations with ATPS, is the toroidal coil design. Unlike the designs discussed in Sections 1.4.2.4.1 and 1.4.2.4.2, the toroidal coil does not employ planetary motion to generate the centrifugal force.

1.4.2.4.3 Toroidal coil centrifuge

The toroidal coil centrifuge consists of tubing wound around a ring so that the overall effect is something of a hollow, circular sausage. Unlike the
previous designs of CCC machine described in Sections 1.4.2.4.1 and 1.2.2.4.2, the coil is rotated only around it’s own centre; there is no planetary motion. As the coil is rotated under the laterally acting centrifugal force field the heavier phase occupies the outer portion of the coil, and the lighter the inner. Eluting the lighter phase as mobile phase toward the direction of rotation, or the heavier in the opposite direction has been shown to produce the greatest partition efficiencies compared to the opposite mode of elution, which seems unusual given the symmetrical nature of the toroidal coil (Ito and Ma 1998). Ito and co-workers (Ito and Ma 1998) investigated the mass transfer rates of five samples of varying size in rotary tube experiments, with the conclusion that the resistance to mass transfer increases with the increasing molecular weight of the sample. Subsequently the coriolis force produced the greatest effect on the partitioning of the larger sample components, highlighting the importance of surface area available for mass transfer for the separation of large macromolecules. The coriolis force results in the path of descending (heavier phase) droplets being shifted against the direction of rotation and that of the ascending droplets being shifted in the direction of rotation. Thus it is desirable to operate the elution mode such that the coriolis force acts parallel to the coil segment resulting in the mobile phase being dispersed into the stationary phase as multiple droplets, thus increasing the area available for mass transfer.

Again such factors as rotational speed, mobile phase flow rate, sample volume and tube length are important in the optimisation of a given separation (Matsuda et al. 1998). Separations of various macromolecules have been reported using ATPS with a toroidal coil CCC (Elles and Sutherland 1980; Qi et al. 1998), but this design has been superseded by the cross-axis design, due to the superior mixing achievable with planet centrifuge designs.
1.4.2.4.4 Selection of countercurrent chromatography system for bio-separations

Historically, ATPS have been successfully used for the partitioning of biological macromolecules, cells and organelles (Rudin and Albertsson 1966; Favre and Pettijohn 1967; Walter et al. 1985; Albertsson 1986; Huddleston et al. 1996; Marcos et al. 1998; Walker and Lyddiatt 1998; Kulkarni et al. 1999; Cole et al. 2000; Ribeiro et al. 2000; Rito-Palomares et al. 2000; Rito-Palomares and Lyddiatt 2000). The use of organic solvents in phase systems is likely to cause denaturation of the product which is highly undesirable (Albertsson 1986; Diamond and Hsu 1992). ATPS are thus the preferred choice of phase system for plasmid separations using CCC. Indeed, the use of both PEG-salt and PEG-dextran ATPS to achieve separations of plasmid from RNA, DNA restriction fragments, proteins and other macromolecules has been previously reported (Elies and Sutherland 1980; Madava and Ito 1988; Shinomiya et al. 1993; Lin and Chu 1995; Qi et al. 1998). In terms of their physiochemical properties, ATPS are known for their low interfacial tension, low phase density difference and high viscosity compared to organic phase systems (Albertsson 1986). This increases the settling time for the phase systems, which in turn can result in the phases failing to separate adequately within a CCC coil. This may result in low stationary phase retention and elution (stripping) of the stationary phase during operation. The J-type design of CCC is intended for use with organic-aqueous phase systems, and is designed to achieve a vigorous mixing regime to maximise the area available for mass transfer. Thus it would be expected that the use of this design with ATPS would be limited due to excessive emulsification of the phases within the coil. Use of a toroidal coil or cross-axis X-type CPC system should improve matters, due to the gentler mixing within the coils enabling the phases to separate more easily at the expense of reducing interfacial area available for mass
1. Introduction

transfer (Sutherland et al. 1987; Ito et al. 1998). Scale up of countercurrent chromatography

To quote the UK Biotechnology and Biological Science Research Council (BBSRC) there is a need for a ‘generation of new, robust and usable techniques for bioprocess intensification and simplification’, and also for technology that can be scaled from laboratory to process scale easily and cheaply. Very few processes can be scaled up from laboratory to industrial scale in a straightforward manner. For instance in the case of high-performance liquid chromatography, scale up is expensive, uses large amounts of solvents, product can become hydrolysed or react with the solid phase within the column inducing conformational changes, and scale up is not linear (Sutherland et al. 1998). In the case of CCC scale up is achieved by simply increasing the bore of the column, and volumetric flow rate appropriately. There is no solid support within the column which should allow 100% sample recovery, the operational process is very simple and can be applied to a broad range of products from different industries. Furthermore it has been calculated that CCC would use 5 – 80 times less solvents than an equivalent preparative scale HPLC operation, in addition to incurring less capital expenditure on hardware for large scale process (Sutherland et al. 1998). Sutherland and co-workers (Du et al. 1999; Sutherland et al. 2001; Booth et al. 2002) have shown that scale up is predictable and performance can easily be analysed theoretically from a laboratory scale chromatographic trace once $S_v$ values at two different mobile phase flow rates for the industrial scale machine have been obtained.

Throughputs of 25 – 50 tonnes per year have been predicted for industrial scale CCC machines (Sutherland et al. 1998) and furthermore the operation of CCC at industrial scale is likely to be considerably less costly than industrial scale HPLC (Sutherland et al. 1998). These predictions, combined with the reports of the separation of nucleic acids discussed in
this section suggest the high-resolution purification of plasmid DNA by CCC may provide a cost effective, high capacity alternative to conventional chromatography operations described to date.

1.5 Aims of the project

The aim of this work is to develop alternative strategies for the downstream processing of naked plasmid DNA, with the intention of improving upon the efficiency of the purification strategies reported in the literature to date.

The capacity of conventional chromatographic techniques for the purification of plasmid DNA has been reported to be low (Collins, personal communication, 1998). Pre-purification of the process stream should increase the capacity of such unit operations (Collins, personal communication, 1998). Initially, therefore, the capacity of nitrocellulose membrane and nitrocellulose powder to adsorb single stranded DNA from a pure model solution will be investigated. An alternative approach to the selective adsorption of chromosomal DNA and other cellular contaminants from the double stranded plasmid DNA model product will be developed using a nitrocellulose membrane as a combined purification and clarification step, intended to be placed before the high-resolution chromatography operations. The efficiency of adsorption of various contaminants will also be considered, as will the potential for regeneration and reuse of the nitrocellulose membrane.

In addition, a novel high-resolution technique utilising countercurrent chromatography for the separation of supercoiled plasmid DNA from contaminant open circular plasmid DNA and chromosomal DNA will be developed. This technique has been shown to be extremely versatile in terms of the variety and nature of the solute and feed streams which have
purified using CCC (Ito and Conway 1996; Sutherland et al. 1998). However the separation of SC plasmid from OC plasmid and chromosomal DNA contaminants has not been previously investigated. Sutherland and co-workers (Sutherland et al. 1998) have forecast both high throughputs and relatively low cost of operation for scaled up CCC. This suggests that the use of CCC for the high resolution purification of pharmaceutical grade plasmid DNA has the potential to overcome the current constraints imposed by the low capacity, and high cost of conventional solid chromatographic matrices, as used in plasmid purification processes reported to date (Horn et al. 1995; Diogo et al. 1999; Ferreira et al. 1999; Varley et al. 1999). Initially a wide range of ATPS are to be investigated, both in terms of their suitability for the separation of SC plasmid DNA from cellular contaminants, and for use in the J-type CCC machine. Preliminary experiments will be carried out to assess the effectiveness of CCC for the purification of plasmid DNA. In the event of achieving a separation of plasmid DNA from other contaminant species, a wider investigation into the influence of such factors as choice of phase system and preparation of the plasmid containing feed, will be carried out.
2. Materials and Methods

2.1 Preparation of commonly used buffers and model solutions

All chemicals were obtained from Sigma Aldrich chemical company (Poole, Dorset, UK) unless otherwise stated. Deionised water was used for the make up of all reagents. The composition of buffers commonly used in this work is listed in Table 2.1 below.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition of buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 x TBE buffer</td>
<td>90 mM Tris-borate, 2 mM EDTA</td>
</tr>
<tr>
<td>0.5 x TBE buffer</td>
<td>45 mM Tris-borate, 1 mM EDTA</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris-HCl, 1 mM EDTA pH 8</td>
</tr>
<tr>
<td>20 x SSC</td>
<td>3 M sodium chloride, 300 mM sodium citrate</td>
</tr>
<tr>
<td>10 x SSC</td>
<td>1.5 M sodium chloride, 150 mM sodium citrate</td>
</tr>
<tr>
<td>5 x SSC</td>
<td>750 mM sodium chloride, 75 mM sodium citrate</td>
</tr>
<tr>
<td>2 x SSC</td>
<td>300 mM sodium chloride, 30 mM sodium citrate</td>
</tr>
<tr>
<td>0.1 x SSC</td>
<td>15 mM sodium chloride, 1.5 mM sodium citrate</td>
</tr>
</tbody>
</table>

Table 2.1. Composition of buffers commonly used in this work.

Calf thymus dsDNA was dissolved in TE buffer to a final concentration of 1 mg DNA mL\(^{-1}\). To obtain a pure ssDNA solution, the dsDNA stock solution was diluted to approximately 20 μg mL\(^{-1}\) and denatured by heating at 95 °C for 10 minutes. This was followed by rapid cooling by addition of an equal volume of ice cold 20 x SSC buffer and subsequent incubation on ice for 5 to 10 minutes. Hereafter the feed prepared in this way will be referred to as pure ssDNA solution. The solution was further diluted with 10 x SSC in some cases to obtain the desired concentration. Bovine serum albumin (BSA) was dissolved to the required concentration in 10 x SSC buffer before use.
2. Materials and Methods

2.2 Preparation of plasmid containing lysate

2.2.1 Plasmid and culture conditions.

The plasmids used in the present study were (I) pSVβ (obtained from Promega Corp., MA, USA), 6.9 kb, (II) pQR150, 20 kb and (III) pMT103 29 kb (Levy et al. 1999b). PQR150 and pMT103 were previously prepared by inserting whole or part of the TOL pathway from Pseudomonas putida into the vectors pBGS18 and pBR322 (Promega Corp) respectively (Jackson 1995; Jackson et al. 1995). Plasmids were transformed and propagated in E. coli DH5α (Gibco-Life Technologies, MD, USA) grown on LB media (5 g L⁻¹ yeast extract, 10 g L⁻¹ bacto-tryptone, 10 g L⁻¹ sodium chloride pH = 7) (Sambrook et al. 1989). E. coli cell paste containing pSVβ was obtained from two fermentations. In the first, bacteria grown in two 2 L baffled shake flasks were used to inoculate a series 2000 LH 75 L bioreactor (Inceltech, Reading, Berks, UK) containing 50 L of LB media with 0.1 mL L⁻¹ polypropyleneglycol and 0.1 g L⁻¹ ampicillin. Bioreactor operating conditions were: temperature, 37 °C; agitation 400 rpm, airflow rate, 30 L h⁻¹, pH 6.95. Bacteria were harvested at a final OD₆₀₀ = 1.6. A volume of 12 L from this culture was then used to inoculate 300 L of LB media contained in a 450 L Chemap bioreactor (Chemap, AG, Maenndorf, Switzerland). The fermentation conditions were: temperature, 37 °C; agitation 250 rpm; air flow rate 150 L h⁻¹, pH 6.95. The bacteria were grown for 10 h to a final OD₆₀₀ = 2.85 (Noites et al. 1999; Levy et al. 2000a).

Bacterial cells were harvested using a semi-continuous Carr Powerfuge P6 (Carr separations Inc., Franklin, MA, USA) at a flow rate of 30 L h⁻¹ and relative centrifugal force of 20 000 g. Total wet cell weight recovered was approximately 797 g and dry cell weight 490 g. The cell paste was double sealed in plastic bags before being stored initially at −20 °C and then at −70 °C for up to 36 months. Agarose gel electrophoresis, as described in Section
2.6.1, of control samples showed no deterioration in the quality of the plasmid over that time. This fermentation shall hereafter be referred to as the 450 L fermentation. Cells from the 450 L fermentation were used to provide pSVβ material for subsequent studies unless otherwise stated. A second 50 L working volume fermentation and cell harvest procedure was carried out under identical conditions to the first, except the final fermentation before harvest was in the 75 L fermenter only. Bacteria were harvested in this case at OD_{625} = 4.94. Total cell weight recovered was approximately 150 g and dry cell weight 91 g. This fermentation shall hereafter be referred to as the 75 L fermentation. Figure 2.1 (courtesy of A. Kay, University College London) shows the dissolved oxygen tension (DOT), oxygen uptake rate (OUR) and carbon emission rate (CER) which was recorded during the course of the 75 L fermentation. The offgas stream was analysed using a mass spectrophotometer (MM8-80S, VG Gas Analysis Ltd, Winsworth, UK) and OUR and CER were calculated and logged using the Propack data acquisition / logging system (Acquisition Systems Ltd, Sandhurst, UK).

![Figure 2.1](image_url)

**Figure 2.1.** DOT, OUR and CER profiles for a DH5α pSVβ 75 L fermentation. Data courtesy of A. Kay (University College London). DOT (---), OUR (-----), CER (- - - - - -).
Bacteria containing pQR150 and pMT103 were grown in 5 L LB media contained in a 7 L Series 210 LH bioreactor (Inceltech UK Ltd., Pangbourne, UK) using culture conditions equivalent to the larger fermentation (temperature, 37 °C; agitation 1000 rpm, air flow rate, 4 L h⁻¹, pH 6.95). Bacterial cells were harvested by batch centrifugation for 30 minutes at 10000 rpm using a Beckman J2-M1 centrifuge (Beckman Instruments Ltd., Progress Road, Sands Industrial Estate, High Wycombe, Bucks, UK). The supernatant was decanted from the resulting pellet, and the cell paste stored initially at -20 °C and then -70 °C for up to 36 months.

2.2.2 Alkaline lysis of E. coli cells and lysate clarification

Cells were lysed using a modified alkaline lysis procedure (Birnboim and Doly 1979). Cells (50 g) were defrosted and resuspended in 500 mL TE buffer. When stated, 0.1 μg mL⁻¹ RNAse A (Qiagen, West Sussex, UK) was added to the resuspension buffer for some applications. Lysis was achieved by addition of 200 mM Sodium Hydroxide (500 mL), containing 1 % w/v sodium dodecyl sulphate (SDS). After 5 minutes incubation, the mixture was neutralised by addition of 3 M potassium acetate pH 5.5 (500 mL). The final reaction volume was typically 1.5 L. Gentle mixing was achieved over a time period of approximately 0.25 min by five consecutive gentle manual inversions of the reaction vessel (a plastic storage vessel 120 mm i.d. x 200 mm in height) following addition of reagents. Removal of the resultant floc and clarification of the lysate was performed by gravity-driven filtration through eight layers of muslin Miracloth (Carbiochem La Jolla, USA) followed by further filtration through a single sheet of Qualitative No.1 filter paper (Whatman, Maidstone, Kent, UK). Typically 1.2 L of clarified lysate was recovered. The plasmid concentration in lysates prepared from cell paste from the 450 L fermentation was typically 81 μg mL⁻¹ (of which 73 % w/w of total plasmid DNA obtained was SC plasmid) whereas 41 μg mL⁻¹ (of which 58 % w/w of total plasmid DNA obtained was SC plasmid) was typical for lysates prepared from the 75 L
2. Materials and Methods

fermentation. The levels of chromosomal DNA contamination were typically 10 % w/w and 23 % w/w for the 450 L and 75 L fermentation's respectively.

2.3 Primary purification of plasmid DNA by batch adsorption using nitrocellulose powder.

2.3.1 An alternate protocol for the preparation of plasmid containing lysate

To investigate the effect of the cation present in the lysate feed on adsorption, for one set of experiments (described later in Section 2.3.3), lysate was prepared as in Section 2.2, except that 3 M sodium acetate was substituted for 3 M potassium acetate in the neutralisation step of the lysis protocol.

2.3.2 Preparation of nitrocellulose powder

Nitrocellulose powder shipped in 30 % w/v isopropanol (described as granular, with 10.9-11.2 % Nitrogen content) was acquired from Aldrich Chemical Company (Poole, Dorset, UK).

2.3.2.1 Room temperature grinding and washing

Before use, the nitrocellulose powder (~ 10 g) was ground to approximately 0.5 mm diameter particles using a mortar and pestle and thoroughly washed four times with an excess of RO water and twice with an excess of 2 x SSC buffer (300 mM sodium chloride, 30 mM sodium citrate). The washed nitrocellulose powder was stored in 2 x SSC. Prior to use, the 2 x SSC buffer was decanted and 10 x SSC added to excess. The powder was equilibrated for 10 minutes in 10 x SSC buffer before use. All operations involving nitrocellulose powder were carried out in a fume hood due to the irritant nature of the dust if inhaled. Used nitrocellulose powder was stored under water in a dedicated container before disposal. At no time was the powder allowed to completely dry out due to the explosion hazard this might cause.
2.3.2.2 High temperature washing protocol

To facilitate the removal of the alcohol the nitrocellulose powder had been shipped in, an initial high temperature washing at 65 °C in order was investigated before the capacity of the nitrocellulose powder to selectively adsorb ssDNA contaminants from plasmid containing lysates was determined. Nitrocellulose powder (approximately 10 g) was first ground as described in Section 2.3.2.1. The powder was washed twice with excess RO water before dewatering by filtration and was then incubated in excess 0.1 x SSC buffer for 22 hours in a shaking 65 °C waterbath. The powder was washed again with an excess of 0.1 x SSC buffer before storage at room temperature in 2 x SSC buffer. The required amounts of wetted powder were equilibrated with 10 x SSC buffer before use.

2.3.3 Stirred batch adsorption experiments

Nitrocellulose powder that had been previously washed as described in Section 2.3.2.1 or 2.3.2.2 was de-watered by centrifugation using a GS-6R Beckman benchtop centrifuge at 3500 rpm for 20 minutes. 1 g nitrocellulose powder was then weighed out into individual 50 mL Falcon tubes, and the appropriate volumes of lysate (approximately 90 µg mL⁻¹ total DNA of which 10 % w/w was chromosomal DNA), prepared as described in Section 2.2 (pretreated or not with RNAse), or pure ssDNA solution (approximately 10 µg mL⁻¹), prepared as in Section 2.1 were added to give ratios of 0.5 - 6 mL lysate or model ssDNA solution g⁻¹ nitrocellulose powder. The tubes were sealed and incubated with continuous inversion for 45 minutes before the nitrocellulose was removed from the reaction mixture by centrifugation of the tubes for 20 minutes at 3500 rpm. Aliquots of the plasmid containing supernatant were taken for further analysis as described in Section 2.6.1.
2.3.4 Regeneration of nitrocellulose powder

In order to study the regeneration of the nitrocellulose powder it was first necessary to carry out an adsorption experiment from clarified lysate as described in Section 2.3.3, using a ratio of 1 mL lysate g⁻¹ nitrocellulose powder for each adsorption experiment. Following the adsorption step the supernatant (obtained by centrifugation using a Beckman GS-6R centrifuge as described in Section 2.3.3) was analysed as described in Section 2.6.1. The nitrocellulose powder recovered was regenerated before a second batch adsorption experiment. Between adsorption experiments the nitrocellulose powder was regenerated as described below.

Two regeneration protocols were subsequently investigated. Following the initial adsorption experiment the powder was de-watered by filtration under vacuum. Two washes of 10 minutes duration at high salt concentration using 1 mL of 10 X SSC buffer g⁻¹ nitrocellulose powder were initially carried out to remove any remaining non-adsorbed nucleic acids. The powder was then split in two. One half was incubated with continuous inversion with 2 mL low salt buffer (0.1 x SSC) per g⁻¹ nitrocellulose powder at room temperature for 22 hours. The other half was incubated with 2 mL low salt buffer (0.1 x SSC) per g⁻¹ nitrocellulose powder with shaking in a water bath at 65 °C 22 hours. Samples from the washing steps were kept for analysis as described in Section 2.6.1. Following equilibration of the powder with 10 x SSC buffer, a second adsorption step (as described previously in this section) was then carried out with fresh lysate (prepared as described in Section 2.2) to enable the comparison of the effectiveness of the two protocols. Supernatant samples from the adsorption experiments were also analysed as described in Section 2.6.1.
2.4 Primary purification of plasmid DNA by integrated filtration-adsorption

2.4.1 Pre-purification of lysate by selective precipitation of contaminants with calcium chloride

The nucleic acids present in 120 mL of lysate containing a 6.9 kb plasmid pSVβ, prepared as described in Section 2.2, were precipitated using 0.7 volumes of isopropanol (Sambrook et al. 1989). Following centrifugation at 10 000 rpm for 30 minutes at 4 °C in a Beckman J2-M1 centrifuge, the supernatant was discarded and the resultant pellet washed with 0.6 volumes of 70 % v/v ethanol. The pellet was again centrifuged for 20 minutes at 10 000 rpm, and the supernatant discarded. The pellet was then allowed to air dry for ten minutes before being resuspended in 60 mL TE buffer. 2.5 M calcium chloride (CaCl₂) was then added to a final overall concentration of 0.5 M CaCl₂ (McHugh and Hoare 2001). The suspension was incubated for 3 hours at room temperature with gentle agitation before the precipitated contaminant species were removed by centrifugation for 30 minutes at 10 000 rpm. Approximately 57 mL supernatant containing plasmid was thus recovered. The pellet was resuspended in 60 mL TE buffer for subsequent agarose gel analysis as described in Section 2.6.1. Nucleic acids present in the supernatant were subjected to a second precipitation using 0.7 volumes of isopropanol (Section 2.6.1.1), and the resultant plasmid contained in the pellet resuspended in 60 mL TE buffer, with the addition of an equal volume of 20 x SSC buffer prior to use.

2.4.2 Membrane modules and filtration conditions

2.4.2.1 Membrane types and equilibration

Nitrocellulose and cellulose acetate membranes of 0.45 μm nominal pore size, 47 mm diameter, were obtained from Whatman (Maidstone, Kent, UK).
All membranes were soaked in pure water and equilibrated in 10 x SSC buffer for ten minutes before use.

2.4.2.2 Dead-end filtration-adsorption

A Millipore (Watford, Herts, UK) sterifil dead-end filtration system was fitted with a single membrane sheet, either nitrocellulose or cellulose acetate, and operated under a low vacuum generated by a Bruckner flask. The unit is comprised of an upper reservoir (maximum volume 50 mL) feeding directly onto the membrane. Permeate was collected in clean 25 mL universals which had been placed in the lower collection reservoir. The membrane area available for filtration was 15.9 cm². 2 mL aliquots of pure ssDNA prepared as described in Section 2.1 were drawn through the membrane and were collected for analysis by spectrophotometry (A²⁶⁰). A control sample of the feed was also kept for subsequent analysis by A²⁶⁰.

2.4.2.3 Tangential-flow filtration-adsorption

Adsorbent nitrocellulose membranes prepared as described in Section 2.4.2.1 were fitted into a custom-built perspex tangential-flow filtration rig (Figure 2.2) being held in place by a rubber o-ring. The total membrane area available for filtration was 12.5 cm² while the diameter of the inlet (feed) and outlet (retentate) ports was 4 mm. The height of the channel above the membrane surface was 1 mm. All the connecting tubing was 5 mm i.d. (8 mm o.d.) silicon. The retentate was circulated around the rig using a pre-calibrated peristaltic pump (Watson Marlow, Falmouth, Cornwall, UK) at flow rates of 1.0 x 10⁻⁶, 1.3 x 10⁻⁶, 2.0 x 10⁻⁶ and 3.8 x 10⁻⁶ m³ s⁻¹ (or 8.0, 10.4, 16.0 and 30.4 m m² membrane area s⁻¹). The corresponding linear velocities in the connecting tubing were 5.1 x 10⁻², 6.8 x 10⁻², 1.0 x 10⁻¹ and 2.0 x 10⁻¹ m s⁻¹ respectively. Pressures were measured using custom built water-filled U-tube manometers connected to the microfiltration rig with a T-piece at the indicated points (Fig 2.2 A).
Approximately 100 mL pure ssDNA solution, partially clarified *E. coli* lysate or lysate pre-purified using CaCl₂, prepared as described in Sections 2.1.1, 2.1.2 and 2.2, and 2.4.1 respectively were utilised as feed. The membrane unit was always operated in concentration mode. A control sample of the initial feed was kept for analysis of nucleic acid composition as described in Section 2.6.1, and for selected samples, quantitative analysis of chromosomal DNA, protein content and endotoxin levels were also determined as described in Sections 2.6.2.1, 2.6.3 and 2.6.4 respectively. Permeate fractions (2 mL) were collected as discreet samples by volume, the time at which collection was complete being recorded. Analysis of nucleic acid composition in each of the permeate fractions was as described in Section 2.6.1, and for selected

---

**Figure 2.2 (A)** Schematic diagram of the tangential flow filtration rig used and **(B)** detail of the membrane housing. Dimensions are given in Section 2.4.2.3.
samples, quantitative analysis of chromosomal DNA; protein and endotoxin levels as described in Sections 2.6.2.1, 2.6.3 and 2.6.4 respectively were subsequently carried out.

2.4.3 Regeneration protocols for the nitrocellulose membrane

Following tangential-flow filtration-adsorption experiments, several strategies were evaluated for the regeneration and reuse of the nitrocellulose membranes. The membranes were removed from the rig, and incubated at room temperature for 5 minutes in 5 mL 10 x SSC buffer under gentle agitation, a sample of the 10 x SSC wash buffer being kept for subsequent analysis by agarose gel electrophoresis as described in Section 2.6.1. The membranes were then replaced within the rig, either in the same orientation as before, or upside down to facilitate removal of the fouling layer from the surface of the membrane by backflushing. The membrane was kept wet at all times. A number of washing solutions were investigated; (I) pure H₂O (room temperature), (II) 0.15 M Triton X-100 (E-phenoxypolyethoxy ethanol, a non-ionic detergent) (room temperature), (III) 1 M Triton X-100 (37 °C) or (IV) 0.1 M NaOH (60-50°C). These were then pumped through the rig at a retentate recirculation rate of 1.3 cm³ s⁻¹ for up to 1 hour. Samples of the permeate from the wash buffer were kept for agarose gel analysis as described in Section 2.6.1.

The pure water flux through the membranes were measured and recorded before the start of the experiment for the fresh membrane, and again after each regeneration operation for comparison. Following regeneration, the membranes were equilibrated as before in 10 x SSC buffer (Section 2.4.2) before a subsequent tangential-flow filtration-adsorption experiment was carried out.
2.5 Plasmid purification by countercurrent chromatography

2.5.1 Concentration and preparation of feed

To further concentrate and purify the plasmid preparation prior to countercurrent chromatography, 200 mL of lysate from the 450 L fermentation (described in Section 2.2.1) containing the pSVβ plasmid was lysed in the presence of RNAse as described in Section 2.2. The resultant lysate was precipitated using 0.7 volumes of isopropanol (Section 2.6.1.1) and the resulting pellet resuspended in 5 mL of TE buffer or mobile phase from the phase system selected for the CCC run (Table 2.2) and used as feed samples for the subsequent CCC experiments. The final concentration of plasmid was determined by the picogreen assay as described in Section 2.6.4 to be 1.3 mg mL⁻¹ for the plasmid resuspended in TE buffer and 0.45 mg mL⁻¹ for the plasmid resuspended in mobile phase.

2.5.2 Composition and separation of aqueous two-phase systems

Polyethylene glycol of average molecular weight 400, 600 and 1000 kDa was obtained from Sigma Aldrich chemical company (Poole, Dorset, UK). Anhydrous potassium di-phosphate (K₂HPO₄) was obtained from Fisher Scientific (Loughborough, Leics, UK). Stock solutions of 40 % w/w K₂HPO₄ and PEG 1000 were individually prepared in deionised water (PEG 600 and PEG 400 are liquid at room temperature and can be utilised in undiluted form), and used immediately to make up the required phase systems.

Prior to use for CCC experiments, phase systems were mixed for 30 minutes and left to equilibrate overnight. Initial screening of effective phase systems for CCC application was based on measuring the time required after phase mixing (performed by inverting the test tube 5 times) for two distinct phases to form (Tₚ). The volume of the upper (Vₗ) and lower (Vₘ) phase were also recorded and used to calculate the volume ratio (P) (P = Vₗ / Vₘ). Five phase systems hereafter referred to as PEG 1000, PEG 600, PEG 600 NaCl, PEG...
600 IPA and PEG 400 were chosen after the initial screening. The composition of each phase system is given in Table 2.2.

<table>
<thead>
<tr>
<th>System</th>
<th>PEG 400</th>
<th>PEG 600</th>
<th>PEG 1000</th>
<th>K$_2$HPO$_4$</th>
<th>NaCl</th>
<th>IPA</th>
<th>H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 400</td>
<td>15 % w/w</td>
<td></td>
<td></td>
<td>20 % w/w</td>
<td></td>
<td>65 % w/w</td>
<td></td>
</tr>
<tr>
<td>PEG 600</td>
<td></td>
<td>12.5 % w/w</td>
<td></td>
<td>18 % w/w</td>
<td></td>
<td>69.5 % w/w</td>
<td></td>
</tr>
<tr>
<td>PEG 600 NaCl</td>
<td></td>
<td>12.5 % w/w</td>
<td></td>
<td>18 % w/w</td>
<td>1 % w/w</td>
<td>68.5 % w/w</td>
<td></td>
</tr>
<tr>
<td>PEG 600 IPA</td>
<td></td>
<td>12.5 % w/w</td>
<td></td>
<td>18 % w/w</td>
<td>2 % w/w</td>
<td>67.5 % w/w</td>
<td></td>
</tr>
<tr>
<td>PEG 1000</td>
<td></td>
<td>16.2 % w/w</td>
<td></td>
<td>18 % w/w</td>
<td></td>
<td>65.8 % w/w</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Composition of selected phase systems used for CCC experiments

2.5.2.1 Construction of binodal phase diagrams

The cloud points for a range of phase systems containing different ratio's of K$_2$HPO$_4$ to PEG 1000 or K$_2$HPO$_4$ to PEG 600 were determined. Each starting phase system was made up from stock solutions (described in Section 2.5.2) and the weights of the constituents recorded. Deionised water was added in a drop-wise fashion into phase forming mixtures for several ratios of PEG 1000 / K$_2$HPO$_4$ and PEG 600 / K$_2$HPO$_4$ (Walter et al. 1985). After each addition of deionised water the phase systems were thoroughly mixed by shaking, and the resultant emulsion formed from the two phases observed. The end of each experiment was reached the first time an emulsion was not observed following the mixing of the diluted phase system. This is the cloud point and signifies that the solutes in the phase system had been diluted to the critical concentration at which a two phases are no longer formed. The total weights of the vessels containing the phase systems were recorded before the start of each experiment, and again once the cloud point had been reached. From this data the weight of water added during each experiment was determined, and the final composition of the system at the cloud point determined. Two similar sets of experiments were carried out except that IPA was introduced into each of the initial phase forming mixtures at two fixed concentrations (2 % w/w and 5 % w/w), this concentration being kept constant by the drop-wise addition of deionised water IPA mixed at the same percentage w/w of IPA as
the starting phase system. The data were then used to construct the binodal phase diagrams shown in Figure 5.1.

2.5.3 Preparation of feed by aqueous two-phase extraction

Lysate prepared without the addition of RNAse and utilising cell paste prepared as described in Section 2.2.1 from the 75 L fermentation was incorporated into 20 g of a phase system in the following proportions: 5 % w/w isopropyl alcohol (IPA), 12.5 % w/w PEG 600, 16 % w/w \( \text{K}_2\text{HPO}_4 \) (from a 40 % w/w stock solution) and 42.5 % w/w lysate. The final concentration of plasmid in the lower phase was determined by the picogreen assay (described in Section 2.6.4) to be 31 \( \mu \text{g mL}^{-1} \).

2.5.4 Equilibrium partitioning of plasmid DNA in ATPS

2.25 g of a wide range of phase systems were initially made up in capped test tubes and 0.25 g lysate prepared using cell paste from the 75 L fermentation as described in Section 2.2 added. The phases were then mixed by inversion for 10 minutes, to allow mass transfer to occur, allowed to settle, and the upper and lower phase volumes measured. The equilibrium partition coefficients \( (K) \) of the various nucleic acids were estimated by measuring the transmittance of the DNA bands from each phase after gel electrophoresis as described in Section 2.6.1. The equilibrium partition coefficient \( (K) \) of both SC and OC plasmid was then calculated according to the equation:

\[
K = \frac{C_t}{C_b}
\]

[1.2]

where \( C_t \) is the estimated concentration (appropriate units) in the upper phase and \( C_b \) is the estimated concentration (appropriate units) in the lower phase.
2.5.5 CCC instrumentation and operation

The CCC machine used was a Brunel Labprep, "J-type" instrument as described in Section 1.4.2.4.1 (BIB, Rhomulus Technologies, Uxbridge, UK). This device was fitted with two equivalent bobbins, to allow balancing of the apparatus. The column used was made from 3.2 mm o.d., 1.6 mm i.d. PTFE tubing spirally wound onto the bobbin from the centre to the periphery in an anti-clockwise manner. The column had a total volume of 94.3 mL and β values (defined in Section 1.4.2.4.1) between 0.83 – 0.86 (Conway 1990). Temperature was controlled with an internal fan and a water-cooled jacket. The stationary and mobile phases were pumped using Dionex P580 high-pressure isocratic and gradient pumps (Dionex, U.K.). Samples were injected through a Rheodyne 7725i valve fitted with a 1 mL loop (Rheodyne, Rohnert Park, USA).

The CCC machine was operated in reverse-phase mode, i.e. with the PEG as the chosen stationary phase, except for experiments in which the PEG 400 phase system was used, in which case the lower K$_2$HPO$_4$ phase was stationary (normal mode). The upper PEG phase was first pumped into the column at a flow rate of 6 mL min$^{-1}$ in a 'head' to 'tail' direction. Once filled with stationary phase, rotation of the bobbins was started in the 'reverse' (clockwise) direction. When a rotational speed of 800 or 600 rpm had been reached the lower (salt) mobile phase was pumped isocratically through the column at a flow rate of 0.5 mL min$^{-1}$, again in the 'head' to 'tail' direction. Eluted stationary phase was collected in a graduated measuring cylinder and used to calculate the proportion (by volume) of stationary phase retained ($S_f$) once hydrodynamic equilibration of the phases had been achieved (taking into account the volume of the inlet and outlet leads).

In initial experiments, the plasmid preparation (Section 2.5.1), was dissolved (1:10 v/v) in the mobile phase and 1 mL injected onto the CCC column. For later experiments, the plasmid preparation (Section 2.5.1), was then prepared in one of three ways. (I) Plasmid resuspended in TE buffer was either injected...
directly onto the column, or (II) diluted in a ratio of 9:1 or 1:1 with mobile phase prior to loading. Alternatively (III) plasmid resuspended in mobile phase was also injected directly onto the column. In each case 1 mL was injected. The eluent from the column was collected at 5 minute intervals for further offline analysis by gel electrophoresis as described in Section 2.6.1. Upon completion of each chromatographic run, the contents of the coil were blown out using compressed nitrogen gas in the head to tail direction. Fractions of the eluted stationary and mobile phases were again collected for further offline analysis as described in Section 2.6.1 and for chosen experiments Section 2.6.2.3 and Section 2.6.5.

In an alternate approach, 10 mL of feed prepared as described in Section 2.5.3 was loaded onto the CCC column. Feed was loaded directly through the mobile phase buffer line at 0.5 mL min⁻¹. Feed prepared as described in Section 2.5.3 was also loaded onto the column immediately after the rotation of the bobbins had been started, i.e. the hydrodynamic equilibrium between the two phases in the coil is set up in the presence of the plasmid solute. 10 mL feed was again loaded at a flow rate of 0.5 mL min⁻¹ through the mobile phase buffer line. In these experiments PEG 600 IPA was the chosen phase system.

The full range of CCC experiments performed is summarised in Table 2.3.
### Table 2.3. Summary of the experiments carried out using the countercurrent chromatograph (composition ATPS used given in Table 2.2). Experiments performed as described in Section 2.5.5.

<table>
<thead>
<tr>
<th>Composition of ATPS used</th>
<th>Bobbin rotational speed (rpm)</th>
<th>Temperature (°C)</th>
<th>Stationary phase for CCC</th>
<th>Volume of feed loaded (mL)</th>
<th>Total plasmid loaded (µg)</th>
<th>Feed loaded after column equilibrated (Y/N)</th>
<th>Feed composition ratio</th>
<th>TE buffer</th>
<th>Mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 1000</td>
<td>800</td>
<td>30</td>
<td>Upper (PEG)</td>
<td>1</td>
<td>100</td>
<td>Y</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG 400</td>
<td>800</td>
<td>30</td>
<td>Lower (K$_2$HPO$_4$)</td>
<td>1</td>
<td>100</td>
<td>Y</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG 600</td>
<td>800</td>
<td>30</td>
<td>Upper (PEG)</td>
<td>1</td>
<td>100</td>
<td>Y</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>450</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>310</td>
<td></td>
</tr>
<tr>
<td>PEG 600 NaCl</td>
<td>600</td>
<td>30</td>
<td>Upper (PEG)</td>
<td>1</td>
<td>100</td>
<td>Y</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>450</td>
<td>1</td>
</tr>
<tr>
<td>PEG 600 IPA</td>
<td>600</td>
<td>30</td>
<td>Upper (PEG)</td>
<td>1</td>
<td>1000</td>
<td>Y</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>450</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>310</td>
<td></td>
</tr>
</tbody>
</table>

Plasmid prepared as ATPS (Section 2.5.2)
2.6 Analytical techniques

All analysis were carried out in triplicate where possible, and a minimum of duplicate

2.6.1 Plasmid DNA analysis by agarose gel electrophoresis

Agarose gel electrophoresis was used as a qualitative technique to identify the nucleic acid species (RNA, chromosomal DNA, supercoiled and open circular plasmid DNA) present, and the approximate proportions of nucleic acids, in each of the samples tested.

2.6.1.1 Precipitation of nucleic acids by isopropanol precipitation

Isopropanol precipitation was used throughout this work to exchange nucleic acids into a suitable buffer and as a concentration step.

Samples were incubated at room temperature for 20 minutes with 0.7 volumes of isopropanol before the precipitated nucleic acids were pelleted by centrifugation for 15 minutes at 13 000 rpm in a Biofuge 13 (Heraeus, Essex, UK), or 30 minutes at 10 000 rpm in a Beckman J2-M1 centrifuge for larger volumes used in the preparation of feed material (Sambrook et al. 1989). For nucleic acids suspended in a low salt buffer (i.e. TE buffer) 0.15 volumes of 3 M sodium chloride solution were included in the incubation mixture to facilitate the precipitation of the nucleic acids (Sambrook et al. 1989). The supernatant was then decanted and the nucleic acid pellet washed with 0.5 to 0.6 volumes of 70 % v/v ethanol before centrifugation for 10 minutes at 13 000 rpm or, for larger volumes, 20 minutes at 10 000 rpm. The supernatant was again decanted, and the remaining ethanol allowed to evaporate from the samples (aided by aspiration with a pipette) before being resuspended in the final volume of desired buffer (Sambrook et al. 1989). Typically samples were resuspended in half the original sample volume of TE buffer prior to gel electrophoresis.
2.6.1.2 Agarose gel electrophoresis conditions

Samples prepared as described in Section 2.6.1.1 were mixed with 5 to 7 µl gel loading buffer (0.25 % w/v bromophenol blue, 40 % w/v sucrose in deionised water). Samples containing the 6.9 kb pSVβ plasmid were loaded onto 0.8 % w/v agarose gels containing 0.05 μg mL⁻¹ ethidium bromide and electrophoresed for 2 hours in 0.5 x TBE buffer at 80 V as described by Sambrook and co-authors (Sambrook et al. 1989). Samples containing the 20 kb plasmid PQR150 or the 29 kb plasmid pMT103 were loaded onto 0.5 % w/v agarose gels containing 0.05 μg mL⁻¹ ethidium bromide and electrophoresed in TBE buffer for 30 minutes at 50 V (to run the DNA into the gel) and then for 16 hours at 15 V. When necessary, gels were deliberately overloaded to enhance the visualisation of contaminant DNA. Gels were density scanned and analysed using UVP 5000 Gel Documentation System and Gelbase™ analysis software (Ultra Violet products Ltd., Cambridge, UK) or the Scion Image for Windows software (Scion Corporation). The accuracy of the technique for quantitative analysis is commonly considered to be in the region of ± 10 % (Levy, personal communication. 1998).

The chromatograms shown in figures 5.2, 5.5 and 5.6, and Appendix B were constructed by expressing the density of the plasmid bands from the collected fractions as a percentage of the density of the corresponding bands from the feed sample.

In the case of fractions obtained from CCC experiments (as described in Section 2.5), samples of 20 µL volume were loaded directly onto the agarose gels for initial analysis, due to the large number of fraction obtained from each run. It was determined that the best results could be obtained using 0.8 % w/v agarose gels containing 0.05 μg mL⁻¹ ethidium bromide and electrophoresed for 2.5 hours in 1.0 x TBE buffer at 80 V.
2. Materials and Methods

2.6.2 Chromosomal DNA analysis

2.6.2.1 Preparation and labelling of the chromosomal DNA probe

Non transformed DH5α cells were streaked out in triplicate on LB agar plates containing 0.1 g L⁻¹ ampicillin and incubated for 24 hours at 37 °C. A single colony was used to inoculate 4 x Bijou vials containing 10 mL LB media with 0.1 g L⁻¹ ampicillin which were then incubated horizontally at 37 °C for 24 hours with shaking at 200 rpm. 1 mL aliquots of bacterial suspension (Qiagen 08/99) were centrifuged using the Biofuge 13 at 13 000 rpm for 15 minutes to pellet the cells. The supernatant was then decanted, and the bacterial pellet stored at –20 °C before use.

The pelleted cells from 1 mL bacterial culture were resuspended and lysed using the protocol recommended by the Qiagen Genomic Tip manufacturer (Qiagen 08/99). Lysis of the bacterial cells was achieved through a combination of enzymatic degradation of the cell wall (due to the addition of lysozyme to the resuspension buffer) and the action of the detergents present in the resuspension buffer supplied with the kit (Qiagen 08/99). Purification of chromosomal DNA was achieved through enzymatic degradation of contaminants such as proteins and RNA, prior to further purification by Qiagen anion-exchange technology.

The chromosomal DNA was eluted from the Qiagen genomic-tip before precipitation with isopropanol as described in Section 2.6.1.1. The pellet was then resuspended in sterile deionised water. The DNA content of samples was determined by spectroscopy at A²₆₀ as described in Section 2.6.4. Aliquots containing 5 μg chromosomal DNA were digested with the restriction enzyme Hae III using the protocol suggested by D. Cooke (Sambrook et al. 1989; Cooke, personal communication. 2000). 5 μg chromosomal DNA in sterile water previously purified by the Qiagen genomic tip protocol was included in a reaction mixture containing 10 μL 10 x Hae III reaction buffer, 3
μL Hae III stock solution and sterile water to a final volume of 100 μL. The reaction mixture was incubated overnight at 37 °C. 2 μL 0.5 M EDTA was added to the reaction mixture at the end of this time to stop the reaction.

Following restriction cutting with Hae III, a 10 μL aliquot of chromosomal DNA suspension was diluted 1: 39 in TE buffer (to give a final volume of 400 μL) before further purification by phenol chloroform extraction (Sambrook et al. 1989). An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1 v/v) was added to the chromosomal DNA suspension, and vortexed until an emulsion formed. The phases were then separated by centrifugation at 13 000 rpm in the Biofuge for 30 minutes. The upper aqueous phase was then removed by pipetting. An equal volume of chloroform was then added to the aqueous phase and subsequently mixed by vortexing, before the two phases were again separated by centrifugation at 13 000 rpm for 30 minutes. The upper aqueous phase was again removed and precipitated by incubation for 15 – 20 minutes with 2 x volumes of ice cold 100 % ethanol. Precipitated DNA was pelleted by centrifugation at 13 000 rpm for 15 minutes. The supernatant was then decanted and the pellet washed with 0.6 - 0.4 volumes of 70 % ethanol before a second centrifugation step at 13 000 rpm for 10 minutes. The supernatant was decanted and the pellet was then resuspended in 12 μL sterile water to give a final chromosomal DNA concentration of approximately 0.25 μg μL⁻¹.

1 μg digested and purified chromosomal DNA was labelled with the steroid hapten digoxigenin (DIG), obtained in the DIG High Prime Labelling and Detection Starter Kit 1 (Boehringer Mannheim, East Sussex, UK). 4 μL of the digested, purified chromosomal DNA fragment suspension was diluted to a final volume of 16 μL with sterile water and denatured by heating for 10 minutes to 95 °C before rapid cooling on ice. 4 μL of DIG High Prime was added and the mixture incubated at 37 °C overnight as per the manufacturers instructions. The concentration of labelled DNA was determined as approximately 13 ng mL⁻¹ using the test strips and protocol supplied with the
2. Materials and Methods

kit. Detection of chromosomal DNA fixed onto a charged nylon membrane was by the colorimetric immunoprecipitation technique recommended by the manufacturer (Boehringer-Mannheim 2000).

2.6.2.2 Chromosomal DNA analysis following nitrocellulose filtration

Samples were concentrated by isopropanol precipitation (Section 2.6.1.1) before being further purified and concentrated using the Qiaspin mini prep kit (Qiagen, West Sussex, UK). RNAse A supplied with the kit is used in the loading buffer to digest RNA present in the samples. Purification of the DNA species present from digested RNA and other cellular contaminants such as proteins is then achieved through non-selective binding of the nucleic acids to an adsorptive membrane at high ionic strength followed by a low ionic strength elution step. The total DNA content of the purified samples was determined spectrophotometrically by measuring the absorbance at 260 nm, as described in Section 2.6.4. The yield of DNA from the Qiaspin column was determined by comparing samples eluted from the column with the corresponding feed sample on an agarose gel prepared as described in Section 2.6.1. The data obtained was used to calculate the percentage of DNA loss at this stage. A factor of 0.85 ± 0.1 was obtained.

For specific detection of chromosomal DNA, 680 ng total DNA from each sample was blotted directly onto a charged nylon membrane (Boehringer-Mannheim), using a manifold slot-blotting apparatus as recommended by the manufacturer. The membrane was then baked for 2 hours at 80°C to fix the nucleic acids to the membrane (Sambrook et al. 1989). A digoxigenin (DIG) labelled probe (Boehringer-Mannheim), specific for E.coli chromosomal DNA had previously been prepared by M.S. Levy (Levy et al. 2000a). Detection was through a colorimetric immunoprecipitation technique recommended by the manufacturer (Boehringer-Mannheim 2000). Blots were scanned as described in Section 2.6.1. Known amounts of DH5α DNA (86 – 2.7 ng DNA) were loaded to provide the calibration curve.
2.6.2.3 Chromosomal DNA analysis following CCC

For quantitative analysis, selected samples were dialysed overnight against TE buffer using Slide-A-Lyzer dialysis cassettes (Perbio Science UK Ltd, Tattenhall, Cheshire, UK) and concentrated by precipitation using 0.7 volumes of isopropanol, and the concentration of DNA present determined by the Picogreen assay described in Section 2.6.4.

For specific detection of chromosomal DNA, 350 ng total DNA of each sample was blotted onto a charged nylon membrane (Boehringer-Mannheim) using a manifold slot-blotting apparatus as recommended by the manufacturer. The membrane was then baked for 2 hours at 80°C to fix the nucleic acids to the membrane (Sambrook et al. 1989). A DIG labelled probe (Boehringer-Mannheim) specific for *E.coli* chromosomal DNA was prepared and as described in Section 2.6.2.1. Blots were scanned as described in Section 2.6.1. Known amounts of DH5α DNA (50 – 0.7 ng) were loaded to provide the calibration curve.

For qualitative analysis, DNA from the analytical gels was blotted onto a charged nylon membrane by the capillary transfer method (Sambrook et al. 1989). Chromosomal DNA was detected using a DIG labelled probe prepared as described above.

2.6.3 Quantitation of purified DNA samples by measuring absorbance at 260 nm

The concentration of purified dsDNA or ssDNA samples can be determined by measuring the absorbance of the sample at 260 nm ($A_{260}$) and assuming 50 μg mL dsDNA has $A_{260} = 1$ and 40 μg mL ssDNA has $A_{260} = 1$ (Sambrook et al. 1989). Pure DNA samples were pipetted into clean quartz cuvette and the absorbance determined against an appropriate blank using a DU 70 Beckman
spectrophotometer. The ratio of $A_{260} / A_{280}$ was determined to ensure the purity of the DENA samples, and was acceptable between 1.7 and 1.9 for all samples (Sambrook et al. 1989).

### 2.6.4 Quantitation of un-purified DNA samples using the picogreen assay

The concentration of dsDNA from samples which had not been previously purified (i.e. using the Qiagen mini prep protocol) was determined using the Picogreen assay, a fluorometric technique. The sample was first diluted to suitable approximate DNA concentration (typically x 400) before loading onto a 96 well microtitre plate. Following addition of fluorescent dye (x 400 diluted from the stock solution) and subsequent incubation for 3 minutes in the dark, the fluorescence at an excitation level of 520 nm was measured using a Packard Fluorocount Microplate fluorometer (Packard Instrument Co. Meriden, CT, USA). Samples containing plasmid at known concentrations were included in each plate to provide a standard curve (1 - 100 µg mL$^{-1}$). A typical calibration curve is shown in Figure 2.3. More details on this technique are given by Levy and co-workers (Levy et al. 2000b).

![Figure 2.3. Typical calibration curve for Picogreen assay using known concentrations of the 6.9 kb plasmid pSV/β as a standard. Calibration prepared as described in Section 2.6.4.](image)
2.6.5 BioRad DC protein assay

Protein assays were performed using the BioRad DC assay (BioRad, Hemel Hemstead, Herts, UK) and reagents. The colour development was determined at 650 nm using a Dynatech MR7000 plate reader. A calibration curve of $A_{650}$ against known concentrations of bovine serum albumin (BSA) was constructed for each assay. Figure 2.4 shows a typical calibration curve. The maximum coefficient of variance of this assay was 5%. Under the conditions used, the lower level of detection of this assay is approximately 0.1 mg mL$^{-1}$ protein.

![Figure 2.4. Typical calibration curve for BioRad DC protein assay using known concentrations of BSA as a standard. Calibration prepared as described in Section 2.6.5.](image)

2.6.6 Endotoxin analysis (Limulus amebocyte lysate gel clot assay)

Pyrotell™ LAL reagent of 0.03 EU mL$^{-1}$ sensitivity (1 endotoxin unit (EU) can be assumed to correspond to 100 pg endotoxin (Petsch et al. 1998)), reconstitution buffer, and sodalime glass reaction tubes certified pyrogen free, were obtained from Associates of Cape Cod (Liverpool, UK). Pyrotell™ was
reconstituted as recommended by the manufacturer. The sensitivity of the Pyrotell™ was confirmed by testing a range of serial dilutions of sample endotoxin. A range of dilutions of the selected samples was prepared using endotoxin free water. The result was considered to be negative (endotoxin levels less than 0.03 EU mL⁻¹ in the dilution) if no gel had formed, or if the gel collapsed upon inversion. This assay has an accuracy of + 0.03 EU mL⁻¹ or – 0.015 EU mL⁻¹ under the conditions used.

Due to the variability in the environmental conditions and facilities available, difficulties were encountered in achieving the required low background levels, so that this assay was successfully performed on only a small number of samples.

2.6.7 Estimation of solids content by measuring absorbance at 600 nm

The reduction solids content of feed and permeate samples following tangential-flow filtration-adsorption, as described in Section 2.4.2.3, was estimated by measuring the absorbance of each sample at 600 nm using a DU 70 Beckman spectrophotometer (Levy, personal communication, 1998). The reduction in solids content for permeate fractions was expressed as a fraction of that measured for the feed samples.
3. Primary purification of plasmid DNA by batch adsorption using nitrocellulose powder

3.1 Aims and introduction

The utility of nitrocellulose powder to selectively remove denatured DNA species from plasmid-containing lysates was reported over 30 years ago (Cohen and Miller 1969; Palchaudhuri and Chakrabarty 1976). The mechanism underlying the adsorption of denatured nucleic acids to nitrocellulose is thought to be hydrophobic interaction, which was discussed in detail in Section 1.4.1. The lysis protocols used by Cohen and contemporaries were not however the alkaline lysis method of Birnboim and Doly (Birnboim and Doly 1979) reported in the late 1970's, which has been used as the cell disruption step for the work reported in this thesis. Batch adsorption in a stirred tank reactor would be a convenient step to follow alkaline lysis, particularly after removal of the floc by flotation and coarse filtration (Theodossiou et al. 1999). Therefore batch adsorption of the contaminants present following the alkaline lysis step using nitrocellulose has been investigated for the primary purification of plasmid DNA.

Following the neutralisation stage of the alkaline lysis operation (described in Section 2.2), plasmid DNA regains the native double stranded form, while chromosomal DNA remains in the single stranded, denatured form. Fragments of denatured chromosomal DNA have proved problematic to remove either by precipitation (Levy et al. 2000a), or chromatography (Prazeres et al. 1998). The ionic strength of the process stream containing the plasmid used for the work described in this thesis (described in Section 2.2) is considerably higher than the conditions under which selective adsorption using nitrocellulose has previously been carried out (Cohen and Miller 1969; Palchaudhuri and Chakrabarty 1976). The exact conditions of the previous adsorption experiments are more fully discussed in Section 1.4.1. The potential of nitrocellulose powder to selectively adsorb single stranded
chromosomal DNA immediately subsequent to the alkaline lysis step is thus investigated in this chapter. The intention is to reduce levels of chromosomal DNA prior to high-resolution purification stages, in order to improve the levels of purity achieved and increase the capacity of the matrices for plasmid products. The capacity of the nitrocellulose powder to adsorb ssDNA from a model solution, and denatured chromosomal DNA from partially clarified \textit{E.coli} lysate, was examined, and the capacity for adsorption in terms of the wet weight of nitrocellulose powder required per volume of partially clarified lysate determined. From this the amount of nitrocellulose powder required upon scale up of this process can be estimated and the feasibility of the process assessed. Potential regeneration protocols, to facilitate reuse of the powder, are also considered.

3.2 Batch adsorption of ssDNA from model solutions and plasmid containing liquors

3.2.1 Batch adsorption of ssDNA from a model solution

Initial stirred batch adsorption experiments were carried out using a pure model solution of ssDNA, prepared as described in Section 2.1. The capacity of nitrocellulose powder to adsorb ssDNA was estimated to be 225 $\mu$g g$^{-1}$ of nitrocellulose powder (results not shown). Before use the nitrocellulose powder was ground to approximately 0.5 mm diameter particles using a mortar and pestle. However it proved very difficult to produce particles of homogenous size using this technique and a wide range of particle sizes resulted which was considered to have unfavourably affected the reproducibility of the experiments, so that accurate quantitation was not possible. Despite these difficulties, the selective adsorption of denatured chromosomal DNA from plasmid containing lysates was still investigated, and qualitative results are given in the following Section 3.2.2.
3. Primary purification of plasmid DNA by batch adsorption using nitrocellulose powder

3.2.2 Capacity of nitrocellulose to adsorb chromosomal DNA from partially clarified lysate.

In order to evaluate the performance of a batch adsorption operation under the process conditions dictated by use of the alkaline lysis procedure, *E. coli* lysates containing a 6.9 kb plasmid, partially clarified as described in Section 2.2, were incubated with nitrocellulose powder. Experiments were set up at various ratios of partially clarified lysate to nitrocellulose powder (0.5 – 6 mL lysate per gram (wet weight) of nitrocellulose), as described in Section 2.4.2.2. To investigate the effect of variation of the cation on the adsorption step, lysate containing 6.9 kb plasmid, but prepared using sodium acetate in place of potassium acetate in the alkaline lysis step (as described in Section 2.2), was also used in the batch adsorption experiments.

From the data illustrated in Figure 3.1A it is evident that for ratios up to 2 mL lysate g⁻¹ nitrocellulose powder, the absence of a chromosomal DNA band on the agarose gel indicates that the majority of chromosomal DNA is adsorbed from the lysate. Replicate experiments with different batches of nitrocellulose powder indicated significant variability in the amount of chromosomal DNA adsorbed, hence no attempt was made to accurately quantify chromosomal DNA adsorption by Southern blotting. The variability in the results is attributed to the wide range of particle sizes present in the nitrocellulose powders, and will be discussed further in Section 3.2.4. From typical levels of chromosomal DNA that would be expected following alkaline lysis (9 µg mL⁻¹ as described in Section 2.2) the capacity for nitrocellulose powder to adsorb chromosomal DNA from partially clarified lysate can be estimated at 18 µg chromosomal DNA g⁻¹ nitrocellulose (wet weight). Some reduction in plasmid and RNA content for high ratios of nitrocellulose powder to lysate is also apparent, indicating other nucleic acids are also adsorbed.

Results for the batch adsorption of chromosomal DNA from lysate prepared using sodium acetate in place of potassium acetate are presented in Figure 3.1B. Although reduced, the chromosomal DNA band is apparent in all
samples in this case. This suggests that the use of Sodium acetate to replace Potassium acetate in the neutralisation step of the lysis operation is detrimental to the ability of nitrocellulose powder to adsorb chromosomal DNA from the resultant lysate. This is contrary to the observations of Popovic and co-workers who found increased adsorption of rRNA to nitrocellulose powder in the presence of different cations (Li⁺ > Na⁺ > K⁺ > Cs⁺ (Popovic and Wintzerith 1983)). The results presented in Figure 3.1 do however agree with the Hofmeister series which describes the increased tendency of proteins to precipitate, in the order NH₄⁺ > Rb⁺ > K⁺ > Na⁺ > Cs⁺ > Li⁺ > Mg²⁺ > Ca²⁺ > Ba²⁺ (Amersham-Pharmacia 1999)), i.e. stronger hydrophobic interaction would be expected in the presence of K⁺ compared to Na⁺ cations. This suggests that further investigation into the composition of the buffer used in the adsorption step may be valuable. However, as discussed in Section 1.3.1.2, an increase in levels of contaminants following alkaline lysis utilising sodium acetate in the place of potassium (Sinnett et al. 1998) may also have adversely affected the capacity of nitrocellulose powder to selectively adsorb chromosomal DNA.

In order to investigate the kinetics of adsorption of the single stranded chromosomal DNA to nitrocellulose powder, a batch adsorption experiment was carried out in which samples of the supernatant were collected for analysis following incubation of the lysate with nitrocellulose powder between 15 and 180 minutes (data not shown). Reduction of the incubation time to as little as 15 minutes did not affect the efficiency of adsorption of the chromosomal DNA.

The gels shown in Figure 3.1 also suggest that both RNA and plasmid DNA are partially adsorbed by the nitrocellulose regardless of which cation is present. With the intention of increasing the capacity of the nitrocellulose powder to adsorb chromosomal DNA contaminants, 0.1 μg mL⁻¹ RNase A was included in the lysis reaction as described in Section 2.2. Agarose gel electrophoresis of samples, which had previously been incubated with nitrocellulose powder, as described in Section 2.3.3, indicated that no
apparent increase in capacity resulted from the digestion of RNA molecules present following alkaline lysis (results not shown). This suggests that either small RNA fragments remaining after RNase digestion are still adsorbed by the nitrocellulose, or that other contaminants (such as endotoxin or protein) are more significant in terms of competition with chromosomal DNA for binding sites on the nitrocellulose powder.

**Figure 3.1.** Agarose gel analysis of feed and incubated lysate supernatant from batch adsorption experiments. (A) Partially clarified lysate prepared as described in Section 2.2. (Potassium acetate for neutralization). Lane 1; feed. Lane 2; 1 mL lysate g⁻¹ nitrocellulose Lane 3; 2 mL lysate g⁻¹ nitrocellulose. Lane 4; 3 mL lysate g⁻¹ nitrocellulose. (B) Partially clarified lysate prepared as described in Section 2.2 (Sodium acetate for neutralization). Lane 1; feed. Lane 2; 0.5 mL lysate g⁻¹ nitrocellulose. Lane 3; 1 mL lysate g⁻¹ nitrocellulose. Lane 4; 2 mL lysate g⁻¹ nitrocellulose. Lane 5; 3 mL lysate g⁻¹ nitrocellulose. Analysis performed as described in Section 2.6.1. Batch adsorption experiments carried out as described in Section 2.3.3.

### 3.2.3 Regeneration of nitrocellulose powders

The capacity of nitrocellulose to adsorb contaminants from plasmid containing process streams suggests that at least 0.5 kg nitrocellulose powder (wet weight) per L of lysate will be required for the selective adsorption of contaminants. Even at modest scale, this will be a large amount. Therefore, it
is desirable that the nitrocellulose powder be re-used to minimise the amount purchased and held in store. Regeneration of the nitrocellulose powder following adsorption of chromosomal DNA from partially clarified lysate was carried out by incubation of the nitrocellulose powder in low-salt buffer overnight as described in Section 2.3.4. In addition, as elevated temperatures are often used to optimise the effect of regeneration steps, both 65°C and room temperature was also investigated.

The regenerated powder was used to in batch adsorption experiments for the selective adsorption of chromosomal DNA contaminants, and these results compared to those achieved with fresh nitrocellulose powder. Figure 3.2 shows agarose gel analysis of samples taken following batch adsorption with fresh nitrocellulose powder, and regenerated nitrocellulose powder (as described in Section 2.3.4). During this experiment the level of chromosomal DNA contamination only been slightly reduced compared to the results presented in Figure 3.1. The chromosomal band was analysed as described in Section 2.6.1.2 and the results from samples subjected to the batch adsorption process compared with that obtained for the feed. Just 20% reduction was observed following the first incubation with nitrocellulose powder, and 16% reduction following incubation with nitrocellulose powder regenerated at room temperature. Following incubation with nitrocellulose powder regenerated at 65°C, there was no detectable decrease in levels of chromosomal DNA contamination. It is evident that adsorbed nucleic acids can be removed from the nitrocellulose powder by incubation of the nitrocellulose powder overnight at 65°C in low-salt buffer, due to the presence of de-sorbed contaminants in Lane 8. However it is also evident that double stranded plasmid DNA has been adsorbed by the nitrocellulose in addition to denatured chromosomal DNA. Less nucleic acids were de-sorbed by regeneration of the nitrocellulose powder at room temperature (Lane 7) than regeneration at 65°C (Lane 8), yet this step appears to have been more effective.
3. Primary purification of plasmid DNA by batch adsorption using nitrocellulose powder

Figure 3.2 Agarose gel analysis of feed and incubated lysate supernatant from batch adsorption experiments with fresh and regenerated nitrocellulose powder. Lane 1; feed. Lane 2; partially clarified lysate incubated with freshly washed nitrocellulose powder in a ratio of 1 mL lysate/1 g nitrocellulose. Lane 3; partially clarified lysate incubated with nitrocellulose powder regenerated at room temperature at ratio of 1 mL lysate/1 g nitrocellulose. Lane 4; partially clarified lysate incubated with nitrocellulose powder regenerated at 65°C in a ratio of 1 mL lysate/1 g nitrocellulose. Lane 5; unbound nucleic acids removed by the initial high salt wash. Lane 6; unbound nucleic acids removed by second high salt wash. Lane 7; nucleic acids desorbed from the nitrocellulose powder during low salt wash at room temperature. Lane 8; nucleic acids desorbed from nitrocellulose powder during low salt wash at 65°C. Agarose gel electrophoresis carried out as described in Section 2.6.1. Preparation of nitrocellulose powder carried out as described in Section 2.3.2. Batch adsorption and regeneration carried out as described in Section 2.3.4.

Generally speaking, hydrophobic interactions are decreased with decreasing temperature (Hjerten 1973), so the room temperature protocol would have been expected to have been more effective for the removal of adsorbed contaminants. That a greater concentration of contaminants are observed to have de-sorbed from the nitrocellulose at high temperature, followed by a loss in capacity to adsorb denatured nucleic acids may be explained by an irreversible reaction at the surface of the nitrocellulose granules as a result of exposure to a high temperature. A high temperature washing protocol, designed to aid the removal of the ethanol in which the nitrocellulose powder had been shipped was also investigated (Section 2.3.2.2). The resultant agarose gel analysis revealed that the capacity of the nitrocellulose to adsorb denatured nucleic acids had been adversely affected, with a ratio of 1 g nitrocellulose per 0.5 mL lysate required for significant reduction of chromosomal DNA contamination to be visible (results not shown). This observation supports the theory that the loss of capacity is due to a reaction at
3. Primary purification of plasmid DNA by batch adsorption using nitrocellulose powder

the surface of the granules, possibly involving the constituents of the 0.1 x SSC buffer used (15 mM NaCl, 1.5 mM sodium citrate) rather than irreversible binding of the contaminant species.

3.2.4 Discussion

Initial results on the binding of nucleic acid species to nitrocellulose powders suggest that the capacity of nitrocellulose to adsorb pure ssDNA from model solutions is much greater than the capacity to adsorb ssDNA contaminants, such as chromosomal DNA from partially clarified lysate. This is probably due to the binding of other contaminants present in the complex lysates. As shown in Figure 3.1, the capacity of nitrocellulose powder to adsorb chromosomal DNA from partially clarified lysates is around 18 µg chromosomal DNA g⁻¹ nitrocellulose (wet weight). Nitrocellulose did not appear to be as effective at adsorbing chromosomal DNA when Sodium acetate had been substituted for Potassium acetate in the lysis procedure Figure 3.1 B). The effect of varying the cation present in the feed stream has been previously described (Popovic and Wintzerith 1983) in terms of the retention of rRNA in a nitrocellulose column. The pattern observed in this work did not follow the order reported by Popovic and co-workers, rather the increase in the strength of hydrophobic interactions was as predicted by the Hofmeister series (Amersham-Pharmacia 1999). This suggests further development of the buffer to be used both in terms of selectivity and increase of hydrophobic interaction, could be advantageous. An understanding of the differences in the behaviour of the nucleic acid species binding to nitrocellulose, as observed in this work, and as reported by Popovic and co-workers, would aid in the modelling, and ultimately design of an adsorption process, but is beyond the scope of this thesis.

Some success was achieved with the regeneration of the nitrocellulose powder during a low salt wash at room temperature as shown in Figure 3.2. Interestingly, washing and regeneration at high temperatures as previously described (Nygaard 1963) was found to be deleterious to the performance of
The reason for this was not investigated in detail, but may be a result of an irreversible reaction at the surface of the nitrocellulose granules reducing the number of hydrophobic binding sites available. Boezi and co-workers have previously reported a protocol for the regeneration of nitrocellulose powder involving an extensive wash step at low ionic strength (Boezi and Armstrong 1967), which indicates that further investigation may result in an acceptable protocol for the regeneration of nitrocellulose powder. However Boezi and co-workers do not indicate the degree of purity of the DNA solution initially applied to nitrocellulose, which may prove significant.

From the results presented in this initial chapter it is also evident that at least 500 g of nitrocellulose powder per litre of lysate will be required to achieve significant purification by batch adsorption of chromosomal DNA contaminants. Nitrocellulose powder is explosive when dry, is very difficult to handle and, in addition, is a respiratory irritant. This makes it an undesirable chemical to handle in the large quantities that would be required at industrial scale. The use of nitrocellulose powder packed into a column for the separation of nucleic acids has been described (Nygaard 1963; Boezi and Armstrong 1967; Popovic and Wintzerith 1983). This would have the advantage of containing the potentially hazardous nitrocellulose powder. Large quantities would still be required (7.8 cm\(^3\) to adsorb 500 \(\mu\)g denatured DNA (Boezi and Armstrong 1967), or 50 mL lysate). However with a crude lysate, the capacity would be low and the column likely to block unless the lysate were highly clarified before loading. Nygaard and Hall (Nygaard 1963) discuss the tendency of nitrocellulose to leak from the column during chromatography, which is an extra consideration for the downstream operations. Also, although Nygaard and Hall (Nygaard 1963) discuss the regeneration of the column, it is to be expected that in an industrial situation it would be necessary to repack the column at regular intervals, which given the quantities of a hazardous chemical required is an operation which would require considerable forethought to ensure that it was carried out in a safe manner. Nitrocellulose powder might alternatively be used as a filter aid, but
3. Primary purification of plasmid DNA by batch adsorption using nitrocellulose powder

this may be hazardous as the risk of the danger of the powder drying out and becoming explosive is increased.

The greatest limitation in the batch adsorption experiments performed here has been the variability of results obtained with different batches of nitrocellulose powder (data not shown). It has proved very difficult to achieve a homogenous particle size using mortar and pestle as described in Section 2.3.1. This may have been a contributing factor to the inconsistency of some of the results. Sieving might be used to grade nitrocellulose powder particles by size, however this is likely to be too intensive a process and also dangerous without specialist equipment. Further development of a more effective strategy for the preparation of nitrocellulose powder in terms of achieving a homogenous range of particle sizes was not undertaken, as this line of investigation was discontinued due to safety concerns.

The results have, however, indicated that the binding of nucleic acid contaminant species to nitrocellulose might be a useful step for the primary purification of plasmid DNA. For this reason, work on adsorption using nitrocellulose membranes is considered in the next chapter. Compared to the use of nitrocellulose powder, membranes have the advantage of being easy to handle, and ultimately could be incorporated in a contained cassette for tangential-flow or dead-end filtration operations.
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption

4.1 Aims and introduction

After the neutralisation step of the alkaline lysis procedure (Sambrook et al. 1989), plasmid DNA regains its native dsDNA conformation while most chromosomal DNA remains in a denatured ssDNA form. Cellular debris, RNA, high molecular weight chromosomal DNA and other impurities precipitate out of solution with the salt-detergent complexes to form an insoluble floc (Birnboim and Doly 1979; Ciccolini et al. 1998; Sinnett et al. 1998). It is important that the floc is exposed to minimum shear levels at this stage to avoid breakage, which would result in contaminants being released back into the plasmid containing liquor. At laboratory-scale, the floc is usually removed by batch centrifugation (Levy et al. 2000c). Larger process volumes may require the use of continuous centrifugation in which shear forces may be unacceptably high (Levy et al. 2000c). Clarification by filtration could provide an attractive option if operating conditions were such that shear degradation of the floc was minimised (Theodossiou et al. 1997; Theodossiou et al. 1999). Single stranded chromosomal DNA fragments that are released into the plasmid DNA containing liquor have been found to be particularly difficult to separate from the plasmid product by PEG precipitation (Levy et al. 2000a), or chromatography (Prazeres et al. 1998).

Under appropriate high ionic strength conditions, nitrocellulose membranes strongly adsorb denatured ssDNA through hydrophobic interaction, but not dsDNA or RNA as discussed in Section 1.4.1. The potential of an integrated unit operation, comprising tangential-flow filtration-adsorption, to simultaneously adsorb contaminants and further clarify an *E. coli* lysate after coarse filtration is investigated in this chapter. The aim is to create a novel unit operation capable of removing key contaminants at an early stage of the process thus maximising the capacity of the subsequent chromatographic
operations. The capacity of the nitrocellulose to adsorb ssDNA from model solutions and denatured chromosomal DNA from partially clarified *E.coli* lysate was compared, and the degree of purification of plasmid from the partially clarified lysate determined. Potential regeneration protocols, to facilitate reuse of the membrane, are investigated, as is the effect of pre-purification of the lysate prior to the tangential-flow filtration–adsorption step.

Some of the results presented in this chapter have been published in: D. Kendall, G.J. Lye and M.S. Levy. 2002 Purification of plasmid DNA by an integrated operation comprising tangential-flow filtration and nitrocellulose adsorption. Biotechnology and Bioengineering. 79(7): 816-822.

4.2 Filtration-adsorption of model ssDNA solutions: determination of membrane capacity and effect of filtration operating mode

4.2.1 Comparison of cellulose acetate and nitrocellulose membranes

The initial experiments were designed to confirm that the mechanism of clearance of ssDNA was by adsorption to the nitrocellulose membrane. These involved dead-end filtration of a model ssDNA solution (prepared as described in Section 2.1) through either a nitrocellulose or cellulose acetate membrane as described in Section 2.4.2.2. Typical results for both membranes are presented in Figure 4.1. In the case of the nitrocellulose membrane (black bars), greater than 95 % w/w ssDNA was removed for the first 24 mL of collected permeate samples, after which breakthrough (defined here as the point at which the ssDNA concentration of the permeate is ≥25 % w/w of that of the feed) was observed. The operational capacity of the membrane was calculated by mass balance assuming adsorption by the membrane accounted solely for the difference in the ssDNA content of the feed and permeate. The calculated membrane capacity in this case was $36 \pm 3 \mu g \ cm^{-2}$. 

In contrast to the results obtained for the nitrocellulose membrane, very little ssDNA binding was observed during filtration through a cellulose acetate membrane, with relatively low adsorptive properties, of identical pore size. The 50 % reduction in ssDNA levels seen for the first fraction can be attributed to a combination of adsorption by the membrane during the early stages of the operation, and dilution effects due to wetting of the membrane during the preparation stages for the experiments. Later fractions showed between 80 % and 100 % transmission of ssDNA. This latter result confirmed that the depletion of ssDNA observed after filtration through nitrocellulose was due to an adsorption phenomenon as discussed in Section 1.4.1 rather than a size exclusion effect.

Figure 4.1. Dead-end filtration-adsorption of a solution of pure ssDNA, prepared as described in Section 2.1 through cellulose acetate (striped bars) or nitrocellulose (solid bars) membranes of pore size 0.45μm. Experiments carried out as described in Section 2.4.2.1. C concentration in permeate; C₀ concentration in feed. The initial concentration of ssDNA in the feed solution was 16 μg mL⁻¹ and 24 μg mL⁻¹ for the cellulose acetate and nitrocellulose experiments respectively. Concentration of ssDNA in permeate fraction determined by measuring absorbance at 260 nm as described in Section 2.6.3
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

4.2.2 Adsorptive properties of nitrocellulose membrane at varying retentate recirculation rates

For use in an industrial environment, operation of micro-filtration processes with crude process streams in a dead-end mode is undesirable. The build up of the filter cake on the membrane surface leads to rapid flux decline and also necessitates frequent membrane cleaning (Doran 1995) such that several pre-filtration steps, amounting to a large amount of membrane surface would be required. As nitrocellulose membrane with larger pore sizes have been shown to have poor adsorptive properties compared to the membrane of 0.45 μm pore size used for these experiments (Wohlhieter et al. 1966), the pre-filters would not be expected to add to the area available for adsorption. Thus a tangential-flow filtration-adsorption step was investigated with the intention of increasing the volume that could be processed per square meter of membrane area. It was not possible to obtain commercially a tangential-flow ultrafiltration cassette containing a nitrocellulose membrane. Tangential-flow filtration-adsorption experiments were thus performed with the model ssDNA solution using a small, purpose-built membrane rig as described previously in Figure 2.1. This had a circular geometry in order to allow the ready use of the nitrocellulose membranes. Experiments were performed at four retentate recirculation flow rates between $1.0 \times 10^{-6}$ and $3.8 \times 10^{-6} \text{ m}^3 \text{s}^{-1}$ (8.0 to 30.4 m m$^{-2}$ membrane area s$^{-1}$). The corresponding linear velocities in the connecting tubing were in the range $1 \times 10^{-2}$ to $2.0 \times 10^{-1} \text{ m s}^{-1}$, while those across the membrane were between 0.06 and 0.25 m s$^{-1}$ at the inlet and outlet to the membrane and between 0.025 and 0.09 m s$^{-1}$ midway along the circular membrane. The corresponding transmembrane pressure drops, determined as described in Section 2.4.2.3 were between 200 and 2000 Pa. The membrane unit was operated in all cases below the critical transmembrane pressure.

Levels of shear for the range of retentate recirculation rates used in this work were calculated from the well known equation:
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

\[ \gamma = 6u / h \]  \hspace{1cm} [4.1]

Where \( \gamma \) is shear force in s\(^{-1} \), \( u \) is linear velocity in m s\(^{-1} \) and \( h \) is the height of the flow channel in m. The maximum shear rates at the inlet and outlet ports were calculated to be between 400 to 1600 s\(^{-1} \), and the average shear rate across the membrane to be between 190 and 780 s\(^{-1} \).

As shown in Figure 4.2 A, between 85 % w/w and 95 % w/w of the ssDNA content of the feed was initially adsorbed during passage of permeate through the membrane for all the recirculation flow rates tested. Between 44 and 68 mL of permeate was collected before breakthrough was seen (concentration of chromosomal DNA in the permeate at \( \geq 25 \) % w/w of that of the feed, as described in Section 4.2.1). From the breakthrough data shown in Figure 4.2 B, an average operational capacity of the nitrocellulose membrane for the adsorption of ssDNA of 45 ± 7 \( \mu \)g cm\(^{-2} \) was calculated by mass balance assuming adsorption by the membrane accounted solely for the difference in ssDNA content in the feed and permeate for all four flow rates. The characteristic profiles of the curves i.e. high ssDNA concentration in the initial fraction, indicate that maximum ssDNA clearance occurs only after the first fraction has permeated the membrane. This may indicate that the formation of a gel layer on the membrane aids the adsorption process. At the slower retentate recirculation rates, due to the lower permeate flux (Figure 4.2 C), a greater processing time was required before breakthrough was observed. However the breakthrough data could be normalised in all cases against the total amount of permeate collected.

As expected, permeate flux rates declined over the time course of the filtration runs, as shown in Figure 4.2 C. This is due to the formation of a gel layer on the surface of the membrane. In this case it is likely that adsorption of ssDNA resulting in blockage of the membrane pores also contributed to the flux decline observed. The decline in permeate flux was more rapid at the higher retentate recirculation rates, due to the compaction of the gel layer at the
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

![Diagram]

Figure 4.2. Tangential-flow filtration-adsorption of a solution of pure ssDNA (prepared as described in Section 2.1) through a nitrocellulose membrane at retentate recirculation rates of $3.8 \times 10^6 \text{m}^3 \text{s}^{-1}$ (black bars and symbols), $2 \times 10^6 \text{m}^3 \text{s}^{-1}$ (horizontal fill bars and symbols), $1.3 \times 10^6 \text{m}^3 \text{s}^{-1}$ (vertical fill bars and symbols) and $1 \times 10^6 \text{m}^3 \text{s}^{-1}$ (unfilled bars and symbols). (A) ssDNA transmission as a function of collected permeate volume; (B) ssDNA transmission over time; (C) permeate flux over time. Experiments carried out as described in Section 2.4.2. Concentration of ssDNA in the permeate fractions determined as described in Section 2.6.3. $C$ concentration in permeate; $C_o$ concentration in feed. The average concentration of freshly denatured ssDNA in the feed for each experiment were 9.9 $\mu$g mL$^{-1}$, 12.3 $\mu$g mL$^{-1}$, 11.2 $\mu$g mL$^{-1}$, 12 $\mu$g mL$^{-1}$ for the flow rates of 3.8, 2, 1.3 and $1 \times 10^6 \text{m}^3 \text{s}^{-1}$ respectively.
Figure 4.3. Tangential-flow filtration-adsorption of a solution of BSA (prepared as described in Section 2.1) through a nitrocellulose membrane at a retentate recirculation rate of $1.3 \times 10^8$ m$^2$ s$^{-1}$. (A) BSA transmission as a function of collected permeate volume; (B) BSA transmission over time; (C) permeate flux over time. Experiments carried out as described in Section 2.4.2 and BSA concentration in the fraction determined as described in Section 2.6.5. $C$ concentration in permeate; $C_0$ concentration in feed. The average concentration of BSA in the feed for each experiment was 2.5 mg mL$^{-1}$. 
resultant higher transmembrane pressures. An accelerated rate of adsorption of ssDNA within pores due to higher permeate flux is likely to have contributed to the rate of flux decline. The final permeate flux is around 40 L m⁻² h⁻¹ in all cases, being reduced to around 20% of the original. Washing the nitrocellulose membrane with pure water as described in Section 2.4.3 resulted in ssDNA breakthrough being observed at an earlier time point during a subsequent filtration-adsorption step, indicating that 85% of the capacity of the membrane to adsorb ssDNA was recovered by the washing step (results not shown).

For comparison, a pure BSA solution (2.5 mg mL⁻¹) in 10 x SSC buffer was also filtered in the tangential-flow rig to investigate protein adsorption to the nitrocellulose membranes. As shown in Figure 4.3, Protein transmission was ~50% w/w during the first 30 seconds of the filtration-adsorption process and increased to a constant value of 80% w/w subsequently. The initial permeate flux was comparable to that observed for the ssDNA solution at the same retentate recirculation rate and permeate flux was observed to decline over the time course of the experiment. Permeate flux reduced from 160 to 100 L m⁻² h⁻¹ over 8 minutes, indicating the formation of a gel layer on the membrane surface. From this data the capacity of the nitrocellulose membrane to adsorb the model BSA solution can be calculated to be 18.9 mg cm⁻².

4.2.3 Discussion

From the results obtained with model ssDNA solutions, the utility of tangential-flow nitrocellulose adsorption for the removal of ssDNA contaminants seems promising. The results obtained from the comparison of adsorption of ssDNA during filtration in a dead-end mode through either an adsorptive nitrocellulose or non-adsorptive cellulose acetate membrane of identical pore size showed that the reduction of ssDNA in the permeate following filtration through nitrocellulose is primarily due to adsorption rather than size exclusion, in the case of model solutions. Typical results are shown in Figure 4.1. The difference in the adsorptive properties of the two membranes is due to the
difference in the membrane chemistries. Subsequent experiments with the filtration-adsorption step operated in tangential-flow mode (typical results shown in Figure 4.2) indicated that this mode of operation was also effective for the reduction of ssDNA. The capacity of nitrocellulose membrane to adsorb ssDNA was calculated to be around 45 μg cm⁻². Characteristic breakthrough curves showing the increase in ssDNA concentration in the permeate as the membrane became saturated, were recorded. As expected the permeate flux was observed to decline with time, due to the formation of a gel layer on the membrane surface, and binding of ssDNA within the membrane pores. Variation of the retentate recirculation rate, between 1.0 x 10⁻⁶ and 3.8 x 10⁻⁶ m s⁻¹ and the inherent variation in the flux rate did not greatly affect the efficiency of the adsorption process. Some adsorption of protein by nitrocellulose membranes during tangential-flow filtration-adsorption from model BSA solution was also observed for early fractions. Proteins often carry hydrophobic patches on the surface of the molecule (Matthews and van-Holde 1990), and therefore would be expected to bind through hydrophobic interaction to nitrocellulose. However, the concentration of BSA in the permeate was reduced at most to 50 % w/w that of the feed stream. In comparison ssDNA concentrations in the permeate were reduced to as little as 5 % w/w of the feed stream, indicating that at the conditions used in these experiments, protein from the model solutions is not as readily adsorbed by the nitrocellulose membrane as ssDNA. This is most readily explained by the hydrophobic nature of the bases of the DNA molecule, which are exposed following denaturation of the molecule resulting in effective adsorption of the ssDNA by the nitrocellulose membrane under the conditions used. Adsorption of the chosen model protein through hydrophobic interaction with nitrocellulose is less effective, presumably as the model protein is relatively hydrophilic compared to the ssDNA. A retentate recirculation rate of 1.3 x 10⁻⁶ m³ s⁻¹ (10.4 m m⁻² membrane area s⁻¹) was chosen for subsequent experiments, due to concerns regarding the shear sensitivity of large contaminant chromosomal DNA molecules, and potentially also of the plasmid product (Levy, personal communication. 1998; Levy et al. 1999b), despite the increased length of time required for the operation due the sub-optimal
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

processing conditions. There was also a requirement to keep the pressures at which the rig was operated low, due to problems with leakage from increased pressure at higher retentate recirculation rates.

4.3 Tangential-flow filtration-adsorption of partially clarified *E. coli* lysates

In order to evaluate the performance of the membrane adsorption operation under real process conditions, *E. coli* lysates containing a 6.9 kb or 20 kb plasmid prepared and partially clarified as described in Section 2.2, were used to perform tangential-flow filtration-adsorption experiments as described in Section 2.4.2.2. From the data obtained earlier with model ssDNA feeds, a retentate recirculation flow rate of $1.3 \times 10^{-6}$ m$^3$ s$^{-1}$ was used in each case. Either 2 mL or 1 mL fractions of permeate were collected. The feed and permeate fractions were then analysed by agarose gel electrophoresis as described in Section 2.6.1.

4.3.1 Filtration-adsorption of partially clarified *E. coli* lysates containing a 6.9 kb plasmid

The first experiments using partially clarified lysates were conducted using the 6.9 kb plasmid, previously examined in Chapter 3. Figure 4.4 shows a typical agarose gel analysis of selected permeate fractions following tangential-flow filtration-adsorption.

As expected, a substantial decrease in the single stranded chromosomal DNA content of the permeate fractions can be observed in the first three 2 mL fractions due to binding of the exposed bases of the DNA molecule to hydrophobic nitrocellulose. Breakthrough of the chromosomal DNA was just evident in the fourth 2 mL fraction collected. Plasmid DNA remains in a double stranded conformation following the lysis reaction, so the hydrophobic bases are shielded and would not be expected to readily adsorb to nitrocellulose.
Densitometric scanning of the gel as described in Section 2.6.1 indicated that SC plasmid transmission was approximately 100% w/w. A decrease in RNA content was also observed in the first permeate fraction (Figure 4.4) indicating that RNA is also adsorbed by the membrane at early stages of the filtration-adsorption process. However due to the chemical differences between RNA and DNA, and the presence of double stranded hairpin structures in some species of RNA (Section 1.2) the interaction is not as strong as seen with ssDNA. The similarity of agarose gel profiles obtained from seven independent experiments (data not shown) demonstrated the reproducibility of the operation.

Figure 4.4. Agarose gel analysis of feed and permeate fractions from a tangential-flow filtration-adsorption run. A partially clarified lysate prepared as described in Section 2.2, containing a 6.9 kb plasmid was processed at a retentate recirculation rate of $1.3 \times 10^5$ m$^3$ s$^{-1}$ as described in Section 2.4.2.3. Lane 1, feed; Lanes 2-5, permeate fractions collected after 2, 4, 6 and 8 mL of permeate had passed through the membrane respectively. CHR, chromosomal DNA; OC, open circular plasmid DNA; SC, supercoiled plasmid DNA. Agarose gel analysis performed as described in Section 2.6.1.
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

Figure 4.5. Southern slot-blot analyses of feed and permeate fractions from a tangential-flow filtration-adsorption run. A partially clarified lysate prepared as described in Section 2.2, containing a 6.9 kb plasmid was processed at a retentate recirculation rate of $1.3 \times 10^6 \text{ m}^2 \text{s}^{-1}$ as described in Section 2.4.2.3. 580 ng total DNA for feed and permeate fractions (collected every 1 mL) were loaded on the blot. Slots: 1A, feed; 2A-6A, permeate fractions collected after 1-5 mL respectively; 7A-12A, E. coli DH5α chromosomal DNA standard curve (2.7, 5.5, 10.5, 21.5, 43 and 86 ng respectively); 1B-6B, E. coli DH5α chromosomal DNA standard curve (86, 43, 21.5, 10.5, 5.5 and 2.7 ng respectively); 7B-9B, permeate fractions 6-8 mL; 10B, retentate; 11B, phage λ DNA (200 ng); 12B, TE buffer. Slot blot analysis carried out as described in Section 2.6.2.2.

Feed and 1 mL permeate fractions corresponding to five independent filtration-adsorption experiments (corresponding fractions from separate experiments being pooled to provide enough volume for subsequent analysis) were subjected to Southern slot-blot analysis using a probe specific for E. coli chromosomal DNA (as described in Section 2.6.2.1). Pure DH5α DNA was loaded to generate a standard curve that represents contamination levels from 0.5 % w/w to 15 % w/w. Under the conditions used, the detection limit was 5.5 ng DNA (1%). A typical slot-blot result is shown in Figure 4.5. Quantitative data obtained from densitometric scanning of the blots, carried out as described in Section 2.6.2, was plotted against filtration-adsorption time and is shown in Figure 4.6 A. The feed had a chromosomal DNA content of 10 % w/w which decreased to 8 % w/w in the first collected fraction and further decreased to <1 % w/w (below the level of detection) in the following fraction; this observation will be discussed further in Section 4.3.4. Considering data obtained from Southern slot-blot analysis and the yield from the Qiaspin columns (as described in Section 2.6.2.2), the feed was estimated to have a chromosomal DNA content of approximately $9 \mu \text{g mL}^{-1}$. Therefore under the conditions used, the operational capacity of the nitrocellulose membrane to absorb chromosomal DNA was calculated as $3.1 \pm 0.4 \mu \text{g cm}^{-2}$ (data from four replicate experiments).
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

Figure 4.6. Permeate flux and composition during the tangential-flow filtration-adsorption of a partially clarified *E. coli* lysate containing a 6.9 kb plasmid at a retentate recirculation rate of $1.3 \times 10^6 \text{ m}^3\text{s}^{-1}$ as described in Section 2.4.2.3. (A) plasmid and chromosomal DNA transmission; data shown represents the mean of 5 independent experiments, error bars reflect standard deviation; (B), protein and solids transmission; (C), permeate flux. C concentration in permeate; $C_0$ concentration in feed. The composition of the feed was typically 9 $\mu$g mL$^{-1}$ chromosomal DNA, 75 $\mu$g mL$^{-1}$ plasmid DNA and 0.74 mg mL$^{-1}$ total protein. Analyses carried out as described in Section 2.6.
Also plotted in Figure 4.6 A is the concentration of plasmid DNA in the permeate fraction obtained from scanning the agarose gels. This shows approximately 100 % w/w transmission of both open circular and supercoiled plasmid into the permeate stream. A five-fold decrease in protein content was initially recorded (Figure 4.6 B) which then gradually increased to 80 % w/w transmission. From this data the amount of protein adsorbed or retained by the membrane can be estimated to be 0.26 mg cm⁻². As expected, permeate flux declined over time. Initial values for the permeate flux were calculated from the time taken to collect the first 1 mL permeate to be approximately 77 L m⁻² h⁻¹. The permeate flux then decreased steadily, until approximately 50 L m⁻² h⁻¹ was recorded for the time point at which breakthrough of the chromosomal DNA was observed. Endotoxin levels were assayed for one filtration-adsorption experiment as described in Section 2.6.6, and were found to be reduced from 4.8 x 10³ EU mL⁻¹ in the feed to 48 EU mL⁻¹ in the first 2 mL permeate collected indicating a 100-fold decrease in endotoxin levels.

4.3.2 Effect of plasmid size on plasmid DNA recovery

Tangential-flow filtration-adsorption experiments using an E. coli lysate containing a 20 kb plasmid were also performed again using a retentate recirculation rate of 1.3 x 10⁻⁶ m³ s⁻¹. Agarose gel profiles for the collected permeate fractions are shown in Figure 4.7 and are similar to those obtained in previous experiments using the 6.9 kb plasmid (Figure 4.4). A substantial decrease in chromosomal DNA content was again observed in the first three permeate fractions breakthrough being evident in the fifth. Densitometric scanning of the gel indicated that plasmid transmission was again approximately 100 % w/w. The proportions of OC and SC plasmid DNA in the permeate fractions are similar to that observed for the feed fractions. This indicates that the environment in the small scale tangential-flow filtration device (Figure 2.2) and passage through the membrane have not resulted in the 20 kb plasmid becoming sheared during the tangential-flow filtration-adsorption operation under the conditions used.
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

Figure 4.7. Agarose gel analysis of feed and permeate fractions from a tangential-flow filtration-adsorption run. A partially clarified *E. coli* lysate containing a 20 kb plasmid prepared as described in Section 2.2 were filtered at a retentate recirculation rate of $1.3 \times 10^6 \text{ m}^3 \text{ s}^{-1}$ as described in Section 2.4.2.3. Lane 1, feed; Lanes 2-6, fractions corresponding to 2, 4, 6, 8 and 10 mL of collected permeate respectively; CHR, chromosomal DNA; OC, open circular plasmid DNA; SC, supercoiled plasmid DNA. Agarose gel electrophoresis were performed as described in Section 2.6.1.

4.3.3 Membrane cleaning and regeneration

A variety of protocols for cleaning the membrane as described in Section 2.4.3 were investigated. This was essential in order to assess the potential for re-use of the membrane, and improve the economics of the tangential-flow filtration-adsorption operation. The pure water flux was initially determined for each fresh membrane before the start of the experiments. The average pure water flux was found to be $346 \pm 32 \text{ L m}^{-2} \text{ h}^{-1}$. Membranes were subsequently fouled by a filtration-adsorption step as described in Section 2.4.2.3 before various cleaning (Section 2.4.3). First an initial wash with 10 x SSC buffer to remove unbound contaminant and product was performed. The main cleaning was subsequently carried out by pumping either pure H$_2$O (room temperature), 0.15 M Triton X-100 (room temperature), 1 M Triton X-100 (37 °C) or 0.1 M NaOH at 50-60°C through the rig at a retentate recirculation rate of $1.3 \times 10^6 \text{ m}^3 \text{ s}^{-1}$ for between 30 minutes and 1 hour. During cleaning the membrane was replaced within the rig either in the same orientation as during
the filtration-adsorption step, or upside down to facilitate removal of the gel layer from the surface of the membrane by backflushing. At no point was the membrane allowed to dry out.

The best results were obtained by backflushing the membrane with water for 1 hour. From agarose gel analysis of the initial tangential-flow filtration-adsorption run a substantial decrease in chromosomal DNA concentration was observed in the first 8 mL of permeate collected. Following regeneration by backflushing with water as described in Section 2.4.3, chromosomal DNA levels were only reduced for the first 4 mL permeate. In this manner approximately 50% of the membrane capacity for single stranded chromosomal DNA adsorption was regained (Figure 4.8, lanes 11-15). However only 4% of the initial pure water flux was regained following the first round of filtration-adsorption and regeneration.

For regeneration protocols involving wash buffers other than pure water, agarose gel electrophoresis (Section 2.6.1) indicated that little or no membrane capacity for single stranded chromosomal DNA was regained. Pure water fluxes were restored to between 8% and 50% of those originally measured. Physical limitations of the small-scale rig used for these experiments meant that high shear rates which might help remove the gel layer could not be achieved. However as the requirement for low shear rates to avoid breakage of the plasmid product is the predominant factor determining the operating conditions, it was decided not to redesign the tangential-flow rig.

A further regeneration step by back flushing with water following a second filtration-adsorption operation (as described in Section 2.4.3), failed to regain any of the capacity of the membrane, and again only 4% of the pure water flux was recovered following regeneration. Figure 4.8, lanes 7-9 and 16-18 show the nucleic acids cleaned from the membrane during the regeneration protocol.
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

Figure 4.8. Agarose gel analyses of feed and permeate fractions from three sequential tangential-flow filtration-adsorption runs, with two regeneration stages comprising an initial wash in 10 x SSC before backflushing with water for one hour as described in Section 2.4.3. A partially clarified lysate containing a 6.9 kb plasmid prepared as described in Section 2.6.1.2 and 2.2 was processed at a retentate recirculation rate of $1.3 \times 10^5$ m$^3$ s$^{-1}$ as described in Section 2.4.2.3. Lane 1, feed; Lanes 2-6, permeate fractions collected during initial tangential-flow filtration-adsorption run after 2, 4, 6, 8 and 10 mL of permeate had passed through the membrane respectively. Lane 7, material removed during high salt (10 x SSC) wash in first regeneration stage; Lanes 8 and 9, contaminants desorbed during H$_2$O backflush in first regeneration stage collected after 2 and 4 mL respectively; Lane 10, feed for second filtration-adsorption step; Lanes 11-15, permeate fractions collected during second tangential-flow filtration-adsorption run after 2, 4, 6, 8 and 10 mL of permeate had passed through the membrane respectively. Lane 16, material removed during high salt (10 x SSC) wash in second regeneration stage; Lanes 17 and 18, contaminants desorbed during H$_2$O backflush in second regeneration stage collected after 2 and 4 mL respectively; Lane 19, feed for filtration-adsorption step; Lanes 20-24, permeate fractions collected during third tangential-flow filtration-adsorption run after 2, 4, 6, 8 and 10 mL of permeate had passed through the membrane respectively. Lane 25, retentate from third tangential-flow filtration-adsorption run. CHR, chromosomal DNA; OC, open circular plasmid DNA; SC, supercoiled plasmid DNA; RNA, contaminant RNA. Agarose gel analysis carried out as described in Section 2.6.1.
Alternative regeneration methods were tried, including 0.1 M NaOH at 50-60°C and 1% Triton X-100 at 37 °C. However none were as successful in terms of recovering the capacity of the membrane for the adsorption of chromosomal DNA as straight forward desorption using water. Indeed, use of caustic at an increased temperature was observed to result in the membrane becoming very brittle following the regeneration process.

4.3.4 Discussion

In tangential-flow filtration-adsorption experiments with real *E. coli* lysates for the conditions and transmembrane pressures investigated, chromosomal DNA contamination could be decreased from around 10% w/w to approximately 8 % w/w in the first collected fraction, and an average of approximately 1 % w/w for the remaining permeate fractions collected prior to chromosomal DNA breakthrough (Figure 4.5). The mechanism for this is selective adsorption of the single stranded chromosomal DNA during passage through the nitrocellulose membrane. Plasmid DNA, which regains the more hydrophilic double stranded conformation following the alkaline lysis step, passes through the membrane with close to 100 % w/w transmission indicating virtually complete product recovery. Some adsorption of other hydrophobic contaminant species, such as RNA, proteins and endotoxin is seen at an early stage of the filtration-adsorption process, however breakthrough for these occurs earlier than for chromosomal DNA. The reason for the extended adsorption of single stranded chromosomal DNA by the nitrocellulose membrane over other contaminants present in the process stream is probably the strong hydrophobic interaction between the exposed bases along the length of the DNA molecule and the nitrocellulose membrane (Oss et al. 1987). Other contaminants such as proteins and RNA do not interact through hydrophobic structures with the nitrocellulose as strongly, due to physical and chemical differences when compared with the single stranded chromosomal DNA (Oss et al. 1987). The formation of a polarised gel layer, most likely in combination with the adsorption of contaminants in the membrane pores, reduces the permeate flux over time. Southern blot analysis
showed that chromosomal DNA levels of the first permeate fraction, although 25% lower than the feed were considerably higher than in the subsequent fractions. This indicates that formation of a gel layer on the surface of the membrane may aid the adsorption process by slowing the permeate flux, or through partial blocking of the membrane pores.

The operational capacity of the membranes for chromosomal DNA adsorption from clarified lysates was modest (3.1 μg cm\(^{-2}\); Figures 4.4 - 4.6), around 10% of the capacity calculated for model systems using pure ssDNA (Figure 4.2), and lower than that achievable with purer process streams (Collins, personal communication 1998; Levy et al. 2000a). Nevertheless, the limited capacity, high cost and in some cases low resolution of chromatographic matrices available for nucleic acids separations (Levy et al. 2000c) means that every reduction in contaminant nucleic acids made at an early stage of the process is particularly desirable for processes at large scale. Chapter 6 includes a discussion on the economic aspects of the tangential-flow filtration-adsorption experiment.

Following filtration of pure ssDNA through nitrocellulose membrane, the capacity of the membrane to adsorb ssDNA could be readily recovered by washing with pure water strength buffer (Section 4.2.2). Various protocols were tested for the regeneration of nitrocellulose following filtration-adsorption of partially clarified lysate, including washing for up to 3 hours with low ionic strength buffer or detergents. These included pure H\(_2\)O (room temperature), 0.15 M Triton X-100 (room temperature), 1 M Triton X-100 (37 °C) or 0.1M NaOH at 50 – 60 °C. However, all protocols proved unsatisfactory, with only a small fraction of the capacity (at most 50% and typically < 20%) of the fresh membrane being recovered. It is suggested that this is due to the irreversible binding of other contaminants present in the lysate such as endotoxin and proteins during the filtration-adsorption process, which cannot then easily be desorbed. The preparation and regeneration of nitrocellulose powders for chromatographic separations of nucleic acids has been reported previously.
but under much harsher conditions (incubation for 16 hours in 0.06 M KCl, 0.01 M Tris, pH 7.3, at 65-78°C) (Boezi and Armstrong 1967). In addition the extent of the preparation and purification that the nucleic acids were subjected to, before loading onto the nitrocellulose column is not described in the text, and may have resulted in problematic contaminant species being removed. An extended washing step may be successful, but will be undesirable at industrial scale. Thus the application of nitrocellulose membranes for adsorption is likely to be applied using disposable membranes. Use of a disposable step will minimise the stringent cleaning validation requirements that will be associated with DNA products for human use, particularly in multiproduct facilities (Butler 1996).

4.4 Effect of lysate pre-purification on membrane capacity

RNA is a major contaminant in plasmid containing lysates due to the proliferation of this species within cells. Given that RNA levels are seen to be reduced in the early permeate fractions during the filtration-adsorption stages (Figure 4.4), the possibility of increasing the capacity of nitrocellulose to adsorb chromosomal DNA by depleting levels of RNA before the filtration-adsorption step was investigated. Two approaches were tried, the addition of RNAse during the lysis stage as described in Section 2.2, and pre-purification of the lysate by CaCl₂ precipitation of contaminants as described in Section 2.4.1.

4.4.1 Effect of RNAse addition during lysis

Two identical batches of lysate were initially prepared from cells containing the 6.9 kb plasmid obtained from the 75 L fermentation. One batch was lysed as per the standard protocol described in Section 2.2.2 with no addition of RNAse to act as a control. The other was lysed with the addition of RNAse as described in Section 2.2.2. A tangential-flow filtration-adsorption experiment was subsequently carried out as described in Section 2.4.2.3 at a retentate
recirculation rate of $1.3 \times 10^{-6}$ m$^3$ s$^{-1}$ for each batch of lysate. The presence of chromosomal DNA was detected in the filtrate fractions at similar stages in the experiments for both batches; Breakthrough was observed after 4 mL of permeate had been collected when lysate not treated with RNase was utilised as feed (Figure 4.9 A), compared to after 3 mL of permeate collected when the lysate which had been treated with RNase was used for the feed (Figure 4.9 B). The pre-treatment of the feed stream with RNase, resulting in the digestion of RNA into small fragments was thus found not to be effective at increasing the capacity of the membrane to specifically bind chromosomal DNA. The fact that chromosomal DNA is actually evident slightly earlier for reactions treated with RNase is likely due to small differences in the levels of contaminants between the two lysate batches.

The failure of RNase pre-treatment to increase the capacity of the membrane to adsorb chromosomal DNA (by reduction in competitive RNA adsorption), may be due to the fact that RNA, when present, does not use a significant proportion of the adsorption capacity of the nitrocellulose membrane, despite the proliferation of RNA in lysate. An alternate interpretation is that the small fragments of RNA, not detected by agarose gel electrophoresis, continue to bind to the membrane therefore taking up adsorption capacity. However given that fragments of DNA < 300 nucleotides in length are not retained by the nitrocellulose membrane, and that RNA molecules are not as readily adsorbed by nitrocellulose (Nygaard 1963; Oss et al. 1987; Sambrook et al. 1989), it seems unlikely that small fragments of nitrocellulose would continue to bind.
Figure 4.9. Agarose gel analysis of feed and permeate fractions from a tangential-flow filtration-adsorption runs carried out as described in Section 2.4.2.3. (A) Bacterial cells from the 75 L fermentation as described in Section 4.2.2, containing a 6.9 kb plasmid were lysed in the absence of RNAse and partially clarified before being filtered through a nitrocellulose membrane at a retentate recirculation rate of $1.3 \times 10^{-6}$ m$^3$ s$^{-1}$. Lane 1: feed; Lanes 2-9: permeate fractions corresponding to 1-8 mL of permeate collected respectively (1 mL fractions). Lane 10: retentate. (B) Bacterial cells from the 75 L fermentation containing a 6.9 kb plasmid were lysed in the presence of RNAse and partially clarified before being filtered through a nitrocellulose membrane at a retentate recirculation rate of $1.3 \times 10^{-6}$ m$^3$ s$^{-1}$. Lane 1: feed; Lanes 2-9: permeate fractions corresponding to 1-8 mL of permeate collected respectively. Lane 10: retentate. CHR, chromosomal DNA; OC, open circular plasmid DNA; SC, supercoiled plasmid DNA. Agarose gel analysis performed as described in Section 2.6.1.
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

4.4.2 Effect of CaCl₂ precipitation

CaCl₂ precipitation has been developed in our laboratory (McHugh, personal communication. 2000; McHugh and Hoare 2001) to selectively precipitate RNA from plasmid containing lysate. RNA and some chromosomal DNA had been observed to precipitate during incubation with CaCl₂ as described in Section 2.4.1, and can be removed as a pellet following centrifugation, while plasmid DNA remains in the supernatant (McHugh, personal communication. 2000; McHugh and Hoare 2001). No information on removal of proteins or endotoxin through selective CaCl₂ precipitation was available. The capacity of tangential-flow filtration-adsorption using a nitrocellulose membrane to selectively adsorb chromosomal DNA present in the plasmid suspension following pre-purification of plasmid contained in the crude lysate by selective CaCl₂ precipitation was investigated.

The pre-purification of lysate with CaCl₂ precipitation of the contaminants as described in Section 2.4.1 significantly increased the volume of lysate that could be processed per unit area of nitrocellulose membrane before chromosomal DNA was detected in permeate fractions by agarose gel electrophoresis. For one experiment 24 mL lysate was processed through the 12.5 cm² nitrocellulose membrane. Subsequent regeneration of the membrane was carried out immediately, on the assumption that by this point the membrane would have become saturated and no longer adsorb contaminants, as observed in previous experiments. However, subsequent analysis revealed this to not be the case, indeed following regeneration, a further 24 mL of permeate passed through the membrane before breakthrough of chromosomal DNA was observed.
Figure 4.10. Agarose gel analyses of feed and permeate fractions from two consecutive tangential-flow filtration-adsorption runs using plasmid previously pre-purified by CaCl₂ precipitation as described in Section 2.4.1 as feed. A retentate recirculation rate of $1.3 \times 10^5$ m$^3$ s$^{-1}$ was used, and tangential-flow filtration-adsorption was carried out as described in Section 2.4.2.3. When 24 ml permeate had been collected, the membrane filter the membranes were regenerated using pure water as described in Section 2.4.3. A second adsorption step was then carried out as before. Lane 1, partially clarified lysate; Lane 2, nucleic acids from contaminant pellet obtained following CaCl₂ pre-purification; Lane 3, CaCl₂ pre-purified feed; Lanes 4-6, permeate fractions collected during initial filtration-adsorption run after 1, 13 and 24 ml of permeate had passed through the membrane respectively; Lane 7, material removed during high salt (10x SSC) wash; Lanes 8-12, contaminants desorbed during H₂O backflush collected after 1, 2, 3, 4 and 5 ml respectively. Lanes 13-15, permeate fractions collected during second filtration-adsorption run after 1, 13 and 24 ml of permeate had passed through the membrane respectively. CHR, chromosomal DNA; OC, open circular plasmid DNA; SC, supercoiled plasmid DNA; RNA, contaminant RNA. Agarose gel analyses of feed and permeate fractions as described in Section 2.6.1

Nucleic acid species selectively precipitated from crude lysate with CaCl₂, as described in Section 2.4.1 were resuspended and loaded onto an analytical gel (Lane 2, Figure 4.10). A purification factor of approximately 6.5 has been estimated for this purification operation (McHugh, personal communication. 2000). RNA is clearly visible as having precipitated from the lysate at high concentrations of CaCl₂. A distinct band of chromosomal DNA, and some plasmid is also visible. Comparison of Lane 1 (untreated lysate) and Lane 3 (nucleic acids remaining in the supernatant following the pre-purification of crude lysate by precipitation of contaminants with CaCl₂) indicates that levels of chromosomal DNA contamination, while visible on an agarose gel, are
considerably reduced following the CaCl₂ precipitation step. RNA levels are also greatly depleted. In addition, a noticeable proportion of plasmid also appears to have been precipitated from the supernatant by the presence of CaCl₂. The depletion in contaminants levels probably explains the increase in the volume of permeate collected following tangential-flow filtration-adsorption through nitrocellulose membranes before breakthrough of the chromosomal DNA is observed on an agarose gel. However, subsequent to the preliminary investigation, it was decided not to quantify the reduction in contaminant levels or plasmid yield, but to use the experimental time remaining to investigate an alternative purification technique, (results from which are reported in Chapter 5). That no breakthrough was evident following regeneration may be due to increased efficiency of the regeneration step due to the reduction in the levels of irreversibly bound contaminant species, or simply due to the overall increase in the volume of lysate which may be processed in this way.

4.4.3 Discussion

In order to try and improve the operational capacity of the nitrocellulose membrane for chromosomal DNA a several potential pre-purification steps were evaluated. Digestion of the contaminant RNA into small, soluble fragments by addition of RNase A into the lysis reaction mixture (as described in Section 2.2.2) had no significant effect on the capacity of the nitrocellulose membrane to adsorb chromosomal DNA (Figure 4.9). However physical removal of RNA and other contaminants (precipitated in the presence of CaCl₂) was effective. In an experiment carried out as described in Section 2.4.1 and 2.4.2, no breakthrough of chromosomal DNA was observed, indicating at least a 3 fold increase in the volume of lysate it would be possible to process in this way. Unfortunately due to the prioritisation of other work, it was not possible to carry out further experiments to determine the composition of the feed following CaCl₂ precipitation and permeate fraction collected after subsequent tangential-flow filtration-adsorption though a nitrocellulose membrane. Thus the capacity of the nitrocellulose membrane to
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

adsorb chromosomal DNA from a feed pre-purified by CaCl₂ precipitation, and the potential for regeneration, was not determined.

It is evident from the qualitative data that levels of chromosomal DNA were reduced following CaCl₂ precipitation, but some chromosomal DNA was still visible on the gel. Following the tangential-flow filtration-adsorption step chromosomal DNA was no longer visible on an agarose gel as indicated in Figure 4.10, and 24 mL pre-purified lysate was processed in this way through 12.5 cm² nitrocellulose membrane. This suggests that should a final polishing step be necessary in a purification process to remove residual chromosomal DNA contamination, then nitrocellulose filtration-adsorption may be suitable.

4.5 Conclusions

In this chapter it has been shown that an integrated filtration-adsorption step offers a new way to reduce major contaminants from process liquors during pharmaceutical grade plasmid DNA processing. This is especially important prior to chromatographic fractionation due to the low capacity of conventional chromatographic matrices. Selective removal of chromosomal DNA is also desirable as chromosomal DNA fragments can be particularly difficult to separate from plasmid DNA due to chemical similarities (Durland and Eastman 1998). The method has been shown to be reproducible and no degradation of the plasmid product was seen under the experimental conditions used with a relatively large plasmid (20 kb), indicating that the method will be applicable to a range of plasmid sizes. Shear damage of DNA during processing may also convert the desirable supercoiled, circular form of the plasmid DNA into undesirable forms such as open circular and linear DNA (Middaugh et al. 1998). For operations further downstream, the shear sensitivity of the plasmid DNA product will determine the choice of unit operation. In this instance, the linear flow rate of the retentate stream, and the dimensions of the flow channel in the membrane module mainly determine the shear forces prevailing during tangential-flow filtration-adsorption.
Shear rates for the membrane rig used were calculated from Equation 4.1 to be in the order of $2.5 \times 10^2$ s$^{-1}$ for retentate recirculation rates of $1.3 \times 10^6$ m$^3$ s$^{-1}$ (10.4 m$^2$ membrane area s$^{-1}$). As a rule of thumb, shear forces present across ultrafiltration membranes during large scale processing can be expected to be in the order of $10^4$ s$^{-1}$ (Davies, personal communication. 2002) although this will vary considerably with the construction of the cartridge and the characteristics of the process stream. Sensitivity to shear forces has been previously found to be critically dependent on plasmid size; experiments with plasmid sizes up to 89 kb indicated that the molecules were sensitive to shear rates above $10^4$ s$^{-1}$ (Levy et al. 1999b). Therefore, an operation similar to that described in the present study would potentially be suitable for processing a wide range of plasmid sizes up to 89 kb, providing shear levels in the larger scale membrane cartridge were kept below this critical level. For each product, the requirement to keep shear levels across the membrane low to avoid damage to the plasmid product, (or to chromosomal DNA fragments) must be balanced against the formation of a gel layer at low retentate recirculation rates, which impact unfavourably on the permeate flux.

Difficulties in cleaning of the membrane following processing of partially clarified lysate, combined with the strict regulatory standards imposed by governing bodies, suggest that this operation is likely to be most applicable as a disposable process. However the low capacity of the nitrocellulose membrane for the processing of crude lysate (Section 4.3.1) will mean that early in the process, the amount of membrane required will be costly. However, the increased capacity of the nitrocellulose membrane for pure ssDNA (Section 4.2) and pre-purified lysate (Section 4.4.2) suggest that this operation may more cost effectively be applied later in the process. For more highly purified process streams, a dead-end membrane adsorption step may be more appropriate for the removal of problematic chromosomal DNA fragments, and possibly endotoxin, subsequent to intermediate purification operations, or as a polishing step following chromatography may be a viable option.
4. Primary purification of plasmid DNA by integrated membrane filtration-adsorption.

Having scanned adsorption processes for the primary purification of plasmid DNA, work in the following chapter will investigate high-resolution purification of SC plasmid DNA by countercurrent chromatography.
5. Countercurrent chromatography for the high resolution purification of plasmid DNA

5.1 Aims and introduction

In the previous chapters, the initial purification of plasmid DNA was considered with a focus on the removal of key contaminants by adsorption. Another key separation challenge in the purification of plasmid DNA for therapeutic applications however is the high-resolution separation of different forms of plasmid DNA. Aqueous two-phase systems have long been known as a tool for the purification of DNA from other cellular components (Albertsson 1965; Rudin and Albertsson 1966; Favre and Pettijohn 1967; Walter et al. 1985). The results of previous research in this liquid-liquid separation technique have been outlined in Section 1.4.2. Conventional multi-stage countercurrent contactors can be used to improve the efficiency of such liquid-liquid separation processes, however the number of equilibrium stages achieved is generally limited to between 5-10 due to the cost and complexity of the centrifugal extraction devices employed (Walter et al. 1985; Diamond and Hsu 1992). Countercurrent chromatography is an emerging low pressure chromatographic technique, which separates molecules on the basis of different partition coefficients between two immiscible liquid phases as described in Section 1.4.2.3 (Conway 1990; Ito and Conway 1996). As discussed in Section 1.4.2.4, there are several different designs of CCC machine available, and the use of toroidal coil and cross-axis models with ATPS to achieve separations of labile biological macromolecules such as proteins has been previously reported (Elles and Sutherland 1980; Madava and Ito 1988; Shinomiya et al. 1993; Lin and Chu 1995).

Although J-type CCC machines are not considered the optimal design for dealing with high viscosity phase systems, the feasibility of using such a device for the high resolution fractionation of plasmid DNA preparations will be described in this chapter. The initial aim of this work is to identify phase systems likely to be suitable for the fractionation of plasmid DNA by CCC. The
speed with which the phase system settles ($T_s$) is considered the major factor in determining if an acceptable level of stationary phase retention ($S_r$) can be achieved for optimal conditions (Conway 1990). For this work, only a J-type rotor is available, therefore, to maximise $S_r$ which can be achieved, a suitably fast settling time will be a major factor in the choice of phase systems. Only ATPS will be considered in this work, aqueous–organic phase systems, despite the superior $S_f$ values which would be expected, were not considered as they have been found in the past to be unsuited to the separation of large labile biological macromolecules (Albertsson 1986). Following the initial screening of potential phase systems, a number of polymer-salt phase systems, and operating conditions are tested during CCC, until an initial separation is achieved. With the intention of maximising throughput, and improving the separation achieved, the effect of feed preparation and loading is then investigated.


5.2 Choice of phase system and hydrodynamic properties

5.2.1 Phase separation and equilibrium plasmid distribution on various ATPS

The hydrodynamic properties of a wide range of aqueous two-phase systems were initially determined. Due to the vigorous mixing present during operation of the J-type CCC machine, a low settling time (preferably < 60 seconds, ideally < 20 seconds) was of paramount importance. In addition, ideal solute partition coefficients for plasmid DNA are between 0.1 and 5, and it is also desirable to use phase systems close to the binodal curve (Walter et al. 1985). A wide range of PEG molecular weights (PEG 8000, 4000, 1000, 600
400 and 200) and salts ((NH₃)₂HPO₄, (NH₃)₂HSO₄, NaH₂PO₄, Na₂HSO₄, KH₂PO₄ and various ratios of K₂HPO₄:KH₂PO₄) were initially tested as described in Section 2.5.4 (results not shown). The use of PEG variants with high molecular weight (PEG 4000 and 8000) resulted in the upper PEG rich phase becoming extremely viscous, which in turn increased the time required for phase separation and were thus excluded from further study. Of the salts tested, only phase systems incorporating K₂HPO₄ exhibited settling times swift enough to be considered for use with the J-type rotor.

Phase settling times, volume ratio of phase systems and partition coefficients for OC and SC plasmid DNA obtained with a range of phase systems based on PEG 1000, PEG 600 or PEG 400 and K₂HPO₄ are listed in Table 5.1. Use of PEG variants of low molecular weight (PEG 400 and 200) resulted in the various plasmid forms partitioning strongly to the upper phase, with little or no difference in partition coefficient. Use of K₂HPO₄ for the salt-rich phase resulted in the fastest phase separation times. Phase separation times were faster at high concentrations of salt (106 seconds were required for the phases to settle with a phase system comprised of 12.5 % w/w PEG 600, 15 % w/w K₂HPO₄, compared to 54 seconds for 12.5 % w/w PEG 600, 18 % w/w K₂HPO₄). However at a concentration of 20 % w/w or more, DNA was observed to precipitate from the phase system, so that the choice of phase system was restricted to a maximum of 18 % w/w salt in phase systems where plasmid did not partition to the upper PEG phase. In general the volume ratio of the phase system reflected the proportions of phase forming chemicals present. For example, the volume of PEG rich upper phase is greater (P = 1.59) for a phase system comprised of 18 % w/w PEG 600, 12.5 % w/w K₂HPO₄, but smaller (P = 0.52) for a system comprised of 12.5 % w/w PEG 600, 18 % w/w K₂HPO₄. The partition coefficients of the plasmid also varies widely, from detection only in the lower, salt rich phase (K(OC) and K(SC) = 0) as seen for the phase system composed of 12.5 % w/w PEG 600, 18 % w/w K₂HPO₄, to detection only in upper PEG rich phase (K(OC) and K(SC) > 100) for the phase system 18 % w/w PEG 600, 18 % w/w K₂HPO₄.
5. Countercurrent chromatography for the high resolution purification of plasmid DNA

<table>
<thead>
<tr>
<th>K$_2$HPO$_4$</th>
<th>PEG 1000</th>
<th>12.5% w/w</th>
<th>15% w/w</th>
<th>16.2% w/w</th>
<th>18% w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ts (s)</td>
<td>P</td>
<td>K (OC)</td>
<td>K (SC)</td>
<td>Ts (s)</td>
<td>P</td>
</tr>
<tr>
<td>12.5% w/w</td>
<td>90</td>
<td>0.73</td>
<td>0</td>
<td>0</td>
<td>120</td>
</tr>
<tr>
<td>15% w/w</td>
<td>90</td>
<td>0.80</td>
<td>0</td>
<td>0.33</td>
<td>80</td>
</tr>
<tr>
<td>16.2% w/w</td>
<td>120</td>
<td>1.25</td>
<td>0</td>
<td>0.24</td>
<td>60</td>
</tr>
<tr>
<td>18% w/w</td>
<td>100</td>
<td>0.96</td>
<td>0</td>
<td>0.00</td>
<td>50</td>
</tr>
</tbody>
</table>

Phase system with 0.15 M NaCl included in the phase system in each case

<table>
<thead>
<tr>
<th>K$_2$HPO$_4$</th>
<th>PEG 800</th>
<th>12.5% w/w</th>
<th>15% w/w</th>
<th>16.2% w/w</th>
<th>18% w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ts (s)</td>
<td>P</td>
<td>K (OC)</td>
<td>K (SC)</td>
<td>Ts (s)</td>
<td>P</td>
</tr>
<tr>
<td>12.5% w/w</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>15% w/w</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>120</td>
</tr>
<tr>
<td>16.2% w/w</td>
<td>82</td>
<td>1.37</td>
<td>-</td>
<td>-</td>
<td>41</td>
</tr>
<tr>
<td>18% w/w</td>
<td>57</td>
<td>1.59</td>
<td>-</td>
<td>-</td>
<td>47</td>
</tr>
</tbody>
</table>

Stable emulsion formed

Table 5.1. Equilibrium plasmid partition coefficients, phase separation times and phase volume ratios for selected aqueous two-phase systems. Experimental methods as described in Section 2.5.2 and 2.5.4. Ts: Time required following phase mixing for two distinct phases to form. P: Volume ratio of phases (volume upper phase / volume lower phase). K: Equilibrium plasmid partition coefficient (concentration in upper phase / concentration in lower phase) for supercoiled (SC) and open circular (OC) plasmid forms. >100 indicates plasmid DNA not detected in lower phase.
Five phase systems, the composition of which is given in Table 2.2, (reproduced below) were chosen after the initial screening as described in Section 2.5.2 and 2.5.4. These phase systems showed fast settling times, and the partition coefficients for the two plasmid forms indicated that it should be possible to obtain separation of the OC and SC plasmid forms.

<table>
<thead>
<tr>
<th></th>
<th>PEG 400</th>
<th>PEG 600</th>
<th>PEG 1000</th>
<th>K$_2$HPO$_4$</th>
<th>NaCl</th>
<th>IPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 400</td>
<td>15 % w/w</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG 600</td>
<td></td>
<td>12.5 % w/w</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG 600 NaCl</td>
<td>12.5 % w/w</td>
<td></td>
<td></td>
<td></td>
<td>1 % w/w</td>
<td></td>
</tr>
<tr>
<td>PEG 600 IPA</td>
<td>12.5 % w/w</td>
<td></td>
<td></td>
<td></td>
<td>18 % w/w</td>
<td>2 % w/w</td>
</tr>
<tr>
<td>PEG 1000</td>
<td></td>
<td></td>
<td>16.2 % w/w</td>
<td></td>
<td>18 % w/w</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Composition of selected phase systems

5.2.2 Stationary phase retention

Phase diagrams for some of the systems selected following the initial screening procedure are shown in Figure 5.1. In general it is desirable to choose phase systems close to the binodal curve, as interfacial tensions increase with increasing polymer concentrations, and more extreme partitioning behaviour has been reported for proteins at higher polymer concentrations (Walter and Johansson 1986). Due to the requirement for a rapid phase separation time, in this particular application it was necessary to compromise and choose phase systems located further from the origin of the phase diagram (Figure 5.1) which had the required separation kinetics. The corresponding phase separation times and partition coefficients for SC and OC plasmid DNA for the selected phase systems are given in Table 5.2.
From the results presented in Table 5.2 it can be observed that in general OC plasmid partitions more strongly to the lower salt rich phase than the SC plasmid, i.e. lower K values are recorded. Therefore if the lower phase is used as the mobile phase in CCC experiments, the OC plasmid would be expected to elute first during a chromatographic run. The molecular weight of the PEG present in the phase system has a distinct effect on the partitioning of the two plasmid forms. From literature results it is known that for PEG – dextran ATPS, there is a general tendency for a decrease in the molecular weight of a polymer in a given phase to increase the partitioning of biological molecules to that phase (Walter and Johansson 1986). The data shown in Table 5.2 for the selected phase systems indicates that the partitioning of plasmid DNA follows this trend. The partition coefficients for both plasmid forms are low when PEG 1000 is present, indicating that the plasmid DNA prefers the lower salt rich phase. With a decrease in the average molecular weight of the PEG the partition coefficients increase, with the SC plasmid showing a greater
tendency to partition into the upper PEG rich phase. The partition coefficient for the OC plasmid is largely unaffected by the increase in PEG molecular weight. The addition of 0.15 M NaCl to the phase forming mixture (which would be expected to partition uniformly to both phases, hence negating charge effects (Walter et al. 1985)) results in both plasmid forms partitioning more strongly to the lower salt phase. The addition of IPA has the opposite effect, with the partition coefficients for both plasmid forms increasing. When PEG 400 is used in the phase forming mixture, both plasmid forms partition strongly to the upper phase, and are not detected in the lower salt rich phase.

The hydrodynamic properties of the chosen phase system in the CCC machine were also examined. Stationary phase retention studies during CCC operation at different rotational speeds and using the various selected phase systems were carried out to determine if sufficient stationary phase would be obtained for a separation to be achieved. These were performed as described in Section 2.5.5 by measuring the volume of stationary phase displaced by the mobile phase during operation before hydrodynamic equilibrium is reached.

### Table 5.2

<table>
<thead>
<tr>
<th>Phase System</th>
<th>Temp (°C)</th>
<th>P (V/Vb)</th>
<th>Tₜ (s)</th>
<th>K SC plasmid</th>
<th>K OC plasmid</th>
<th>Bobbin rotation (rpm)</th>
<th>Sᵢ (% v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 1000</td>
<td>30</td>
<td>0.7</td>
<td>60</td>
<td>0.12</td>
<td>0.02</td>
<td>800</td>
<td>56</td>
</tr>
<tr>
<td>PEG 600</td>
<td>30</td>
<td>0.6</td>
<td>45</td>
<td>0.34</td>
<td>0.01</td>
<td>800</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>600</td>
<td>33</td>
</tr>
<tr>
<td>PEG 600 NaCl</td>
<td>30</td>
<td>0.6</td>
<td>41</td>
<td>~ 0</td>
<td>~ 0</td>
<td>600</td>
<td>37</td>
</tr>
<tr>
<td>PEG 600 IPA</td>
<td>30</td>
<td>0.6</td>
<td>45</td>
<td>1.13</td>
<td>0.36</td>
<td>600</td>
<td>36</td>
</tr>
<tr>
<td>PEG 400</td>
<td>33</td>
<td>0.7</td>
<td>45</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>800</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 5.2. Equilibrium plasmid partition coefficient, phase separation times and stationary phase retention of selected aqueous two-phase systems. Phase system compositions are given in Table 2.2. Tₜ: Time required following phase mixing for two distinct phases to form. P: Volume ratio of phases (volume upper phase / volume lower phase). K: Equilibrium plasmid partition coefficient (concentration in upper phase / concentration in lower phase). >100 indicates plasmid DNA not detected in lower phase. Sᵢ is the percentage of the column occupied by the stationary phase, calculated as described in Section 2.5.5. Data obtained as described in Section 2.5.2 and 2.5.4. Mobile phase flow rate of 0.5 mL min⁻¹ used in all experiments.
(stationary phase no longer elutes from the column). A high degree of stationary phase retention (> 30 % v/v) is generally required for efficient peak resolution (Ito and Conway 1996), and values of up to 95 % v/v have been achieved in J-type machines using aqueous-organic two-phase systems (Ito and Conway 1996; Booth et al. 2002). As expected, the \( S_f \) values obtained using ATPS were much lower, being between 33 – 56 % v/v (Table 5.2) depending on the phase system used and the speed of rotation. Based on literature studies these \( S_f \) values should however produce sufficient stationary phase within the coil for chromatographic separations to be achieved. During most of the CCC experiments carried out at 600 rpm, some stripping of the stationary phase (i.e. small droplets of stationary phase being continually eluted in the mobile phase) was observed.

5.3 Initial results on CCC fractionation of plasmid DNA

5.3.1 CCC fractionations carried out at 800 rpm

Despite the relatively low \( S_f \) values obtained for the selected ATPS, chromatographic runs in the CCC apparatus were subsequently performed. The conditions used for each experiment are summarised in Table 2.3. Initially, gel electrophoresis of eluted mobile phase fractions (as described in Section 2.6.1) obtained during a run using the PEG 1000 system (800 rpm, \( S_f = 56 \) % v/v) of 3.5 hours duration revealed that no DNA had eluted from the column (Figure 5.2 A). Rather, the bulk of the plasmid DNA was found to have been retained within the CCC column when the coil was pumped out following the cessation of revolution. In particular, SC plasmid was observed in the latest fractions of the mobile phase collected (corresponding to positions close to the point of sample injection near the head of the column) while OC plasmid had moved a little distance along the column. It was hypothesised that either the high viscosity of the stationary phase (\( \sim 10^3 \) cP), coupled with the large size of the plasmid DNA molecules, had restricted mass transfer of the plasmid back into the mobile phase during column rotation, or that the SC
plasmid DNA had become associated with the large interfacial area generated between the two phases during phase mixing at high rotational speed. Both of these hypotheses would result in increased retention of the plasmid, much greater than would be what would be predicted solely on the basis of the measured K values (Table 5.2).

With the aim of reducing the viscosity of the upper phase, the PEG 600 system was next evaluated during CCC operation. Following CCC with the upper PEG phase again stationary and the coil rotated at 800 rpm, analytical agarose gel electrophoresis of resulting fractions revealed that plasmid again had been retained in the column, but was present in both phases, and had moved considerably further along the column to the tail end of the coil (Figure 5.2 B). OC plasmid forms were present in the pumped out stationary phase at a relatively high concentration close to the tail, with the SC form predominating as the column was emptied toward the tail. Some plasmid, mainly the SC form was present in the pumped out mobile phase at the head end of the column. With the intention of allowing the plasmid to actually elute from the CCC column, the time allowed for the CCC run was increased from 3.5 hours to 7 hours. However, this did not result in the elution of either of the plasmid DNA forms. Indeed plasmid was again observed in a similar position relative to the head and tail of the column (data not shown). PEG 400 was also tried as a phase system. In this case the lower K$_2$HPO$_4$ phase was chosen as the stationary phase in the CCC column due to the high K values (>100 for OC and SC plasmid as indicated in Table 5.2. Following agarose gel analysis of the fractions collected for a chromatographic run, plasmid was observed to have eluted in the mobile phase (Figure 5.2 C). However elution was at the solvent front (CV = 1) and no separation of the plasmid forms was observed. A significant proportion of both plasmid forms had also been retained throughout the column, which is contrary to the result, which would be expected from the high K value.
Figure 5.2. CCC chromatograms showing plasmid DNA elution for various ATPS at a mobile phase flow rate of 0.5 mL min⁻¹ and CCC operation at 800 rpm as described in Section 2.5.5. Dashed line: OC plasmid. Solid line: SC plasmid. Red indicates plasmid eluted in upper PEG phase. P: point at which pumping out of the column initiated. Chromatograms estimated from scanning of agarose gels as described in Section 2.6.1, Y axis normalized to 50 % of the signal from the feed sample. (A) Phase system comprised of 16.2 % w/w PEG 1000 (stationary phase) and 18 % w/w K₂HPO₄ (mobile phase). (B) Phase system comprised of 12.5 % w/w PEG 600 (stationary phase) and 18 % w/w K₂HPO₄ (mobile phase). (C) Phase system comprised of 15 % w/w PEG 400 (mobile phase) and 20 % w/w K₂HPO₄ (stationary phase). Feed composition 35 μg mL⁻¹ OC plasmid, 95 μg mL⁻¹ supercoiled plasmid (as a 1/10 dilution of concentrated plasmid in TE buffer with mobile phase).
Under identical operating conditions, but between the various ATPS investigated, the $S_f$ values obtained increased with increasing molecular weight of the PEG species present in the phase system, being 56% v/v, 50% v/v and 45% v/v for PEG 1000, PEG 600 and PEG 400 systems respectively. Interestingly the settling times (shown in Table 5.2) followed the opposite trend (being the highest for PEG 1000 (60 seconds) and 45 seconds for PEG 600 and PEG 400. This discrepancy from the expected results is most probably due to the relatively high viscosity of PEG 1000 upper phase resulting in increased retention.

Although some separation of the plasmid forms had been observed, elution of neither form had been achieved. It was considered that the vigorous mixing of the two phases in the CCC column at 800 rpm, and the large interfacial area created, could be retarding the progress of the plasmid DNA. In subsequent experiments, the speed of revolution of the column was reduced, with the intention of reducing the interfacial area created between the two phases within the CCC column.

### 5.3.2 CCC fractionations carried out at 600 rpm

In order to decrease the degree of mixing experienced by the two phases present in the CCC column, the speed of revolution of the coil was decreased to 600 rpm. The tendency of plasmid DNA to partition to the interface in ATPS has been recently reported (Ribeiro et al. 2000). By decreasing the intensity of phase mixing, less interfacial area should be present within the column. A PEG 600 system was initially used in a 3.5 hour run. Under the operating conditions used, $S_f$ was calculated to be low (33% v/v) which is generally barely sufficient for a separation to be achieved (Conway 1990). Gel electrophoresis of the eluted mobile phase fractions (Figure 5.3, lanes 2 and 3) indicated that the majority of the OC plasmid eluted in a single peak between 1.1 and 1.29 column volumes. A small amount of SC plasmid was also visible in the fractions between 1.2 and 1.29 column volumes. Analysis of the phases recovered following pumping out of the column after 1.75 column
volumes revealed that the majority of SC plasmid DNA had again been retained during CCC operation, but had partitioned back into the mobile phase during pumping out of the coil. The SC plasmid was present in a peak 88 - 98% along the length of the column (Figure 5.3, lanes 4 and 5). Chromosomal DNA was clearly visible analytical gels as having been retained in the stationary PEG phase, near the point of sample injection (data not shown) indicating that this had been effectively separated from both forms of plasmid.

Figure 5.4 shows a CCC chromatogram constructed using results from the picogreen assay (as described in Section 2.6.2.3) for both the eluted mobile phase fractions and the fractions recovered following pumping out of the column. The proportions of SC and OC plasmid were determined by scanning of the corresponding agarose gels. The chromatogram shows a good separation between the various plasmid forms. The OC plasmid begins to elute near the solvent front at 1.1 CV, however given that a $K$ value of 0.01 was obtained in equilibrium experiments (Table 5.2) for the OC plasmid this is slightly later than would be expected. The SC plasmid is strongly retained in the column, again contrary to the result expected from the partition coefficient ($K = 0.34$ for SC plasmid). That the OC plasmid elutes later than expected is probably due to a combination of the dilution of the phase system following the introduction of the feedstock (9 : 1 ratio mobile phase to TE buffer), and the variation of the phase composition in the environment of the column, due to differences in the ratio of the two phases present. That the majority of the SC plasmid is retained within the column, given the relative mobility of the OC plasmid, is most probably due to the SC plasmid DNA becoming more associated with the interfacial area generated between the two phases during phase mixing. Chromosomal DNA had been observed to strongly partition to the upper PEG phase during equilibrium phase partitioning experiments as described in Section 2.5.4. (results not shown). This is in line with the detection of chromosomal DNA in the stationary phase pumped out from the column following the CCC experiments.
5. Countercurrent chromatography for the high resolution purification of plasmid DNA

Figure 5.3. Agarose gel analysis as described in Section 2.6.1 of feed and selected fractions collected during a CCC run carried out using a phase system comprised of 12.5% w/w PEG 600 (stationary phase) and 18% w/w K₂HPO₄ (mobile phase) at 600 rpm and a mobile phase flow rate of 0.5 mL min⁻¹. Lane 1; feed (0.02 dilution of original). Lanes 2 and 3; mobile phase fractions eluted during chromatography after 1.23 and 1.29 column volumes respectively. Lanes 4 and 5; fractions collected during pump out of the column at 2.45 and 2.48 column volumes respectively (88 and 98% v/v of the column cleared, x2 concentration of the original). Experiments carried out as described in Section 2.5.5.

Figure 5.4. CCC chromatogram of plasmid DNA eluted during a CCC run carried out as described in Section 2.5.5. Dashed line; OC plasmid. Solid line; SC plasmid. P; point at which pumping out of the column initiated. Feed composition 35 μg mL⁻¹ OC plasmid, 95 μg mL⁻¹ supercoiled plasmid (as a 1/10 dilution of concentrated plasmid in TE buffer with mobile phase). Total concentration of dsDNA determined using picogreen assay as described in Section 2.6.2.3. Proportions of SC and OC plasmid estimated from scanning of agarose gel as described in Section 2.6.1.
Comparable results obtained following further CCC runs under identical conditions indicated that an efficient and reproducible separation of OC, SC and chromosomal DNA had been achieved.

Partition coefficients for both forms of plasmid had been observed (Table 5.2) to be significantly lower for the PEG 600 NaCl system than for the PEG 600 system used to produce the chromatogram shown in Figure 5.4. It was therefore anticipated that use of this system for CCC experiments would result in both plasmid forms being eluted. Following CCC with the PEG 600 NaCl system (Appendix B.1), OC plasmid was observed to elute in a peak between 1.09 and 1.33 CV, while very faint bands of SC plasmid were visible as having eluted between 1.16 and 1.53 CV. A second peak containing both plasmid forms, (considerably diminished in intensity compared to that detected in the run shown in Figure 5.4) was also present having been retained in the column between 2.43 and 2.54 CV. In addition, unlike the previous run with PEG 600, quite distinct bands of SC plasmid were observed in stationary phase stripped from the column during CCC operation (1.2 to 1.43 CV), indicating that a considerable proportion (~13 % w/w) of SC plasmid eluted had partitioned to the upper phase which chromosomal DNA had also been noted to prefer. It might be possible to selectively partition SC plasmid back into the lower phase, hence removing the bulk of chromosomal DNA contamination present in the stationary phase stripped from the column, but an extra step, with the attendant loss in yield, is not desirable. Despite the fact that both plasmid forms had eluted, the retention of the majority of SC plasmid within the coil and the dilution of the small amount of SC plasmid eluted was such that it was considered wise to look at alternative systems.

A short series of retention studies were also carried out to investigate the effect of temperature on stationary phase retention. It is to be expected that the upper stationary phase would be less viscous at higher temperatures. Increasing the temperature at which CCC operation was subsequently carried out did appear to have a favourable influence of $S_f$, which increased from 33 % v/v to 37 % v/v when the temperature was increased from 30 °C to 43 °C.
A CCC run was carried out at 40°C to investigate the effect of the increased temperature on the separation. However plasmid was again completely retained in the column (Figure 5.5), probably due to increased area of interface present between the phases due to greater mixing of the lower viscosity phases.

**Figure 5.5.** CCC chromatogram of plasmid DNA eluted during CCC carried out at a temperature of 40°C using a phase system comprised of 12.5 % w/w PEG 600 (stationary phase) and 18 % w/w \( \text{K}_2\text{HPO}_4 \) (mobile phase) at 600 rpm and a mobile phase flow rate of 0.5 mL min\(^{-1}\) (as described in Section 2.5.5). Feed composition 35 μg mL\(^{-1}\) OC plasmid, 95 μg mL\(^{-1}\) supercoiled plasmid (as a 1/10 dilution of concentrated plasmid in TE buffer with mobile phase). Dashed line; OC plasmid. Solid line; SC plasmid. P; point at which pumping out of the column initiated. Chromatograms estimated from scanning of agarose gel as described in Section 2.6.1, Y axis normalized to 50 % of the signal from the undiluted feed sample.

In general it would be desirable for the SC plasmid form to elute from the CCC column. This would facilitate continuous operation, i.e. consecutive samples loaded and purified without the need to equilibrate the column with fresh phase system between runs. As attempts to get both forms of plasmid to elute
had not been entirely successful, a strategy to reduce the time per batch was investigated. In this case the feed sample was loaded onto the column immediately after the column had been filled with stationary phase, and rotation of the bobbin (600 rpm) started. Compared to the previous studies, the phases had not reached hydrodynamic equilibrium prior to sample injection. CCC runs for both PEG 600 and PEG 600 NaCl systems were carried out using this strategy (Appendix B.2). In the case of the PEG 600 system the OC plasmid eluted between 1.2 and 1.35 CV, and a sharp peak of SC plasmid elutes between 1.25 and 1.4 CV (data not shown). However the SC plasmid was still detected retained in the column, in two distinct peaks. The first at the tail end of the column (1.52 – 2.42 CV) and the second at the head of the column (2.75 – 3 CV). For the PEG 600 NaCl system the eluted plasmid forms were rather better separated, if the peaks are somewhat broader and the relative concentration of plasmid lower (data not shown). OC plasmid was seen to elute in the mobile phase between 1.0 and 1.2 CV, while most SC plasmid was retained in the column, in a diffuse peak between 1.6 and 2.2 CV.

5.3.3 Discussion

Initial experiments reveal that effective separation of OC and SC forms of plasmid DNA can be achieved using a J-type CCC machine in conjunction with ATPS. When the upper PEG rich phase is the chosen stationary phase, some of the DNA species are retained within the column at high revolution speeds of the bobbin (800rpm – Figure 5.2 A and 5.2 B) even though K values determined from batch partition experiments (Table 5.2) suggested both forms of the plasmid should have eluted. When the lower K$_2$HPO$_4$ rich phase was chosen as the stationary phase (Figure 5.2 C), some plasmid did elute at 1CV, (although no separation was evident), and the remainder was found to be retained at low concentrations throughout the length of the column. Again the partition coefficients (Table 5.2) suggested that both would elute strongly in the PEG phase.
The high viscosity of the stationary (PEG) phase, coupled with the large dynamic volume of the DNA molecule (Ito et al. 1998) may have the effect of reducing the rate of DNA mass transfer out of the PEG phase during chromatography. Plasmid DNA has also been observed to accumulate at the interface in aqueous two-phase systems (Ribeiro et al. 2000), thus the large interfacial area present within the column, due to the vigorous mixing action of the J-type machine, could also result in the retention of the plasmid DNA. Reducing the speed of revolution of the bobbin (from 800 to 600 rpm) had the effect of reducing the degree of phase mixing in the CCC Coil such that OC plasmid species could be eluted from the column (Figure 5.4). For the first experiments, in which the amount of sample loaded was small (0.1 mg plasmid onto a 94.6 mL column), SC plasmid, could only be recovered at the end of the chromatographic run once the column had been pumped out. That the SC plasmid did not elute may be due to a number of factors such as the size of the plasmid, or tendency to partition to the interface as discussed above. Approximately 76 % w/w of the SC form of the plasmid was recovered in the main SC peak, while the overall recovery of SC plasmid was ~90 % w/w. SC plasmid was recovered in the mobile phase, separated from the chromosomal DNA which had partitioned to the stationary phase. Ultimately the SC plasmid is the form required for therapeutic applications and could transferred into a buffer suitable for therapeutic usage by diafiltration.

Increasing the temperature of the phase systems during CCC would be expected to reduce the viscosity of the PEG phase. In this way elution of the SC plasmid might be evident if the rate if plasmid mass transfer was limited due to the viscosity of the PEG phase. However, that both plasmid forms were again seen to be retained in the column supports the hypothesis that the plasmid is accumulating at the interface between the phases during chromatography. The interfacial area would also be expected to be larger at higher temperatures due to the reduced viscosity of the stationary phase. The addition of 0.15 M NaCl to the phase system (PEG 600 NaCl) will negate charge effects, and also reduce the partition coefficient for both plasmid species in equilibrium experiments (Table 5.2). In this case, with the PEG rich
phase stationary, it would be expected that both plasmid forms would elute earlier in the K$_2$HPO$_4$ rich mobile phase. Both species were seen to elute (Appendix B.1), however the peaks were very broad and the concentration of plasmid low. Some SC plasmid was also found to partition to the stationary phase stripped from the column, so the investigation of this phase system was discontinued in later work.

Since limited success had been realised for elution of the SC plasmid, so that the column would need to be set up and equilibrated for each run, and alternative approach was tried in which the feed was injected onto the column immediately after rotation of the column was started. The aim of this approach is to reduce the batch time. In these cases, both plasmid forms were seen to elute soon after the mobile phase broke through, although the separation of plasmid forms eluted was not good when this strategy was tried with PEG 600 (Appendix B.2). When the immediate injection strategy was tried with PEG 600 NaCl (Appendix B.2) the separation between OC and SC plasmid was better, but again, SC plasmid was found to be retained in a diffuse peak throughout the column.

In the next section various strategies for the preparation of the plasmid containing feed are investigated. The effect of the sample suspension buffer composition and plasmid DNA concentration loaded on the resolution achieved, and stationary phase retention, is reported.

5.4 Effect of feed composition and solute loading

5.4.1 Effect of plasmid concentration

In the CCC experiments described so far plasmid was prepared from an alkaline lysis step by precipitation and resuspension in TE buffer, followed by dilution at a ratio of 1:9 with equilibrated mobile phase. While this is effective for a laboratory scale separation of plasmid DNA using PEG 600, it greatly
restricts the concentration of plasmid which can be loaded onto the column. Experiments were therefore carried out in which plasmid was loaded onto the column, containing either the PEG 600 or PEG 600 IPA phase system highly concentrated (up to 1 mg mL\(^{-1}\) in an injection volume of 1 mL) in TE buffer. However, the liquid-liquid disruption to the hydrodynamic equilibrium in the column resulting from the introduction of sample suspended in TE buffer (Figure 5.6 A, B, E & F) resulted in a great deal of stripping of the stationary phase; the stationary phase retention is typically reduced to 11 % v/v in these experiments. The two plasmid forms were observed to elute together at the solvent front in a sharp peak between 1.0 and 1.1 CV suggesting that they moved with the injection buffer as it passed straight through the column. Due to the disruption of the hydrodynamic equilibrium and the lack of separation of the plasmid forms observed, it was not possible to draw conclusions as to whether the upper loading limit of the system had been reached.

5.4.2 Effect of buffer composition

With the intention of increasing the amount of plasmid which could be loaded on the column, while reducing the disruption to the hydrodynamic equilibrium seen as a result of loading plasmid in 100 % TE buffer (Section 5.4.1), the plasmid preparation in TE buffer was diluted with an equal volume of mobile phase. This reduced the amount of stripping observed for both types of system used, with final values of \(S_f\) at 25 % v/v and 30 % v/v for PEG 600 and PEG 600 IPA systems respectively. These values can be compared to 11 % v/v observed for plasmid injected in 100 % TE buffer as described in Section 5.4.1). When plasmid which had been resuspended in the mobile phase following precipitation and concentration as described in Section 2.5.1 was used as feed, only a small amount of stripping occurred (\(S_f > 30\) % v/v at the end of both CCC runs). When PEG 600 was the phase system used for the CCC run, very little separation of the two forms was observed for either feed preparation (Figure 5.6 C). Plasmid prepared as a 1:1 dilution of plasmid suspension in TE buffer and mobile phase resulted in OC plasmid being eluted between 1.15 and 1.35 CV and SC plasmid between 1.15 and 1.8 CV
5. Countercurrent chromatography for the high resolution purification of plasmid DNA

(Figure 5.6 C). A similar result was observed for plasmid loaded in 100 % mobile phase; OC plasmid eluted between 1.09 and 1.25 CV, and a sharp SC plasmid peak between 1.12 and 1.25 CV (Figure 5.6 D), with SC plasmid detected on an agarose gel in later fractions, but at low concentrations.

When the PEG 600 IPA system was used, a more marked separation of the plasmid forms was observed. When the plasmid was prepared as a 1:1 dilution of plasmid suspension in TE buffer and mobile phase (Figure 5.6G), the majority of OC plasmid was eluted in a peak between 1.16 and 1.4 CV, while the SC plasmid was again retained in the column, and was only detected once the stationary phase was pumped out. When plasmid was loaded in 100 % mobile phase (Figure 5.6 H), both forms of plasmid were observed to elute, the majority of OC plasmid in a peak between 1.27 and 1.47 CV, while SC plasmid eluted in a broad peak between 1.3 and 2.3 CV, inclusive of SC plasmid that not eluted from the column.

Overall, the PEG 600 IPA system gave the best CCC results in terms of resolution of the two plasmid forms. Resolution was calculated from the well known formula (Doran 1995);

\[ R = \frac{T_2 - T_1}{0.5 \times (W_1 + W_2)} \]  

[5.1]

Where R is the resolution between the plasmid peaks, \( T_1 \) and \( T_2 \) are the time of elution of OC and SC plasmid respectively (consistent units), and \( W_1 \) and \( W_2 \) are the width at the base of the OC and SC plasmid forms respectively (consistent units). The resolution achieved was calculated only for the chromatograms shown in Figure 5.6 G and Figure 5.6 H, as the other chromatograms presented in Figure 5.6 did not show appreciable separation of the OC and SC plasmid peaks. For Figure 5.6 G and Figure 5.6 H, only the main peaks observed were considered in the calculations that follow. The resolution of the two plasmid forms was less when plasmid was resuspended.
Figure 6.6. CCC chromatograms of plasmid DNA eluted during a selection of runs using feed prepared with different buffer compositions and concentration of plasmid. 1 mL plasmid feed was loaded in each case. CCC carried out at a bobbin rotational speed of 600 rpm and a mobile phase flow rate of 0.5 mL min⁻¹ as described in Section 2.5.5. (A) Phase system comprised of 12.5 % w/w PEG 600 and 16 % w/w K₂HPO₄. Feed prepared by precipitating plasmid with IPA and resuspending (40 x concentrated) in TE buffer. Feed composition 310 μg mL⁻¹ OC plasmid, 990 μg mL⁻¹ SC plasmid. (B) Phase system comprised of 12.5 % w/w PEG 600 and 18 % w/w K₂HPO₄. Feed prepared by precipitating plasmid with IPA and resuspending (40 x concentrated) in TE buffer before further dilution with an equal volume of TE buffer. Feed composition 155 μg mL⁻¹ OC plasmid, 495 μg mL⁻¹ SC plasmid. (C) Phase system comprised of 12.5 % w/w PEG 600 and 18 % w/w K₂HPO₄. Feed prepared by precipitating plasmid with IPA and resuspending (40 x concentrated) in TE buffer before dilution with an equal volume of the lower (mobile) phase of the selected system for the run. Feed composition 155 μg mL⁻¹ OC plasmid, 495 μg mL⁻¹ SC plasmid, 1 mL feed loaded. (D) Phase system comprised of 12.5 % w/w PEG 600 and 18 % w/w K₂HPO₄. Feed prepared by precipitating plasmid with IPA and resuspending (40 x concentrated) in the lower (mobile) phase of the selected system for the run. Feed composition 100 μg mL⁻¹ OC plasmid, 350 μg mL⁻¹ SC plasmid. (E) As (A) except phase system comprised of 2 % w/w IPA, 12.5 % w/w PEG 600 and 18 % w/w K₂HPO₄. (F) As (B) except phase system comprised of 2 % w/w IPA, 12.5 % w/w PEG 600 and 18 % w/w K₂HPO₄. (G) As (C) except phase system comprised of 2 % w/w IPA, 12.5 % w/w PEG 600 and 18 % w/w K₂HPO₄. (H) As (D) except phase system comprised of 2 % w/w IPA, 12.5 % w/w PEG 600 and 18 % w/w K₂HPO₄. Chromatograms estimated from scanning of agarose gel as described in Section 2.6.1. Y axis normalized to 67 % of the signal from the undiluted feed sample for A, D, G and H. Dashed line; OC plasmid. Solid line; SC plasmid. P; point at which pumping out of the column initiated.
in 100 % mobile phase (Figure 5.6 H) than was seen for the plasmid feed resuspended in TE buffer and diluted with mobile phase (Figure 5.6 G), being 0.75 and 1.05 respectively. As a rule of thumb, a resolution value of 1 calculated for two solute peaks equates to 98 % separation (Doran 1995). SC plasmid retained in the column for both experiments could readily be recovered by pumping out the column. Increasing the length of time for the run may also allow the SC plasmid to elute fully. It is desirable that both plasmid forms elute in order to facilitate continuous operation. For experiments carried out using plasmid resuspended first in TE buffer, then diluted with mobile phase (Figure 5.6 G), contaminant OC plasmid forms were detected only in the early fractions of the SC plasmid peak (1.59 - 1.81 CV). This represents a reduction of ~16 % w/w OC plasmid in the early fractions, or 9 % w/w overall. CCC runs carried out using a plasmid feed sample prepared by resuspending the precipitated plasmid in the mobile phase (Figure 5.6 H) resulted in the proportion of contaminating plasmid forms being reduced from 22 % w/w in the feed, to 10 % w/w in the main peak. Unfortunately, plasmid was not readily resuspended in the mobile phase, and 65 % w/w loss of plasmid resulted from preparation of the feed sample in this way.

Southern slot blot analysis was carried out on the feed samples prepared by resuspension of precipitated plasmid in either TE buffer, or the mobile phase. The chromosomal DNA contamination was calculated to be to 23 % w/w for plasmid resuspended in TE buffer. However, plasmid DNA from the same lysis reaction, resuspended in mobile phase contained only 9 % w/w chromosomal DNA (Figure 5.7). Given the difficulties experienced in the resuspension of plasmid DNA in the mobile phase, the most likely reason for this reduction is low solubility of chromosomal DNA in the mobile phase. Contaminant chromosomal DNA was not detected in any fractions by qualitative Southern blot analysis of the analytical gels for either experiment carried out using plasmid suspended in 100 % mobile phase as feed. A similar analysis for samples from an experiment using plasmid resuspended in TE buffer (Figure 5.10) revealed that chromosomal DNA was readily
detected in the stationary PEG phase (Lanes 1, 2 & 4), but not in the mobile salt rich phase (Lane 3).

**Figure 5.7.** Southern slot-blot analysis of different plasmid DNA feed preparations carried out as described in Section 2.5.1. 350 ng total DNA for feed and eluted fractions (determined by picogreen assay) was loaded on the blot. Slots 1–7 E. coli DH5α standard curve (1.5, 3, 6, 12, 25, 50 and 100 ng respectively). Slot 8; unprocessed lysate. Slot 9; plasmid resuspended in mobile phase. Slot 10; plasmid resuspended in TE buffer. Analysis carried out as described in Section 2.6.2.3

**Figure 5.8.** Southern blot as described in Section 2.6.2.3 of analytical gel (as described in Section 2.6.1) for feed and selected fractions using a probe specific for E. coli chromosomal DNA. The chromosomal DNA smear in fractions from the stationary PEG phase of the system is illustrated. CCC run carried out with feed composition 310 μg mL⁻¹ OC plasmid 990 μg mL⁻¹ SC plasmid, prepared by precipitating plasmid with IPA and resuspended (40 x concentrated) in TE buffer as described in Section 2.5.1. 1 mL feed loaded. Lane 1; feed (40 x diluted before loading onto gel). Lane 2; stationary phase fraction stripped from column at 1.12 column volumes. Lane 3; mobile phase containing plasmid eluted at 1.12 column volumes. Lane 4; stationary phase recovered during pumping out of column at 2.19 column volumes (approximately 65 % v/v of column cleared).

### 5.4.3 Discussion

In all the experiments reported in Section 5.4, a small amount of plasmid DNA was detected in fractions corresponding to the point of injection at the head of the column, and in some cases spread along the column with a second peak of retained DNA near the tail. It was noticeable that those CCC runs in which the greatest amount of stripping occurred (plasmid feed prepared in 100 % TE buffer) resulted in the smallest proportions of plasmid DNA being retained at
the head of the column. This suggests that plasmid may be carried along at the interface of the stationary phase stripped from the column and the mobile phase. Partial dilution or resuspension of the plasmid with the mobile phase decreases the amount of stripping which occurs, and in these cases more plasmid was seen to be retained within the column. When PEG 600 IPA was used as the phase system for CCC, reasonable separation of the OC and SC plasmid forms was observed.

A major problem in the preparation of feed sample to load onto the CCC column has been obtaining highly concentrated plasmid in a suitable buffer. Use of buffers in which DNA is readily soluble (TE buffer) resulted in unacceptable levels of stripping of the stationary phase following injection with no separation of plasmid forms achieved. Also the DNA proved difficult to resuspend in the mobile phase of the ATPS resulting in 65 % w/w losses. This approach will also prove difficult to scale up, and although ultrafiltration and diafiltration may replace precipitation and resuspension, problems may still be encountered with the plasmid precipitating out at high concentrations. It is likely that plasmid would prove to be more soluble in a (slower settling) phase system located closer to the binodal curve on a phase diagram (Figure 5.1). Chromosomal DNA levels were also reduced following resuspension in the mobile phase from 23 % w/w chromosomal DNA contamination in the crude lysate to 9 % w/w following resuspension.

Although removal of chromosomal DNA contaminants was not quantified, qualitative data from southern blot analysis of the analytical gels (Figure 5.8) revealed that chromosomal DNA was readily detected in the PEG phase fraction recovered, while plasmid partitioned preferentially to the salt phase. This suggests that CCC can readily deplete chromosomal DNA contaminants present in plasmid containing process streams. Assays were carried out as described in Section 2.6.5 to determine the levels of protein present in the phases eluting from the CCC column, but protein concentrations were below the limit of detection for all samples tested. However results from equilibrium partition experiments using a model protein solution prepared as described in
section 2.1 (data not shown) indicated that the model protein partitions strongly to the upper PEG phase in both the PEG 600 and PEG 600 IPA phase systems.

Plasmid preparations used for feed sample so far have been partially purified. For a large scale process this is not an ideal situation, indeed one of the major advantages of CCC is the ability to deal with crude samples due to the absence of a densely packed solid matrix. However the results so far have clearly demonstrated that there is a need to get the feed sample into a buffer with a similar composition to the mobile phase to avoid unacceptable levels of stripping of the stationary phase following sample injection. To this end, the potential of adding phase forming agents to the lysate directly in order to create a feed sample is described in the following section. This approach also has the advantage that preparation of the feed sample is minimised.

5.5 Use of plasmid feed prepared by aqueous phase extraction of a crude *E. coli* lysate

5.5.1 Preparation of plasmid feed by batch aqueous phase extraction of crude *E. coli* lysate

With the aim of reducing the amount of pre-purification of the plasmid feed before loading onto the CCC column, *E. coli* lysate containing the plasmid pSVβ prepared as described in Section 2.2, was subjected to an initial aqueous two-phase extraction step, as described in Section 2.4.2. This approach does not utilise animal derived enzymes to digest RNA, or large quantities of organic solvent, and was designed to test the ability of CCC to purify SC plasmid from a relatively crude sample. Cell paste from the 75 L fermentation (Section 2.1) was used in these experiments to prepare sample for CCC. 20 g of a phase system in the following proportions. 5 % w/w isopropyl alcohol (IPA), 12.5 % w/w PEG 600, 16 % w/w K₂HPO₄ (from a 40 % w/w stock solution) and 42.5 % w/w lysate was prepared and allowed to
settle. 10 mL of the lower (salt rich) phase was loaded as feed for subsequent CCC runs. The final total concentration of plasmid in the lower phase was determined by the picogreen assay as described in Section 2.6.2.2 to be 31 \( \mu g \text{ mL}^{-1} \), corresponding to approximately 75 % w/w plasmid from the lysate partitioning to the lower phase. Neither RNA nor chromosomal DNA was detected in the lower phase by agarose gel electrophoresis. No nucleic acids were detected in the upper phase, and so it is assumed that the nucleic acids unaccounted for in the mass balance were present in the white precipitate which formed at the interface of the system.

### 5.5.2 CCC using plasmid feed prepared by aqueous phase extraction

Figure 5.9 shows a typical chromatogram (calculated from data obtained using the picogreen assay) when 10 mL of the lower phase of the plasmid containing ATPS was loaded onto the CCC column following equilibration of the column as described in Section 2.5.5. The phase system used was the PEG 600 IPA system and the column was spun at 600 rpm with a mobile phase flow rate of 0.5 mL min\(^{-1}\). Some stripping occurred during operation (from 1.55 to 1.92 CV), and \( S_f \) was reduced from the initial value of 45 % v/v to 40 % v/v following injection of the sample. Both plasmid forms were seen to elute from the column, however the time taken for elution was greater than previously seen, with the first OC plasmid eluting between 215 minutes and 240 minutes (1.78 to 2.0 CV) with SC plasmid eluting slightly later in a peak between 1.82 and 2.30 CV. This compares to plasmid elution after 120 minutes when the feed was concentrated by IPA precipitation as described in Section 5.4. The resolution between OC and SC is 0.3, less than described in Section 5.4 (0.75 and 1.05 for Figures 5.8 H and 5.8 G respectively), however a much larger volume of relatively dilute feed was loaded. In addition lysate prepared from cells from the 75 L fermentation contain proportionally less plasmid in the SC form than lysate prepared from cells from the 450 L fermentation (73 % w/w SC plasmid DNA compared to 58 % w/w SC plasmid for lysates prepared from cell paste from the 450 L and 75 L fermentations respectively). The feed sample contained 58 % w/w SC plasmid DNA and 42
% w/w contaminant plasmid forms (mainly OC plasmid). The proportion of SC plasmid present in the eluted fraction is 2 % w/w, 19 % w/w, 79 % w/w, 82 % w/w, 75 % w/w and 82 % w/w at 1.81, 1.85, 1.88, 1.92, 1.96 and 2.00 CV respectively. Figure 5.10 shows an analytical agarose gel illustrating the proportion of plasmid variants as detected for each sample. Quantitation of total plasmid eluted by picogreen analysis indicated that close to 100 % w/w of both plasmid forms had eluted, with only a small amount detected in the column after the run.

**Figure 5.9.** CCC chromatogram of plasmid DNA eluted using a phase system comprised of 2 % w/w IPA 12.5 % w/w PEG 600 (stationary phase) 18 % w/w K₂HPO₄ (mobile phase). CCC carried out at 600 rpm with mobile phase flow rate 0.5 mL min⁻¹ as described in Section 2.5.5. Feed prepared as the lower phase of a phase system comprising 12.5 % w/w PEG 600, 16 % w/w K₂HPO₄ (as 40 % w/w stock solution) 5 % w/w IPA and 37.5 % w/w E. coli lysate containing plasmid as described in Section 2.5.3. Composition of feed 13 μg mL⁻¹ OC plasmid, 18 μg mL⁻¹ supercoiled plasmid. 10 mL feed loaded. Total concentration of dsDNA determined using picogreen assay as described in Section 2.6.2.3. Proportions of SC and OC plasmid estimated from scanning of agarose gel as described in Section 2.6.1. Dashed line OC plasmid, solid line SC plasmid. P, point at which pumping out of column initiated.
Chromosomal DNA was not detected in any of the fractions collected by qualitative Southern blot analysis of the analytical gel (data not shown). However quantitation of selected fractions by Southern slot blot analysis indicated that the proportion of contaminating chromosomal DNA was just 3 % w/w of total DNA in the feed, compared to 23 % w/w chromosomal DNA in the unprocessed lysate. As discussed in Section 2.5.1, this reduction is due to the majority of chromosomal DNA precipitating at the interface of the phase system during the batch aqueous extraction operation used to prepare the feed. Following CCC, chromosomal DNA levels are reduced to 1 % w/w, 2 % w/w, 2 % w/w, 0.3 % w/w and 1 % w/w at column volumes 1.81, 1.85, 1.88, 1.92, and 2.00 CV respectively (Figure 5.11). Protein was below the limit of detection of the assay used (< 0.01 mg mL⁻¹). However partition experiments with model protein solution (BSA) indicated that protein partitioned strongly to the top phase and so it is hypothesised that the majority of cellular proteins precipitated to the interface, or were either removed with the upper phase during the initial preparation of the feed sample by aqueous phase extraction.
5. Countercurrent chromatography for the high resolution purification of plasmid DNA

5.5.3 Discussion

Preparation of the feed by a batch aqueous phase extraction of unprocessed lysate was found to be effective for removing the bulk of chromosomal DNA (chromosomal DNA contamination reduced from 23 % w/w in the lysate to 3 % w/w (Figure 5.11)). Contaminant chromosomal DNA will be present as

With the intention of shortening the time required for a batch run, the feed was loaded onto the column as the phase system was equilibrated. This reduces the time required to carry out a run by one third. The bulk of OC plasmid eluted between 1.48 and 1.68 CV, while the majority of SC plasmid was retained throughout the length of the column (Appendix B.3). The feed loaded onto the column was 59 % w/w SC plasmid, which is increased to 82 % w/w in the SC plasmid retained in the column. A considerable amount of stripping of the stationary phase was observed, S being reduced to just 12 % v/v at the end of the run.

Figure 5.11. Southern slot-blot analysis of feed and eluted fractions containing plasmid collected during CCC using a phase system comprised of 2 % w/w IPA 12.5 % w/w PEG 600 (stationary phase) 18 % w/w K$_2$HPO$_4$ (mobile phase). CCC carried out at 600 rpm with mobile phase flow rate 0.5 mL min$^{-1}$ as described in Section 2.5.5. Feed prepared as the lower phase of a phase system comprising 12.5 % w/w PEG 600, 16 % w/w K$_2$HPO$_4$ (as 40 % w/w stock solution) 5 % w/w IPA and 37.5 % w/w E. coli lysate containing plasmid as described in Section 2.5.3. Composition of feed 13 μg mL$^{-1}$ OC plasmid, 18 μg mL$^{-1}$ supercoiled plasmid. 10 mL feed loaded. Conditions as described in Figure 5.7. 350 ng dsDNA for feed and eluted fractions was loaded on the blot. Slots A 1- 7 E. coli DH5α standard curve (100, 50, 25, 12, 6, 3 and 1.5 ng respectively). Slot B1; unprocessed lysate. Slot B2 Feed prepared by batch extraction in ATPS. Slots B 3-7 fractions eluted in mobile phase at 1.81, 1.85, 1.88, 1.92, and 2.00 column volumes respectively, as described in Section 2.6.2.3.

With the intention of shortening the time required for a batch run, the feed was loaded onto the column as the phase system was equilibrated. This reduces the time required to carry out a run by one third. The bulk of OC plasmid eluted between 1.48 and 1.68 CV, while the majority of SC plasmid was retained throughout the length of the column (Appendix B.3). The feed loaded onto the column was 59 % w/w SC plasmid, which is increased to 82 % w/w in the SC plasmid retained in the column. A considerable amount of stripping of the stationary phase was observed, S being reduced to just 12 % v/v at the end of the run.

5.5.3 Discussion

Preparation of the feed by a batch aqueous phase extraction of unprocessed lysate was found to be effective for removing the bulk of chromosomal DNA (chromosomal DNA contamination reduced from 23 % w/w in the lysate to 3 % w/w (Figure 5.11)). Contaminant chromosomal DNA will be present as
fragments of varying size and morphology at this point in the process. Denatured chromosomal DNA fragments may have regained double stranded conformation, either through the formation of hairpin loops, or partial reannealment with other fragments during processing. Size, hydrophobicity and charge are all factors that influence the partitioning of solutes in a phase system (Walter et al. 1985), so the partitioning behaviour of different chromosomal DNA fragments can also be expected to vary. Following CCC, levels of contaminant chromosomal DNA in eluted fractions were again reduced (0.3 - 2 % w/w) with the highest concentration of chromosomal DNA eluting in a peak slightly earlier than the SC plasmid peak, and roughly corresponding to the OC plasmid peak. This result was reproducible and approaches the levels of purity likely to be required by the FDA for therapeutic plasmid DNA (Marquet et al. 1997b). However the chromosomal DNA fragments remaining in the eluted mobile phase fractions are likely to be those most similar in size and hydrophobicity to the plasmid and therefore may prove difficult to further reduce in a polishing step. However further improvement of the feed preparation and CCC protocol may improve the separation of both OC plasmid and chromosomal DNA from SC plasmid. Upstream operations (i.e. fermentation and alkaline lysis) may also be optimised to reduce the levels of chromosomal DNA in the process stream, thus increasing the final purity of the product following high resolution separation by CCC.

Preparation of the plasmid feed by batch aqueous phase extraction resulted in 25 % w/w of plasmid losses, which is comparable to 30% w/w yield loss reported for intermediate purification by precipitation (Ferreria et al. 1998). Following CCC using the PEG 600 IPA system approximately 100 % w/w of plasmid forms were recovered in the eluted fractions. In this case plasmid eluted considerably later than in the previous runs, probably due to the difference in the solutes present in the plasmid preparation altering the composition of the phase systems contained within the column, and therefore the partition properties of the plasmid forms. The resolution achieved in this manner was not high, however the loaded sample was dilute and a large
volume (10 mL) was loaded onto the column (94.3 mL). It may be possible to further concentrate the sample using ultrafiltration before loading, which may improve the separation achieved. Some stripping of stationary phase was observed as the solvent front from the loaded sample began to elute, but the amount was not significant. As the majority of plasmid was seen to elute, it may be possible to operate this separation in a continuous rather than batch fashion, (i.e. loading a series of samples onto the column) if levels of stripping remain low enough that the resolution is not unduly affected. This would have the effect of reducing the time required overall for each run.

From this study it is evident that the preparation of the feed is also an important factor to be considered when designing a CCC protocol. The introduction of new solutes in the mobile phase is known to alter the hydrodynamics of the phase system within the column and hence the separation will be affected (Walter et al. 1985; Rito-Palomares and Cueto 2000). In this case, considerable clearance of contaminants through the pre-treatment of the plasmid sample was achieved at the expense of product yield loss. This may be due to the high salt concentrations of the phase systems used rendering some contaminants insoluble as much as a partitioning effect, and this pre-purification may not be as effective with systems positioned nearer to the binodal curve. Optimisation of the feed preparation for initial clearance of contaminants, product yield and efficient partitioning and phase separation within the column will be required to optimise the CCC protocol for resolution and yield.

5.6 Conclusions

At present the low capacity of conventional chromatographic matrices (typically around 50 μg plasmid mL⁻¹ solid matrix (Green et al. 1997; Theodossiou et al. 2000)) is prohibitive for the purification of plasmid DNA for therapeutic applications. In this work, several examples of separations between OC and SC forms of plasmid by CCC were demonstrated, and evidence for the significant depletion of other contaminants, such as
chromosomal DNA and protein, to levels approaching those likely to be required by the FDA (Marquet et al. 1997b) found. Up to 33 \( \mu \text{g} \text{ plasmid mL}^{-1} \) stationary phase was loaded onto the CCC column (Section 5.4); however the upper limit for loading was not established due to problems with the solubility of the plasmid in the buffer. The partitioning of high molecular weight DNA has been reported at concentrations of 500 \( \mu \text{g} \text{ DNA mL}^{-1} \) ATPS (Walter et al. 1985). The cost of the ATPS used in this work is likely to be several orders of magnitude less than the cost of the same volume of conventional matrix, indicating that CCC is likely to be more cost effective as a high resolution operation for plasmid DNA purification. There is considerable scope for optimisation of the operation of CCC for the purification of SC plasmid DNA. In particular it is desirable to eliminate stripping of the stationary phase during CCC operation, and hence increase the stationary phase retention achieved. The proportion of the stationary phase retained within the column affects both the capacity and efficiency of the CCC operation (Du et al. 1999). It has been shown in our laboratory that increasing the bore of the column upon scale up resulted in greater stationary phase retention with aqueous-organic phase systems (Booth et al. 2002). This allows scope for further optimisation of the CCC operation through balancing increased flow rate (possible because of greater stationary phase retention with increased column bore) with increased column length (and therefore increased resolution). Should the same prove to be true for ATPS, then the resolution achieved through this technique can also be expected to improve upon scale up.

In all cases, the range of phase systems that could be used with the J-type machine available was extremely limited, due to the requirement to choose systems with relatively rapid phase separation times. Use of an alternate type of CCC machine, such as cross-axis (Shinomiya et al. 1993; Shibusawa et al. 2001) or toroidal column (Ito et al. 1998) designs, specifically designed for use with ATPS is likely to drastically improve the separations achieved. The high laterally acting force field in these designs tends to suppress emulsification of the two phases, but at the cost of efficient mixing. This should result in higher
S_f values, which in turn will result in higher resolution (Du et al. 1999), and reduce batch-processing times, as the volume of stationary phase within the column will be reduced. Better separation of the phases during CCC will reduce levels of stripping of the stationary phase during CCC and will facilitate continuous operation. In addition the reduced interfacial surface area may reduce the amount of plasmid retained in the column. Also, in addition to improvement of the CCC operation in terms of enhancing the operating conditions, increasing the range of phase systems available (as fast settling times will be a less important factor in phase system selection) may enable improvements in terms of plasmid partition coefficients observed and the capacity of the phase system used. It may also be possible to change the composition of the mobile phase system used during the CCC operation, thus allowing the plasmid forms and other contaminants to be selectively moved between the phases, through the use of step gradients. In addition to facilitating separations, it may be possible to concentrate the SC plasmid in the stationary phase before elution in this manner.

Further investigation is required to determine the maximum loading possible. Other studies carried out in our laboratory indicated that loading of up to 10 mg solute mL^{-1} mobile phase are possible (Booth and Lye 2001). This compares very favourably to 50 μg SC plasmid mL^{-1} solid stationary phase loaded onto a reversed phase HPLC column (Green et al. 1997; Theodossiou et al. 2000), and 400 μg plasmid mL^{-1} purified using a 10 mL Mustang Q anion exchange membrane adsorber (Nochumson et al. 2000). Throughputs of 25 – 50 tonnes per year have been predicted for industrial scale CCC machines (Sutherland et al. 1998). Furthermore the operation of CCC at industrial scale is likely to be considerably less costly due to lower initial capital expenditure requirements and reduced operating costs (i.e. smaller volumes of solvent required), compared to industrial scale HPLC (Sutherland et al. 1998).
6. Final discussion and future work

6.1 Final discussion

Two distinct strategies for the purification of pharmaceutical grade plasmid DNA have been investigated in this work; the selective adsorption of contaminants using nitrocellulose (carried out using both nitrocellulose powder and adsorbent nitrocellulose membrane), and the high resolution separation of plasmid DNA by CCC, combined with aqueous two-phase extraction. Table 6.1 summarises the results for the purification techniques investigated.

<table>
<thead>
<tr>
<th>Purification specification</th>
<th>Tangential-flow filtration-adsorption using nitrocellulose membrane</th>
<th>Batch extraction using aqueous two-phase system</th>
<th>CCC using aqueous two-phase system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal DNA (≤1 % w/w)</td>
<td>Feed 10 % w/w Purified fractions 1 % w/w</td>
<td>Feed 23 % w/w Purified fractions 3 % w/w</td>
<td>Feed 3 % w/w Purified fractions 1.3 % w/w</td>
</tr>
<tr>
<td>Protein (undetectable)</td>
<td>80 % w/w reduction (0.2 to 0.6 mg mL⁻¹)</td>
<td>&lt;0.01 mg mL⁻¹</td>
<td>&lt;0.01 mg mL⁻¹</td>
</tr>
<tr>
<td>RNA (undetectable)</td>
<td>Some reduction, but not quantified</td>
<td>None detected</td>
<td>None detected</td>
</tr>
<tr>
<td>Endotoxin (&lt; 0.1 EU μg⁻¹ plasmid)</td>
<td>Feed 48 EU mL⁻¹ or 60 EU μg⁻¹ plasmid First purified fraction 0.48 EU mL⁻¹ or 0.6 EU μg⁻¹ plasmid</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>SC plasmid (not specified)</td>
<td>Feed 73 % w/w Purified 73 % w/w</td>
<td>Feed 58 % w/w* Purified 58 % w/w</td>
<td>Feed 58 % w/w* Purified 82.5 % w/w</td>
</tr>
<tr>
<td>Yield</td>
<td>100 %</td>
<td>75 %</td>
<td>~ 100 %</td>
</tr>
<tr>
<td>Approximate time required for processing</td>
<td>5 minutes</td>
<td>~ 30 minutes settling</td>
<td>4 hours per cycle</td>
</tr>
</tbody>
</table>

Table 6.1. Comparison of the novel plasmid DNA purification techniques investigated. Target contaminant levels are from the published specification for the release of Allovectin-7 plasmid DNA, taken from (Marquet et al, 1997b). See Section 4.2 for results from tangential-flow filtration-adsorption of contaminants using nitrocellulose membrane. See Section 5.5 for results from batch extraction using aqueous two-phase system and CCC for the separation of plasmid DNA.

*Levels of chromosomal DNA and SC plasmid present in the lysate prepared from the 75 L fermentation (Section 2.1) (used as the source of plasmid DNA for these CCC and extraction experiments) were high compared to lysate prepared using cells from the 450 L fermentation, (as used for adsorption experiments).
All the techniques investigated achieved the purification of plasmid DNA from contaminating chromosomal DNA fragments. As discussed in Section 3.2.4, work with nitrocellulose powder was discontinued after a short period of research due to safety concerns. Nitrocellulose membranes, however, were found to partially adsorb RNA, protein and endotoxin from crude lysate streams in addition to chromosomal DNA fragments (Section 4.3.1). The capacity of the nitrocellulose membrane for the adsorption of chromosomal DNA from crude lysate was lower than observed for other, purer feed streams (Section 4.2.2 and Section 4.4.2) suggesting that this unit operation may more cost effectively be used as a final polishing step.

Aqueous two-phase extraction and CCC were both effective for the purification of plasmid DNA from contaminants such as chromosomal DNA, RNA and proteins (Section 5.4.3). In addition the SC form of the plasmid was partially resolved from the OC form following CCC (Section 5.5.3). The scope for further optimisation of the CCC operation is discussed in Section 5.6

6.1.1 Initial economic evaluation of process options

For the purposes of an approximate economic evaluation of the novel techniques investigated in this thesis, a 2000 L fermentation has been considered. Details of the evaluation are tabulated in Appendix A. This evaluation is intended to provide a rough comparison between the various techniques, and has not been expanded to include other expenses such as the cost of additional buffers or cleaning solutions, utility usage (i.e. the cost of meeting water for injection (WFI) demands for buffer make-up or phase systems requirements), cleaning and sterilisation, initial outlay on equipment (CCC rotor, ultrafiltration rig, conventional chromatography column and bioprocessor) manpower, other intermediate operations (i.e. concentration by ultrafiltration or diafiltration), and the cost of shipping and storage of flammable liquids.
The following assumptions have been made for the product of the fermentation and lysis steps:

- 2000 L of fermentation broth will yield 68 L lysate containing 80 mg plasmid L\(^{-1}\) or 6.0 g plasmid total (Section 2.2).
- 10 % w/w chromosomal contamination (Section 2.2), equal to 0.6 g chromosomal DNA in total.

The following assumptions have been made for the pre-purification of plasmid DNA using the tangential-flow membrane adsorption:

- Disposable cartridge
- Capacity of 3.1 \(\mu\)g chromosomal DNA cm\(^{-2}\) nitrocellulose membrane, with approximately 90 % chromosomal DNA contaminants adsorbed (Section 2.4, and Section 4.2).
- Estimated cost of the nitrocellulose membrane is £1 200 m\(^2\) (Murrell, personal communication. 2002).

The following assumptions have been made for the pre-purification of plasmid by aqueous two-phase extraction and high resolution purification of plasmid DNA by CCC:

- A cost of £10 kg\(^{-1}\) for the potassium phosphate, £12.50 kg\(^{-1}\) for the PEG 600 and £12.50 kg\(^{-1}\) for the IPA. These prices were obtained by taking the Sigma catalogue values for kg quantities of the chemicals (£20 kg\(^{-1}\) for the potassium phosphate, £25 kg\(^{-1}\) for the PEG 600 and £25 kg\(^{-1}\) for the IPA) and assuming that for bulk purchases, a discount of at least 50 % could be obtained.

The following assumptions have been made for the purification of plasmid DNA by anion exchange chromatography, previously described for the purification of plasmid DNA (Prazeres et al. 1998):
• The cost of anion exchange matrix, was taken to be £ 500 L\(^{-1}\) (Murrell personal communication. 2002).

• The capacity of conventional matrices for plasmid is 0.05 g L\(^{-1}\) (Theodossiou et al. 2000).

• The matrix is reused for 50 cycles (Murrell, personal communication. 2002).

Pre-purification of plasmid DNA using the tangential-flow filtration-adsorption process with a disposable nitrocellulose membrane cartridge, can be estimated to cost approximately £ 21 000 per 2000 L fermentation in terms of the membrane required. By contrast, Pre-purification of the lysate by aqueous two-phase partitioning costs £ 600 in terms of the polymer, salt and IPA required to make up the phase system. Although the duration of the operation is greater, at this early stage in the purification process, aqueous two-phase extraction is both more cost effective and achieves superior clearance of contaminants (Table 6.1) than selective adsorption using nitrocellulose membrane.

Data from Chapter 5 indicates that a minimum of 0.04 g plasmid L\(^{-1}\) stationary phase can be loaded onto the column, and S\(_f\) values of 35 % can be achieved. The volume of mobile phase required (approximately 3 CV to equilibrate the column, and consequently carry out the CCC run) will dictate the amount of phase system to be prepared. Therefore a single cycle on a 400 L CCC column should be sufficient to purify 5.4 g of plasmid DNA from a 2000 L fermentation. The cost of phase forming solutes sufficient for this CCC run would be £ 5 000. In contrast, 120L anion exchange matrix, (an initial outlay of approximately £ 60 000), is required to purify 5.4 g plasmid at a cost to £ 1200 per cycle assuming 50 cycles of reuse. With these conditions, conventional chromatography appears the most cost effective, as long as multiple cycles of reuse (a minimum of 12 cycles) can be achieved. However, as previously discussed in this Section, not all the potential costs involved
with the unit operations have been considered. In addition, the feed stream loaded onto a conventional chromatography column must be highly clarified to prevent blinding of the packed bed, whereas only minimal pre-preparation was necessary before CCC. Furthermore, the yield predicted for the CCC operation is in excess of that expected from conventional chromatography.

Due to time constraints, it was not possible to optimise the CCC protocol to achieve the highest possible throughputs. As discussed in Section 5.6, it is probable that the performance of the CCC step can be improved, both in terms of throughput and the resolution between plasmid forms. Improvements to the CCC protocol that increase throughput will also improve economic performance. Consideration is given below to a number of scenarios where throughput is increased, and the economic impact:

- **Stationary phase retention is increased to 50%** (Ito and Conway 1996). The size of the column required is 300 L, and the cost of the phase forming polymers required is approximately £3,000.
- **The mass of plasmid loaded per cycle is increased by the concentration of the prepared plasmid prior to CCC**, (for example by ultrafiltration). 0.4 g plasmid kg⁻¹ stationary phase is loaded (Walter and Johansson, 1986). Assuming the stationary phase retention is 50%, the size of the column required is 30 L, and the cost of the phase forming chemicals is £350.
- **The mass of plasmid loaded per cycle is further increased to 4 g plasmid L⁻¹ stationary phase** (Booth et al, 2002). The cost of the phase forming chemicals is now just £36 (assuming 50% stationary phase retention). This compares very favourably with £1,200 for the cost of matrix required for conventional chromatography.

To further reduce the costs, recycling of phase forming chemicals as described in Section 1.4.2.2 could be investigated (Rito-Palomares et al. 2000). The effect of recycling the polymer on the partitioning behaviour of the nucleic acids, and cost of the recycling operations (i.e. ultrafiltration or back extractions), must first be evaluated (Rito-Palomares et al. 2000).
Figure 6.1 illustrates a conventional plasmid purification process, and in addition a theoretical novel process is also proposed. The rationale behind the selection of the unit operations for the novel process is as follows.

6.1.2 Evaluation of a novel process for the purification of plasmid DNA

Pre-purification of lysate by aqueous two-phase extraction (Section 5.5.1):
- The plasmid partitions to the lower phase, which can be loaded directly onto the CCC column.
- Plasmid DNA in the lower phase may be concentrated by ultrafiltration before the next stage.
- A range of contaminants (Chromosomal DNA fragments, Protein, RNA) are depleted in this initial step (Table 6.1).
- Approximately 25% yield losses are expected.

High resolution purification of plasmid DNA by CCC (Section 5.5.2):
- The OC plasmid form is resolved from the SC plasmid product.
- The majority of the remaining chromosomal DNA fragments are separated from the plasmid product (Table 6.1).
- Other contaminants (Protein, RNA) remaining are also expected to be separated from the plasmid DNA during this step.
- Close to 100% yield can be expected.
- It may be necessary to modify the composition of the plasmid containing fractions before proceeding to the final polishing step.

Selective adsorption of chromosomal DNA using nitrocellulose (Section 4.3.1 and Section 4.4.2):
- If required, the level of chromosomal DNA contamination is further reduced by the final polishing step, using selective adsorption during filtration through nitrocellulose membranes.
- Capacity of nitrocellulose membranes to adsorb chromosomal DNA is expected to be significantly higher for the purified feed stream than was observed for crude lysate, hence the membrane area required should be reduced.
- Close to 100% yield is expected.
- A buffer exchange step is likely to be required before final filling.

The major advantages of the theoretical novel process over the conventional process illustrated in Figure 6.1 are the relatively high yield expected (approximately 75% w/w compared to 37% w/w reported by Ferreria et al (1999)), and (assuming the throughput can be increased as expected) the low cost of the phase forming chemicals used for the pre-purification and high resolution stages. In addition, it is possible the process could be made fully disposable (as discussed in Section 6.2.2), and it has been shown that the OC and SC plasmid forms can be separated by CCC, while this is not possible using anion exchange chromatography.
6. Final discussion and future work

6.2 Future Work

6.2.1 Studies on the selective adsorption of contaminants

Nitrocellulose in both the powder and membrane composition was investigated for the selective adsorption of contaminants from plasmid containing liquors. Due to difficulties in handling the nitrocellulose powder and initial poor results (Section 3.2.4), the utility of this form was not investigated in the same detail as the nitrocellulose membrane. Following the establishment of a safe method of producing a homogenous mixture of nitrocellulose particles within a predictable range of sizes, the following topics would be investigated:

- Quantification of the adsorption of contaminants other than chromosomal DNA (i.e. protein, endotoxin, RNA).
- Investigate the effect of particle size on capacity.
- Compare the use of nitrocellulose powder from other sources, in terms of capacity and ease of handling.
- Investigate alternative protocols, such as use of nitrocellulose powder as a filter aid or the use of a packed column.

There is a great deal of scope for further research into the selective adsorption of contaminants, using either adsorptive powders or membranes. Further work on the regeneration of nitrocellulose (Section 3.2.3 and Section 4.3.4), or use of nitrocellulose membrane to adsorb contaminants remaining from purified feed streams (Section 4.4.3) has already been discussed. In particular, the use of selective adsorption as the final polishing step following the purification of plasmid DNA by CCC (as discussed in Section 6.1.2) should be investigated. Additional avenues of exploration for further work on selective adsorption of contaminants are outlined below:
6. Final discussion and future work

- Investigate the influence of buffer composition on adsorption as discussed in Section 1.4.1.2 (Popovic and Wintzerith 1983).
- Compare other adsorptive membrane chemistries and alternative adsorptive materials as discussed in Section 1.4.1.2 (Horn et al, 1996; Poly et al, 2000; Theodossiou et al, 1997).
- Quantify the adsorption of RNA onto nitrocellulose.
- Consider the use of membrane stack chromatography as discussed in Section 1.4.1.2 (Gebauer et al, 1997; Henrickson 1996).

6.2.2 Studies on Countercurrent chromatography and aqueous two-phase extraction

The main aim of the work presented in Chapter 5 was to show that plasmid DNA can be purified by CCC. Having established an appropriate methodology, there was no time for a detailed program to optimise the protocol. Some of the parameters, which would benefit the protocol by further characterisation, are detailed below.

- Optimisation of the pre-preparation of the sample by aqueous two-phase extraction (i.e. composition of the phase system, and time allowed for settling (Heywood-Waddington et al. 1986)).
- Investigate the concentration of the prepared sample prior to loading (i.e. by ultrafiltration, with the intention of increasing throughput by achieving the maximum loading possible (Section 5.6).
- Full characterisation of the effect of varying the rotational speed and mobile phase flow rate.
- Studies on the scale up of the CCC operation, to characterise the effect of increased bore and to identify if the scalability of the system mimics that seen with aqueous-organic systems as discussed in Section 5.6 (Booth et al, 2002).
- Use of an alternative design such as the cross axis or toroidal coil planet centrifuges to increase throughput and resolution achieved (as discussed
in Section 1.4.2.4. and Section 5.6), and consideration of phase systems with slower settling times.

In addition to the optimisation of the basic protocol established in this work (Chapter 5), there are a number of alternative approaches, which may also be worthy of investigation for the purification of plasmid DNA.

- Use of affinity ligands, charged polymers and additives such as urea
- Use of the aqueous two-phase partitioning step prior to hydrophobic interaction chromatography, or selective adsorption of contaminants through hydrophobic interactions.
- Test and develop theoretical models; for example to describe the behaviour of the plasmid DNA and associated contaminants within the phase system, or to predict the robustness of the method for application to plasmids of varying morphology.

For use in the production of pharmaceutical grade plasmid DNA, several additional factors will have to be considered in addition to the purification achievable. For instance the parameters under which the machine operates, such as temperature and bobbin rotational speed, will need to be easily set, and deviations avoided. Limits also need to be set for the variables applicable to CCC operation, and the performance of the machine between these will need to be validated. Cleaning and sterilisation of the column between batches will also be required. These design considerations, and the validation of the cleaning and controls will need to be considered and implemented in conjunction with the manufacturer of the machine before commercial use will be possible. However the cleaning of conventional chromatography columns is restricted to sanitation using chemicals, which, over time, can result in an undesirable accumulation of bioburden. In contrast it should be possible to design a steam sterilised CCC column constructed using stainless steel tubing, which will eliminate such problems. Alternatively, for use with ATPS, it might prove possible to design a machine, which used a disposable PTFE column, which would be replaced after each use. This would eliminate
problems that might be encountered for the cleaning and sterilisation of the columns, and would be particularly useful in multi-product facilities. In addition to the considerations of design, control and purification achieved, removal of residual phase forming chemicals, in particular IPA will need to be validated to stringent requirements before the application of this technique is likely to be acceptable to regulatory bodies such as the FDA. However, such exercises must be carried out for each new product, whether the purification process is conventional or novel.

To conclude, while further work is needed to develop the novel techniques described in this work, the results presented indicate that the application of aqueous two-phase extraction, countercurrent chromatography or selective adsorption to the purification of pharmaceutical grade plasmid DNA should have significant advantages over conventional processes.
Appendices

Appendix A tabulates the calculations to determine the relative cost of a conventional chromatography operation, a tangential-flow membrane-adsorption step, aqueous two-phase extraction and countercurrent chromatography separation sized for a 2000 L fermentation, and should be read in conjunction with the economic evaluation detailed in Section 6.1.1.

Appendix B illustrates the chromatograms for the separation of the OC and SC plasmid DNA forms obtained following countercurrent chromatography, as described in Section 2.5 and should be read in conjunction with Section 5.3.2 and Section 5.5.2.
Appendix A

Assumptions (from 450 L fermentation data)

<table>
<thead>
<tr>
<th>Operation</th>
<th>Assumptions</th>
<th>Cost of material</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000 L fermentation</td>
<td>5400 g cell paste (wet weight) recovered</td>
<td></td>
</tr>
<tr>
<td>68 L lysate recovered</td>
<td>0.08 g plasmid L^1 lysate</td>
<td></td>
</tr>
<tr>
<td>3.40 g total DNA</td>
<td>5.40 g plasmid in total</td>
<td></td>
</tr>
<tr>
<td>0.60 g chromosomal DNA</td>
<td>0.60 g chromosomal DNA</td>
<td></td>
</tr>
<tr>
<td>6.00 g total DNA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Operation</th>
<th>Assumptions</th>
<th>Cost of material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional chromatography</td>
<td>0.05 g L^1 (plasmid / matrix)</td>
<td>£ 500 L matrix</td>
</tr>
<tr>
<td>Single step</td>
<td>108 L matrix required</td>
<td>£ 60000 for 150 L matrix</td>
</tr>
<tr>
<td>120 L CV - 1x cycles</td>
<td>120 L matrix required</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Operation</th>
<th>Assumptions</th>
<th>Cost of material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tangential-flow filtration-adsorption</td>
<td>0.031 g chromosomal DNA m^2</td>
<td>£ 1200 per m^2</td>
</tr>
<tr>
<td>of contaminants using</td>
<td>nitrocellulose membrane</td>
<td>£ 20903 Total membrane area</td>
</tr>
<tr>
<td>as a disposable step</td>
<td>90 % chromosomal DNA adsorbed (0.54 g)</td>
<td></td>
</tr>
<tr>
<td>Intermediate purification of Plasmid DNA using ATPS</td>
<td>12.5% PEG 600</td>
<td>£ 12.5 per kg</td>
</tr>
<tr>
<td>(No recycling of phase forming chemicals)</td>
<td>16% K_2HPO_4</td>
<td>£ 248 Total for PEG 600</td>
</tr>
<tr>
<td></td>
<td>5% IPA</td>
<td>£ 12.5 per kg</td>
</tr>
<tr>
<td></td>
<td>43% Lysate</td>
<td>£ 99 Total for IPA</td>
</tr>
<tr>
<td></td>
<td>158.82 kg Phase system</td>
<td>£ 602 for phase forming chemicals</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Operation</th>
<th>Assumptions</th>
<th>Cost of material</th>
</tr>
</thead>
<tbody>
<tr>
<td>High resolution purification of plasmid DNA by CCC</td>
<td>0.04 kg^1 stationary phase (plasmid / PEG 600)</td>
<td>£ 12.5 per kg</td>
</tr>
<tr>
<td></td>
<td>35% retention</td>
<td>£ 2188 Total for PEG 600</td>
</tr>
<tr>
<td></td>
<td>135 kg stationary phase</td>
<td>£ 10 per kg</td>
</tr>
<tr>
<td></td>
<td>386 L coil</td>
<td>£ 254 Total for K_2HPO_4</td>
</tr>
<tr>
<td></td>
<td>140 L stationary phase</td>
<td>£ 12.5 per kg</td>
</tr>
<tr>
<td></td>
<td>260 L mobile phase (equil.)</td>
<td>£ 99 Total for IPA</td>
</tr>
<tr>
<td></td>
<td>520 L mobile phase (elution)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.62 volume ratio (V/V_b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>780 mobile phase (3 CV_m)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1264 L phase system</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1400 kg phase system in total</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.5% PEG 600</td>
<td>£ 12.5 per kg</td>
</tr>
<tr>
<td></td>
<td>18% K_2HPO_4</td>
<td>£ 10 per kg</td>
</tr>
<tr>
<td></td>
<td>2% IPA</td>
<td>£ 2520 Total for K_2HPO_4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>£ 12.5 per kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>£ 350 Total for IPA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>£ 5058 Total for phase system</td>
</tr>
<tr>
<td>Amount of DNA</td>
<td>Stationary Phase</td>
<td>CCC Details</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>0.04 g kg⁻¹</td>
<td>0.44 kg stationary phase (plasmid / PEG 600)</td>
<td>50% retention</td>
</tr>
<tr>
<td>0.4 g kg⁻¹</td>
<td>13.5 kg stationary phase</td>
<td>50% retention</td>
</tr>
<tr>
<td>4 g kg⁻¹</td>
<td>1.35 kg stationary phase</td>
<td>50% retention</td>
</tr>
</tbody>
</table>
Appendix B.1. CCC chromatogram of plasmid DNA eluted during CCC carried out using a phase system comprised of 12.5% w/w PEG 600 (stationary phase) and 18% w/w K₂HPO₄, 0.15 M NaCl (mobile phase) at 600 rpm and a mobile phase flow rate of 0.5 ml min⁻¹ as described in Section 2.5.5. P; point at which pumping out of the column initiated. Feed composition 35 μg mL⁻¹ OC plasmid, 95 μg mL⁻¹ supercoiled plasmid (as a 1/10 dilution of concentrated plasmid in TE buffer with mobile phase). Dashed line; OC plasmid. Solid line; SC plasmid. Red indicates plasmid eluted in upper PEG phase. P; point at which pumping out of the column initiated. Chromatograms estimated from scanning of agarose gel as described in Section 2.6.1. Y axis normalized to 50% of the signal from the undiluted feed sample.
Appendix B.2. CCC chromatogram of plasmid DNA eluted during a CCC runs carried out with a bobbin rotational speed 600 rpm and a mobile phase flow rate of 0.5 ml min$^{-1}$ as described in Section 2.5.5 were used in both experiments. 1 ml feed injected at the start of the CCC run, before coil had been equilibrated. (A) Phase system comprised of 12.5% w/w PEG 600 (stationary phase) and 18 % w/w K$_2$HPO$_4$ (mobile phase). (B) Phase system comprised of 12.5% w/w PEG 600 (stationary phase) and 18.5% w/w K$_2$HPO$_4$, 0.15 M NaCl (mobile phase). P; point at which pumping out of the column initiated. Feed composition 35 µg mL$^{-1}$ OG plasmid, 95 µg mL$^{-1}$ supercoiled plasmid (as a 1/10 dilution of concentrated plasmid in TE buffer with mobile phase). Dashed line; OG plasmid. Solid line; SC plasmid. Red indicates plasmid eluted in the upper PEG phase. P; point at which pumping out of the column initiated. Chromatograms estimated from scanning of agarose gel, Y axis normalized to 50 % of the signal from the undiluted feed sample.
Appendix B.3. CCC chromatogram of plasmid DNA eluted using a phase system comprised of 2 % w/w IPA 12.5 % w/w PEG 600 (stationary phase) 18 % w/w K₂HPO₄ (mobile phase). CCC carried out at 600 rpm with mobile phase flow rate 0.5 ml min⁻¹ as described in Section 2.5.5. Feed prepared as the lower phase of a phase system comprising 12.5 % w/w PEG 600, 16 % w/w K₂HPO₄ (as 40 % w/w stock solution) 5 % w/w IPA and 37.5 % w/w E. coli lysate containing plasmid as described in Section 2.5.3. Composition of feed 13 µg mL⁻¹ OC plasmid, 18 µg mL⁻¹ supercoiled plasmid. 10 mL feed injected immediately at the start of the run, i.e. before coil had been equilibrated. Total concentration of dsDNA determined using picogreen assay as described in Section 2.6.2.3. Proportions of SC and OC plasmid estimated from scanning of agarose gel as described in Section 2.6.1. Dashed line OC plasmid, solid line SC plasmid. P, point at which pumping out of column initiated.
7. Nomenclature

Cb  Concentration of solute in lower phase (appropriate units)
Ct  Concentration of solute in upper phase (appropriate units)
CV  Volume of CCC column available for flow of mobile phase (mL)
D   HETP value at minimum critical flow rate for CCC (m)
h   Height above membrane available for flow (m)
K   Partition coefficient of given solute (dimensionless)
P   \(= \frac{V_t}{V_b}\) (dimensionless)
R   Resolution of solute peaks during chromatography (dimensionless)
S_f Stationary phase retention (% v/v)
T   Temperature (K)
T_s Phase separation time (s)
T_1  Time of elution of first (OC plasmid) peak during chromatography (appropriate units – same as W_1)
T_2  Time of elution of first (SC plasmid) peak during chromatography (appropriate units – same as W_2)
u   Linear velocity (m s^{-1})
V_b Volume of lower phase
V_t Volume of upper phase
W_1 Width of first peak to elute at the baseline (appropriate units – same as T_1)
W_2 Width of second peak to elute at the baseline (appropriate units – same as T_2)
\(\gamma\) Shear rate (s^{-1})
\(\Delta G\) Gibbs free energy (J)
\(\Delta H\) Enthalpy (J)
\(\Delta S\) Entropy (J K^{-1})
8. References


Boehringer-Mannheim. 2000. DIG High Prime labelling and detection starter Kit: For color detection with NBT/BCIP.


Butler, VA.1996. Points to consider on plasmid DNA vaccines for preventative infectious disease indications, Food and Drugs Administration.


Cole, KD, Tellez CM and Blakesley RW. 2000. Separation of different physical forms of plasmid DNA using a combination of low electric field strength and flow in porous media: Effect of different field gradients and porosity of the media. Electrophoresis 21: 1010-1017.


8. References


McHugh, P. 2000. Personal communication.


Petsch, D, Deckwer W-D, Anspach FB, Legallais C and Vijayalakshmi M. 1998. Endotoxin removal with poly(ethyleneimine)-immobilised adsorbers:


Qiagen. 08/99. Genomic DNA handbook.


Purification of Plasmid DNA by an Integrated Operation Comprising Tangential Flow Filtration and Nitrocellulose Adsorption

D. Kendall, G. J. Lye, M. S. Levy

The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, London, WC1E 7JE, United Kingdom; telephone: +44 0207 6793246; fax: +44 0297 9163943; e-mail: myriom.levy@ucl.ac.uk

Received 28 September 2001; accepted 6 March 2002

DOI: 10.1002/biit.10325

Abstract: There is an increasing interest in the development of scalable and reproducible plasmid DNA purification protocols for vaccine and gene therapy. The use of an integrated unit operation, comprising tangential flow microfiltration coupled with the adsorption of contaminants onto nitrocellulose membranes as a single processing step was examined in this work. Experiments were performed using a custom-built tangential flow microfiltration rig (membrane area = 12.5 cm²). Tangential flow filtration-adsorption of E. coli lysates containing a plasmid product removed most solids (>75%) and decreased chromosomal DNA contamination by 75% w/w. Total plasmid DNA concentration and supercoiled content of the permeate were virtually identical to those of the feed, indicating a recovery yield of 100% (transmission equal to 1). Results were similar for E. coli lysates containing either a 6.9 kb or a 20 kb plasmid. Significant reductions in RNA, endotoxin, and protein levels were also observed. The reproducibility and potential for scale up of this integrated filtration-adsorption operation makes it an attractive option for downstream processing of plasmid DNA consists of alkaline lysis, flotation, and removal of the resultant floe, intermediate purification steps such as precipitation or ultrafiltration, and finally chromatographic separation (Ferreira et al., 2000; Levy et al., 2000a; Murphy, 1999). Chromatographic separations can be by conventional HPLC or FPLC (Green et al., 1997; Horn et al., 1995) or by new techniques such as liquid–liquid countercurrent chromatography (Kendall et al., 2001).

A recent communication by Kahn and co-workers (Kahn et al., 2000) reported the use of tangential flow filtration for plasmid DNA purification using a pore size such that the membrane retained the plasmid DNA. Here we examine the use of an integrated unit operation, tangential flow filtration-adsorption, as a single-processing step to further clarify an E. coli lysate after coarse filtration. In contrast to the approach of Kahn and co-workers, we aim to retain contaminants by specific adsorption and size-exclusion mechanisms while recovering the partially purified plasmid DNA in the permeate fractions. Our strategy is to remove key contaminants at an early stage of the process thus maximizing the capacity of the subsequent chromatographic operations. Such membrane adsorption processes have previously been applied to proteins (Champluvier and Kula, 1992) and we have recently reported the use of membrane adsorption to remove contaminant nucleic acids from laboratory scale plasmid preparations (Levy et al., 2000b). Adsorption of nucleic acids and proteins...
to nitrocellulose is thought to occur via hydrophobic interaction (Lui et al., 1996). Under moderate-to-high ionic strength conditions, single-stranded DNA (ssDNA), RNA, and proteins but not double-stranded DNA (dsDNA) are adsorbed to nitrocellulose (Boezi and Armstrong, 1967). After the neutralization step of the alkaline lysis procedure, plasmid DNA molecules regain their native dsDNA conformation, by contrast E. coli chromosomal DNA remains in the denatured ssDNA form (Sambrook et al., 1989). The differential hydrophobicity of plasmid DNA and its process contaminants has been exploited by Diogo and co-workers (Diogo et al., 1999) using hydrophobic interaction chromatography to separate the more hydrophobic ssDNA, RNA, and lipopolysaccharides from double stranded plasmid DNA.

MATERIALS AND METHODS

Pure DNA and Protein Solutions

Calf thymus DNA and bovine serum albumin (BSA) were obtained from Sigma Aldrich Chemical Company (Poole, Dorset, UK). To model chromosomal contamination, a solution of calf thymus ssDNA at a concentration and ionic strength similar to that of chromosomal DNA in plasmid process streams was prepared. A DNA stock solution was first diluted to approximately 80 µg/mL in TE buffer (1 mM Tris-HCl, pH 8; 5 mM EDTA). The DNA was then denatured by heating at 95°C for 10 min, followed by rapid cooling by addition of an equal volume of ice cold 20 × SSC buffer (3M NaCl, 300 mM sodium citrate, pH 7) and subsequent incubation on ice. Hereafter, this feed will be referred to as pure ssDNA solution. If necessary, the solution was further diluted with 10× SSC to obtain a sufficient volume for circulation around the microfiltration rig (described in Section 2.4). BSA was dissolved to 1.5 mg/mL in 10× SSC buffer before use.

Plasmids and E. coli Culture Conditions

Plasmids used in the present study were: pSVβ (Promega Corp., MA), 6.9 kb and pQR150 (Jackson et al., 1995), 20 kb. Plasmids were propagated in E. coli DH5α (Gibco-Life Technologies, MD); LB media (Sambrook et al., 1989) was used for all cultures. Bacteria were grown in 7-L and 450-L bioreactors as described previously (Levy et al., 2000b; Levy et al., 2000c; Noites et al., 1999). Bacterial cells from the 450-L fermentation were harvested using a Carr Powerfuge (Carr Separations Inc, Franklin, MA), while cells from the 7-L fermentations were harvested by batch centrifugation for 30 min at 10,000 rpm using a Beckman J2-M1 centrifuge (Palo Alto, CA). The resulting cell paste was stored initially at −20°C and then at −70°C.

Alkaline Lysis of Escherichia coli

Cells were lysed using a modified alkaline lysis procedure (Birnbom and Doly, 1979). Cells (50 g) were defrosted and resuspended in 500 mL TE buffer. Lysis was achieved by addition of 500 mL of 200 mM NaOH, containing 1% w/v SDS. After 5 min incubation, the mixture was neutralized by addition of 500 mL of 3M potassium acetate, pH 5.5. The final reaction volume was typically 1.5 L. Gentle mixing was achieved by inversion of the reaction vessel following addition of reagents. Removal of the floc and partial clarification of the lysate was performed by gravity-driven filtration through eight layers of Miracloth (Calbiochem, La Jolla, CA) followed by further filtration through a single sheet of qualitative no.1 filter paper (Pore-size 110 µm; Whatman, Maidstone, Kent, UK). Typically, 1.2-L lysate were recovered. When required, lysates were stored at −20°C for up to 3 months before use.

Tangential Flow Filtration-Adsorption

Nitrocellulose membranes of 0.45-µm pore-size (Whatman) were fitted into a custom-built tangential flow filtration rig (Fig. 1A), designed to house commercially available circular membranes. The total membrane area available for filtration was 12.5 cm². The diameter of the
inlet and outlet ports was 4 mm while the height of the channel above the membrane surface was 1 mm (Fig. 1B). The retentate was circulated around the rig using a precalibrated peristaltic pump (Watson Marlow, Falmouth, Cornwall, UK) at flow rates of 1.0, 1.3, 2, and 3.8 cm$^3$/s$^{-1}$ (the corresponding linear velocities in the connecting tubing were $5.1 \times 10^{-2}$, $6.8 \times 10^{-2}$, $1.0 \times 10^{-1}$ and $2.0 \times 10^{-1}$ m s$^{-1}$, respectively). All the connecting tubing was 5 mm I.D. (8 mm O.D.) silicon. Pressures were measured using water-filled U-tube manometers connected to the microfiltration rig with a T-piece at the indicated points (Fig. 1A). The membrane unit was always operated in concentration mode.

**Endotoxin Analysis**

Pyrotell™ LAL reagent [0.03 EU/mL$^{-1}$ sensitivity, where 1 endotoxin unit (EU) can be assumed to correspond to 100 pg endotoxin (Petsch et al., 1998)], reconstitution buffer, and sodalime glass reaction tubes, certified pyrogen free, were obtained from Associates of Cape Cod (Liverpool, UK). Pyrotell was reconstituted as recommended by the manufacturer. The sensitivity of the Pyrotell was confirmed by testing a range of serial dilutions of sample endotoxin. A range of dilutions of the selected samples was prepared in triplicate using endotoxin-free water. The result was considered to be negative (endotoxin levels less than 0.03 EU/mL$^{-1}$ in the...

**Analytical Techniques**

**Turbidimetric Measurements**

The concentration of solids in the feed and permeate fractions was determined by turbidimetric measurements at 600 nm using a spectrophotometer (Beckman Instruments, Bucks, UK).

**Agarose Gel Electrophoresis**

Feed and permeate fractions were precipitated with 0.7 V of isopropanol and washed with 70% v/v ethanol. Samples were analyzed by 0.6-0.8% agarose gels as previously described (Levy et al., 2000b; 2000c).

**Chromosomal DNA Analysis**

Samples were precipitated using 0.7 V of isopropanol before being further purified and concentrated using the QiaSpin mini prep kit (QiaGen, West Sussex, UK), total DNA content was determined spectrophotometrically by absorbance at 260 nm. The yield from the QiaSpin columns was determined as 85% w/w through comparison of the isopropanol precipitated and QiaSpin purified samples on an agarose gel. 580 ng total DNA of each sample was blotted onto a charged nylon membrane (Boehringer-Mannheim, East Sussex, UK) following the protocols recommended by the manufacturer. A DIG-labeled probe (Boehringer-Mannheim) specific for *E. coli* chromosomal DNA was prepared and used as described previously (Levy et al., 2000b). Under the conditions used the level of detection was 5.5 ng.

**Protein Analysis**

Protein assays were performed using the BioRad DC assay kit (Hemel Hempstead, Herts, UK). A calibration curve was made with BSA standards (Sigma). The maximum coefficient of variance of this assay was 10%. All samples were assayed in duplicate. Under the conditions used, the level of detection of this assay was 0.1 mg/mL$^{-1}$ protein.
dilution) if no gel had formed, or if the gel collapsed upon inversion. This assay has an accuracy of $+0.03$ EU/mL or $-0.015$ EU/mL.

**RESULTS**

**Processing of Model ssDNA and Protein Solutions**

A series of preliminary tangential flow filtration–adsorption experiments were performed using model feed solutions of ssDNA and proteins using a small, purpose-built membrane rig (Fig. 1). Experiments were performed at four retentate recirculation flow rates between 1.0 and 3.8 cm$^3$/s. Corresponding linear velocities across the membrane were between 0.06 and 0.25 m/s at the inlet and outlet to the membrane and between 0.025 and 0.09 m/s midway along the circular membrane. The corresponding transmembrane pressure drops were between 200 and 2000 Pa (permeate outlet at atmospheric pressure). The membrane unit was operated in all cases below the critical transmembrane pressure.

As shown in Figure 2A, between 85–95% w/w of the ssDNA content of the feed was initially adsorbed during passage through the membrane for all the recirculation flow rates tested. Between 44 and 68 mL of permeate was collected before breakthrough was seen (in this work breakthrough is arbitrarily defined as the point at which chromosomal DNA concentration of the permeate is $>25\%$ w/w of that of the feed). From the data shown in Figure 2B, an average capacity of the nitrocellulose membrane for the adsorption of ssDNA of $45 \pm 7$ μg/cm$^2$ was calculated for all four flow rates. The characteristic profiles of the curves indicate that maximum clearance occurs only after the first fraction has permeated the membrane, indicating that early fouling of the membrane may aid the separation process. At the slower retentate recirculation rates a greater processing time was required before breakthrough was observed due to the lower permeate flux. However, the data could be normalized against the total amount of ssDNA adsorbed by the membrane in each case.

As expected, permeate flux rates declined over the time course of the filtration runs (Fig. 2C) due to adsorption of ssDNA resulting most probably in blockage of the membrane pores and formation of a gel layer on the surface of the membrane. The decline in permeate flux was more rapid at the faster retentate recirculation rates. This may be related to the higher permeate flux accelerating saturation of the membrane and pore blockage. Also, the higher pressures measured at the higher flow rates will result in greater compaction of any gel layer on the membrane surface. The final permeate flux was between 125 and 20 L · m$^{-2}$ · h$^{-1}$ over the four retentate recirculation rates, being reduced to around 20% of the original. For operational convenience, a retentate recirculation rate of 1.3 cm$^3$/s$^{-1}$ was chosen for all subsequent experiments.

For comparison, a pure BSA solution (1.5 mg/mL) in 10x SSC buffer was also filtered in the tangential flow rig to investigate protein adsorption. Protein transmission was $=50\%$ w/w during the first 30 s of the filtration–adsorption process and increased to a constant value of $80\%$ w/w subsequently. The initial permeate flux was comparable to that observed for the ssDNA solution at the same retentate recirculation rate and followed a similar pattern for the flux decline. Permeate flux dropped from 160 to 100 L · m$^{-2}$ · h$^{-1}$ over 8 min, indicating the adsorption of the protein within the membrane pores combined with the formation of a gel layer may be responsible for the retardation of the permeate flux as with the ssDNA feed stream.

![Figure 3. Agarose gel analysis of feed and permeate fractions from a tangential flow filtration–adsorption run.](image-url)
Processing of *Escherichia coli* Lysates Containing 6.9 kb or 20 kb Plasmids

To evaluate the performance of the membrane filtration-adsorption operation under real process conditions, *E. coli* lysates (containing a 6.9 kb plasmid) were processed at a retentate flow rate of 1.3 cm$^2$/s. Either 2 mL or 1 mL fractions of permeate were collected (permeate fractions of 1 mL were subsequently pooled with corresponding fractions from identical experiments to obtain enough sample for subsequent analysis). The feed and permeate fractions were then analyzed by agarose gel electrophoresis.

A typical agarose gel is shown in Figure 3. A substantial decrease in chromosomal DNA content was observed in the first three (2 mL) permeate fractions with breakthrough of the chromosomal DNA just being evident in the fourth. Densitometric scanning of the gel indicated that SC plasmid transmission was approximately 100% w/w. A decrease in RNA content was also observed in the first permeate fraction (Fig. 3) indicating that RNA is also adsorbed by the membrane at early stages of the filtration-adsorption process. The similarity of the agarose gel profiles obtained for the seven independent experiments performed (data not shown) demonstrated the reproducibility of the operation.

To further estimate the removal of chromosomal DNA, feed and 1-mL permeate fractions corresponding to five independent filtration-adsorption experiments were subjected to Southern slot-blot analysis using a probe specific for *E. coli* chromosomal DNA. Pure DH5α DNA was loaded to generate a standard curve that represents contamination levels from 0.5 to 15%. A typical slot-blot result is shown in Figure 4. Quantitative data obtained from densitometric scanning of the blots was plotted against process time and is shown in Figure 5A. The feed had a chromosomal DNA content of 9 pg/mL with respect to total DNA, which decreased to 8% w/w in the first collected fraction and further decreased to < 1% w/w (below the level of detection) in the following fraction; this observation will be discussed further in the following section. Typically, the feed was estimated to have a chromosomal DNA content of $\approx 9$ pg/mL$^{-1}$. Therefore under the conditions used, the operational capacity of the nitrocellulose membrane to absorb chromosomal DNA was calculated as $3.1 \pm 0.4$ pg/cm$^{-2}$ (data from four replicate experiments). Also plotted in Figure 5A is the concentration of plasmid DNA in the permeate obtained from scanning of the agarose gel which shows complete recovery of the desired plasmid product. A fivefold decrease in protein content was initially recorded (Fig. 5B) which then gradually increased to give a 20% reduction indicating significant protein transmission. The solids content of the filtrate fractions was reduced by > 75% (Fig. 5B). Endotoxin levels were assayed for one filtration-adsorption experiment, and were found to be reduced from $4.8 \times 10^3$ EU/mL$^{-1}$ in the feed to 48 EU/mL$^{-1}$ in the first 2 mL permeate collected indicating a 100-fold decrease in endotoxin levels.
Finally, a tangential flow filtration-adsorption experiment using an E. coli lysate containing a 20 kb plasmid was also performed again at a retentate recirculation rate of 1.3 cm/s. Agarose gel profiles were similar to those obtained in previous experiments (data not shown). A substantial decrease in chromosomal DNA content was again observed in the first three permeate fractions breakthrough being evident in the fourth. Densitometric scanning of the gel indicated that plasmid transmission was again 100% w/w with no evidence of shear damage occurring during the operation.

**DISCUSSION AND CONCLUSIONS**

We describe in this study the use of tangential flow filtration-adsorption as an integrated operation to clarify and decrease contaminant levels in plasmid DNA containing process streams. Over the range of flow rates and transmembrane pressures investigated, chromosomal DNA contamination could be decreased from around 10% w/w to an average of 2.5% w/w in fractions collected prior to breakthrough during passage through the nitrocellulose membrane. As expected due to their hydrophobic characteristics (Bressler et al., 1983; Liu et al., 1996; Petch and Ansprach, 2000), adsorption of other contaminant species, such as RNA, proteins and endotoxin was also seen at an early stage of the filtration-adsorption process. Breakthrough for these, however, occurred earlier than for chromosomal DNA. The adsorption of contaminants on the membrane probably resulted in the formation of a gel-fouling layer, which reduced the permeate flux. Southern blot analysis showed that chromosomal DNA levels of the first permeate fraction, although 25% lower than the feed, were which occurred in each experiment, may aid the adsorption process by slowing the permeate flux, or through partial blocking of the membrane pores. Plasmid DNA, which regains the more hydrophilic double stranded conformation following the alkaline lysis step, passes through the membrane with close to 100% w/w transmission.

The reproducibility and potential for scale-up of the operation described here makes it an attractive option for pilot and manufacturing scales. Clearly, the design of the membrane housing would be changed to a more standard configuration at larger scales thus allowing a better characterization of the hydrodynamic features of the module. The improved control over both transmembrane pressure and liquid shear rate at the membrane surface should also enable the rapid initial rate of flux decline to be reduced. The operational capacity of the membranes for chromosomal DNA adsorption from clarified lysates was modest (3.1 μg/cm²), Figs. 4-6, around 10% of the capacity calculated for model systems using pure ssDNA (Fig. 2). This result suggests that one option to overcome this capacity limitation could be to place the operation further downstream in the process. Alternatively, other types of membranes with stronger hydrophobic characteristics may provide attractive options to retain contaminants at an early stage of the process. The high hydrodynamic diameter of plasmid DNA molecules significantly limits the capacity of conventional chromatography matrixes in comparison to smaller proteins due to diffusion limitations. Thus, every reduction in closely related contaminants made at an early stage of the process is particularly desirable. This is critical in the case of chromosomal DNA, which due to chemical similarities with plasmid DNA, competes for binding sites in high-resolution chromatographic matrixes.

UCL is the EPSRC Innovative Manufacturing Research Centre (IMRC) for Bioprocessing. The council’s support is gratefully acknowledged. DK would also like to thank BBSRC for the provision of a studentship and GJL would like to thank Esso and the Royal Academy of Engineering for the award of an Engineering Fellowship. We would also like to thank Jon Postlethwaite for the design and construction of the cross-flow filtration device and Ronan O’Kennedy for fermentation products.

**References**


Separation of supercoiled and open-circular plasmid DNA by liquid-liquid counter-current chromatography

D. Kendall, A.J. Booth, M.S. Levy & G.J. Lye*
The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, London WC1E 7JE, UK
*Author for correspondence (Fax: +44 (0)20 7209 0703; E-mail: g.lye@ucl.ac.uk)

Received 8 February 2001; Accepted 16 February 2001

Abstract

We report for the first time the use of liquid-liquid counter-current chromatography (CCC) for the preparative scale fractionation of plasmid DNA. Almost complete fractionation of supercoiled and open circular plasmid DNA (6.9 kb) could be achieved using a phase system comprising 12.5% (w/w) PEG 600 and 18% (w/w) K$_2$HPO$_4$. Experiments were carried out on a Brunel J-type CCC machine (100 ml PTFE coil) at a mobile phase flow rate of 0.5 ml min$^{-1}$ and a rotational speed of 600 rpm. Compared to conventional HPLC techniques the capacity of CCC is not limited by the surface area of resin available for adsorption.

Symbols: $C_b$, Concentration of plasmid in lower phase ($\mu$g ml$^{-1}$); $C_t$, Concentration of plasmid in upper phase ($\mu$g ml$^{-1}$); $C_V$, Total volume of mobile phase present in the coil and connecting leads (ml); $K$, Equilibrium solute partition coefficient ($K = C_t/C_b$); $C$ OC, Open circular plasmid; SC, Supercoiled plasmid; $S_f$, Percentage stationary phase retention ($S_f = V_s/V_t$); $t_s$, Time for phase separation (s); $V_b$, Volume of bottom phase (ml); $V_c$, Coil volume (ml); $V_m$, Volume of mobile phase present in coil at equilibrium (ml); $V_r$, Volume ratio of two phases ($V_r = V_t/V_b$); $V_s$, Volume stationary phase present in coil at equilibrium (ml); $V_t$, Volume of top phase (ml); $V_{tot}$, Total volume of phase system (ml).

Introduction

At the present time there is considerable interest in the use of DNA products as vaccines and for gene therapy applications (Mountain 2000, Robinson et al. 1997). Consequently it is desirable to develop readily scaleable and economic processes for the purification of such DNA based therapeutics (Durland & Eastman 1998, Levy et al. 2000b, Marquet et al. 1995, Prazeres et al. 1999). A variety of methods are also under development for the delivery of DNA to the target tissue.

Non-viral vectors that employ bacterial plasmids to carry the therapeutic gene are gaining favour due to the safety concerns associated with viral vectors (Marquet et al. 1995; Prazeres et al. 1999). It is also clear that the regulatory authorities will set stringent standards for the reduction of levels of contaminants such as host chromosomal DNA, proteins and lipopolysaccharides, and for the transfection efficiency of such vectors. The majority of plasmid DNA isolated from the host cell will be in the supercoiled (SC) form. However during industrial scale processing, chemical and shear degradation may convert the SC form to the open circular (OC) and linear forms (Levy et al. 1999). It is desirable to remove OC forms of the plasmid from the final product to ensure consistent potency and stability of the final dosage form (Bergan et al. 2000, Bonilla et al. 1991, Middaugh et al. 1998).

Several processes have been reported for the production and high resolution separation of plasmid DNA. However these often rely on operations which are likely to be difficult to scale up (Horn et al. 1995, Levy et al. 2000b, Li & Huang 2000, Murphy 1999, Schleef 1999, Wils et al. 1997). Ion-pairing reversed-phase HPLC is efficient for the purification of SC plasmid DNA from host cell contaminants, including OC forms of the plasmid (Green et al. 1997). How-
ever, the operation of an HPLC column at industrial
scale is both expensive and hazardous, due to the low
capacities of the available adsorbents (Green et al.
1997) and the large quantities of organic solvents re­
quired. Aqueous two-phase systems (ATPS) have long
been known as a tool for the purification of DNA from
other cellular components (Albertsson 1965, Favre &
1985) and would overcome the need for organic
solvents. Counter-current extraction techniques can be
used to improve the efficiency of such liquid-liquid
separation processes. However the number of equi­
librium stages is generally limited to between 5 and
10 due to the cost and complexity of the centrifugal
extraction devices usually employed (Diamond & Hsu

Counter-current chromatography (CCC) is an
emerging low pressure chromatographic technique,
which separates molecules on the basis of different
partition coefficients between two immiscible liquid
phases (Conway 1990, Ito & Conway 1996). Within
the CCC column, one liquid phase (the stationary
phase) is held in place by centrifugal forces created
as a result of spinning a spirally wound coil, i.e.,
the CCC column. A second liquid phase (the mo­
bile phase) is then continuously pumped through the
column and is subjected to multiple stages of phase
mixing (during which solute mass transfer occurs) and
settling with the stationary phase. Those components
having a higher affinity for the mobile phase are eluted
first. The alternate stages of mixing and separation
are a result of the cardiodial path that the coil fol­

Materials and methods

Plasmid and culture conditions

The plasmid used in the present study was pSVβ
(Promega Corp., MA, USA), 6.9 kb. Plasmids were
transformed and propagated in E. coli DH5α (Gibco­
Life Technologies, MD, USA) grown on LB media
(Sambrook et al. 1989). Bacteria were grown in 7.5 l
and 450 l bioreactors as described previously (Levy
et al. 2000a, Noites et al. 1999). Bacterial cells were
harvested using a Carr Powerfuge (Carr separations
Inc., Franklin, MA, USA). The resulting cell paste was
stored initially at −20 °C and then at −70 °C.

Alkaline lysis of E. coli cells, lysate clarification and
concentration

Cells were lysed using a modified alkaline lysis pro­
cedure (Birnboim and Doly 1979). Cells (50 g) were
defrosted and resuspended in 500 ml TE buffer (1 mM
Tris-HCl, pH 8; 5 mM EDTA) containing RNase A
(Qiagen, West Sussex, UK) at 0.1 μg ml⁻¹. Lysis
was achieved by addition of 500 ml 200 mM NaOH,
containing 1% (w/v) SDS. After 5 min incubation,
the mixture was neutralized by addition of 500 ml
3 M potassium acetate pH 5.5. The final reaction vol­
ume was typically 1.5 l. Gentle mixing was achieved
by inversion of the reaction vessel following addition
of reagents. Removal of the floc and clarification of
the lysate was performed by gravity-driven filtration
through 8 layers of muslin cloth followed by further
filtration through a single sheet of qualitative No. 1 fil­
ter paper (Whatman, Maidstone, Kent, UK). Typically
1.2 l lysate was recovered. Finally to further purify
the plasmid preparation prior to CCC, 200 ml lysate
was precipitated using 0.7 volumes 2-propanol and the
resulting pellet resuspended in 5 ml TE buffer. The
final concentration of plasmid was determined by the
picogreen assay to be 1 mg ml⁻¹.
Composition and separation of aqueous two-phase systems

Polyethylene glycol (PEG) of average molecular weight 600 or 1000 was obtained from Sigma Aldrich chemical company (Poole, Dorset, UK). Anhydrous K_2HPO_4 was obtained from Fisher Scientific (Loughborough, Leics, UK). Stock solutions of 40% (w/w) PEG 1000 or PEG 600, and 40% (w/w) K_2HPO_4, were prepared and used immediately to make up the required phase systems. Prior to use, phase systems were mixed for 30 min and left to equilibrate overnight. Initial screening of effective phase systems for CCC application was based on measuring the time required after phase mixing (performed by inverting the test tube 5 times) for two distinct phases to form. Two phase systems were eventually chosen for use in the CCC experiments: 16.2% (w/w) PEG 1000, 18% (w/w) K_2HPO_4 and 12.5% (w/w) PEG 600, 18% (w/w) K_2HPO_4. The cloud points for several ratios of PEG 1000 or PEG 600/K_2HPO_4 were determined and the data used to construct binodal phase diagrams (Walter et al. 1985).

Equilibrium phase partitioning

2.25 g of a wide range of phase systems were initially made up in capped test tubes and 0.25 g lysate was added. The phases were then mixed by inversion for 10 min, to allow mass transfer to occur, allowed to settle and the upper and lower phase volumes were measured. The equilibrium partition coefficients (K = C_t/C_b) of the various nucleic acids were estimated by measuring the transmittance of the DNA bands after gel electrophoresis.

CCC instrumentation and operation

The CCC machine used was a Brunel Labprep, ‘J’ type design (BIB, Rhomulus Technologies, Uxbridge, UK). Full details of the design and operation can be found elsewhere (Booth & Lye 2001). The column used was made from 3.2 mm outer diam., 1.6 mm internal diam. PTFE tubing spirally wound onto the bobbin from the center to the periphery in an anti-clockwise manner. The column had a total volume of 94.3 ml and β values between 0.83–0.86 (Conway 1990).

The CCC machine was operated in reverse-phase mode, i.e., with the PEG as the chosen stationary phase. The upper PEG phase was first pumped into the column at 6 ml min^{-1} in a ‘head’ to ‘tail’ direction. Once filled with stationary phase, rotation of the bobbins was started in the ‘reverse’ (clockwise) direction. When a rotational speed of 600 or 800 rpm had been reached the lower (salt) mobile phase was pumped isocratically through the column at a flow rate of 0.5 ml min^{-1}, again in the ‘head’ to ‘tail’ direction. Eluted stationary phase was collected in a graduated measuring cylinder and used to calculate the proportion (by volume) of stationary phase retained (S_f) once hydrodynamic equilibration of the phases had been achieved (taking into account the volume of the inlet and outlet leads).

The plasmid preparation was then dissolved (1:10 v/v) in the mobile phase and 1 ml injected onto the CCC column. The eluent from the column was collected at 5-min intervals for further off-line analysis. The full range of CCC experiments performed is summarized in Table 1. Upon completion of each chromatographic run, the contents of the coil was pumped out using compressed nitrogen gas in the head to tail direction. Fractions of the eluted stationary and mobile phases were again collected for further off-line analysis.

Analytical techniques

Feed and eluted fractions of the mobile phase were purified using the Qiaspin mini prep kit (Qiagen, West Sussex, UK), the final volume of purified sample being equal to that loaded. For quantification of DNA concentration it was assumed that the yield from the Qiaspin columns was 100% (w/w). Samples were loaded onto 0.8% (w/v) agarose gels containing 0.05 μg ml^{-1} ethidium bromide and electrophoresed for 2 h in Tris/borate electrophoresis buffer (Sambrook et al. 1989). Where necessary gels were scanned and analysed using UVP 5000 Gel Documentation System and Gelbase analysis software (Ultra Violet Products Ltd., Cambridge, UK). The coefficient of variance for the gel scanning technique for quantitative analysis is ±10%. The concentration of dsDNA was determined using the picogreen assay (Cambridge Bioscience, Cambridge, UK) as described previously (Levy 2000).
Table 1. Plasmid partition coefficients and hydrodynamic properties for selected aqueous two-phase systems.

<table>
<thead>
<tr>
<th>Phase system</th>
<th>VTs</th>
<th>Ts (s)</th>
<th>K SC</th>
<th>K OC</th>
<th>Bobbin rotation (rpm)</th>
<th>Sf</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.2% PEG 1000</td>
<td>0.7</td>
<td>60</td>
<td>0.12</td>
<td>0.02</td>
<td>800</td>
<td>56.4</td>
</tr>
<tr>
<td>18% K2HPO4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5% PEG 600</td>
<td>0.6</td>
<td>50</td>
<td>0.34</td>
<td>0.01</td>
<td>800</td>
<td>50.0</td>
</tr>
<tr>
<td>18% K2HPO4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results

ATPS selection: plasmid partitioning and phase separation

The partitioning of plasmid DNA in a wide range of ATPS was initially examined. For efficient CCC fractionation equilibrium solute partition coefficients of between 0.1–5 are usually desirable (Ito & Conway 1996). It is also generally desirable to use phase systems close to the binodal curve on the phase diagram (Walter et al. 1985). However, given the high level of liquid-liquid mixing that can be achieved by the J-type CCC machine, a low settling time (preferably <60 s and ideally <20 s) was considered to be of paramount importance for this particular application. Ultimately it was necessary to compromise and choose phase systems located further from the origin of the phase diagram (Figure 1), which had the desired phase separation kinetics. The two systems finally chosen from the initial screening experiments consisted of 16.2% (w/w) PEG 1000, 18% (w/w) K2HPO4 and 12.5% (w/w) PEG 600, 18% (w/w) K2HPO4 (hereafter referred to as PEG 1000 and PEG 600 systems respectively). The phase separation times and equilibrium partition coefficients of SC and OC plasmid DNA in these two systems are given in Table 1.

Stationary phase retention

Stationary phase retention studies using the chosen PEG 1000 and PEG 600 phase systems in the CCC machine were subsequently carried out. A high degree of stationary phase retention (high Sf values) is normally required for efficient chromatography (Ito & Conway 1996) with values up to 95% being achievable in J-type machines using aqueous-organic two-phase systems (Ito & Conway 1996). As shown in Table 1, however, the Sf values obtained when using ATPS were considerably lower, being between 33.2–56.4% depending on the phase system used and the speed of rotation. For the PEG 600 phase system, some stripping of the stationary phase was observed (i.e., small droplets of the PEG rich stationary phase were present in the eluted mobile phase) during subsequent operation of the CCC machine at 600 rpm.

CCC fractionation of plasmid DNA

Despite the low Sf values obtained for the two selected ATPS, chromatographic runs in the CCC machine were still performed. Initially, gel electrophoresis of eluted mobile phase fractions obtained during a run using the PEG 1000 system (800 rpm) of 3.5 h duration revealed that no DNA had eluted from the column. Rather, the bulk of the plasmid DNA was found to have been retained within the column when
Fig. 2. Agarose gel analysis of feed and selected fractions collected during CCC carried out using a phase system comprised of 12.5% (w/w) PEG 600 (stationary phase) and 18.5% (w/w) K$_2$HPO$_4$ (mobile phase) at 600 rpm and a mobile phase flow rate of 0.5 ml/min$^{-1}$. Lane 1: feed (0.02 dilution of original). Lanes 2 and 3: fractions eluted during chromatography after 1.23 and 1.29 column volumes respectively. Lanes 4 and 5: fractions collected during pump out of the coil at 2.45 and 2.48 column volumes respectively (88% and 98% of the column cleared).×2 concentration of the original).

![Fig. 2](image)

the coil was emptied. In particular SC plasmid was observed in the latest fractions collected (corresponding to positions close to the point of sample injection near the head of the column) while OC plasmid had moved a little distance toward the tail (results not shown). It was hypothesized that either the high viscosity of the stationary phase ($\sim 10^3$ cP), coupled with the large size of the plasmid DNA molecules, had restricted mass transfer of the plasmid back into the mobile phase, or that the SC DNA had become associated with the large interfacial area generated between the two phases during phase mixing at high rpm, both of which would result in increased retention.

Subsequently, in order to decrease the degree of mixing of the two phases present in the coil, the speed of revolution of the bobbin was decreased to 600 rpm. A PEG 600 system of lower viscosity was also used in a 3.5-h run. Gel electrophoresis of the eluted fractions (Figure 2, lanes 2 and 3) indicated that the majority of the OC plasmid eluted in a peak after 1.1 and 1.29 column volumes. A small amount of SC plasmid was visible in the fractions between 1.2 and 1.29 column volumes. Analysis of the phases recovered following pumping out of the coil revealed that the majority of SC plasmid DNA had again been retained, but had partitioned back into the mobile phase during the pumping out process. The SC plasmid was present in a peak 88%-98% along the length of the column (Figure 2, lanes 4 and 5). Chromosomal DNA was clearly visible in the stationary PEG phase near the point of sample injection. Figure 3 shows a CCC chromatogram constructed using results from the picogreen assay for both the eluted mobile phase and the material recovered following pumping out of the column. The proportions of SC and OC plasmid were determined by scanning of the agarose gels. The chromatogram shows a good separation between the various plasmid forms. Comparable results obtained following a second CCC run under identical conditions indicated that an efficient and reproducible separation of OC, SC and chromosomal DNA had been achieved.

![Fig. 3](image)

**Discussion**

CCC using ATPS has been shown to be an efficient method for the fractionation of various DNA species. Analysis by agarose gel electrophoresis indicates that levels of chromosomal DNA and OC plasmid have been significantly reduced in the main SC plasmid fractions (2.45-2.48 CV). Further investigation is required to determine the absolute levels of purity achieved and also the degree of endotoxin removal. Approximately 76% (w/w) of the SC form
of the plasmid was recovered in the main SC peak, while the overall recovery of SC plasmid was ~90% (w/w). In this work the amount of sample loaded was small (0.1 mg plasmid onto a 94.6 ml coil) hence further investigation is required to determine the maximum loadings possible. Other studies carried out in our laboratory indicated that loadings of up to 10 mg solute ml\(^{-1}\) mobile phase are possible (Booth & Lye 2001). This compares very favourably to 0.04 mg SC plasmid ml\(^{-1}\) solid stationary phase loaded onto a reversed phase HPLC column (Green et al. 1997), and 0.4 mg plasmid ml\(^{-1}\) purified using a 10 ml Mustang Q anion exchange membrane adsorber (Nochumson et al. 2000). Throughputs of 25–50 tonnes per year have been predicted for industrial scale CCC machines (Sutherland et al. 1998) and furthermore the operation of CCC at industrial scale is likely to be considerably less costly than industrial scale HPLC (Sutherland et al. 1998).

Interestingly, some of the DNA species are retained within the column even though K values determined from batch partition experiments (Table 1) suggested both forms of the plasmid should have eluted. The high viscosity of the stationary (PEG) phase, coupled with the large dynamic volume of the DNA molecule (Ito et al. 1998) may have the effect of reducing the rate of DNA mass transfer out of the stationary phase during chromatography. Plasmid DNA has also been observed to accumulate at the interface in aqueous two-phase systems (Ribeiro et al. 2000), thus the large interfacial area present within the coil, due to the vigorous mixing action of the J-type machine, could also result in the retention of the plasmid DNA. Reducing the speed of revolution of the bobbin (from 800–600 rpm) had the effect of reducing the degree of mixing in the coil such that SC plasmid species could be eluted from the column. SC plasmid, however, could still only be recovered at the end of the chromatographic run, having partitioned back into the mobile phase once the phases had settled. That the SC plasmid does not elute may be due to a number of factors such as the morphology or surface activity of the plasmid. Ultimately the SC plasmid is the form required for therapeutic applications and could easily be recovered from the mobile phase by diafiltration.

While we have shown the potential of CCC for the fractionation of DNA species, the type of CCC machine used in this study severely limited the choice of phase system that could be used. The degree of stationary phase retention achieved at the low rotation speeds required for elution of the OC plasmid was much lower than is normally desirable. The suitability of cross-axis (Shibusawa et al. 2001, Shinomiya et al. 1993) and toroidal coil (Ito et al. 1998) CCC designs for use with aqueous two-phase systems has been demonstrated, which suggests that our DNA separations could be significantly improved if a more suitable design of CCC machine were used.

Conclusions

In this work we have shown that efficient separation of SC, OC and chromosomal DNA can be achieved by CCC using aqueous two-phase systems. The potential loading capacity and throughput of the CCC machine is significantly greater than HPLC techniques currently available. Some of the limitations of the J-type CCC machine used in the present study could readily be overcome by the use of a toroidal coil or cross-axis CCC machine, suggesting further improvements in separation and throughput are possible.

Acknowledgements

University College London (UCL) hosts the Biotechnology and Biological Sciences Research Council (BBSRC) sponsored Advanced Centre for Biochemical Engineering and the council's support is gratefully acknowledged. DK and AJB would also like to thank the BBSRC for provision of research studentships. GJL would like to thank Esso and the Royal Academy of Engineering for the award of an Engineering Fellowship and the Nuffield Foundation for financial support (NUF-NAL).

References


