UPTEC X 04 009
FEB 2004

ISSN 1401-2138

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New chromatographic media for plasmid DNA purification

Master’s degree project

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 04 009	**Date of issue 2004-02**

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Title (English)

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Title (Swedish)

Abstract

The interest in plasmid DNA for therapy and vaccination has rapidly evolved during recent years. Chromatography is the most common large scale purification technique for plasmid DNA. There is, however, currently no good chromatographic media available since most media are optimized for small or medium sized molecules. New media for large molecules such as plasmid DNA are therefore needed. In this report, several new prototype media are evaluated. Specifically how the media's pore and bead size as well as surface extenders affect the purification of plasmid DNA has been investigated. The conclusions from this study are that a good media for plasmid DNA purification should consist of a media where the plasmid DNA can move freely (*i.e.* in or on the media). Suitable media could be either small semisolid particles with extender arms such as dextran or somewhat larger particles with macro-pores.

Keywords

Plasmid DNA, liquid chromatography, chromatographic media, capacity

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Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **36**

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New Chromatographic Media for Plasmid DNA Purification

Karin von Post

Sammanfattning

Genterapi och DNA vacciner är två medicinska områden som kan komma att ha stor betydelse i framtiden. En defekt gen kan göra att ett ämne i kroppen produceras för mycket eller för lite och därmed blir orsak till olika sjukdomar. Med genterapi hoppas man kunna bota många svåra sjukdomar genom att ersätta den defekta genen med en frisk gen. DNA vaccin är en typ av vaccin som anses vara billigare att tillverka samt säkrare att föra in i kroppen än andra nu tillgängliga vacciner. Både genterapi och DNA vaccin bygger på att DNA förs in i kroppens celler vilket kan göras med hjälp av plasmid DNA. Nackdelen med användandet av plasmid DNA är att det kan behövas stora mängder. Det kommer därför att finnas ett behov av en storskalig process för rening till ett relativt lågt pris.

Idag finns det ett flertal metoder för att rena plasmid DNA. Däremot finns det inget bra kromatografiskt processmedia att rena fram det på. I denna studie optimeras ett kromatografiskt reningssteg i en komplex reningsprocess. Optimeringen syftar till att göra det möjligt att fånga upp så mycket plasmid DNA som möjligt på så lite media som möjligt. Resultaten visar att framtidens reningsmaterial skulle kunna bestå av stora mängder små runda semisolida partiklar där plasmid DNA binder på utsidan eller av större partiklar med större porer där plasmid DNA kan gå in i partiklarna. Slutligen kan man tänka sig att mediet har ”fångstarmar” som plasmid DNA:t fastnar på.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet Februari 2004

Table of Abbreviations

A_s	peak asymmetry factor
$AgNO_3$	silver nitrate
bp	base pairs
C	Celsius
CIP	Cleaning-In-Place
Cl^-	chloride ion
cm/h	centimetres per hour
Da	Dalton
D_{eff}	effective diffusion constant
DNA	deoxyribonucleic acid
E. *coli*	escherichia coli
EDTA	ethylene diaminetetraacetic acid
dsDNA	double stranded deoxyribonucleic acid
g	gramme
HCl	hydrochloric acid
HIC	hydrophobic interaction chromatography
HNO_3	nitric acid
HR	High Resolution
IEC	ion exchange chromatography
kbp	kilo base pairs
LPS	lipopolysacharides
M	molar
mAU	milli absorbance units
ml	millilitre
mM	millimolar
NaCl	sodium chloride
NaOH	sodium hydroxide
Na_2SO_4	sodium sulphate
$(NH_4)_2SO_4$	ammonium sulphate
nm	nanometer
NMR	nuclear magnetic resonance
OAc^-	acetate ion
PAOD	peripheral artery occlusive disease
PEG	polyethylene glycol
pDNA	plasmid deoxyribonucleic acid
pH	$-\log a_{H^+}$
Q	quaternary ammonium
Q_{B20}	20 % breakthrough
RNA	ribonucleic acid
RPC	reversed phase chromatography
R_s	chromatographic resolution
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
ssDNA	single stranded deoxyribonucleic acid
TAC	thiophilic aromatic chromatography
THAC	triple helix affinity chromatography
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
µl	microlitre
µm	micrometer
µmol	micromole

1 Introduction

The interest in plasmid DNA for therapy and vaccination has rapidly evolved during recent years. Today there are more than 600 gene therapy clinical trials completed or under progress (Homepage: Gene therapy clinical trials). A robust and safe strategy for plasmid DNA production and purification is therefore needed to enhance further commercial development (Werner *et al.*, 2002).

1.1 Gene Therapy

Gene therapy is the process of introducing nucleic acids into human cells in order to change their genetic repertoire for therapeutic purposes. The nucleic acid most commonly used is double-stranded DNA (dsDNA), which replaces the defective genes in the diseased tissues. Other nucleic acids also used are antisense RNA and single-stranded DNA (ssDNA) binding to targeted sequences in the host cells and thereby inhibiting a gene's expression (Ferreria *et al.*, 2000a; Ferreria *et al.*, 2001; Eon-Duval, 2003). Cancer is the most common target disease (63.4%) for the gene therapy protocols that have reached clinical trials today (Homepage: Gene therapy clinical trials).

The vector, which transfers the nucleic acids into the human cell can either be viral or nonviral. Examples of viral vectors are adenovirus, retrovirus and herpes simplex and an example of a nonviral vector is plasmid DNA. Viral vectors are more efficient than nonviral vectors, but they have higher toxicity and immunogenicity which have led to regulatory and safety concerns (Eon-Duval, 2003). The viral vectors also raise concerns about the possible activation or deactivation of oncogens and tumour repressor genes (Ferreria *et al.*, 2001). Nonviral vectors such as plasmid DNA have the advantage of being easier and cheaper to manufacture (Eon-Duval, 2003).

1.2 DNA Vaccination

The plasmid DNA expresses, when used as DNA vaccine, an antigen on the cell membrane's surface and thereby stimulates and enhances the immune response and memory (Ferreira *et al.*, 2000a). DNA vaccination was first described in 1992 and is now used in the control of infectious diseases (such as HIV and malaria), immune therapy for cancer, specific treatment for autoimmune disease and immunomodulation for allergic diseases (Reimann *et al.*, 2001).

The main advantages of DNA vaccination compared to other types of vaccination (for example weakened bacteria or virus, modified exotoxins or modified attenuated viral vaccines) are that the large scale production of plasmid DNA is more cost-effective and has less demanding storage conditions as well as being generally regarded as safer (Ferreira *et al.*, 2000a; Reimann *et al.*, 2001; Benjamini *et al.*, 2000). The main disadvantage with DNA vaccine is, however, that it is less effective than its viral counterparts and it is estimated that milligram amounts may be needed for full treatment (Ferreira *et al.*, 2000a). Doses with amounts of up to 16 mg has been tested on human patients with sever peripheral artery occlusive disease (PAOD) without complications in a Phase 1 clinical trial (Meyer, 2003).

1.3 Plasmid DNA

Plasmid DNA is an extrachromosomal, double stranded and circular nucleic acid. Naturally occurring plasmids encode for a variety of functions that are not essential for the survival of the host, but gives them higher adaptability to atypical environments (Schmidt *et al.*, 2001).

The size of a naturally occurring plasmid in eucaryotes and procaryotes varies from 200-100,000 bp whereas engineered plasmid vectors have a typical size of less than 10 kbp. Plasmids replicate independently of the cell and have different copy numbers in different types of cells and environments (Schmidt *et al.*, 2001). The plasmid DNA has an overall negative net charge equal to the number of bases in the molecule. This is due to the negatively charged phosphate groups on the nucleic acid backbone of the plasmid, which contributes with one negative charge per base at pH>4 (Ferreira *et al.*, 2001).

Supercoiled and open circular are the two main topological structures of circular plasmid DNA (see Figure 1). Supercoiled plasmid DNA is covalently closed circular DNA, which can be either negatively or positively supercoiled. Nicking (breaking) one of the DNA strands of the supercoiled plasmid DNA could occur due to nuclease activity or mechanical stress and results in relaxation of the supercoiled DNA and thereby forms open circular plasmid DNA. The most common form (by far) of plasmid DNA isolated from bacterial cells is the negatively supercoiled form (Schmidt *et al.*, 2001). The supercoiled form of plasmid DNA is also the one with the highest transfection rate during gene delivery. Because of this other forms of plasmid DNA should be kept at a minimum (Werner *et al.*, 2002). The regulatory agencies have recommended that at least 95 % of the plasmid DNA is in its circular form (open circular or supercoiled) for therapeutic cancer clinical trials (Levy *et al.*, 2000). It should be pointed out that the regulatory agencies have set their recommendations for impurities and contamination before any pharmaceuticals have reached the market and that these requirements will therefore most likely be stricter after the first gene therapy or vaccine is approved for launch.

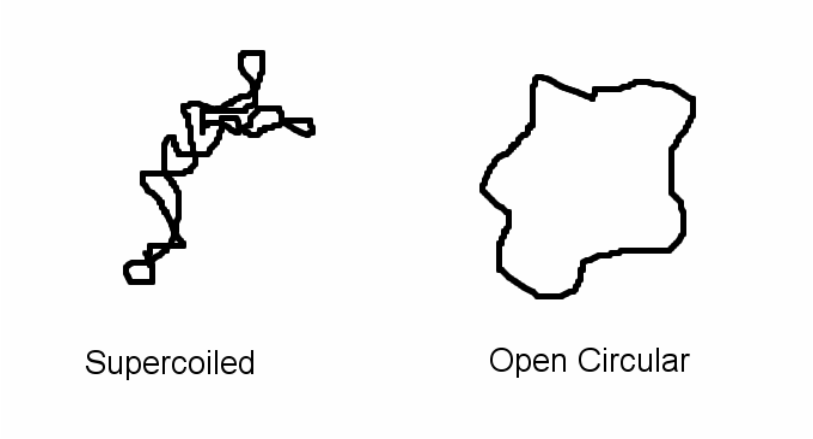


Figure 1. *Schematic representation of supercoiled and open circular plasmid DNA.*

1.4 Plasmid DNA Manufacturing

The manufacturing of plasmid DNA can be divided into two steps: upstream processing and downstream processing (Ferreira *et al.,* 2001). Figure 2 (page 9) shows an example of a flow sheet for plasmid DNA production and the elimination of different impurities.

1.4.1 Upstream Processing

The manufacturing process of plasmid DNA starts with the construction and selection of an appropriate expression vector and host organism, followed by production of the microorganism strains. The preferred plasmid has a high copy number, several endonuclease cleavage sites, small size, genetic stability and specific selection markers (Ferreira *et al.*, 2001). The next step after the plasmid has been introduced into the host organism is the fermentation process where the host organism is cultivated to produce large amounts of plasmid DNA. It is of importance for the use of plasmid DNA in clinical trials that the fermentation process is robust, reproducible, scalable and produces a large amount of material in a short period of time (Werner *et al.*, 2002). The fermentation step previously focused on the production of recombinant proteins, a new science around the production of plasmid DNA is now developing (Schmidt, 2003).

1.4.2 Downstream Processing

The downstream processing follows the upstream processing and includes a series of unit operations. The aim of every unit operation is to either eliminate the impurities or to concentrate the plasmid DNA solution in order to produce a final plasmid DNA preparation, which complies with the approval specifications of the regulatory authorities. The downstream process is greatly affected by the impurities and contaminants present due to the upstream processes and fermentation conditions (Ferreira *et al.*, 2001; Ferreira *et al.*, 2000a).

It is of great importance that the final plasmid preparation has the right quality in terms of safety, potency and purity because of the intended pharmaceutical use. Guidelines for the allowed content in the final plasmid preparation are provided by regulatory agencies such as the Word Health Organization (WHO) and the United States Food and Drug Administration (USFDA). Three impurities, which need to be removed during the downstream process are host nucleic acids (genomic DNA and RNA), proteins and endotoxins. Endotoxins are the most common pyrogen (fever causing substance) and are highly charged lipopolysacharides (LPS) (Ferreira *et al.*, 2001). See Figure 2 for examples of where in the plasmid DNA production impurities and contamination are removed.

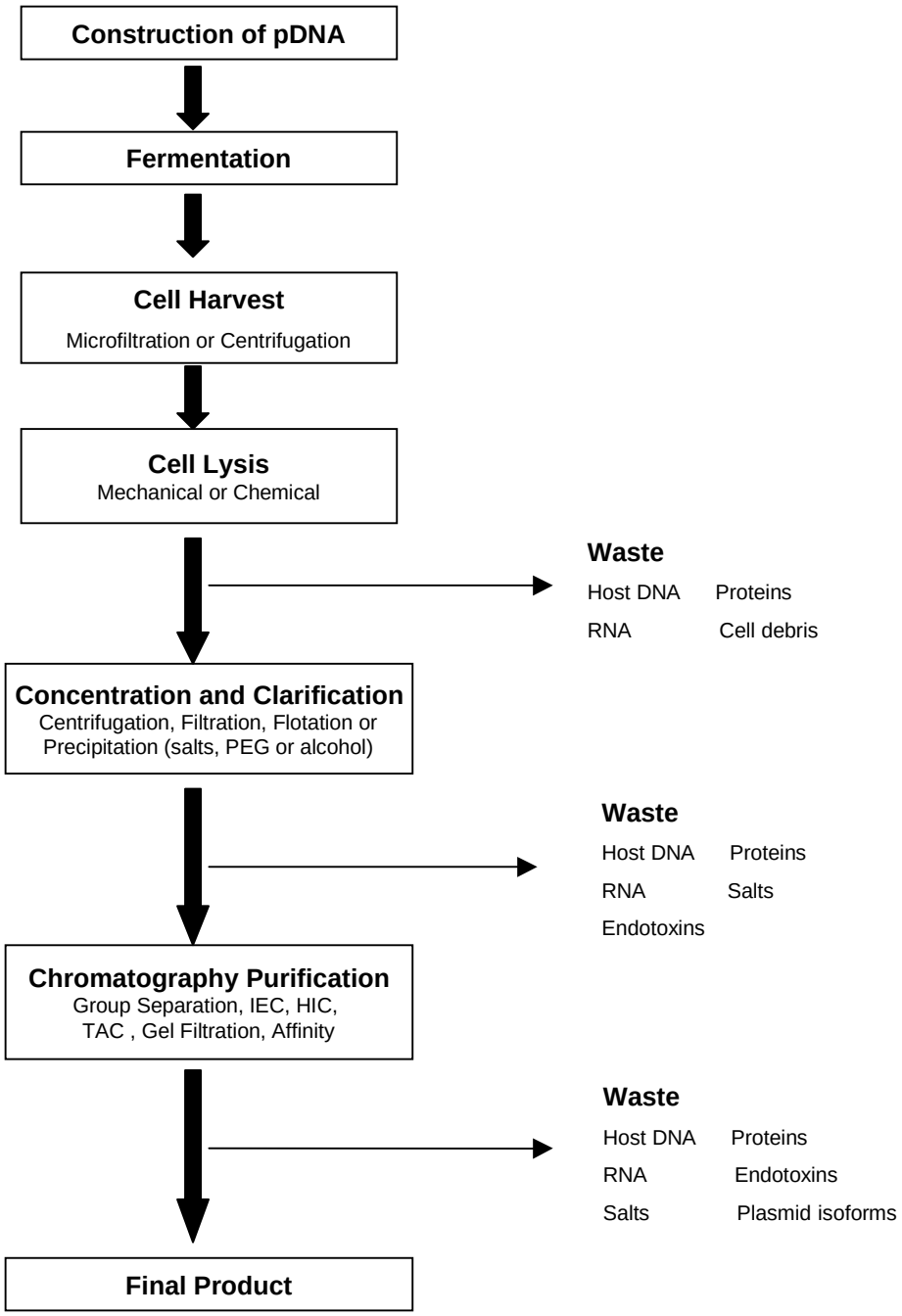


Figure 2. *Example of a process flow sheet for plasmid DNA purification. The unit operations as well as eliminated impurities are indicated.*

1.4.2.1 Cell Lysis

Cell lysis is the first step in the plasmid DNA purification process. The purpose of cell lysis is to disrupt the cell membrane and thereby to release the plasmid DNA from its host (Eon-Duval, 2003). Two examples of cell lysis used in plasmid DNA purification are mechanical disruption and alkaline lysis.

Mechanical disruption of the cell membrane is used in microfluidization and bead milling. The advantage with mechanical disruption is that harmful chemicals are not needed. One

disadvantage is, however, that the plasmid DNA is sensitive to shear forces and it is thereby difficult to keep the plasmids intact at the same time as disrupting the cells (Ferreira *et al.*, 2001). Another disadvantage is that mechanical disruption of the cells generates fragments of chromosomal DNA which are difficult to remove during the purification process.

Alkaline lysis is the most common cell lysis procedure used for plasmid DNA purification. Adding a detergent and an alkali to attain a high pH disrupts the cell in alkaline lysis. The chromosomal DNA is irreversibly denaturated while the plasmid DNA is reversibly denaturated at high pH (pH 12.0-12.5). The plasmid DNA is completely renaturated after neutralisation while the chromosomal DNA aggregates and precipitates. One advantage of using alkaline lysis is that most genomic DNA is removed, which otherwise can be difficult to separate from the plasmid DNA, but the major disadvantage is that it has been difficult to scale up (Eon-Duval, 2003). It is difficult to scale up alkaline lysis since plasmid DNA is sensitive to shear forces. The shear forces could also lead to partial breakdown of the long genomic chromosomal DNA chains, which results in shorter chains of genomic chromosomal DNA that will be very hard to separate from chemically identical plasmid DNA with a similar weight and charge. Furthermore, adding the alkali to a large-scale process could result in pH extremes (resulting in irreversible deterioration of plasmid DNA). Recent technical progress has, however, been made and solutions to large-scale alkaline lysis (1 kg biomass) have been presented (Urthaler, 2003). Alkaline lysis is used in this study since it is the most common lysis technique and has been used in other studies on plasmid DNA at Amersham Biosciences.

1.4.2.2 Clarification and Concentration

Clarification and concentration are unit operations that aim to enrich the plasmid DNA sample and to remove colloidal debris and micelles causing haziness before the chromatographic purification. These steps are needed because plasmid DNA normally represents only 2 % of the total nucleic acids in an E. *coli* lysate. Examples of clarification and concentration methods are centrifugation, filtration and precipitation (using polyethylene glycol (PEG), alcohol or salts) (Ferreira *et al.*, 2000a).

1.4.2.3 Chromatographic Purification

Examples of chromatographic purification steps used for plasmid DNA purification are reversed-phase (RPC), hydrophobic interaction (HIC), ion exchange (IEC), triple helix affinity (THAC), size exclusion (SEC) (Ferreira *et al.*, 2000a) and thiophilic aromatic chromatography (TAC) (Lemmens *et al.*, 2003).

Hydrophobic interaction and reversed-phase chromatography both separate the molecules in the solution upon their surface hydrophobicity. There are several nucleic bases, which are hydrophobic and thereby contribute to the plasmid DNA's hydrophobicity (Eon-Duval, 2003). The major factors affecting the adsorption of nucleic acids in RPC and HIC are their size, base composition and secondary structure. The supercoiled plasmid DNA can be separated from RNA, genomic DNA and linear plasmid DNA using these chromatography methods (Ferreira *et al.*, 2000a). The main difference between HIC and RPC is that the ligand density is much higher for RPC than for HIC, which results in a continuous hydrophobic phase for RPC, whereas the ligands on HIC interact individually with the solutes (Janson and Rydén, 1989). The need to use organic solvents to elute the plasmid DNA from reverse-phase

columns is a major disadvantage of this purification technique whereas high concentrations of salts are needed for hydrophobic interaction chromatography (Ferreira *et al.*, 2000a).

Ion exchange chromatography exploits the difference in the molecules surface charge density at a given pH. There are essentially two different types of ligands used for ion exchangers: anion and cation. Anion exchangers have a positively charged matrix while cation exchangers have a negatively charged matrix (Scopes, 1994). Anion exchange chromatography is used for plasmid purification, because of the high negative charge of plasmid DNA. The advantage with ion exchange chromatography is the fast kinetics and thereby that a high flow rate can be used (Lemmens, 2004). The main disadvantages are, however, the poor selectivity and recovery (Ferreira *et al.*, 2000a).

Triple helix affinity chromatography uses the possibility of forming triple DNA helices between an oligonucleotide covalently linked to the chromatography media and a duplex sequence present on the plasmid DNA (Wils *et al.*, 1997). The formation of triple helices are possible since the structure of DNA contains sites for hydrogen binding in both the major and minor grooves (Ferreira *et al.*, 2000a). Only plasmids with the engineered target sequence are captured while the genomic DNA, RNA, proteins and endotoxins flow straight through the column (Wils *et al.*, 1997). THAC shows a very high selectivity but major problems are the slow kinetics of the plasmid DNA – ligand interaction and the high costs.

Size exclusion chromatography separates plasmid DNA from RNA by size. The somewhat smaller molecules such as RNA, proteins and endotoxins have longer retention times whereas larger molecules such as plasmid DNA and genomic DNA have short retention times in the media. The main advantage with size exclusion chromatography is that complete resolution has been achieved. The disadvantages with size exclusion chromatography for plasmid DNA purification are the high dilution factors and low productivity (Ferreira *et al.*, 2000a). The productivity can be defined as: mg product per ml gel per unit time (Sofer and Hagel, 1997). One advantageous example of size exclusion chromatography for plasmid DNA purification is group separation. The substances that are separated during group separation are of large differences in size and high resolution is therefore not required. One example of group separation, which is used in this study, is desalting where plasmid DNA is separated from salt. The advantages with group separation, is that more sample can be loaded on the column (0.3 instead of 0.02 column volumes of sample) as well as that a higher flow rate can be used. The disadvantage is the lower resolution compared to preparative size exclusion chromatography.

Thiophilic aromatic chromatography separates RNA, genomic DNA and plasmid DNA isoforms by the difference in conformational changes in the presence of water-structuring salts. It has been suggested that the aromatic ring structure may participate in an intercalating hydrophobic (π-π) type of interaction with the supercoiled double helix form of plasmid DNA, while the sulphur moiety may assume an electron donating (charge transfer) role during interaction with specific nucleotides (Lemmens *et al.*, 2003). The advantages with this chromatography method are that it has very high selectivity (can separate the different plasmid DNA topological structures) as well as that lower amounts of salts are needed than for HIC. The main disadvantages are, however that this chromatographic method has slow elution kinetics and low yield.

2 Purpose and Theory

2.1 Purpose

There are several different purification techniques for purifying plasmid DNA but currently no good chromatographic media is available. Most chromatographic media of today are optimized for small or medium sized molecules. New media for large molecules such as plasmid DNA are needed. The purpose of the project is therefore:

> To prepare new chromatographic media and to evaluate the new media and other prototypes which could be useful for plasmid DNA purification. The goal for this study is to find a media with:
> 1) high plasmid DNA binding capacity
> 2) high recovery
> 3) good selectivity for plasmid DNA.

2.2 Theory

The same chromatographic principle, anion exchange chromatography, is used in all experiments. Anion-exchange chromatography is used because it is a straightforward and well-defined model system. Bead and pore size, elution buffer, elution gradient and flow rate influence the capacity, selectivity and resolution for anion exchange chromatography. The theory about anion exchange chromatography as well as different chromatographic media and the concept of resolution will be discussed here.

2.2.1 Anion-exchange Chromatography

The binding of the plasmid DNA to the positively charged ligands on the chromatography media is achieved by an electrostatic interaction between the negatively charged phosphate groups on the nucleic acid backbone and the ligand (Eon-Duval, 2003). A salt gradient was used to elute the charged molecules from the ion exchange column. Increasing ionic strength in the buffer weakens the electrostatic interaction between a charged molecule and the ligand in two different ways. Either by directly displacing the charged molecule by binding to the ligand or by interacting with the charged molecule and thereby weakening the attraction between the molecule and the ligand (Scopes, 1994). The chromatographic media used in this study are gels consisting of small spherical beads. The different chromatographic media are made of different materials and are of various bead and pore sizes. An example of an anion exchange column is shown in Figure 3 (page 13).

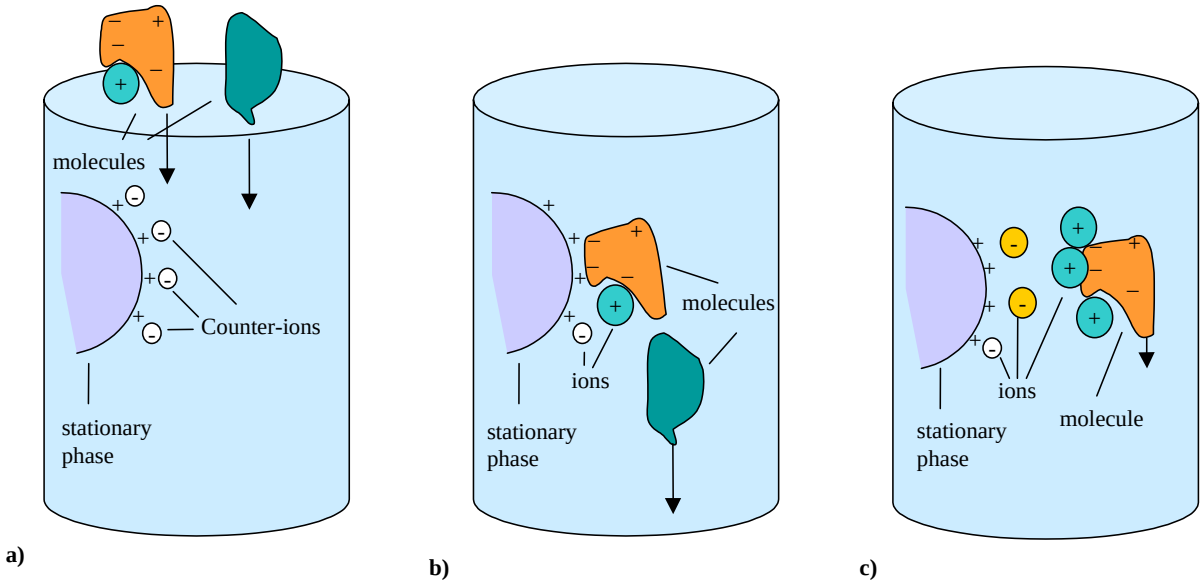


Figure 3. A schematic picture of an anion exchange column: *a) the charged ligands interact with counter-ions before the sample is injected. b) when only a few ions are present, the charges of the molecule interact strongly with the charged ligands on the stationary phase, causing retention or binding. c) higher ionic strength in the buffer shields the charges on both the stationary phase and charged molecules, causing weaker interactions and finally elution.*

Most commercially available anion exchange chromatographic media were originally designed for protein purification and show low capacity for plasmid DNA (Ferreria *et al.*, 2000b; Eon-Duval, 2003). A media's capacity reflects the amount of plasmid DNA that can bind to the media. A media with high capacity is preferred since more plasmid DNA can be purified during each run, which makes the purification process more cost-effective (Lemmens, 2004). Capacity is a function of several parameters such as media composition and media pore structure, bead diameter and bead size distribution profile, the sample's molecular weight and solubility, kinetic constants for the binding reaction (between ligand and charged molecule) and diffusion constants (Janson and Rydén, 1989). The pore size and structure of the media has an effect on the accessibility of adsorptive sites for the solutes and the kinetics of the adsorption/desorption process. Adsorption/desorption kinetics are often fast and the diffusive mass transport of solutes is therefore the primary limiting factor. Too small pores might, therefore, affect kinetics negatively, caused by blocking in the pathways (Sofer and Hagel, 1997).

Experiments have shown that the capacity for plasmid DNA on existing gels decreases with increasing bead diameter as well as that plasmid DNA binding only occurs at the surface of the particles (Ljunglöf *et al.*, 1999; Ferreria *et al.*, 2000b). It can therefore be concluded that a smaller particle radius results in a larger active surface since only the ligands on the surface of the beads are easily accessible to the plasmid DNA.

Large plasmid DNA molecules are partially excluded from particle pores, which results in diffusion constraints of the macromolecules through the particles' pores (Ferreria, 2001). The plasmid DNA is not only restricted from diffusing into the particles but can also get 'stuck' in the pores by only a part of the plasmid DNA being able to enter the pore. It would most likely be more difficult to elute the plasmid if only a part of the plasmid enters the pore since more

ligands could bind to the plasmid in the pore at the same time than if the plasmid binds to the bead surface (see Figure 4). Plasmid DNA, which get stuck in the pores might also result in more complex kinetics since the environment inside and outside the particle could be different due to the plasmid possibly blocking the salts from entering the pore.

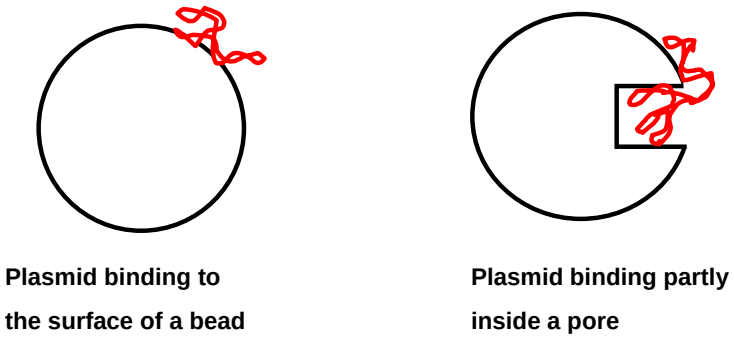


Figure 4. *Representation of possible interactions with a chromatographic media particle and supercoiled plasmid DNA.*

Nucleic acid molecules have one negative charge per base, which results in an overall net charge equal to the number of bases in the molecule. The expected elution profile is therefore that the nucleic acid molecules with higher molecular weight elute at higher ionic strength. The elution profile does not only depend on molecular weight but also on the nucleic acid's bending. The nucleic acid's bending promotes a better fit within the pore curvature, enabling more charges to interact with the solid phase, which result in longer retention times. Another physical explanation to stronger binding for bent double stranded DNA is that the bent DNA has a dipole character and therefore has a highly concentrated charge. Supercoiled plasmid DNA is more bent than opencircular and therefore has a better fit in the pores and has a higher local charge density, which results in a longer retention time (Ferreria *et al.*, 2001).

2.2.2 Chromatographic Media

All chromatographic media in this study are positively charged with a Q ligand. The Q group is a quaternary amino group and is a strong ion exchanger with full charge over the whole pH range (Protein purification, 1999). The different prototype media that will be evaluated in this study are Q Compact Beads, Q Sephacryl (500, 1000 and 2000) and Q Superdex 75 prep grade. Q Sepharose High Performance and SOURCE 15Q (Amersham Biosciences, Uppsala, Sweden) are commercially available media and were used as reference gels.

Q Sepharose High Performance (Amersham Biosciences, Uppsala, Sweden) is made of highly cross-linked spherical 34 µm (mean diameter) 6 % agarose beads. The media is used as reference gel because of the similar size to the evaluated media. Furthermore, Compact Beads are made from the same material.

SOURCE 15Q (Amersham Biosciences, Uppsala, Sweden) media is based on 15 µm, monodisperse, rigid, polystyrene divinyl benzene beads (Figure 5). SOURCE 15Q is used as reference gel since it has similar size as the smaller fractions (<20 µm) of Q Compact Beads.

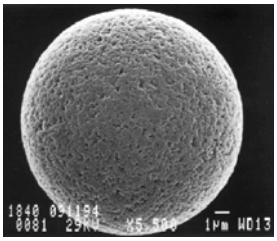


Figure 5 *Scanning electron microscopy picture of SOURCE bead. Illustration used with permission from Philippe Busson, Amersham Biosciences, Uppsala, Sweden.*

Q Compact Beads are shrunken Sepharose High Performance beads, which are then Q coupled. The five times shrinking of the Sepharose High Performance beads makes the beads more compact and thereby less porous. The idea behind the use of Compact Beads is that smaller/fewer pores should lead to less plasmid DNA getting 'stuck' in the pores and simpler binding mechanisms leading to faster kinetics would occur. A more compact bead would also result in a larger active surface for ligand-plasmid DNA interactions. The larger active surface would occur since the bead surface after shrinkage contain more media per surface area, which could result in higher capacity. See Figure 6 for schematic figure of the synthesis of Compact Bead.

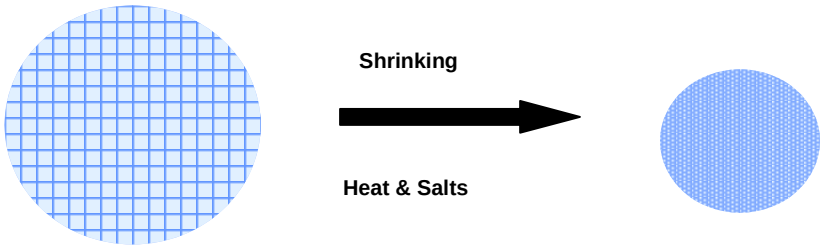
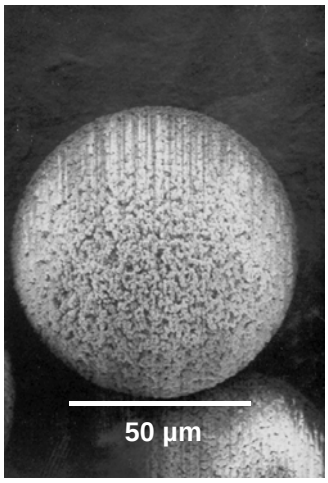


Figure 6. *Schematic figure of the synthesis of Compact Beads, showing the smaller and more dense bead after shrinking.*

Q Sephacryl (500, 1000, 2000) High Resolution is based on the gel filtration media Sephacryl High Resolution, which has been Q coupled (see Figure 7 page 16). Sephacryl High Resolution is a cross-linked copolymer of allyldextran and N.N'-methylene bisacrylamide with a mean diameter of 50 µm. The Sephacryl (500, 1000, 2000) High Resolution media are very porous which is described by their fractionation range. The fractionation range is the molecular weight interval of the molecules, which can be separated on the media and describes the media's selectivity (referring to size). The higher the end value of the fractionation range is the larger are the molecules that can be separated on the media (due to larger molecules being able to enter the pores). The Sephacryl 500 High Resolution has a fractionation range between $4 \times 10^4 - 2 \times 10^7$ Da and Sephacryl 1000 High Resolution's fractionation range is 5×10^5- > 10^8 Da for dextrans. The fractionation range for the Sephacryl 2000 High Resolution media is even higher than for the Sephacryl 1000 High Resolution media and has thereby even larger pores. These three gels are tested in order to evaluate how the porosity affects plasmid DNA purification.

a) 

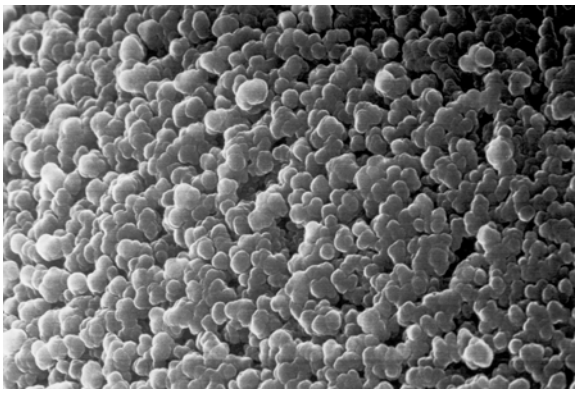
b) 

Figure 7. Scanning electron microscopy pictures of Sephacryl 2000 High Resolution: *a) An overview of a bead with the pores clearly visible. b) A close up on the surface of a bead. Illustration used with permission from Dag Lindström, Amersham Biosciences, Uppsala, Sweden.*

Superdex 75 and 200 prep grade are both commercially available media with highly cross-linked porous agarose particles to which dextran has been covalently bonded (see Figure 8). The Superdex 75 prep grade media was Q-coupled as described in 3.3 (page 20). The dextran increases the accessible area for interactions for the plasmid DNA and could thereby increase the capacity. The fractionation range for dextrans on the Superdex prep grade media is $5 \times 10^2 - 3 \times 10^4$ Da for Superdex 75 prep grade and $1 \times 10^3 - 1 \times 10^5$ Da for Superdex 200 prep grade. Superdex 75 prep grade was chosen instead of Superdex 200 prep grade because of the availability in the laboratory. The difference in porosity (fractionation range) between the two media was most likely too small to have a large effect on the media evaluation since the plasmid DNA is much larger than the porosity of either media. Prep grade describes the size of the beads, which are between 22-44 µm in diameter and with a mean diameter of 34 µm.

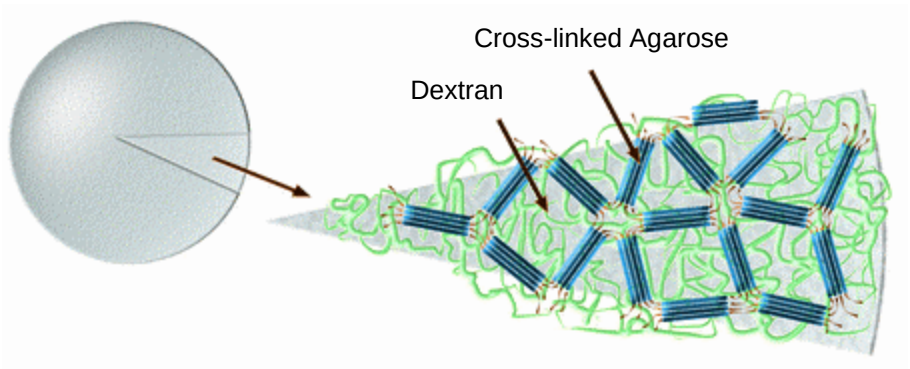


Figure 8. *The dextran chains are covalently linked to highly cross-linked agarose matrix. The figure shows a schematic picture of a section through a Superdex particle. Illustration used with permission from Amersham Biosciences, Uppsala, Sweden.*

2.2.3 Resolution and Peak Asymmetry

Good resolution is needed to be able to separate different peaks of a complex sample since resolution is a measurement of how well two peaks are separated. The resolution between two solutes is determined by their elution volumes/times and peak width. Chromatographic resolution between two peaks is defined by:

$$R_s = \frac{V_{R2} - V_{R1}}{\frac{1}{2}(w_{b1} + w_{b2})}$$

R_s is the distance between the peak maxima divided by the mean peak width (see Figure 9) (Janson and Rydén, 1989). The two peaks are incompletely separated when $R_s < 1.5$ and are completely separated when $R_s > 1.5$. Optimal separation conditions are reached when $R_s > 1.5$ for all compound pairs of interest (Ion exchange chromatography, 2002).

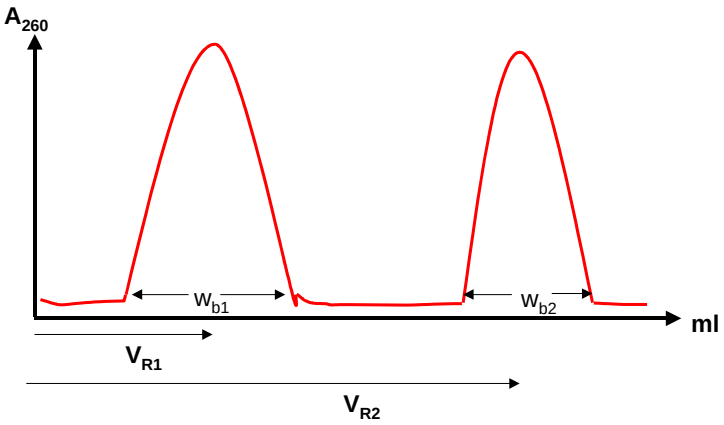


Figure 9. *Peak widths and retention volumes for definition of resolution.*

A sharp peak is desired in this study for two main reasons. Firstly because sharper peaks result in higher resolution and secondly because a sharper peak results in an enriched sample of the molecule of interest.

Peak asymmetry factor is used here as a measure of a packed column. For this, a small amount of acetone or salt is injected onto a column and the following elution profile is analysed. Peak asymmetry factor is calculated according to the following equation:

$$A_s = \frac{b}{a}$$

The definitions of a and b are shown in Figure 10. The calculations of a and b are performed at 10 % of the peak height to minimise the effect of background noise.

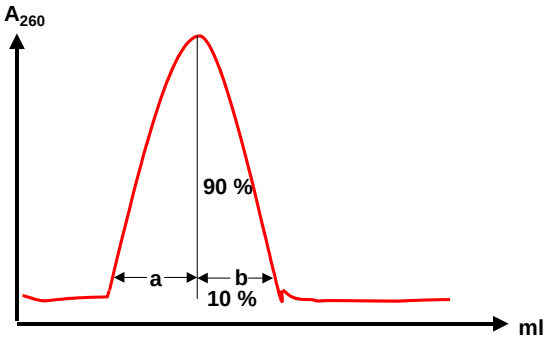


Figure 10. *Peak distances for definition of asymmetric factor.*

Leading peaks have an asymmetry factor less than 1.0 (a larger than b) and indicates channelling in the column bed if the sample elutes before void volume. This can be due to over-packing of the column (packed at too high pressure or flow rate). Tailing peaks, on the other hand, have an asymmetry factor greater than 1.0 (b larger than a) and indicates under-packing of the column or uneven sample application (Gel filtration, 2002).

3 Materials and Methods

The different chromatographic media that are tested in this study were synthesized to different degrees of ion exchange capacities before they were packed in columns and tested. The plasmid DNA sample used for testing the different media was lysed, centrifuged, filtrated, group separated and most often desalted before being injected on the different columns.

The evaluation of the different chromatographic media was performed on an ÄKTAexplorer 10XT (Amersham Biosciences, Uppsala, Sweden). RNA removal and desalting of the plasmid DNA sample was performed on an ÄKTAexplorer 100 system. UNICORN 4.11 (Amersham Biosciences, Uppsala, Sweden) software was used in all three cases for control of the chromatographic system and evaluation of the results.

3.1 Q Compact Beads

Q Compact Beads were synthesized, Q coupled and packed in Tricorn 5/50 columns before the media was evaluated.

3.1.1 Syntheses

Shrinking: First approximately 1.5 l gel (Sepharose High Performance non cross-linked and unsieved) was washed with 10 bed volumes of deionized water before a 75 % slurry (gel and water mixture) was made. Salts were then added to the 75% slurry to get a final concentration of 0.5 M Na_2SO_4 and 0.5 M $MgSO_4$. The last step of the shrinking procedure was to heat up the reaction mixture to 96°C for 30 minutes with stirring before cooling down to under 30°C with ice bath. The salts in the slurry were then removed by washing the gel with 10 bed volumes of deionized water on a sinter glass filtration funnel.

Cross-linking: The cross-linking was performed to make the beads more rigid and was in this study made according to the production protocol for Sepharose 6 Fast Flow which is based on the protocol described by Porath (1982). The only deviation from the protocol was that four times more Epichlorohydrin and NaOH were added to compensate for the more dense gel particles after shrinkage.

Q Coupling: Q-groups were coupled according to the production protocol for Q coupling to Sepharose High Performance. The reaction is similar to the one described by Antal and Micko (1992). The protocol was adjusted when a lower ligand concentration was desired. Titration of Cl^- with 0.1 M $AgNO_3$ was performed after the Q coupling to measure the amount of Q ligand on the Compact Beads. Consequently the Q-groups' counter-ion was changed to Cl^- by washing 10 ml slurry with 20 ml 0.5 M HCl and then with 20 ml 1 mM HCl. Exactly 1.00 ml gel was measured up before the sample was diluted by adding 20 ml deionized water. Finally 2 drops of concentrated HNO_3 were added before titration of Cl^- with 0.1 M $AgNO_3$.

3.1.2 Column Packing

Q Compact Beads were packed in Tricorn 5/50 columns with deionised water as packing liquid. The columns were first packed at a flow rate of 306 cm/h for approximately 15

minutes and then at a flow rate of 764 cm/h for approximately 15 minutes. The column packing was tested by measuring the peak asymmetry after injecting 25 µl 0.4% acetone at a flow rate of 76 cm/h. Columns with a peak asymmetry between 0.8 to 1.8 were accepted for use in this study.

3.2 Q Sephacryl High Resolution

The Sephacryl 1000 and 2000 High Resolution are prototype media whereas Sephacryl 500 High Resolution is commercially available. The Sephacryl High Resolution media was Q coupled in a similar way as the Compact Beads.

3.2.1 Column Packing

The peak asymmetry was measured as in 3.1.2 with a flow rate of 61 cm/h (24 cm/h for Q Sephacryl 1000 High Resolution).

Q Sephacryl 500 High Resolution was packed in Tricorn 5/50 columns with deionized water as packing liquid. The columns were first packed at a flow rate of 31 cm/h for 2 hours and then at a flow rate of 92 cm/h for 20 minutes.

Q Sephacryl 1000 High Resolution was packed in Tricorn 5/50 columns with deionized water as packing liquid. The columns were packed at a flow rate of 37 cm/h for 40 minutes.

Q Sephacryl 2000 High Resolution was packed in Tricorn 5/50 columns with deionized water as packing liquid. The columns were packed at a flow rate of 92 cm/h for 15 minutes.

3.3 Q Superdex 75 prep grade

Q Superdex 75 prep grade was synthesized by Q coupling Superdex 75 prep grade in a similar way as Compact Beads was Q coupled.

3.3.1 Column Packing

Q Superdex 75 prep grade was packed in Tricorn 5/50 columns with deionized water as packing liquid. The columns were first packed at a flow rate of 92 cm/h for 30 minutes and then at a flow rate of 306 cm/h for 15 minutes. The column packing was tested in the same way as the Q Sephacryl High Resolution media.

3.4 Preparation of Plasmid DNA

A 6125 base pair recombinant pUC19 plasmid was used for this study. The plasmid was transfected and grown in E. *coli* TG1α in accordance with protocol by Sambrook and Russel (2001).

3.4.1 Alkaline Lysis

Clarified alkaline lysate was prepared according to Horn *et al.* (1995) by the following procedure. The cellpaste (20 g) was first resuspended in 200 ml of suspension buffer (61 mM glucose, 10 mM Tris-HCl, 50 mM EDTA, pH 8.0) while stirring. The cells were lysed by mixing 200 ml of buffer P2 (0.2 M NaOH, 1% SDS) under gentle stirring after the cellpasta was completely resuspended. The mixture was left for 7 min at room temperature under gentle stirring before neutralization by adding 200 ml ice-cold buffer P3 (5M potassium acetate, pH 5.5 with glacial acetic acid). The solution was mixed gently to assure complete mixing (a large white precipitate was formed) before incubation on ice for at least 20 minutes. The mixture was then incubated in two centrifuge bottles at 4 °C overnight. Finally the solution was centrifuged (at 9500 rpm) for 30 min and filtered through a paper filter (80-200 µm).

3.4.2 RNA Removal

RNA removal from the clarified alkaline lysate was performed on the gel filtration media Sepharose 6 Fast Flow (Amersham Biosciences, Uppsala, Sweden) in XK50/30 column. A buffer containing 1.5 M $(NH_4)_2SO_4$, 10 mM EDTA and 100 mM Tris-HCl at pH 7 was used during the RNA removal procedure and a flow rate of 100 cm/h (Lemmens *et al.*, 2003).

3.4.3 Desalting

The plasmid DNA was further prepared by removing the $(NH_4)_2SO_4$ from the sample. The buffer was changed to 10 mM EDTA and 100 mM Tris-HCl at pH 7.0 by performing a desalting on a XK50/30 column with Sepharose 6 Fast Flow media. The gel filtration was performed at a flow rate of 30 cm/h.

3.5 Evaluation of Chromatographic Media

Binding capacity, yield, recovery and peak symmetry are four characteristics, which need to be taken in to consideration when evaluating a chromatographic media. The capacity was here calculated from the amount of plasmid DNA, which had been loaded on to the column at 20 % breakthrough (Q_{B20}). Plasmid DNA leakage occurred during sample loading for a few runs. The leakage was then subtracted from the amount of loaded plasmid DNA when the capacity was calculated.

Yield and recovery were both calculated from the amount of plasmid DNA that was eluted during the run. The yield was calculated as the percentage of the total amount of loaded plasmid DNA that was eluted during the salt (NaCl) gradient. Recovery was instead calculated as the percentage of the total amount of loaded plasmid DNA, which was eluted during both the salt gradient and the Cleaning-In-Place (CIP, performed by incubating the gel for 30 min in 1 M NaOH) (Figure 11).

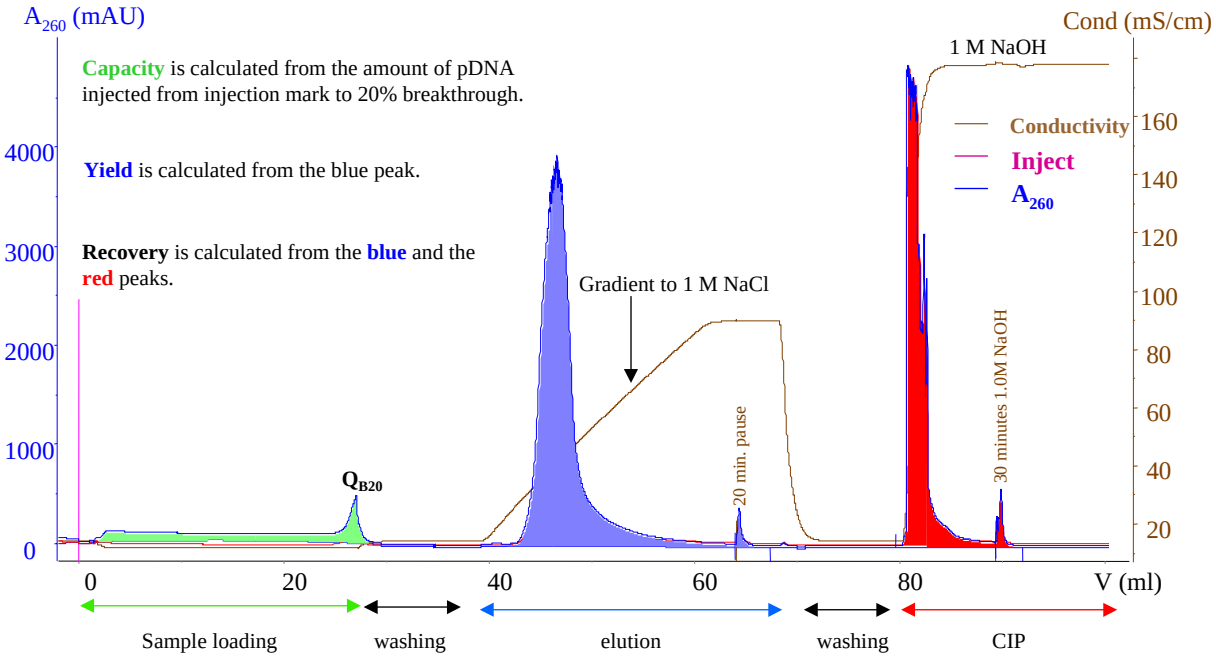


Figure 11. *An example of a chromatogram with NaCl elution gradient and 1M NaOH CIP as well as description of capacity, yield and recovery.*

High capacity is needed for the chromatographic media to be interesting for industrial plasmid DNA purification. Low capacity requires very large columns or many runs and makes the purification process time-consuming and thereby expensive. Because of the high viscosity of highly concentrated plasmid DNA solutions it was estimated that the highest practical binding capacity for plasmid DNA on a gel was approximately 10 mg plasmid DNA/ ml media. The goal for this study was 5 mg plasmid DNA/ml gel.

The yield reflects the amount of purified plasmid DNA, which can be further processed after the purification step. The chromatographic method as well as media affects the yield. The goal in this study was set to 85 % since the anion exchange ligand was known to bind strongly to plasmid DNA. An acceptable yield depends also on the capacity and is not an absolute value. From the recovery the amount of plasmid DNA that is left on the media after the CIP can be calculated. Too much plasmid DNA left on the gel can pollute later runs as well as blocking ligands from binding new plasmid DNA (thereby lowering the capacity). It is also important that the gel can be used repeatedly for the purification process to be cost effective. Recovery should, therefore, preferably be above 95 %.

The columns were first equilibrated with buffer A (0.1 M NaCl, 2.5 mM EDTA, 25 mM Tris-HCl, pH 7) for 10 column volumes before the sample was injected. The different chromatographic media were evaluated (Figure 11) by loading plasmid DNA sample (approx. 72 µg plasmid DNA/ ml) until 20% breakthrough occurred (20 % of the plasmid DNA, which is loaded on the column runs straight through). The column was set on buffer A by injecting 10 column volumes after the sample loading.

The next step of the media evaluation procedure was the elution. A 10 column volume salt gradient with NaCl (final concentration 1 M or 2 M) was therefore applied before the column was incubated for 20 minutes at the final salt concentration. The column was then set on buffer A before loading 1 M NaOH on to the gel followed by 30 minutes of incubation (performed a CIP). The amount of eluted plasmid DNA was measured using an UV detector.

The chromatogram was a result of the UV-detector after the column. Each peak's fractions were pooled together before UV absorbance was measured at 260 and 280 nm in a spectrophotometer. This was performed to get a more accurate measurement of the amounts of plasmid DNA in the peaks. The amount of plasmid DNA was calculated from the following formula:

$$pDNA\ (\mu g) = A_{260} * dilution\ factor * \varepsilon * V$$

Where A_{260} is the 260 nm absorbance, ε is the extinction's coefficient (50µg plasmid/ ml mAU (Ferreria *et al.*, 2000b)) and V is the volume of the fractions of the peak.

4 Results

4.1 Q Compact Beads

Both Sepharose High Performance (non-crosslinked, not sieved) and Sepharose 6B (Amersham Biosciences, Uppsala, Sweden) (non-crosslinked) were used for Compact Beads synthesis. Both the Sepharose 6B beads and the Sepharose High Performance beads shrunk 5-6 times in volume (Figure 12).

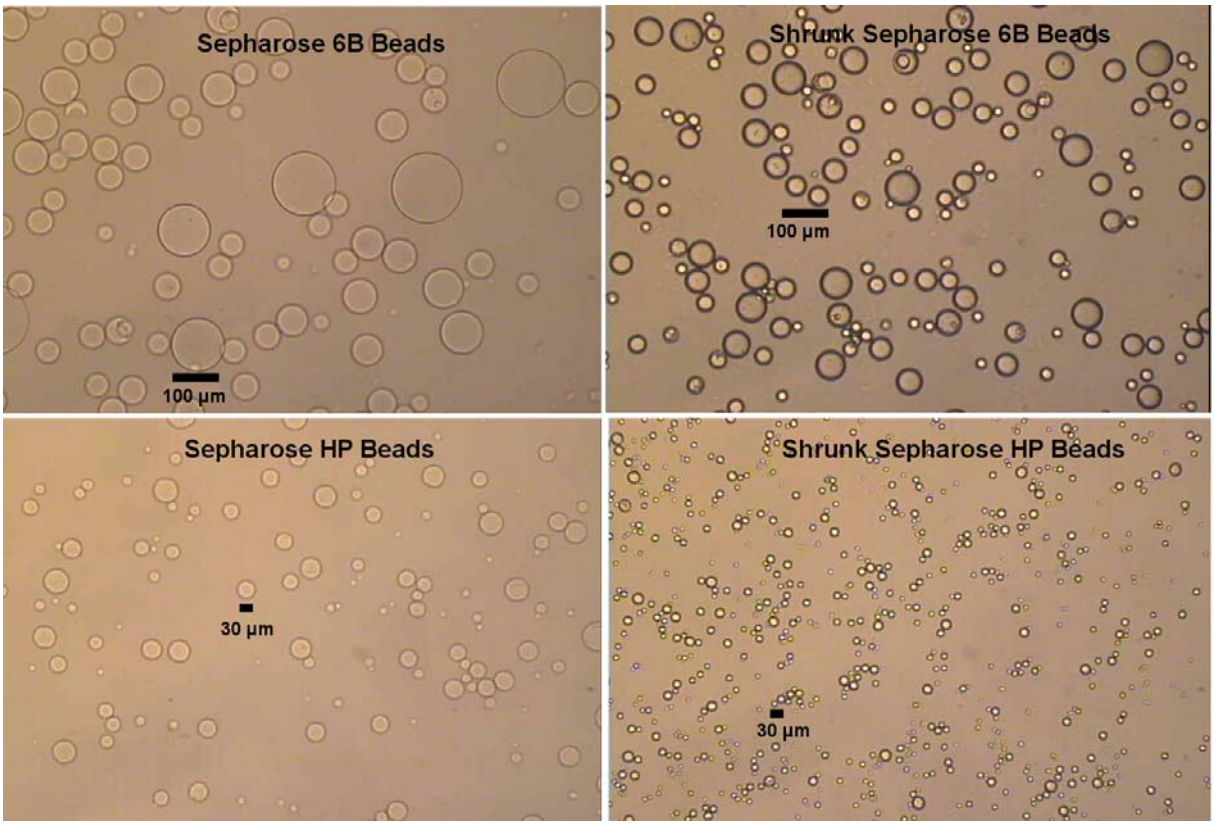


Figure 12. *Microscopy pictures of Sepharose beads before and after shrinking.*

The beads' porosity was also measured by performing an NMR experiment (Figure 13). D_{eff} is the effective diffusion constant and reflects the porosity. The higher the D_{eff} value is, the less hindered is the diffusion. A higher D_{eff} reflects thereby a more porous media. The results show that the porosity decreases as expected after shrinkage and crosslinking. The D_{eff} value of the shrunken and cross-linked Sepharose High Performance beads should probably be slightly lower. Practical problems measuring the sample occurred most likely due to the experimental conditions not being optimized for particles of such high density.

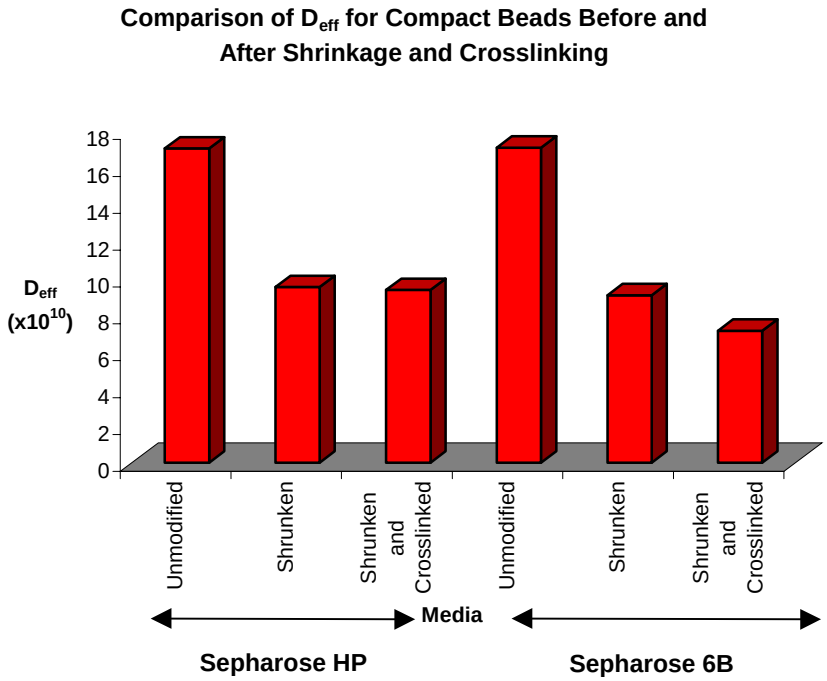


Figure 13. *The diagram shows a decrease in D_{eff} (effective diffusion constant) after shrinkage and cross-linking of Sepharose beads. The reduced D_{eff} indicates a decreased porosity.*

The Compact Beads made from Sepharose High Performance were Q coupled using the previously described method. Compact Beads with two different concentrations of Q ligands were synthesized. The Compact Beads with 675 µmol Q/ ml gel were sieved after Q coupling whereas the 194 µmol Q/ ml Compact Beads were sieved before Q coupling (size 20-40µm). The sieving was performed using first a 40 µm steel sieve and then a 20 µm steel sieve. The beads larger than 40µm and aggregates were discarded whereas beads sized 20-40 µm and < 20µm were collected. The Q Compact Beads, which were sieved after they had been Q coupled, were sieved in 0.3 M Na_2SO_4 to avoid electrostatic interactions between the sieve and media whereas the Compact Beads before Q coupling were sieved using deionized water.

The first plasmid DNA experiments on Q Compact Beads were performed with plasmid DNA sample containing salts and without changing the Q ligands counter-ions to Cl^- at a flow rate of 100 cm/h. See Table 1 for results. The counter-ions were most likely, before the HCl wash, a mixture of acetate ions (OAc^-) and other negative ions.

Table 1: *The table presents the results obtained from media, which had not been pre-treated.*

*Media**	*Size (µm)*	*Conc. Q (µmolQ/ ml gel)*	*Counter-ion*	*Elutes with 1 M NaCl Yield*	*Elutes with 1 M NaOH*	*Tot. Recovery*	*Capacity (mg pDNA/ ml gel)*	*Comments*
Q Compact	20-40	675	OAc^- ?	46 %	26 %	72 %	1.22	
Q Compact	20-40	194	OAc^- ?	? %	? %	? %	? %	Most pDNA flows straight through column
Q Compact	<20	675	OAc^- ?	39 %	33 %	72 %	2.89	Pressure problems
SOURCE 15 Q	15	177	Cl^-	35 %	47 %	82 %	2.28	
Q HP	22-44	170	Cl^- ?	32 %	ca. 0 %	32 %	1.12	

* Q Compact = Q Compact Beads, Q HP = Q Sepharose High Performance.

Pressure problems were experienced during run with Q Compact Beads, 675 µmol Q/ml gel size < 20 µm, and the results are therefore not reliable.

The fact that the strongly negatively charged plasmid DNA surprisingly did not bind to the positively charged Q Compact Beads, 194 µmol Q/ml gel size 20-40 µm, suggested that the experimental conditions were not optimal. The counter-ion to the Q-ligand was therefore changed to Cl^- by washing the gel first with 5 column volumes of 0.5 M HCl and then with 10 column volumes of 1 mM HCl (Table 2).

Table 2: *The table presents the results obtained from media, having Cl^- as counter-ion.*

*Media**	*Size (µm)*	*Conc. Q (µmolQ/ ml gel)*	*Counter-ion*	*Elutes with 1 M NaCl Yield*	*Elutes with 1 M NaOH*	*Tot. Recovery*	*Capacity (mg pDNA/ ml gel)*	*Comments*
Q Compact	20-40	675	Cl^-	32 %	26 %	58 %	3.28	Pressure problems
Q Compact	20-40	194	Cl^-	34 %	51 %	85 %	1.6	
Q Compact	<20	675	Cl^-	5 %	8 %	13 %	> 1.21	Pressure problems

* Q Compact = Q Compact Beads

Pressure increased with both Q Compact Beads, 675 µmol Q/ml gel size 20 - 40 µm, and Q Compact Beads, 675 µmol Q/ml gel size < 20 µm. It is therefore difficult to estimate their capacity since the pressure increased too much to be able to calculate the capacities correctly. Especially the capacity for Q Compact Beads, 675 µmol Q/ml gel size < 20 µm, is dubious since the injection had to be stopped before any breakthrough occurred. The capacity for Compact Beads size 20 – 40 µm (both ligand concentrations) increased substantially when the counter-ion was changed to Cl^-.

The pressure problems could have been due to the viscosity of the plasmid DNA sample since it contained a high concentration of salts (1.5 M $(NH_4)_2SO_4$). The plasmid DNA sample was therefore desalted for further experiments (Table 3). The pressure problems could also be due to local precipitation of plasmid DNA at the filter or at the surface of the beads. The local precipitation of plasmid DNA could block the flow path and thereby contribute to the pressure problems.

Table 3: *The table presents the results obtained from media with Cl^- as counter-ion and a desalted plasmid DNA sample.*

*Media**	*Size (µm)*	*Conc. Q (µmolQ/ ml gel)*	*Counter-ion*	*Elutes with 1 M NaCl Yield*	*Elutes with 1 M NaOH*	*Tot. Recovery*	*Capacity (mg pDNA/ ml gel)*	*Comments*
Q Compact	20-40	675	Cl^-	29 %	48 %	77 %	1.5	Pressure peak
Q Compact	20-40	194	Cl^-	53 %	57 %	110 %	1.6	Flow rate 60 cm/h
Q HP	22-44	170	Cl^-	26 %	3 %	29 %	0.99	

* Q Compact = Q Compact Beads, Q HP = Q Sepharose High Performance.

The problems with high pressure were reduced but not solved by desalting the plasmid DNA. The Q Compact Beads, 675 µmol Q/ml size 20 - 40 µm, were still compressed during the run and the pressure peaked at a higher pressure than is preferred. An illustration of two different pressure profiles for Compact Beads with different ligand concentrations, sample preparations and flow rates is shown in Figure 14. Figure 14 shows furthermore that the pressure is reduced with desalted plasmid DNA sample, lower ligand concentration and reduced flow rate.

Table 2 and 3 shows higher yield and recovery for media with lower ligand concentrations.

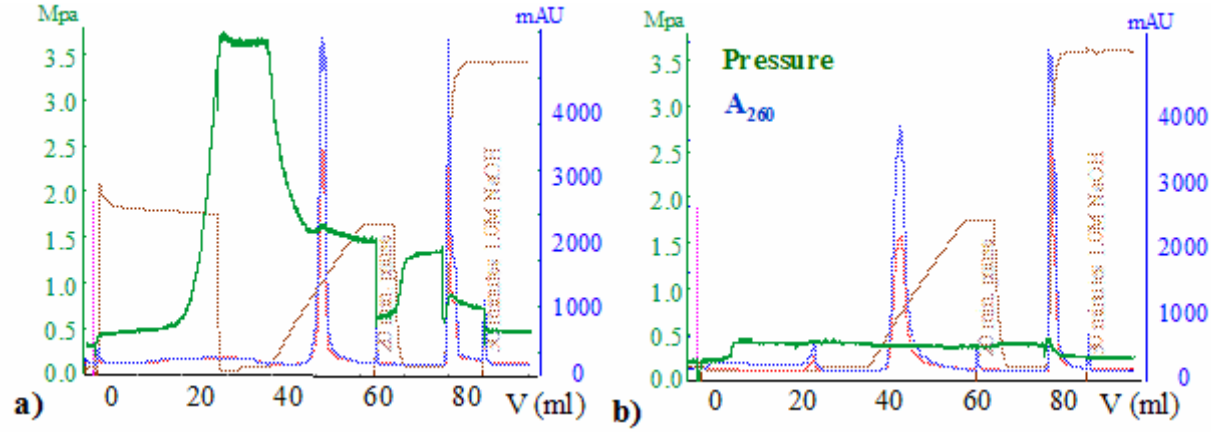


Figure 14. Pressure profile at two different ligand concentrations, flow rates and sample preparations: *a) Compact Beads with 675 µmol Q/ ml, size 20-40 µm, Cl^- as counter-ion, flow rate 100 cm/h and with a plasmid DNA sample that was not desalted. Pressure problems occurred during the run and the sample injection was stopped before Q_{B20} was reached. b) Compact Beads with 194 µmol Q/ ml, size 20-40 µm, Cl^- as counter-ion, flow rate 60 cm/h and with a desalted plasmid DNA sample. The run is completed without pressure problems and Q_{B20} was reached.*

4.2 Q Sephacryl High Resolution

Q Sephacryl 500, 1000 and 2000 High Resolution were tested in the same way as Q Compact Beads. The counter-ion was changed to Cl^- and a desalted plasmid DNA sample was used. The only difference was that the flow rate was changed to 25 cm/h for Q Sephacryl 1000 High Resolution and to 60 cm/h for Q Sephacryl 500 and 2000 High Resolution. Lower flow rates were used on the Q Sephacryl High Resolution media than on Q Compact Beads since the

media was packed at a lower flow rate as well as being more sensitive to pressure. The results are presented in Table 4. The recovery above 100 % could be due to the plasmid DNA being denaturated at 1 M NaOH, altering the extinction coefficient. This change of extinction coefficient was not taken into account while calculating the amount of plasmid DNA eluted during the CIP.

Table 4: *The table presents the results obtained from Q Sephacryl High Resolution media with Cl^- as counter-ion and a desalted plasmid DNA sample.*

Media	*Size (µm)*	*Conc. Q (µmolQ/ ml gel)*	*Counter-ion*	*Elutes with 1 M NaCl Yield*	*Elutes with 1 M NaOH*	*Tot. Recovery*	*Capacity (mg pDNA/ ml gel)*	*Comments*
Q Sephacryl 500 HR	25-75	34	Cl^-	61 %	56 %	117 %	0.76	
Q Sephacryl 1000 HR	25-75	40	Cl^-	64 %	58 %	122 %	1.9	
Q Sephacryl 2000 HR	25-75	59	Cl^-	57 %	4 %	61 %	3.86	

Figure 15 shows the different runs on the Q Sephacryl High Resolution media as well as a comparison of the elution peaks.

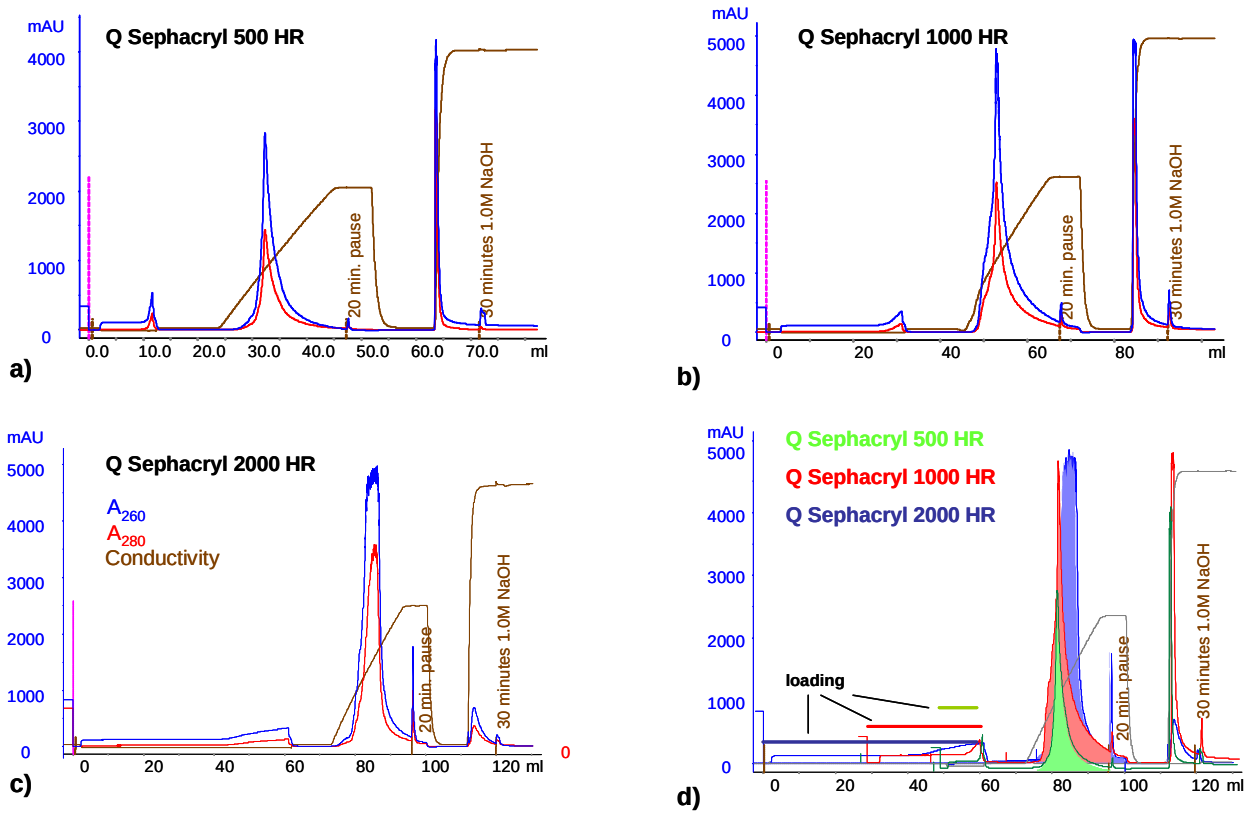


Figure 15. Comparison of the chromatograms from the three Q Sephacryl High Resolution media: *a) chromatogram from a run on Q Sephacryl 500 High Resolution media. b) chromatogram from a run on Q Sephacryl 1000 High Resolution media. c) chromatogram from a run on Q Sephacryl 2000 High Resolution media. d) overlay of the three different peaks from the different media showing the peaks difference in peak tailing and amount of loaded plasmid DNA sample.*

The Q Sephacryl 2000 High Resolution showed a relatively low recovery the first chromatographic run with plasmid DNA. The same gel was then tested for five consecutive runs to investigate the recovery, capacity and yield more closely. Figure 16 illustrates the chromatograms from the first and fifth run on the same Q Sephacryl 2000 High Resolution column. The recovery increased after the first run sometimes to above 100 % (similar to the other Sephacryl High Resolution media) at the same time as capacity and yield decreased somewhat (yield to approximately 39 % and capacity to approximately 2.5 mg pDNA/ml gel, see Table 5). The recovery above 100 % could be due to the plasmid DNA being denaturated at 1 M NaOH, as previously described. Another explanation to the increasing recovery could be that the plasmid DNA left on the gel from previous runs was eluted.

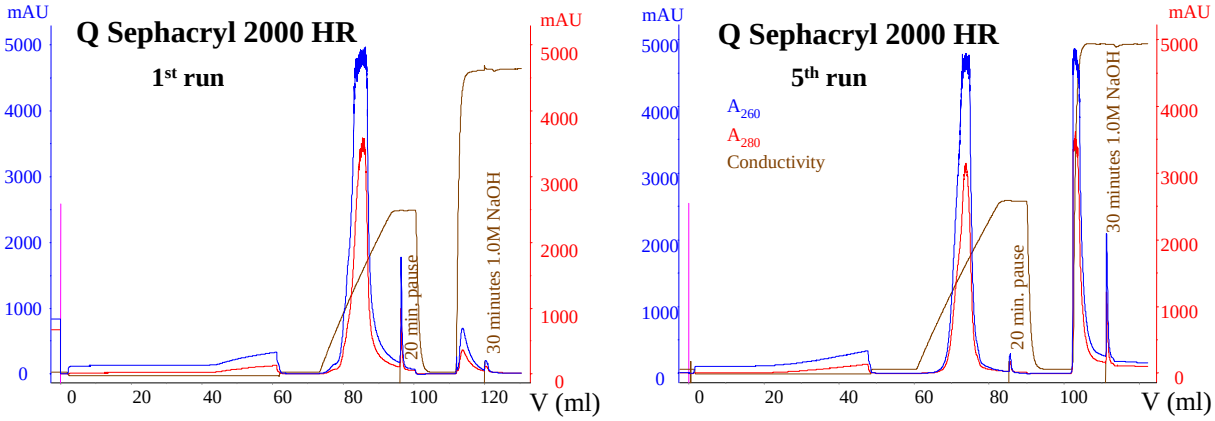


Figure 16 *Chromatogram from the 1st and 5th run on a Q Sephacryl 2000 High Resolution gel.*

Table 5: *The table presents the results obtained from Q Sephacryl 2000 High Resolution media. The same column was tested for five consecutive runs.*

Media	*Size (µm)*	*Conc. Q (µmolQ/ml gel)*	*Counter-ion*	*Elutes with 1 M NaCl Yield*	*Elutes with 1 M NaOH*	*Tot. Recovery*	*Capacity (mg pDNA/ml gel)*	*run*
Q Sephacryl 2000 HR	25-75	59	Cl^-	42 %	5 %	47 %	4.16	1
Q Sephacryl 2000 HR	25-75	59	Cl^-	37 %	58 %	95 %	2.13	2
Q Sephacryl 2000 HR	25-75	59	Cl^-	37 %	58 %	95 %	2.65	3
Q Sephacryl 2000 HR	25-75	59	Cl^-	40 %	63 %	103 %	2.49	4
Q Sephacryl 2000 HR	25-75	59	Cl^-	40 %	66 %	106 %	2.83	5

The Q Sephacryl 2000 High Resolution gel was compressed after every run and gaps were formed at the top of the column. The top of the column was adjusted before injecting 0.4 % aceton and evaluating the peak asymmetry to control the column packing. The asymmetry was 0.84 before and 0.94 after the first run and 1.22 after five runs. The compression of the gel is most likely not due to high pressure since the pressure was quite constant and low during all runs. One explanation to the compressed gel could be that the CIP at 1 M NaOH is at a higher concentration of NaOH than the gel can withstand. Another explanation could be that the column was not packed hard enough.

4.3 Q Superdex 75 prep grade

Two different ligand concentrations were tested on the Q Superdex 75 prep grade; 324 µmol Q/ml and 216 µmol Q/ml. The counter-ion was changed to Cl^- and a desalted plasmid DNA sample was used in the same way as previous tests and the flow rate was 60 cm/h. The results are shown in Table 6.

Table 6: *The table presents the results obtained from Q Superdex 75 prep grade media with Cl^- as counter-ion and a desalted plasmid DNA sample.*

*Media**	*Size (µm)*	*Conc. Q (µmolQ/ ml gel)*	*Counter-ion*	*Elutes with 1 M NaCl Yield*	*Elutes with 1 M NaOH*	*Tot. Recovery*	*Capacity (mg pDNA/ ml gel)*	*Comments*
Q Super 75	22-44	324	Cl^-	12 %	13 %	25 %	>7.44	Stop injection high pressure
Q Super 75	22-44	216	Cl^-	12 %	82 %	94 %	3.73	

* Q Super 75 = Q Superdex 75 prep grade

A summary of the chromatograms for the different media tested in this study is shown in Figure 17 (page 31).

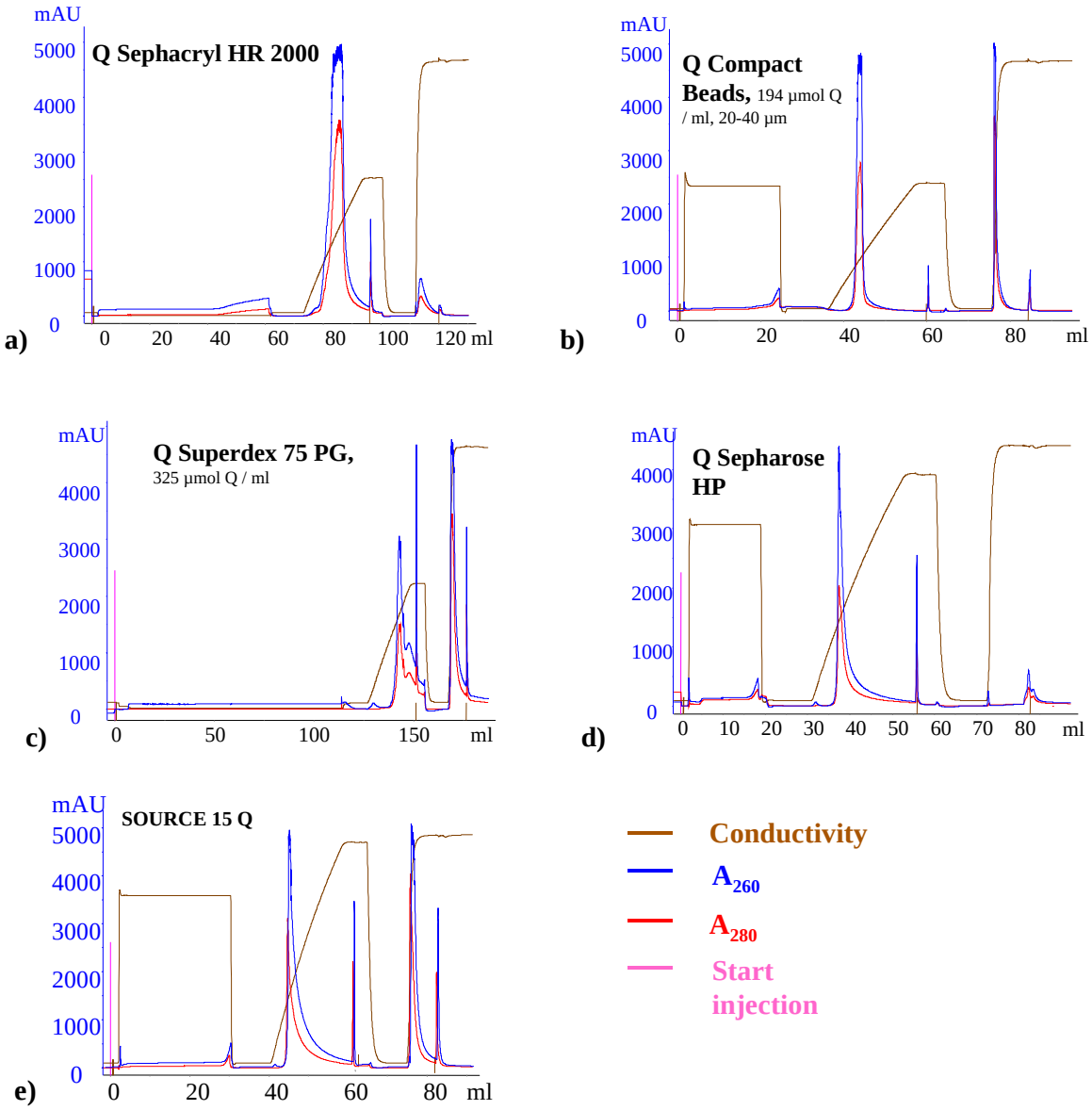


Figure 17. Examples of chromatograms from the different media, which have been tested in this study. *The chromatograms illustrate the conductivity as well as the 260 nm and 280 nm absorbance from which the capacity, yield and recovery can be calculated. In a) and c) desalted plasmid DNA sample and 1 M NaCl gradient was used. In b) the plasmid DNA was not desalted and 1M NaCl gradient was used. In d) and e) plasmid DNA was not desalted and 2 M NaCl gradient was used.*

5 Discussion

5.1 Q Compact Beads

Of the Q Compact Beads media tested the one with 194 µmol Q/ml and a size between 20 – 40 µm is preferable as less problems with pressure were experienced and a higher recovery was obtained. The capacity for plasmid DNA on Q Compact Beads, 194 µmol Q/ml size 20 - 40 µm, is approximately 50 % better than for the original media (Q Sepharose High Performance). Q Sepharose High Performance beads are about the same size as the Q Compact Beads and have about the same amount of Q ligands (170 compared to 194 µmol Q/ml). The higher capacity might therefore be due to the less porous surfaces of the Compact Beads (illustrated in Figure 13), which results in a larger active surface for ligand plasmid DNA interactions. Higher ligand concentration or smaller beads could achieve an even higher capacity. Those changes would probably result in pressure problems if Q Compact Beads were used in the same way as in this study. Pressure problems would also make the media unsuitable for large-scale processes.

The pressure problems could be due to fines in the Compact Bead gel. Fines are very small beads which can block the space between the Compact Beads and thereby the flow path for the plasmid DNA, or block the filter at the end of the column. The occurrence of fines in the Compact Beads media (shrunken Sepharose High Performance) is shown in Figure 12. The sieving method was not optimal and very time consuming which led to the presence of fines in both the 20-40 µm and < 20 µm fractions of Compact Beads. A better classification procedure is therefore needed to be able to evaluate Compact Beads properly. One possible classification procedure could be elutriation using expanded bed column.

The yield for Q Compact Beads is between 30 – 50 %, which was substantially lower than the desired 85 %. One possible reason for the low yield could have been hydrophobic interactions between the plasmid DNA and the media. To check this, a 25 % less cross-linked Compact Bead matrix was tested. 1 or 5% EtOH in the solutions was also tested (data not shown). No substantial improvement in results was noted.

The biggest improvement obtained by shrinking the original Q Sepharose High Performance media to the Q Compact Beads media is the increased recovery and a sharper elution peak (see Figure 17 and Table 3). The Q Sepharose High Performance media had very poor recovery compared to Q Compact Beads which could be due to the plasmid DNA getting ‘stuck’ in the Q Sepharose High Performance pores, a principle discussed earlier.

5.2 Q Sephacryl High Resolution

Increasing porosity for the Q Sephacryl High Resolution gels showed increasing capacities (see Table 4). This effect is most likely due to larger pores, which increase the media surface accessible to the plasmid DNA since the molecules can enter further into the beads. The large increase in capacity from Q Sephacryl 1000 High Resolution to Q Sephacryl 2000 High Resolution might be caused by the ability of the plasmid DNA to diffuse through the larger pores more freely. This combined with also having a larger available surface area makes more ligand accessible to the plasmid DNA.

The elution peak during the salt gradient is also sharper (less tailing) for Q Sephacryl 2000 High Resolution than for the other Sephacryl gels (see Figure 15), which indicates a simpler binding mechanism leading to faster kinetics. This agrees with the previous observation that the pores might be large enough for the whole plasmid DNA to enter and not only a part of the plasmid.

5.3 Q Superdex 75 prep grade

Q Superdex 75 prep grade with ligand concentration 324 µmol Q/ml gel was tested first (see Table 6). The Plasmid DNA sample was injected until the pressure passed the maximum value acceptable for the gel. The capacity was therefore not calculated from 20 % breakthrough but as the amount of plasmid DNA loaded on the gel before the pressure became too high. The gel was compressed during the last part of the sample injection, most likely due to the high viscosity because of the large amount of plasmid DNA in the column, but expanded almost completely after the CIP. The capacity is very high (> 7.44 mg plasmid DNA/ ml gel) at the same time as the recovery and yield are very low. One possible explanation is that the Q ligands bound too strongly to the plasmid DNA by more Q-groups binding to the same plasmid DNA molecule than for previously tested media. Another explanation could also be that the poor yield and recovery are due to the plasmid DNA becoming entangled in the dextrans tentacle like structure.

The Q ligand concentration was lowered (to 216 µmol Q/ml gel) for the next Q Superdex 75 prep grade that was synthesized. The lower ligand concentration was used to examine whether this had a positive effect on the yield and recovery as well as having no large decrease of the capacity (Table 6). The lower ligand concentration had a positive effect on the recovery (increased from 25 % to 94 %), but a negative effect on capacity (almost half the previous capacity) and did not have any effect on the yield. The lower capacity was expected since the lower ligand concentration decreases the number of binding sites. The increase in recovery could be due to less ligands binding to the same plasmid DNA molecule. The lower ligand concentration did not affect the yield, which might indicate that the dextran density and/or the media's porosity affect the yield more than the ligand concentration. The low yield could be due to the beads still having pores covered with dextran, which the plasmid DNA can still enter partly and get stuck in.

5.4 Conclusions

The prototype media Q Compact Beads, Q Sephacryl High Resolution and Q Superdex 75 prep grade were evaluated during this study. The Q Superdex 75 prep grade media with 324 µmol Q/ml gel has by far the highest capacity. It has, however, a low yield and recovery and also a wide elution peak. The Q Sephacryl 2000 High Resolution has relatively high capacity, yield and recovery at the same time as it has a sharp elution peak. The Q Compact Beads media with 194 µmol Q/ml gel has also a very sharp elution peak and relatively high yield (but still far from 85%) and recovery but has a binding capacity, which is lower than for the other prototype media.

Both the Q Sephacryl 2000 High Resolution and the Q Compact Beads media result in sharper elution peaks during the salt gradient than the Q Sepharose High Performance, SOURCE 15Q and Q Superdex 75 prep grade (Figure 17). This could be explained by a simpler binding

mechanism, which leads to faster kinetics for media with hardly any pores or very large pores due to plasmid DNA not partly entering the pores. The Q Sephacryl High Resolution and the Q Compact Beads media also show very small elution peaks after 30 min of incubation in NaCl. This supports the theory of plasmid DNA getting stuck in the pores in media with medium sized pores, since the small peaks after incubation indicates that the elution of plasmid DNA is not dependent on the time the salts has to diffuse into the beads.

The goal is to find a chromatographic media with a binding capacity of 5 mg plasmid DNA/ ml gel and with a yield > 85 %. The media needs also to be scalable since it is of interest for use in a large-scale plasmid DNA purification process. The goals were not met in this study but the results indicate possible solutions to the problem of finding a chromatographic media for plasmid DNA purification.

One possible solution could be to increase the pores to even larger than the pores in Q Sephacryl 2000 High Resolution media. The recovery after the first run could thereby be improved at the same time, as the capacity for the following runs might not be lowered.

Another possible solution to a new chromatographic media for plasmid DNA purification could be to bind Q coupled dextran chains to Compact Beads. The Q coupled dextran chains would increase the accessible area for ligand – plasmid DNA interactions. The use of Compact Beads could at the same time avoid plasmid DNA from getting enboxed in the pores as well as retaining the Compact Beads' favourable flow properties.

A third way to come closer to meeting the set goals for a chromatographic media for plasmid DNA purification could be to optimize the ligand. None of the tested media (either prototype or reference) have shown a high yield (> 85%) in this study. An optimization of the ligand and ligand concentration could result in higher yields, since the ligand as well as the media affects the yield. The Q ligand tested in this study binds strongly to negatively charged plasmid DNA (thereby making it hard to elute the plasmid DNA) and might be a contributing factor to the low yield. A ligand with weaker binding to the plasmid DNA could therefore be preferred.

6 Acknowledgements

My deepest thanks to my supervisors David Buckley and Raf Lemmens for guidance, encouragement and support during experiments and the report writing. I also wish to thank Lena Sandberg and Annika Andersson for helping me out in the lab. Thanks Josef Vasi for supplying me with cellpaste, Dag Lindström for supplying me with Sephacryl High Resolution media (and pictures), Ulrika Skarp for picture and Fredrik Elwinger for helping me with the NMR experiments. Thanks also to Tomas Nyhammar and Philippe Busson for helpful suggestions, proofreading and pictures. Special thanks to Elisabet Linde, Rudolfo Kurt-Fuentes and Helena Hedlund for support and encouragement during my time at Amersham Biosciences. Finally, I wish to thank Jörgen Samuelsson and my scientific reviewer Mikael Berg.

7 References

Antal, M., Micko, M. (1992), Preparation of microcrystaline cellulose amino derivatives, *Carbohydrate Polymers*, **19**(3), 167-169.

Benjamini, E., Coico R. & Sunshine, G. (2000), *Immunology a short course* (4th Edition). Wiley-Liss, New York.

Eon-Duval, A. (2003), Large-Scale manufacturing of plasmid DNA for gene therapy and DNA vaccination -- Part 1: The suitability of current techniques to purify plasmid without adding rNase, *BioPharm International* (Aug.), 48-56.

Ferreria, G. N. M., Monterio, G. A., Prazeres, D. M. F. & Cabral, J. M. S. (2000a), Downstream processing of plasmid DNA for gene therapy and vaccine applications, *Trends in Biotechnol.* **18:9**, 380-388.

Ferreria, G. N. M., Prazeres, D. M. F., Cabral, J. M. S & Schleef, M. (2001), Plasmid manufacturing – an overview. In M. Schleef (Ed) 2001. *Plasmids for therapy and vaccination*. Wiley-VCH, Weinheim.

Ferreria, G. N. M., Cabral J. M. S. & Prazeres D. M. F. (2000b), Studies on the batch adsorption of plasmid DNA onto anion-exchange chromatographic supports, *Biotechnol. Prog*. **16**, 416-424.

Gel filtration: principles and methods. 2002: Amersham Biosciences. Handbook Code No: 18-1022-18.

Horn N. A., Meek, J. A., Budahazi, G. & Marquet, M. (1995), Cancer gene therapy using plasmid DNA: purification of DNA for human clinical trials, *Human Gene Therapy*, **6**(5), 565-573.

Ion exchange chromatography – principles and methods. 2002: Amersham Biosciences. Handbook Code No: 18-1114-21.

Janson, J-C., Rydén, L. (1989), *Protein purification: principles, high resolution methods and applications*. VCH Publishers, New York.

Levy, M. S., O'Kennedy, R. D., Ayazi-Shamlou, P. & Dunnill, P. (2000), Biochemical engineering approches to the challenges of producing pure plasmid DNA, *Trends in Biotechnology* **18**, 296-305.

Lemmens, R (2004) Amersham Biosciences. Personal communications.

Lemmens, R, Olsson, U., Nyhammar, T. & Stadler J. (2003), Supercoiled plasmid DNA: selective purification by thiophilic/aromatic adsorption, *J Chromatogr. B* **784**, 291-300.

Ljunglöf, A, Bergvall, P., Bhikhabhai, R. & Hjorth, R (1999), Direct visualization of plasmid DNA in individual chromatography adsorbent particles by confocal scanning laser microscopy, *J. Chromatogr. A,* **844**, 129-135.

Meyer, F. (2003), Gene based therapies for the treatment of severe chronic diseases, oral presentation at 6th Interlaken conference on advances in production of biologicals, March 25-28, 2003. Interlaken, Switzerland.

Porath, J. (1982), Gel product for separation, patent EP 81-850244.

Protein purification. 1999: Amersham Pharmacia Biotech. Handbook Code No: 18-1132-29.

Reimann, J., Kwissa, M. & Schirmbeck, R. (2001), Genetic vaccination with plasmid vectors. In M. Schleef (Ed) 2001. *Plasmids for therapy and vaccination*. Wiley-VCH, Weinheim.

Sambrook, J., Russel, D. W. (2001), Molecular cloning: a laboratory manual, Cold Spring Harbor Press, New York.

Schmidt, T., Friehs, K. & Flaschel E. (2001), Structures of plasmid DNA. In M. Schleef (Ed) 2001. *Plasmids for therapy and vaccination*. Wiley-VCH, Weinheim.

Scopes, R. K. (1994), *Protein purification principles and practice* (3rd Edition). Springer-Verlag, New York.

Sofer, G, Hagel L. (1997), *Handbook of process chromatography: a guide to optimization, scale-up, and validation*. Academic Press, San Diego.

Urthaler, J. (2003), *Large scale preparation of pDNA from* E. *coli by a continuous combination of cell lysis, clarification and capturing*, poster presented at the 6th Interlaken conference on advances in production of biologicals, March 25-28, 2003. Interlaken, Switzerland.

Werner R. G., Urthaler, J., Kollmann, F., Huber, H., Necina, R. & Konopitzky, K. (2002). pDNA- From process science to commercial manufacture, *Contract Service Europe* March, 34-40.

Wils, P., Esricou, V., Warnery, A., Lacroix, F., Lageneaux, D., Ollivier, M., Crouzet, J., Mayaux, J. F. & Sherman, D. (1997), Efficient purification of plasmid DNA for gene transfer using triple-helix affinity chromatography, *Gene Therapy* **4**, 323-330.

Gene therapy clinical trials: http://www.wiley.co.uk/genetherapy/clinical/, (29 Dec. 2003).