Nucleic Acids: Innovative Methods for Recovery, Clarification and Purification

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Abstract:

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The lysis process is the first critical step, since it can be detrimental to the nucleic acids and it also can release all the content from the cells by its disruption. A new approach was developed in order to minimize this effect and reduce the main host cell contaminants. The electropermeability approach for extraction of small nucleic acids from bacteria avoids complete cell disruption and thereby reduces contaminants for the next stages.

The clarification and concentration steps are normally done to remove most of the contaminants from the cell lysis steps. In this thesis an aqueous two-phase system (ATPS) approach based on poly(ethylene glycol)/poly(acrylate)/salt two phase system is described. This method allows the handling of large volumes, which is important in the preparative industrial scale. ATPS resulted in a pure plasmid DNA directly from crude cell lysates. In addition, smaller DNA fragments from Polymerase Chain Reaction (PCR) can be isolated using this method.

The regular downstream process is normally finished by chromatographic approaches, which often are the main final purification step. DNA molecules harbour some intrinsic chemical properties that render them suitable for chromatographic separations. These include a negatively charged phosphate backbone as well as a hydrophobic character originating mainly from the major groove of DNA which exposes the base pairs on the surface of the molecule. In addition, single stranded DNA often allows for a free exposure of the hydrophobic aromatic bases. In this thesis, multimodal chromatography (MMC) was evaluated as an alternative tool for complex separations of nucleic acids. MMC embraces more than one kind of interaction between the chromatographic ligand and the target molecules. This resin demonstrated a superiority for DNA isolation, including in the purification of pDNA from crude cell lysate and for DNA fragments from PCR samples.

Key words: DNA, plasmid DNA, lysis, electropermeability, aqueous two-phase system, multimodal chromatography.

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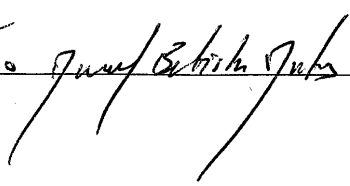
Signature  Date 2013-12-11

Nucleic Acids:

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"Não sou nada,
"I'm nothing,
Nunca serei nada,
I'll never be anything,
Não posso querer ser nada.
I can't wish to be anything.
À parte disso, tenho em mim todos os sonhos do mundo."
Apart from that, I have in me all the dreams of the world."

Álvaro de Campos / Fernando Pessoa

Popular Summary - *English*

...GTAAAAATTAAGCACAGTGGAAGAATTTCATTCTGTTCTCAGTTTTCCTGGAT TATGCCTGGCACCATTAAAGAAAATATCTTTGGTGTTTCCTATGATGAATATAGATACA GAAGCGTCATCAAAGCATGCCAACTAGAAGAG[1].... The string of these block letters (A, T, C and G) is a tiny part of the human DNA sequence, which is more than 3 billion letters long. The combination of these block letters carries genetic information, similar to how the combination of letters form words. Genes, long DNA molecules, are how living organisms receive features from their ancestors. The sequence of DNA molecules of many different organisms is now known, including the human genome completed in 2003. The studies of the human genome are revealing disease origins and other biochemical mysteries. As a consequence of this, scientists came closer to new and better means of diagnosing and treating genetic disorders. The genetic disorders are diseases caused by one or more abnormalities in the genes, from hereditary origin and/or caused by the environment. The appearance of gene therapy, i.e. the insertion in the cells of the new genes missing, or by the expression of a precise gene sequence that produces a specific protein missing, opened new perspectives of treatments. The common process for the production of these therapeutic genes is bacteria-based. Therefore, efficient methods for recovery and purification of these products are needed in order to fulfill basic patterns for being acceptable for gene therapy use. Starting with electropermeability extraction of nucleic acids as a recovery approach, by clarification with aqueous two-phase systems and multimodal chromatographic methods for DNA purification were the aims of this thesis.

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1 The sequence presented in the beginning of the text corresponds to a region of the genome that differs in people who have the genetic disorder *cystic fibrosis*. The most common mutation happens with the loss of three consecutive Ts from the gene sequence, resulting in a single amino acid within a protein chain of 1480 amino acids.

Resumo Científico – *Português*

...GTAAAAATTAAGCACAGTGGAAGAATTTCATTCTGTTCTCAGTTTTCCTGGATTATGC
CTGGCACCATTAAAGAAAATATCTTTGGTGTTTCCTATGATGAATATAGATACAGAAGC
GTCATCAAAGCATGCCAACTAGAAGAG[1]....A cadeia destas letras (A, C, T e G) representa uma pequena parte da sequência de ADN humano, que é constituido por mais de 3 biliões de letras de comprimento. A combinação deste bloco de letras transporta a informação genética, semelhante à combinação de letras na formação de palavras. Os genes, longas moléculas de ADN, são a forma como os organismos recebem características dos seus antecessores. A sequência das moléculas de ADN de vários organismos são agora conhecidos, incluindo o genoma humano completo em 2003. Estudos realizados no ADN humano revelam a origem de doenças e outros mistérios bioquímicos. Como consequência destas descobertas, é possivel agora uma nova e melhor interpretação de diagnósticos e tratamentos de doenças de ordem genética. Doenças de ordem genética são doenças causadas por uma ou mais anormalidades nos genes, de origem hereditária e/ou causadas pelo meio ambiente. O aparecimento da terapia génica, a inserção de novos genes em falta ou a expressão de um específico gene que leva à produção de uma determinada proteína em falta, abriu novas possibilidades de tratamento. O processo comum de produção desses genes terapêuticos é baseado em produção em bactérias. Por isso, é necessário métodos eficientes para recuperar e purificar esses produtos, preenchendo os requisitos básicos para serem aceites em tratamentos de terapia génica. Os objectivos desta tese iniciaram-se com extracções de ácidos nucleicos por electropermeabilização de bactérias, passando pela clarificação de amostras por sistemas de duas fases aquosas e finalmente pela purificação das mesmas por cromatografia multimodal.

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1 A sequencia apresentada no inicio do texto corresponde a uma região do genoma que difere entre pessoas que possuem uma alteração genética chamada *fibrose cística.* A mutação mais comum acontece com a perda de três Ts consecutivos na sequência génica, resultando na perda de um amino ácido numa proteína com uma cadeia total de 1480 amino ácidos.

Abstract

The importance of nucleic acids in pure form for preparative and analytical perspectives has increased constantly, demanding the development of new and more efficient methods for their recovery and isolation. This thesis describes a series of different innovative methods for recovery and purification of these biomolecules. In a general overview of a downstream process there are several critical steps that may influence the yield and quality of the final product, especially in the lysis, clarification and purification steps.

The lysis process is the first critical step, since it can be detrimental to the nucleic acids and it also can release all the contents from the cells by its disruption. A new approach was developed in order to minimize these effects and to reduce the main host cell contaminants. The electropermeability approach for extraction of small nucleic acids from bacteria avoids complete cell disruption and thereby reduces contaminants for the next stages.

The clarification and concentration steps are normally done to remove most of the contaminants from the cell lysis steps. In this thesis an aqueous two-phase system (ATPS) approach based on poly(ethylene glycol)/poly(acrylate)/salt two phase system is described. This method allows the handling of large volumes, which is important in the preparative industrial scale. ATPS resulted in a pure plasmid deoxyribonucleic acid (pDNA) directly from crude cell lysates. In addition, smaller DNA fragments from a Polymerase Chain Reaction (PCR) can be isolated using this method.

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List of Papers

This thesis is based on the following publications, referred to in the text by Roman numerals. The publications and manuscripts are appended to the thesis.

I. **Nucleic acids and protein extraction from electropermeabilized *E. coli* cells on a microfluidic chip.** T. Matos; S. Senkbeil, A. Mendonça, J. A. Queiroz, J. P. Kutter; L. Bülow. Analyst 138 (7347-7353) 2013.

II. **Plasmid DNA partitioning and separation using poly(ethyleneglycol)/poly(acrylate)/salt aqueous two-phase systems.** H. Johansson, T. Matos, J. Luz, E. Feitosa, C. C. Oliveira, A. Pessoa Jr, L. Bülow, F. Tjerneld. Journal of Chromatography A 1233 (30-35) 2012.

III. **Isolation of PCR DNA fragments using aqueous two-phase systems.** T. Matos, H. Johansson, J. A. Queiroz, L. Bülow. Separation and Purification Technology 122 (144-148) 2014.

IV. **Plasmid DNA purification using a multimodal chromatography resin.** T. Matos, J. A. Queiroz, L. Bülow. Journal of Molecular Recognition – Accepted 02-12-2013.

V. **Binding and elution behaviour of small deoxyribonucleic acid fragments on a strong anion-exchanger multimodal chromatography resin.** T. Matos, J. A. Queiroz, L. Bülow. Journal of Chromatography A 1302 (40-44) 2013.

VI. **CaptoTM resins for DNA binding and elution: A tiny difference in ligand composition makes a big impact in separation of guanidyl containing fragments.** T. Matos, E. T. Mohamed, J. A. Queiroz, L. Bülow. Submitted 2013.

VII. **Preparative isolation of PCR products using mixed-mode chromatography.** T. Matos; G. Silva, J. A. Queiroz, Leif Bülow. Submitted 2013.

Papers not included on this thesis:

VIII. **Unwinding of supercoiled plasmid DNA by human haemoglobin.** S. Chakane; T. Matos; L. Bulow. Manuscript.

IX. **Mapping conditions for plasmid DNA downstream processing: pH, salt and time effects.** T. Matos; E. T. Mohamed; C. Dicko; K. Schillén; J. A. Queiroz; L. Bülow; Manuscript.

My contribution to the papers:

Paper I — I took a major part in planning, performed the experimental work after chip production and wrote the manuscript with assistance from the co-authors.

Paper II — I took part in the pDNA production and experimental analysis and co-wrote the manuscript.

Paper III — I took substantial part in planning, took part in experimental design/work and wrote the manuscript with assistance from the co-authors.

Paper IV — I took major part in experimental design/work and wrote the manuscript with assistance from the co-authors.

Paper V — I took major part in experimental design/work and wrote the manuscript with assistance from the co-authors.

Paper VI — I took major part in experimental design/work, supervised the experimental work and wrote the manuscript.

Paper VII — I took major part in experimental design/work, supervised the experimental work and wrote the manuscript with assistance from the co-authors.

Abbreviations

AC – Affinity Chromatography

AEX – Anion Exchange Chromatography

ATPS – Aqueous Two-Phase System

AU – Arbitrary Units

B. Burgdorferi – *Borrelia Burgdorferi*

BCA assay – Bicinchoninic Acid assay

CMV – Cytomegalovirus

C. tetani – *Clostridium tetani*

DNA – Deoxyribonucleic acid

dNTP – Desoxyribonucleotide triphosphate

dsDNA – double-stranded deoxyribonucleic acid

E. coli – *Escherichia coli*

FDA – Food and Drug Administration agency

gDNA – genomic DNA

GFP – Green Fluorescent Protein

GT – Gene therapy

HIC – Hydrophobic Interaction Chromatography

HIV – Human Immunodeficiency Virus

IEX – Ion-Exchange Chromatography

LAL – Lymulus amebocyte lysate

LC – Liquid Chromatography

LMCV – Lymphocytic Choriomeningitis Virus

MMC – Multimodal Chromatography or Mixed-Mode Chromatography

M. Tb. – Mycobacterium Tuberculosis

mRNA – messenger Ribonucleic Acid

NaPAA – Sodium Polyacrylate

oc pDNA – open circle plasmid DNA

PCR – Polymerase Chain Reaction

pDNA – plasmid DNA

PEG - Polyethylene Glycol

Poly dA – Polydeoxyadenylate

Poly dC – Polydeoxycytidylate

Poly dG – Polydeoxyguanylate

Poly dT – Polythymidylate

RNA – Ribonucleic acid

rRNA – ribosomal Ribonucleic acid

S. typhi – *Salmonella typhi*

sc pDNA – supercoiled plasmid DNA

SDS-Page – Sodium Dodecyl Sulphate Polyacrylamide-gel electrophoresis

SEC – Size Exclusion Chromatography

sRNA – small Ribonucleic Acid

ssDNA – single-stranded Deoxyribonucleic Acid

Toxo. gondii – *Toxoplasma gondii*

tRNA – transfer ribonucleic acid

Contents

Introduction and Aims

In 1869, Doctor Friedrich Miescher, a young Swiss doctor working at the University of Tübingen, Germany, performed experiments on leukocytes in order to identify chemical compounds in the cell nucleus. At that time, this young scientist discovered a new substance hitherto unknown[1,2]. This substance was rich in phosphorous and nitrogen atoms, and its particularly acidic characteristics led him to conclude that it was neither a lipid nor a protein. Since those characteristics were unique, and the compound was present in the nucleus, Miescher termed the enigmatic compound "nuclein"[2]. Back then, Miescher believed that the presence of the nuclein in the nucleus created an important chemical difference that set the nucleus apart from the cytoplasm. Later in 1874, his publication stating the presence of nuclein in the sperm of various vertebrates caused some interest in the scientific community[2].

The work on nuclein continued in many scientific laboratories and most of the scientists followed Miescher. Later on, Richard Altman, a student of Miescher, developed a new method for extraction of nuclein in larger quantities. This new method for nucleic acids extraction, as he named it later, allowed further studies on the molecular level[3]. Later, Albrecht Kossel discovered the nucleic acid composition: four bases and sugar molecules. His work earned him a Nobel Prize in Medicine[2]. However, it was Phoebus Levene, in 1919, that identified the base, sugar and phosphate nucleotide unit, suggesting that deoxyribonucleic acid (DNA) consisted of a string of nucleotide units linked together by phosphate groups[4]. Although those results were remarkable, at that time the scientific community still believed that proteins were the main target to study, since they were combined of 20 different amino acids, while nuclein was made up of only four different nucleotide combinations[2].

The interest in DNA revived in the 1940s and early 1950s. At that time, many scientists presented interesting results demonstrating that DNA was the carrier of genetic information[5,6]. In 1953, James D. Watson and Francis Crick determined the structure of DNA and its possible role in the transfer of genetic information[7]. Moreover, Singer *et al.* cracked the genetic code, and this was the point where it became clear how the information for creating the variety of organisms could be encoded in a single molecule composed of only four different building blocks[2]. DNA became known as we know it today, a linear polymer of desoxyribonucleotides linked by phosphodiester bonds. The twisting of two anti-parallel DNA strands, which are connected by hydrogen bonds between complementary nucleotides in each strand, results in a right-handed double helix structure. This structure presents an average of 10.5 base pairs (bp) per turn, with highly hydrophobic grooves accessible to solvent and ligand molecules[8,9].

Science erupted around DNA and many scientific fields are dependent on it, such as molecular biology, biochemistry, molecular evolution, forensic biology, diagnosis of hereditary diseases, genome mapping, sequencing projects, gene therapy (GT), DNA vaccination and others[10–13].

In all these fields, there is a need of obtaining pure nucleic acid molecules to allow efficient and effective analyses and applications. This demand for new and efficient approaches for isolation and purification of nucleic acids, clearly indicates needs for generation of new strategies. Pure products, e.g. for therapeutic agents or substances developed for research, generally require multi-step purifications. In addition, the cost of such a process is usually a major contributor to the total production cost. Thus, the creation and development of novel methods for recovery and purification of nucleic acids molecules are instrumental for gene technology and related technologies.

The improvements to existing methods, or the development of new techniques, belong to the aims of this thesis. Based on a typical downstream processing, new methods have been developed in each step of the process as demonstrated in Figure 1.

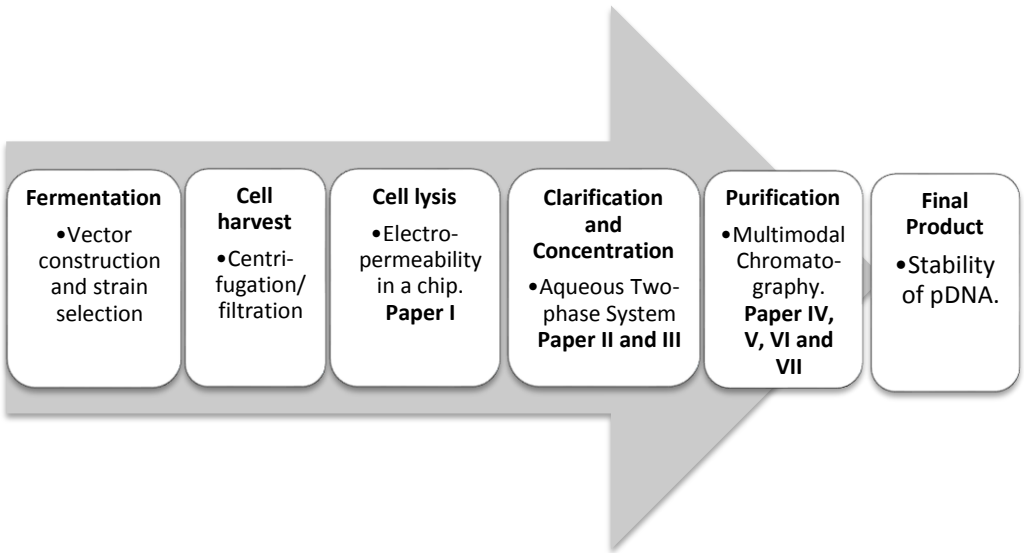

Figure 1 - Downstream processing scheme showing general techniques in the array. In bold, the newly developed methods and the references to the papers in this thesis.

1. Target biomolecules and techniques

Gene therapy and Nucleic Acids

Many techniques are nowadays based on our knowledge of nucleic acids. The recombinant DNA technology and gene cloning developed during the 80's resulted in an increase in genomics data during the 90's. This contributed to the definition of some disease-causing genetic factors and to the exploration of new treatments based on engineered genes and cells[14,15]. Gene Therapy (GT) was one of the new techniques emerging from this molecular biology and biotechnology revolution[14].

GT consists of transfer of nucleic acids to somatic cells of a human patient in order to promote a therapeutic effect, either by correcting genetic defects or by overexpressing proteins that are therapeutically useful[16,17]. It represents an innovative drug delivery system making use of the technical and scientific advantages of, among others, microbiology, virology, organic chemistry, molecular biology, biochemistry, cell biology, genetics, and genetic engineering. It is more than "gene transfer", which is only a part of the complex multiphase process of identification, manufacturing, preclinical and clinical testing of GT products[18]. The theoretical advantages of GT are undisputable, but the practical results have not lived up to the expectations. After almost 10 years of many promising trials, one chocking case occurred in Philadelphia, known as the "Gelsinger case". Jesse Gelsinger's death was caused by a severe immune reaction to the adenoviral vector, the same vector used in 372 other trials at the time[19]. Not only the scientific community was taken aback with such a tragic case, also Food and Drug Administration agency (FDA) and other federal governments took measures to re-examine the entire process to prepare better and safer alternatives[20]. The toxic side-effects, limitation on the transgene size, and potential insertional mutagenesis started to be considered

problematic[21]. Until that time, the existing technologies could not meet the practical needs for such GT experiments. The efficiency and safety of GT are largely dependent on the development of a vector or vehicle that can selectively and efficiently deliver a gene to target cells with minimal toxicity.

The vectors are divided into two main classes: viral and non-viral. The first attempts of GT treatments were based on the ability of viral vectors to deliver genetic information to human cells[22]. The most commonly used viral vectors are Adenoviruses, Adeno-associated viruses, Retroviruses (including Lentiviruses), and to a lesser extent, Herpes simplex virus and *Vaccinia* virus[23]. Although gene transfer by genetically modified viruses is advantageous due to its high transfection efficiency and stability[24], the safety concerns remain present.

Several novel non-viral vectors approaches have been developed, resembling the efficiency of viruses[25]. The simplest approach to nonviral delivery systems is the direct gene transfer with naked plasmid DNA (pDNA). Initially, pDNA was thought to function simply as a shuttle system for the gene, resulting in the *in situ* production of antigen (for vaccines) or therapeutic protein (for GT applications)[17]. Wolff *et al.* [26] were the first demonstrating the long-term gene expression in mouse skeletal muscle with direct intramuscular injection of pDNA encoding a viral antigen in 1990[16].

The DNA vaccine is the prime example of a modern genetic vaccine. It is a non-protein-based vaccine exploiting mechanisms other than traditional vaccines. In order to induce appropriate immune response from an antigen, the DNA vector encoding the antigen, is injected into the human body. The genetically engineered DNA vaccine is then "transcribed" and produces specific proteins (antigens) of a disease. Since the produced pathogenic proteins are not recognized as their own, the immune system is alerted, which then triggers a range of immune responses. After a primary immune response to destroy the pathogen, an immunity memory for the disease is acquired[9].

One of the major advantages associated with the use of pDNA-based vaccines is that DNA vaccination stimulates both the antibodies and cell-mediated components of the immune system, whereas conventional protein vaccines usually stimulate only the antibody response[17]. However, the technologies for generating immunogenicity in humans

still need optimization. The "explosion" in the scientific literature around DNA-based vaccines proves that pDNA is easy to manipulate and hence rapid to construct, a critical attribute for making vaccines, for example against cancer[16] or an emerging pandemic threat[17]. There are to date, 780 completed or active trials utilizing DNA vaccines (*NIH,* http://www.nih.gov*, accessed June 28th, 2013*). Some DNA-based vaccines have demonstrated immunogenicity and/or protection/therapy in preclinical models as a single agent[17], some of which are in Table 1. Yet, the approved treatments are still only for animals[27,28].

Table 1 - Examples of diseases for which DNA-based vaccines have been evaluated. Adapted from Liu et al.[17]

Viruses	HIV; Influenza; Rabies; Hepatitis B, C; Ebola; Herpes simplex; Papilloma; CMV; Rotavirus; Measles; LCMV; St. Louis encephalitis; West Nile virus;
Bacteria	B. Burgdorferi; C. Tetani; M. Tb.; S. Typhi;
Parasites	Malaria; Mycoplasma; Leishmania; Toxo. gondii; Taenia ovis; Schistosoma;
Cancer	Breast (Her2/neu); Colon; Prostate; Myeloma; E7-induced; Lymphoma; Fibro sarcoma;
Allergy	House dust mite; Experimental airway hyper responsiveness (Asthma); Peanut;
Autoimmune diseases	Diabetes; EAE (Multiple sclerosis model)

The existing synthetic vectors, such as naked DNA, cationic liposomes, and others, are far from being perfect delivery systems[29], especially due to the low efficiency. This demands a large-scale production of pDNA for treatments, especially since the recent GT progresses in genetic vaccination trials, which became even more important with the European approval for first treatments[30–32]. When successful, it is believed that DNA vaccines will be able to mimic live viral infections and it is hoped that it will have an important impact on future vaccination.

Polymerase Chain Reaction

The polymerase chain reaction (PCR)[33,34] is one of the most useful techniques for the amplification of genetic sequences[10,35,36], originally developed by Kary Mullis in 1983, who later was awarded the Nobel Prize in Chemistry together with Michael Smith in 1993[33]. This technique is today used in many fields, such as molecular evolution, molecular biology, forensic biology, diagnosis of hereditary diseases, genome mapping and sequencing projects[10,13]. The technique is based on *in vitro* DNA amplification using several polymerization cycles. This consists of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. It is based on three temperature-dependent steps: DNA denaturation, primer-template annealing, and DNA synthesis by a thermostable DNA polymerase.

Many optimizations of the technique have been described to achieve higher specificity and to extend the area of application[35–38]. One of main problems after the process is the purity level of the target product. If possible, the DNA amplified during the PCR process should be totally pure, avoiding the contamination from the rest of primers, desoxyribonucleotide triphosphate (dNTPs), Taq polymerase and other associated proteins. Some commercial kits are available on the market allowing the purification of these products. However, all of them are time-consuming and the economic aspects of using these kits must also be considered.

Papers III and **VII** present two different alternatives for PCR product purification and they will be described in Chapters 3 and 4, respectively.

Plasmid DNA

The plasmids in bacteria can provide vital proteins and enzymes to the cell, and spread the genetic information from an individual bacterial cell to a population of bacteria. Plasmids are also used in laboratories as DNA vehicles to insert new genes into bacteria in order to produce recombinant proteins. By definition, pDNA are extrachromosomal molecules in which the two ends of the double-stranded DNA are covalently linked, forming a closed loop[39]. Each strand of the pDNA molecule is a linear polymer of desoxyribonucleotides linked by phosphodiester bonds. The winding of the two anti-parallel DNA strands around each other and around a common axis generates the classic right-handed double helix structure, which is stabilized by hydrogen bonds between adenine-thymine (AT) and guanine-cytosine (GC) base pairs and by stacking forces. The inside of the double helix is highly hydrophobic due to the close packing of the aromatic bases, while the phosphate groups turns the surface negatively charged at pH above 4 (Figure 2)[39].

The pDNA vectors used for GT and DNA vaccination are typically in the range between two and several hundred kilo base pairs (kbp), meaning that they are very large molecules[39]. The pDNA molecules can adopt different topologies and vary in size and structure. The most common form is a highly ordered structure, denominated as supercoiled plasmid DNA (sc pDNA). This structure is formed when the circular DNA molecule is under- or overwound around its molecular axis, resulting in superhelix structures with different topological conformations (topoisomers)[8]. The sc pDNA is tight and compact and both strands are intact. It is often considered as a nanoparticulate material with the length in the order of hundreds of nanometers and a diameter between 9.9 and 13.4 nm[9]. The degree of supercoiling depends on the action of the supercoiling enzymes present in the cells, typically DNA gyrase (a type II topoisomerase). The bacterial cells utilize circular DNA and exploit supercoiling as a space-saving mechanism. Furthermore, negative supercoiling is a way of preparing the DNA for replication[40]. The breaking of one of the strands creates an open circular plasmid DNA (oc pDNA), and if both strands are broken, linear pDNA is formed. The nicking can occur by mechanical shearing, nucleases or by high temperature. However, the oc pDNA can also be

generated without strand damage, just from relaxing of the sc pDNA[9]. The pDNA can also adopt different multimeric forms, concatemers, such as when two or more sc pDNA (or oc pDNA) join[9].

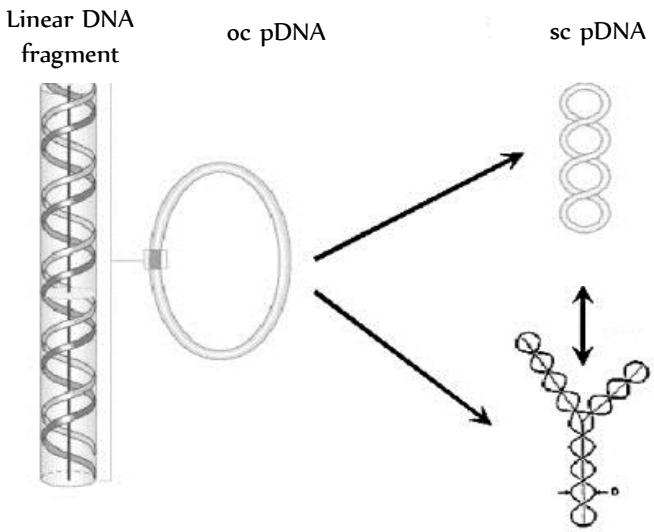

Figure 2 - Different topological structures of pDNA: the oc and the sc isoform. Reprinted from Ghanem A. *et al.*[9].

A common pDNA construct consists of an origin of replication that controls the pDNA copy number per bacterial cell, a gene coding for antibiotic resistance and a complete eukaryotic expression cassette composed of the transcribed region domain inserted between an enhancer and terminator[41]. The region is flanked by restriction sites, allowing the insertion of the desired genes. This formulation leads to an increase of protein production or enhancement of the immune response when GT applications are considered. Additionally, the conformation of the pDNA may contribute significantly to increasing the biological activity of the expressed protein(s)[42].

Plasmid production and downstream processing

Plasmid DNA is commonly produced in *Escherichia coli* (*E. coli*) cells, followed by a downstream process for its purification. The early process of designing a pDNA vector and choosing a host must consider the demands for obtaining high yields. This often results in large-scale production and needs to anticipate some of the recurrent obstacles during fermentation and downstream processing[43]. For instance, the choice of a high-copy-number plasmid as a starting vector has a great impact on the plasmid yield. Furthermore, the influence of a well-developed fermentation process together with a well-defined host strain can improve the yield of pDNA. In addition, it also reduces the amount of contaminants, such as RNA and genomic DNA (gDNA)[44,45], and improves the stability of the pDNA. It is also essential to select strains that produce low levels of nucleases[46].

The major bottlenecks in the production of pDNA are often encountered during the downstream process operations, which are essentially aimed to eliminate other cellular components of the host strain. An overview of the downstream process for purification of pDNA is presented in Figure 3.

Downstream processing starts with the recovery of cells from the broth by centrifugation or microfiltration[47]. The cells are then resuspended and concentrated in an appropriate buffer for the following step, the lysis. The lysis process is basically a disruption in order to release the cellular contents. This will be described further in Chapter 2. The lysis results in a complex mixture of all cellular constituents including some cell debris. Most of the initial processes developed for pDNA recovery are already adapted to reduce and avoid many of these contaminants. The clarification and concentration steps are thus designed to remove most non-desirable nucleic acids and proteins from the host, and to reduce the volume prior to the chromatography steps. Many systems have been developed and adapted for this step, such as isopropanol precipitation and ammonium sulphate precipitation of proteins[48], membrane filtration[49] or aqueous two-phase systems, that will be described in Chapter 3.

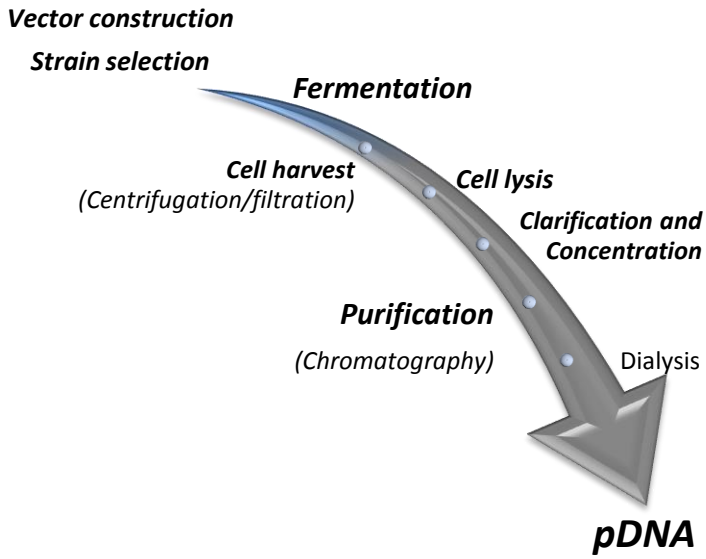

Figure 3 - Process flow sheet for large-scale purification of plasmid DNA.

Although the clarification and concentration steps provide a cleaner and smaller process stream, it is still necessary to include a final purification step. This will permit a higher degree of purity of the final product, especially considering the regulatory agencies demands. At this stage of the process, the majority of impurities are RNA, gDNA fragments, endotoxins and different pDNA variants. The similarity between all these molecules renders the purification more demanding. Chromatography is therefore commonly used as a final step of pDNA purification, and this will be discussed in detail on Chapter 4.

Even though there is not one universally accepted definition of purity, it is most correct to talk about removal of specific contaminants in the context of nucleic acids purification. Indeed, the regulatory agencies specify and recommend specific assays assessing the sample purity, safety and potency in order to obtain a pure pDNA sample, as shown in Table 2.

Table 2 - FDA demands for pDNA purity.

Requirement	Analytical assay	Specification
Proteins	BCA assay SDS-PAGE	Undetectable <0.01 µg/dose
RNA	Agarose gel electrophoresis	Undetectable
Endotoxins	LAL assay	<0.1 EU/µg plasmid
Plasmid homogeneity	Agarose gel electrophoresis	>90% supercoiled
gDNA	Agarose gel electrophoresis Southern blot	Undetectable <0.01 µg/dose
Plasmid identity	Restriction mapping Sequence homology	Expected restriction Sequencing, PCR
Plasmid potency	Cell transfection efficiency	According to application

Stability of plasmid DNA

Whatever techniques are used to produce and purify pDNA, another critical aspect is the stability and conformation of the final sample. The sc pDNA is naturally present in the cytoplasmic space of the host cell, and is involved during replication, recombination and transcription[50,51]. Likewise, the sc isoform is regarded as the most active and efficient form for transfection and gene expression in the eukaryotic cells, since it is the intact, smaller and undamaged form of pDNA[52,53]. A nick in one or both of the sc pDNA strands leads to the total relaxation of the structure or to linear pDNA,

respectively[52]. In this case, the resulting isoforms are less efficient in the gene expression induction, especially if a nick has occurred in the promoter or in the gene coding regions[52]. The steps during the downstream process must to be taken in account in order to ensure the maintenance of the sc pDNA structure. For instance, it is generally established that the genomic material is adaptive under high temperatures in prokaryotic cells, leading to nucleic acid thermostability and stability[54]. Yet the same does not occur with the stability of the pDNA structures. For instance, the temperature increase from 4 to 24°C rises the internal motion promoting the unwinding of the structure and thereby resulting in harmfully effects on the structure[55]. On the other hand, as described by Kunugi *et al.*[56], high hydrostatic pressurization *in vitro* can positively affect the formation of sc pDNA. Indeed, the high pressurization induced supercoiling of relaxed pDNA and the associated compaction of sc pDNA, suggests that this approach can be a potential technique for preparation of pDNA suitable for GT[57].

The effects that organic solvents, temperature, salts and pH variations have on the sc pDNA structure can be irreversible[58]. These severe treatments can lead to the relaxation of the structure and the final product thereby does not fulfil the regulatory agencies' demands and consequently cannot be further used for pharmaceutical purposes. Some studies have reported on the denaturation of DNA structures under very low pH values and/or high temperatures, leading to the disintegration of the DNA structure and the release of free nucleotides[59]. Furthermore, the alkaline lysis process can promote adverse effects on the pDNA structure. If the pH is maintained below 12.5, the sc pDNA starts to unwind as a consequence of the alkaline-promoted hydrogen bond disruption. Still, the anchor base pairs remain intact and prevent the complete separation. The renaturation is done by pH neutralization. However, at pH values higher than 12.5, the anchor base pairs may be lost, leading to irreversible denaturation[60].

Structure and stability of the single- and double-stranded DNA

Oligodeoxynucleotides, in single- and double-stranded forms (ss- and dsDNA), can generate different structures and conformations depending on their nucleotide composition. As described previously, polydeoxyadenylate (poly(dA)) spontaneously forms a helix while polydeoxyguanylate (poly(dG)) generates a quadruplex structure[61]. Similarly, polydeoxycytidylate (poly(dC)) molecules are folded into i-tetraplexes while polythymidylate (poly(dT)) shows extensive regions of hairpins by intra-strand base-paring, exposing the negative charged phosphates of the DNA backbone[62]. These structures influence the charged and hydrophobic characteristics of the molecules and thereby their behaviour on a chromatographic material.

The structural stability presented by ss- or dsDNA is different. Firstly, the stability of a double-stranded DNA is known to be higher compared with a single-stranded version with the same nucleic acid composition in a water-content buffer[63]. The different degrees of hydrophobic exposure of the single- and double-stranded DNA promote different reorientation, and as well, different interactions with a chromatographic ligand. This specific difference of behaviour was explored during the work of this thesis. The multimodal chromatography approach (Chapter 4.3) explores the difference of charge density and hydrophobicity, resulting in ionic and hydrophobic interactions between DNA molecules and the chromatographic ligand. The resulting conformational changes lead to different hydrophobic and ionic expositions of ss- and dsDNA[64]. As a result of these biophysical differences, the separation between them using this chromatography modality turns possible. These separations are presented in **Paper V** and **Paper VI**.

Ribonucleic acid

Besides DNA, also ribonucleic acid (RNA) is part of the nucleic acids group. The main differences between DNA and RNA are the presence of ribose instead of deoxyribose (due to a hydroxyl group attached to the pentose ring in the 2' position) the largely single-stranded constitution and the presence of uracil instead of thymine. The hydroxyl groups make RNA less stable since it becomes more prone to hydrolysis, and often with a shorter half-life after being extracted from tissues or cells[3]. Although RNA is assembled as a chain of nucleotides, it is usually present as a single-stranded structure. In agreement with DNA, most biologically active RNAs contain self-complementary sequences that allow parts of the RNA to fold and pair with itself thereby forming regions with double helices. Highly structured molecules can therefore be formed[65,66]. Whenever RNA forms a double helix, the presence of this conformation causes the helix to adopt to the A-form geometry rather than the B-form which is most commonly observed in DNA (A- and B-form differ significantly in their geometry and dimensions, although they still form helical structures)[67]. This results in a very deep and narrow major groove and a shallow and wide minor groove[68,69].

RNA represents a large family of biological molecules involved in multiple vital roles. There are several types of naturally occurring RNA including ribosomal RNA (rRNA), messenger RNA (mRNA), transfer RNA (tRNA)[3] and small RNA (sRNA). The RNA molecules play an active role within cells by catalysing biological reactions, controlling gene expression, or identifying and communicating these responses into cellular signals. For instance, the protein synthesis is one of the main roles for RNA in the cell, which is initiated by reading the mRNA molecules in the ribosomes. Each codon is detected by anticodon present on the tRNA that carries and delivers the corresponding amino acid. The rRNA promotes the link between amino acids and forms the proteins, which is biosynthesized from N-terminus to C-terminus.

In addition, the sRNA, including the pathways of RNA interference[70] (RNAi – involved in central eukaryotic posttranscriptional regulatory mechanisms[71]) and the microRNA (miRNA - regulating gene expression pathways), defends the organisms against transposable elements and viruses and guides genome rearrangements. The rRNA

and sRNA have become new targets in the design of therapeutic products and for the development of novel gene-based therapies, especially due to their role played in many biological processes. For instance, the tRNA is a key drug target for antibacterial treatments. The rRNA component has been known to be a suitable target receptor for antibiotics in clinical practice. Indeed, it can be used as therapeutic target for gene silencing[71–73], instead of the traditional protein treatments[74,75]. Although many mechanisms are known, there are several additional phenomena involving different kinds of RNA that still not have been fully explained[76,77].

RNA production and downstream process

Just as in the case of pDNA, the advances in our understanding of the RNA roles call for the development of new strategies for higher efficiency in RNA isolation and purification. When RNA production is intended for large-scale, the main process is largely following the same procedure as for pDNA (Figure 4). RNA needs special care and precautions for its isolation, since it is highly susceptible to degradation. RNA is especially unstable due to the ubiquitous presence of RNases, enzymes present in blood, tissues and as well in most basteria[3].

The recovery of RNA is initiated by opening of the cells, often *E. coli* cells, by conventional lysis processes. However, RNA is not isolated by the same method as pDNA. The first report of attempting RNA purification dates back to 1960, when Kurland and colleagues performed the first RNA isolation[78]. Nowadays, the most common protocol for RNA extraction is called acid guanidinium thiocyanate-phenol-chloroform. The guanidinium thiocyanate is a strong protein denaturant, that inhibits the RNase activity and leads to high yields of the final RNA product. In this process, the DNA is removed after multiple centrifugations[78]. Although the method is often used, it is time-consuming. Since RNAs are highly sensitive molecules, especially due to the presence of RNAses in solutions, a fast process is needed to avoid degradation. A faster process thus often leads to better results for the purity of the final product, including higher yields and activity levels.

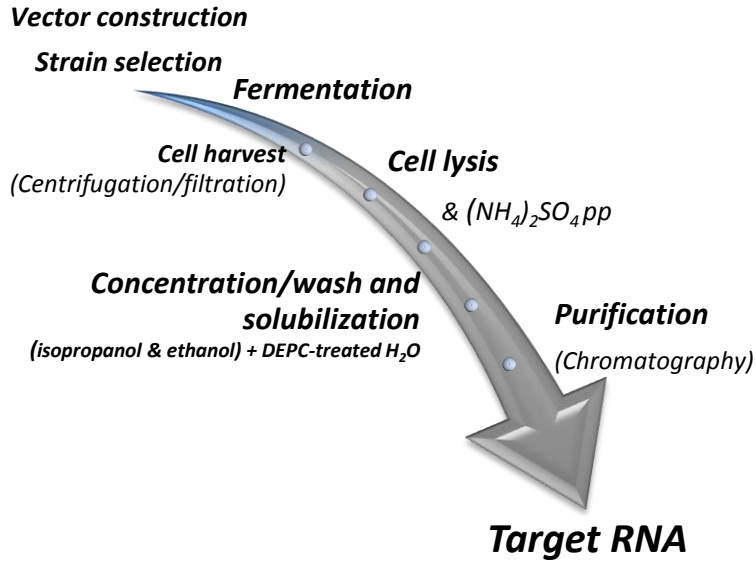

Figure 4 - Process flow sheet for the large-scale purification of RNA.

In **Paper I,** a simple, fast and efficient way for small RNA molecules extraction, which can be followed by a chromatographic step for different RNA molecules separation[79], is presented. The extraction is achieved by electropermeability (Chapter 2.1), avoiding most of the contaminants from a conventional cell disruption such as sonication, French press or enzymatic cell opening. In addition, by promoting the recovery of the RNA molecules in a fast process, it is also possible to secure the stability of the molecules.

Proteins

Proteins are large biological molecules consisting of one or more polypeptide chains, formed by combination of 20 different amino acids and linked together by peptide bonds between the carboxyl and amino groups of the adjacent amino acid residues. The amino acid sequences of proteins are dictated by the nucleotide sequence of the genes and the translation process, resulting in a three-dimensional structure that determines its activity. Shortly after or even during synthesis, the residues in a protein are often chemically modified by posttranslational modifications. This alters the physical and chemical properties, such as folding, stability, activity, and ultimately, the function of the proteins. Proteins perform a variety of functions in living organisms such as catalysing metabolic reactions, replicating DNA, stimuli responding and transport of molecules. In this thesis focus is put on a particular protein, a variant of the green fluorescent protein (GFP), the GFPuv, which will be described in the following subtopic.

GFP, first reported in 1955, was originally isolated from the jellyfish *Aequorea victoria*[80]. Nowadays it is used in a variety of applications, for example as a marker for gene expression[81], proteins [82], protein expression reporter[83], specific cells or tissues marker[84]. A substantial number of GFP mutants have been constructed, via single or multiple gene modifications. GFPuv, used in **Paper I,** is a GFP variant, with three amino acids substituted. It is composed of 238 amino acids (26.9 kDa) and exhibits bright green fluorescence (508 nm) upon excitation by long-wavelength UV light (399 nm)[85]. This variant is 18-fold brighter than the wild-type GFP. Furthermore, GFPuv is efficiently expressed in *E. coli* and soluble[86], in contrast to native GFP, much of which frequently is found as inclusion bodies. The three-dimensional structure of GFPuv consists of tightly packed β-sheets, which form the walls of a cylinder, and a helix running diagonally through the can. The fluorophore, or chromophore, which is responsible for the fluorescent properties, is present in the center of the protein. By enclosing the chromophore in the cylinder, it promotes protection and this strategy is presumably responsible for the stability of GFP[87]. The overall protein structure is shown in Figure 5.

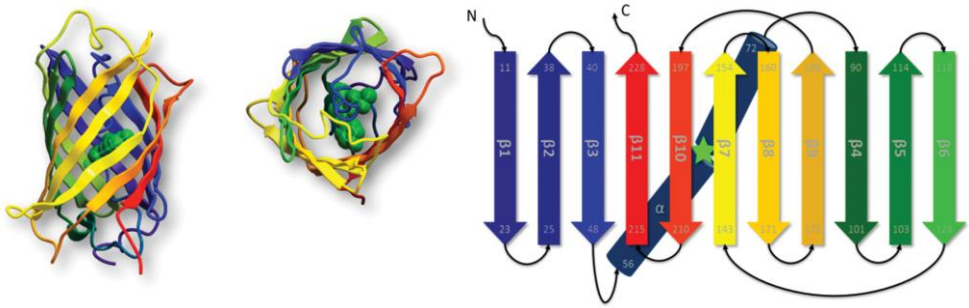

Figure 5 - The three-dimensional structure of GFP. The dense β-sheet cylinder surrounds the fluorescent fluorophore. The very well-defined structure has a cylinder diameter of 30Å and a length of 40Å. Reprinted from Crone *et al.*[88].

GFP is resistant to heat, alkaline pH, detergents, photobleaching, chaotropic salts, organic salts, and many proteases[87]. The robust character of the protein makes it a good target for modification and development of new purification steps. Many approaches have therefore been used for the production and purification of GFP or GFP-protein complexes.

The main lysis process is based on techniques that often are time-consuming, a factor that has the most important influence on protein stability, activity and yield values. In **Paper 1** a novel method is presented for extraction of small nucleic acids and proteins based on electropermeability in a chip, which avoids the long lysis processes. If GFPuv is released from the cells, it is thereby also possible to estimate the minimum size of the pores that is generated after exposed to the electric field. In addition, due to the well-defined fluorescent properties of this protein, the fluorescent signal can be used to quantify the total extraction of protein from the cells. Under specific voltage conditions, the migration of GFPuv can occur to the surrounding medium without complete cell disruption. This leads to an easier purification process, since only channels are formed on cell membranes and most of the contaminants present in conventional processes can be avoided. Moreover, this innovative method turns the process faster and easier, and as a result, it turns the next purification steps more simple. Further information is described on Chapter 2 and **Paper 1**.

2. Bacterial cell disruption

When developing purification methods for biomolecules it is essential to consider the starting material, usually a bacterial suspension. The type of cells involved and the disruption method are the main critical issues for the downstream process.

Based on the cell wall structure, bacteria are divided in to gram-positive and gram-negative. The cell wall promotes not only the protection of the cell but, as well, works as a regulation agent for the exchange of products between the cell and the surrounding environment[89]. The chemical and structural compositions of the cell wall are in both types of bacteria similar, although it is much thinner in gram-negative bacteria. The peptidoglycan (murein) layer is responsible for the strength of the wall[90]. The gram-negative bacteria have a two-layer membrane structure with a periplasmatic space between them. The outer membrane is composed of proteins, phospholipids and lipoproteins while the inner membrane has lipopolysaccharides covering a rigid peptidoglycan layer[91]. When it concerns gram-positive bacteria, multiple layers are associated by a small group of amino acids and amino acid derivatives, forming the glycan-tetrapeptide, which is repeated many times through the wall and which is connected by penta-glycine bridges[92].

There are several developed protocols for cell disruption, which are normally chosen depending primarily on the type of bacteria and which is the target biomolecule or component[93]. Figure 6 illustrates schematically what kinds of cell disruption methods that are available. Although mechanical methods are easily scalable, they are less specific and useful for isolation of biomolecules, since they are dependent on high energetic inputs, leading to elevated temperatures. In many instances, this also results in partial of full denaturation of the biomolecules.

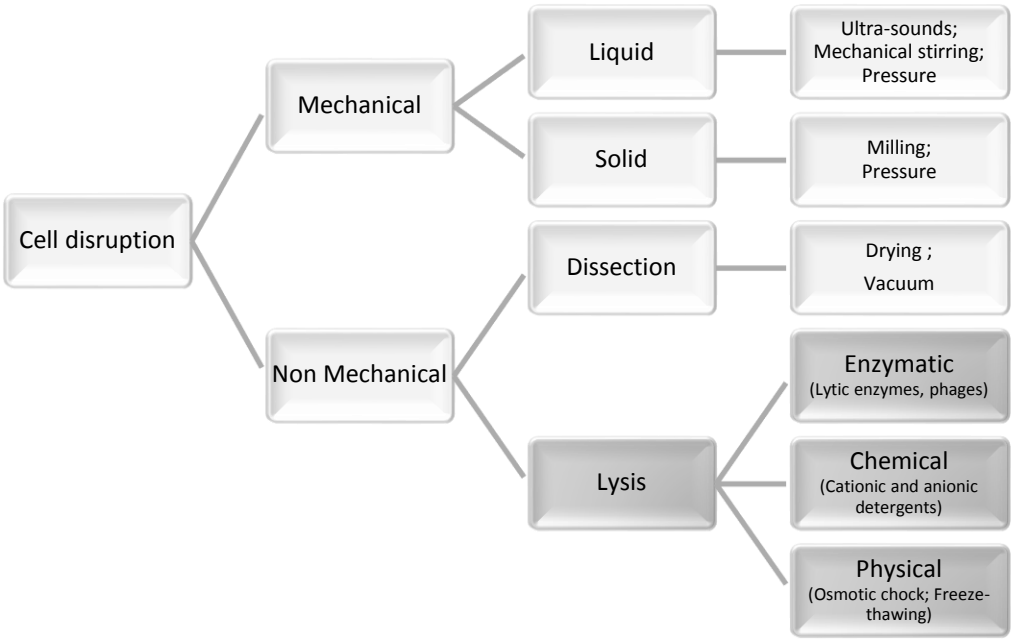

Figure 6 - Schematic model for cell disruption with some examples.

Non-mechanical processes appear to be the easiest approach for sensitive biomolecules. In the case of nucleic acids and proteins, which are large but fragile molecules, gentle disruption methods need to be employed[94]. The lysis processes have been developed to minimize denaturation effects. This especially holds true for the enzymatic, physical and chemical techniques[91], which make them more attractive for large-scale purposes. Enzymatic processes are attractive since they are more selective for the main target biomolecule and due to the high yields that often can be achieved. The enzymes act directly on the cell membrane under specific pH and temperature conditions. Yet, this is a complex process, and a mixture of different kinds of enzymes is needed.

Chemical processes promote the breakage of cell walls, due to the addition of acid or alkaline solutions, surfactants and/or organic solvents. These compounds promote the dissolution of the cell membrane. However, it must to be taken into consideration which

secondary effects that can be conferred on the target biomolecule, especially shifts in pH can be detrimental to nucleic acids. Due to the intrinsic characteristics of the bacterial envelope, the modified alkaline lysis developed initially by Birnboim *et al.*[95], has become the most commonly explored method for cell lysis in order to extract pDNA. This method involves the use of high pH values and detergents to dissolve the lipid cell membrane and release of the cellular compounds from cytoplasm. A few additional steps are also included in order to precipitate proteins and gDNA.

Physical methods using osmotic, temperature or electrical approaches have also become popular. For instance, in the case of stable proteins, freeze-thawing and sonication are commonly used methods.

In a smaller scale, micro- and nano-scale techniques have been developed for isolation of nucleic acids. In some cases have even steps for separation and purification[96] been combined. Basically they consist of microfluidic chips for cells electro-lysis. These nano- and micro-techniques lead to a more directed approach to a specific cell sort, resulting in a more selective lysis. This appears to be initiated by the destabilization of the lipid bilayer of the bacterial cells, leading in the end to a total disruption. These methods have previously been described both for purification of DNA[97] and low molecular weight RNAs[98]. However, these processes can sometimes be disadvantageous due to the sensitive character of nucleic acids.

Other alternative non-mechanical methods, developed for the same propose, are listed in Table 3.

Table 3 - Overview of methods for cells lysis.

Method	Principle	Characteristics
Osmotic shock lysis	Shifting from high to low osmotic values.	Used mostly for proteins and nucleic acids from blood cells. Simple and inexpensive, but yields are often low.
Chaotropic salts lysis	Cell membrane disruption by creating less hydrophilic environment and weaking hydrophobic interactions.	Can also be used for primary nucleic acid isolation. Denaturation of nucleases and proteases.
Enzymatic digestion	Digestion of cell wall together with osmotic disruption.	High yield values but slow and expensive. Is often combined with freeze-thaw cycles.
Detergent lysis	Detergents solubilize cell membranes	High yield values.

Electropermeabilization

Due to the recurrent limitations, especially due to time and costs involved, there is a need for development of more versatile approaches, especially for sensitive biomolecules such as nucleic acids and proteins. In **Paper 1** a novel approach for RNA, GFPuv and pDNA extraction is described. The electropermeability method presented, is based on electroporation, a procedure well known since 1980s[99–102], and which has been a widely explored[103–105].

The electroporation technique was initially developed for cell transformation and pore formation[102,106]. However, it has recently been adapted for other fields, for example

in tumour ablation[107]. The electroporation is based on a membrane destabilization phenomenon, that has been described as Maxwell deformation[102]. When voltage is applied, the lipids will reorganize and a channel is formed. Due to the presence of water, exposure of the hydrophobic sites on the molecules can thereby be minimized. The transient channels created then allow for the migration of molecules from the cytoplasm to the surrounding medium. These channels have recently been studied by Heimburg[108]. Membranes exposed to voltages lead to the formation of hydrophilic pores with sizes reaching 1 nm in diameter, allowing ion and water migration[108]. These pores are reversible as described previously[109]. For example, Pogodin *et al.* have characterized the permeation of small molecules through lipid membranes, where permeability is particularly dependent on the size of the target molecule[110].

Using a simple microfluidic channel with integrated gold electrodes, and using a very low applied voltage, destabilization of the membrane lipid chain is promoted (Figure 7) (**Paper 1**). The pore formation leads to migration of the intracellular compounds, which move freely in the cytoplasm. To this group of compounds belong the diffusible small RNA molecules, the small proteins produced in the cell, such as GFP, and other small molecules and metabolites. Since it is a reversible process, the pores retrocede after the electrical destabilization. This leads to a cleaner starting extract of the stated molecules since no total cellular disruption occurs. Thereafter, further purification steps can be done easily since most of the contaminants will not be present, such as cell debris and other large contaminants.

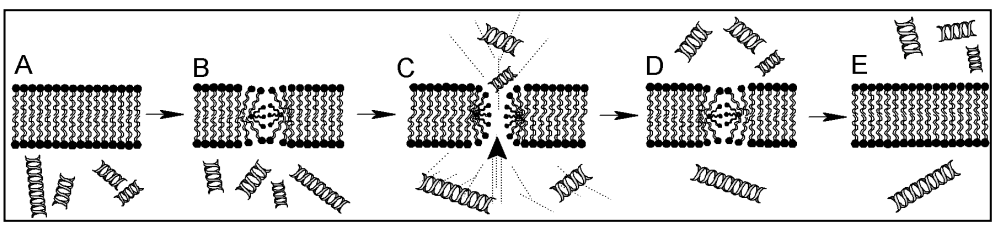

Figure 7 - Chemical state transition scheme for the molecular rearrangements of the lipids in the pore edges of the lipid vesicle membrane. A: denotes the closed bilayer state. The external electric field causes ionic interfacial polarization of the membrane dielectrics analogous to condenser plates. B: The induced membrane field, leading to water entrance in the membrane to produce pores. In the pore, the lipid molecules are turned to minimize the hydrophobic contact with water. C: The open pore will allow the transitions of molecules and ions from both sides of the membrane. D: The membrane stabilizes slowly after moving out from the electrical field. E: The membrane returns to the initial conformation. Reprinted with authorization from Paper I.

However, the electropermeability is limited to a given size of a biomolecule. In case of larger molecules such as pDNA, the approach was also successfully applied, however it occurred only by increasing the voltage over the cells, which leads to cell disruption. In this case, other contaminants are present, and additional purification steps are needed.

This method represents a way for extracting small RNAs and proteins. These biomolecules are sensitive for extended recovery and purification methods. In case of RNA molecules, the release of RNases in traditional methods is cumbersome. The new method presented can be cleaner and faster. Furthermore, denaturation processes can largely be avoided, since the samples can be directly purified by e.g. chromatography. This way, it is possible to achieve a faster process increasing the yields of the desirable protein, as shown for GFP. The adaptive method for cell disruption shows that it is possible to use the same system with different molecules. The larger molecules can hence be obtained by lysing the cells in a faster way compared to a regular method, while the smaller can be released by transient pores.

3. Aqueous Two-Phase Systems

Much attention has been paid to the development of separation methods suitable for both preparative and analytical studies of biochemical systems. In this particular perspective, gentle methods, especially based upon simple physical phenomena have proved particularly useful when dealing with biomolecules. For instance, filtration and centrifugation, solid-liquid separation techniques, are strongly dependent on the particle size and density. However, this becomes a limitation for small bacteria and microorganisms after cell disruption. These techniques must be complemented by methods in which other biophysical properties, such as surface properties, comprise a separation parameter. One of these methods is partitioning in a liquid-liquid two-phase system.

Processes based on liquid-liquid separation can improve the efficiency and yield of the process and bypass previous limitations. The principle of these separation systems was discovered by Beijernick who observed agar and gelatine forming two phases when mixed[111]. However, it was much later, in 1956, that Albertsson directed the knowledge from the two-phases to partitioning of molecules[112].

The aqueous two-phase systems (ATPS) are formed when two water-soluble substances are mixed above certain concentrations, but with a high water content (>75%). The aqueous conditions are suitable especially for particles and macromolecules with biological origin[113]. These systems can be based on different components, including organic solvents or polymers. However, organic solvents may also promote denaturation of the target biomolecules[113], which can be avoided by adding different components such as polymers, e.g. dextran and poly(ethyleneglycol) or poly(acrylate) and poly(ethyleneglycol). One of the most important factors for the success of this technique is the possibility to easy scale-up the system. As a primary recovery step, ATPS allows handling of large volume of particular matter which is especially important for

pharmaceutical purposes. Besides the high efficiency when handling soluble and insoluble particles[90,113], it is also a simple and benign technique. Likewise, it is a fast process with a rapid mass transfer (low interfacial tension) that can permit selective separation (affinity partition). A wide variety of biomolecules have been purified using ATPS, such as proteins[114–117], pDNA[118,119], cells[112] and organelles[120].

The basis for separation by two-phase systems is the selective distribution of substances between the phases. For soluble substances, distribution takes place mainly between the two bulk phases, and the partitioning is characterized by the partition coefficient, *K*

$$K = \frac{Ct}{Cb}$$

where *Ct* and *Cb* are the concentrations of the partitioned substance in the top and bottom phase, respectively. Ideally, the partition coefficient is independent of concentration and also independent of the volume ratio of the phases. It is mainly a function of the properties of the two phases, the partitioned substance and temperature[121]. The interface between the phases should, however, also be considered, since it has a certain capacity for adsorption of the partitioned substance. Therefore, in the separation of cellular particles or molecules there are in fact three "phases" to consider: the upper, the inter- and the lower phase. It is the selective distribution between these phases which forms the basis for separation of particles by a two-phase system[121].

The choice of a suitable phase system is the key step in all partitioning work. However, problems arise when a phase system has to be selected for biogenic particles and macromolecules. The choice of phase system must consider water content, ionic composition, osmotic pressure, ability to elute out substances from the particles, denaturing effects, etc[121]. In principle, most of the hydrophilic naturally occurring or synthetic polymers miscible in water will show phase separation in a mixture with a second polymer or salt. Yet, the two-phase system will only arise when the constituents are present within a certain range of proportions.

The constituent compositions at which phase separation occurs may be represented by a biphasic phase diagram of a mixture of two polymers or a polymer and salt, as shown in Figure 8.

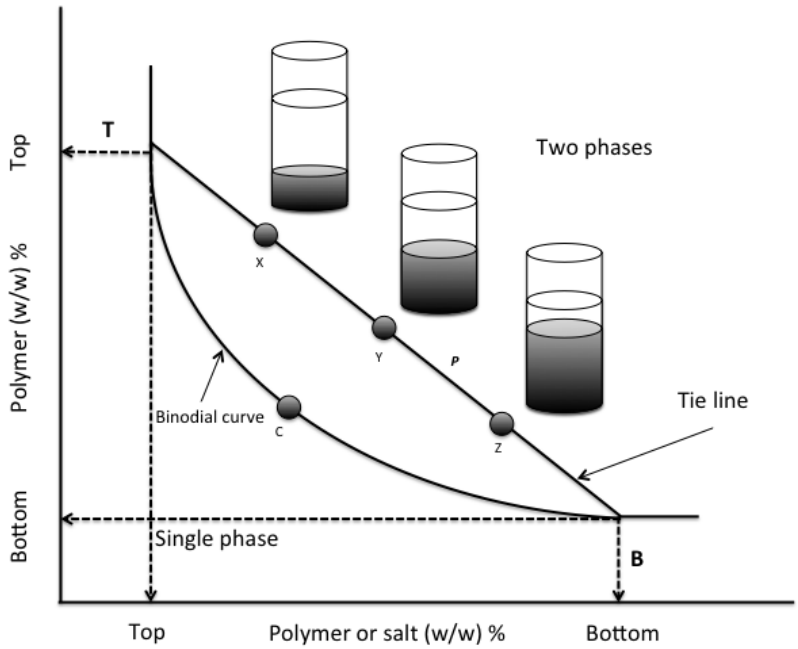

Figure 8 – A typical phase diagram for polymer-polymer or polymer-salt ATPS. In the figure: C - critical point; T - composition of the top phase; B - composition of the bottom phase; X, Y and Z - total composition of ATPS.

The mixture of immiscible polymer-polymer, or polymer-salt, at a certain concentration (P) above the binodal curve, will create a separation of phases, into one top phase and one bottom phase. Below the *binodal* curve, there is no phase formation. The more the composition of the phase system approaches point *C* (critical point), the smaller is the difference between the two phases. At the critical point the compositions and the volumes of the two phases theoretically become equal. The *tie line* connecting the two nodes at the *binodal* curve gives the polymer concentration in the top phase (T) and bottom phase (B). The total mass of the phase system corresponds to the length of the *tie line* and thus the mass of the top phase is the distance between B and P, while

the distance between T and P corresponds to the mass of the bottom phase. However, they all have the same top phase equilibrium composition and the same bottom phase equilibrium composition. Thereby, is possible to combine the system in order to obtain more or less concentrated samples, depending on the volume and top phase or the bottom phase where the biomolecule have migrated.

The partition is to a large degree dependent of three factors: effective hydrophobicity, density of the phases and electrochemical driving force. Qualitatively it can be described as: when a particle or molecule is suspended in a phase, it will interact with the surrounding molecules in a complex manner. Various bonds, such as hydrogen, ionic and hydrophobic bonds are involved, together with other weak forces. Their relative contributions are difficult to estimate, however, their net effect is likely to be different in the two phases[121–124].

The PEG/salt systems are less expensive, but rather low molecular weight PEGs (typically less than 600 g/mol) must be used for pDNA partitioning to the PEG-rich phase. Recently, a two-phase system composed of PEG, sodium polyacrylate (Na-PAA) and salt was developed and used as a separation tool for proteins[114,116]. Contrary to the PEG–dextran system, this system is dependent on pH and salt content and is relatively insensitive to temperature. The driving force for separation seems to be an enthalpic effective repulsion between the charged carboxylate groups of polyacrylate and ethylene oxide units of PEG. The addition of salt is necessary to allow compartmentalization of the polyelectrolyte into one of the phases. Although this system contains more complex driving forces of enthalpic and entropic nature than the PEG–dextran system, it has the advantage of being much less expensive and more practical, for instance by the relatively low polymer content necessary to induce two-phase formation.

Influence of pH, salt, temperature and other biomolecules

In order to find a suitable system for the biomolecule of interest, a number of parameters can be varied, such as electrochemical, hydrophobicity, size, pH, temperature, salt, biomolecule conformation and concentration. The partitioning of a specific biomolecule can thus be affected by introducing a salt or by varying pH of the system[113]. In case of charged molecules, the pH has a direct effect by altering the ratio of charges, and thereby influencing partitioning, or even in the polymer-polymer complexation, which in case of NaPAA is sensitive to pH variations[125,126]. In case of pDNA, which is negatively charged above pH 4, pH has a profound effect on the partitioning. The pDNA is preferentially partitioned into the top PEG-rich phase, whereas at pH 5.4 or 5.8 a more uniform partitioning is observed (**Paper II**). The type of salt can also influence the partitioning[114]. Depending on the charged biomolecule, the partition coefficient factor will be affected by increasing or decreasing of salt. In case of neutral salts such as NaCl, the effect is not drastic at low concentrations (<1 M). However, at higher values the phase diagram alters the partition coefficient due to the differential distribution of salt between the phases[127]. Yet, it can as well increase directly the partitioning of the compound[114]. Moreover, the biomolecule properties have a direct impact on the partitioning, due to e.g. the hydrophobicity level on the surface of the molecule.

Another aspect which needs to be considered, is the influence of temperature, since in some cases lower temperatures allow faster phase separation[121]. In case of polymer-polymer systems, the temperature influence is negligible. In systems close to the binodial curve, the temperature effect must to be examined and controlled in order to achieve reproducible partitioning. In other hand, the polymer-salt systems are relatively insensitive to temperature changes.

Phase system properties

The partitioning of a biomolecule in an ATPS is highly dependent not only on the type of polymer-polymer or polymer-salt used, but as well on the molecular weight of the polymer. Higher molecular weight polymers are expected to create higher viscosity. However, this effect is partly compensated by the fact that lower concentrations are required for phase separation[127]. Also, a larger difference in molecular size between the two polymers generates an asymmetrical binodial curve[121].

Another way of changing the partitioning of a substance in an ATPS is to increase the polymer concentration, leading to an increase of the tie-line length. This will lead to a more extreme partitioning of the biomolecules towards one of the phases, mainly due to the fact that polymer phases differ more in composition at higher concentrations than close to the binodal curve[121]. This principle was used in **Paper III**. In a polymer-salt system, the partitioning of biomolecules is governed by volume exclusion effects (polymer-rich) as well as salting-out effects (salt-rich). In case of high molecular weight polymers and high salt concentrations, the partitioning of the biomolecules is made to the interphase due to both volume exclusion and salting out[121,127](**Paper III**). The small DNA fragments can be directed to the top phase and be separated from other contaminants, which are moved to the other phase. Besides being a fast process, this system can achieve yields higher than 95%. In addition, it is easy to scale-up and scale-down, simply be adapting to the intended and desired volumes.

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4. Chromatographic modalities for DNA and nucleotide isolation

Liquid chromatography, being a high-resolution method, is considered essential for both large-scale purification of supercoiled pDNA as well as for analytical characterization of nucleic acids[128–130]. The overall process must result in a pDNA product with specific quality levels (as described before in chapter 1.2). Although there are some strategies using chromatography directly from crude lysate, it is normal to use chromatography after the clarification and concentration operations, and sometimes to include a double chromatography step[131]. The main different chromatographic modalities are: size exclusion chromatography (SEC)[132], anion-exchange chromatography (AEX)[133,134], hydrophobic interaction chromatography (HIC)[135] and affinity or pseudo-affinity chromatography (AC)[39,136–138]. These modalities can be used alone or together with other modes. Some examples of commonly used chromatography ligands from each chromatography modality are presented in Figure 9.

With exception of gel filtration, which always operates in a flow-through mode, pDNA chromatography should in principle operate in a positive mode. For example, adequate matrices should be used to bind pDNA molecules and allow separation of impurities in the flow-through fractions or upon elution under suitable buffer conditions. In case of a negative mode, the target pDNA passes the column under nonbinding conditions[47]. However, this approach leads to a dilution of the target fractions.

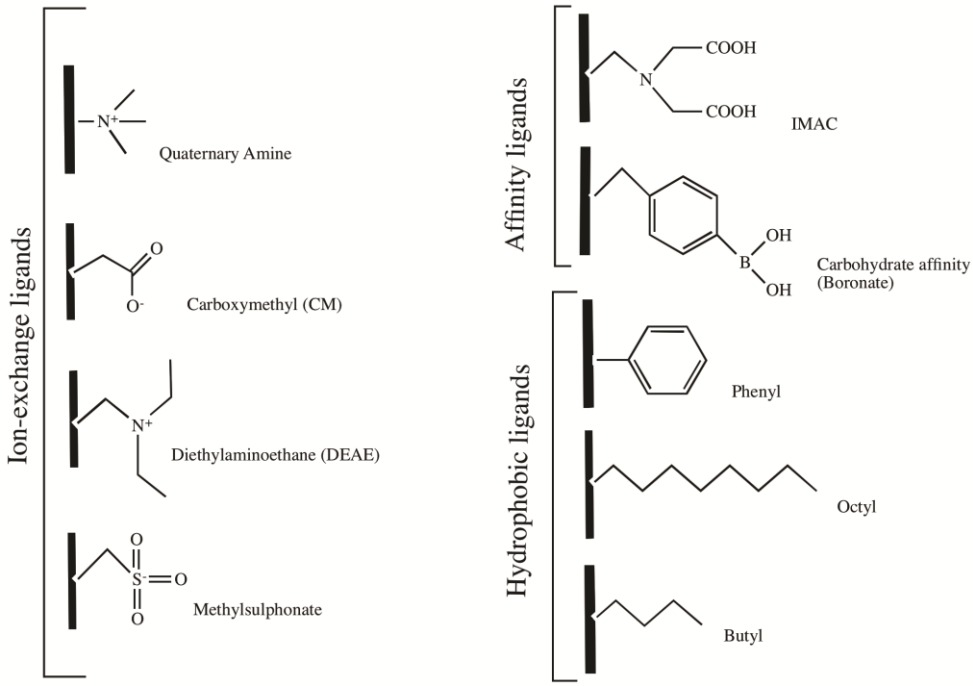

Figure 9 – A selection of the most commonly used chromatography ligands from each chromatography type.

Although chromatography dominates the final purification stage, chromatography is faced with a number of limitations that mostly are deeply linked to the structural nature of the stationary phases and the molecules involved. Poor selectivity and coelution are attributed to similarities between pDNA and impurities derived from *E. coli* such as RNA, gDNA and lipopolysaccharide aggregates, which all are negatively charged and with comparable sizes. Still, these limitations can be reduced with procedures before the chromatographic step, such as precipitation[48], filtration[139,140] and aqueous two-phase systems (**Paper II**). In addition, the main hurdles are associated with the choice and design of the stationary phases. Commercially available chromatographic supports are not suited for large biomolecules, such as pDNA[141]. These supports have mainly been designed and developed to increase the binding capacity for proteins, and usually have pores smaller than 30 nm in diameter. However, the most serious limitations in pDNA chromatography are associated with the slow internal diffusion and the low capacity[142] due to the shape and large molecular weight of these molecules. Plasmids are large

molecules; therefore they have substantial difficulty in penetrating narrow pores. A plasmid of 5-10kbp size intended for use in GT, which corresponds to a molecular weight of 3175-6350 kDa, can present a hydrodynamic radius of 150-250 nm[143]. Other more versatile chromatographic approaches have appeared recently with an increase of capacity, such as monolithic columns[144–149], microparticles[150], nonporous silica fibers[151] or super porous beads[152,153].

The slow diffusion of the pDNA molecules into pores leads to a slow mass transfer[154], resulting in broad peaks and low recovery[155]. However, the size-diffusion barrier can be partially overcome by using multivalent cations or compaction agents. This decreases repulsion between DNA phosphates and thus condense plasmid molecules from an elongated coiled state to a compact globular state, leading to a homogenization of pDNA molecules, which in turn facilitates the access to the pores[156]. The size effect is visible on **Paper IV** and **V**, where pDNA molecules isolation is different when they are alone (**Paper IV**) or mixed with small DNA fragments (**Paper V**). The small DNA fragments exhibit a faster diffusion, resulting in a faster interaction compared with large pDNA molecules. This results in a lower access of the pDNA molecules, reducing the capacity of the column for them, and forcing them to pass through the column in a flow-through mode principle.

Anion-exchange chromatography

Positively charged anion-exchange chromatography (AEX) is the most popular chromatographic modality used for DNA purification. The most prevalent ligand consists of a quaternary amine[157]. However, tertiary-amine ligands[158] and polymeric-amine ligands have also been described in literature[159]. During AEX chromatography, the separation of pDNA is based on the interaction between the negatively charged phosphate groups of the pDNA backbone and the positively charged groups on the stationary phase. The different interaction levels between diverse molecules will then be a result of the conformation or topologies of the pDNA molecules. The topologies presented by the pDNA molecules exhibit different charge densities, leading to the separation ability. The overall interaction between the pDNA and the stationary phase is based on the local

attraction generated by opposite charges. Therefore, the isoforms will have different retention times when pH is varied and/or with an increasing salt gradient[160]. Indeed, the increase in salt concentration promotes the elution in the order of the chain length. This is directly related to the number and local density of charged phosphate groups[9]. This means that sc pDNA elutes lastly when compared with oc pDNA, since it presents a higher charge density and promotes a higher degree of electrostatic interactions. In Figure 10 is a schematic representation of AEX chromatography.

In addition to charge-density effects, the chromatographic separation of linear pDNA is also affected by physico-chemical properties. For instance, small double-stranded DNA (ds DNA) may form random coil in solution, which affords greater elasticity. This increases the ability to interact with the stationary phase, whereas pDNA is more static[128]. Other additional parameters like dispersive forces, hydrogen bonding, dipole-dipole attraction and adenine-thymine content can also influence charge-density[9].

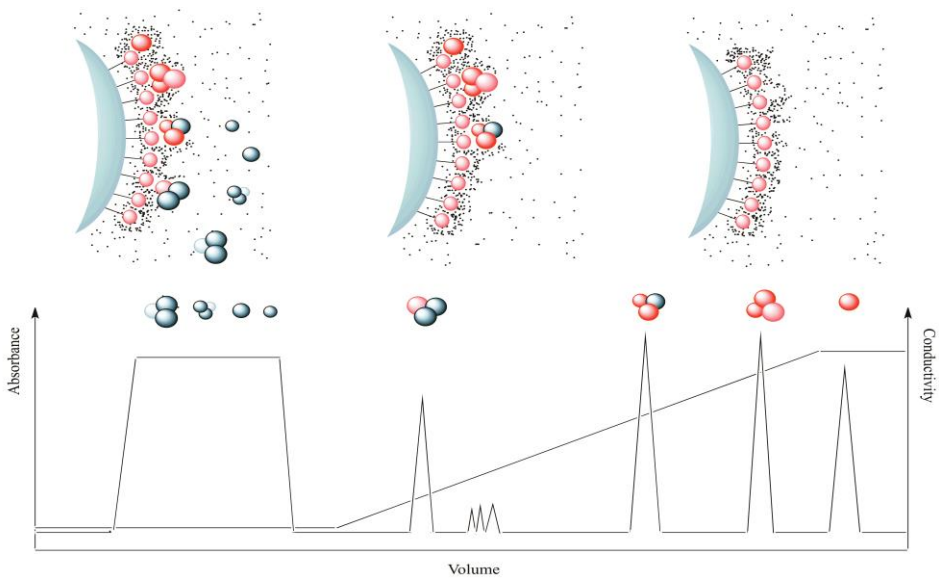

Figure 10 - Schematic model for AEX chromatography. Elution of the different molecules done by increasing salt concentration (conductivity increases). The elution is achieved in the order that represents the different interactivity between molecules and ligands.

Another parameter that favours the binding of the nucleic acids molecules to AEX is the optimal interaction with the curvature of the particle pores, which leads to higher retention factors[128]. Due to the pDNA size, the outer surface is more likely for interaction and the diffusion is limited into the pores[142]. Furthermore, the ligand density of the stationary phase can influence the retention and selectivity during the process. By increasing the ligand density, the affinity to the DNA can be reduced, or increase when the ligand density is reduced, when compared with conventional charged patches as shown by Chen *et al.*[161].

Several AEX chromatographic column studies have been performed to characterize the interactions between DNA and stationary phases[154,162,163]. Many different AEX approaches have also been examined for pDNA purification, but it is most common that this modality is mainly used as a primary purification step. Normally this first step aims at eliminating most of the contaminants, followed by a secondary chromatographic step which polishes the sample into a pure target isoform.

In **Paper VI**, the AEX ligand *Capto Q ImpRes*, is compared with a multimodal chromatography ligand used in **Paper V** and **VII**. This study allowed us to compare and to justify the more efficient multi-modality approach for DNA and nucleotide separations. More information is available in Chapter 4.3.

Hydrophobic interaction chromatography

Hydrophobic Interaction Chromatography (HIC) is another modality explored for pDNA purification. This modality has been used especially for protein purification and its behaviour has been explained in several different theoretical models[164–167].

The solubility of biomolecules depends, among other things, on the salt concentration of the solution and the hydrophobicity. In a non-salt solution, the biomolecule solubility is low due to the interactions between the charged surfaces. However, in low salt concentrations, the charged groups of the biomolecules will instead interact with salt in solution. This is known as salting-in. At higher salt concentrations,

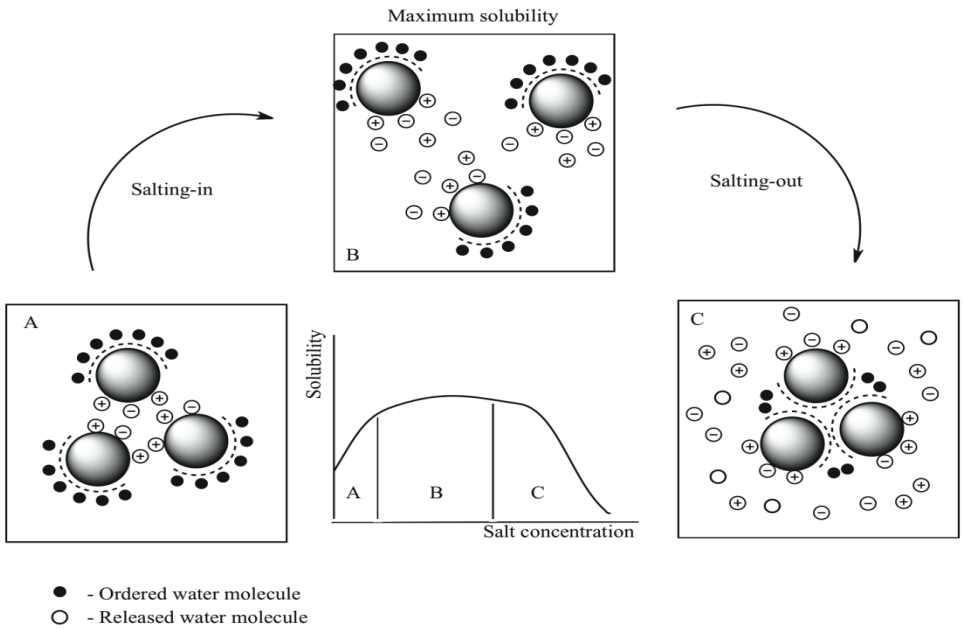

Figure 11 - Salting-in and Salting-out phenomenon. Increasing the salt concentration increases the solubility of the molecule (salting-in) to a maximum value of solubility. A further increase in salt concentration decreases the solubility (salting-out). The dashes represent the hydrophobic regions on the molecule surface. The small black dots represent the ordered water molecules.

the solubility of the biomolecule reaches a plateau for the maximum biomolecule solubility (Figure 11). Around the hydrophobic regions of the biomolecules, the water molecules are arranged in an ordered fashion because of their inability to form hydrogen

bonds in all directions with other water molecules. This creates a thermodynamically unfavourable situation due to a decrease in entropy. As the salt concentration is increased, more water molecules are needed for hydration of the salt ions. To increase the entropy and the disorder of water molecules, the hydrophobic regions will associate, thus releasing the water molecules to the salt ions, resulting in precipitation as there are insufficient molecules to interact with the biomolecules[168]. This phenomenon is known as salting-out effect.

The HIC, known as well as salt-promoted adsortion[169], uses a hydrophobic ligand to promote interactions with the target molecule. In liquid chromatography, the compounds in solution are retained differently depending on the non-polar surface-exposed regions[170]. The ionic strength of the solvent promotes the salting-out effect when close to the precipitation point of the target molecule. The compounds that precipitate at such high salt concentration will pass through the column while other molecules will be retained longer or totally. Those compounds are then eluted by decreasing the ionic strength of the mobile phase. Some approaches using HIC have been developed for pDNA purification, relying on the different hydrophobicities of the pDNA, single-stranded nucleic acids and endotoxins[171]. The process is achieved in a negative mode,

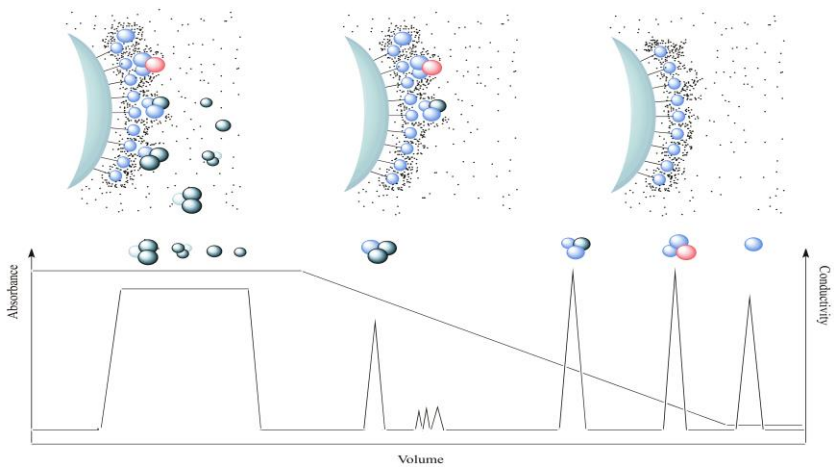

Figure 12 – Schematic model for HIC. Elution of the different molecules by reducing salt concentration (conductivity decreases) and it is achieved in the order of lower to higher hydrophobicity interaction. Blue represents the hydrophobic groups. Red represents the charged groups. Grey represents the neutral groups.

where pDNA isoforms pass through the column while contaminants will be retained[172–174]. In Figure 12 is a HIC chromatogram schematically represented. Some approaches have been done to achieve separation of the pDNA isoforms using this technique, depending for instance on the AT-rich regions. These rich AT-rich regions can increase exposure of DNA bases leading to possibilities for additional hydrophobic interactions[155].

Multimodal chromatography

The chromatographic procedures are usually categorized according to the main type of interaction available between the solute and the stationary phase, and the multimodal or mixed-mode chromatography (MMC) is not an exception. With MMC, resolution relies upon at least two different types of interactions between the solute and the stationary phase. In order to provide solute resolution by mixed interactions, it is necessary that the stationary phases are multifunctional in character, and the secondary interaction cannot be too weak[175]. Although multimodal chromatography has become widely used nowadays, the separation phenomenon is not entirely new. The first use of MMC was done in the 80's, when Regnier's group synthesized a silica-based anion-exchanger stationary phase that also displayed characteristics of hydrophobic interaction chromatography for protein separation[165]. Many chromatographic matrices were based upon a rigid support such as cellulose, agarose, polyacrylamide or silica gel that included modifications to introduce a specific functionality onto the surface of the support. Often this functionality characterizes the chromatographic modality, but the solutes introduced to such a stationary phase experience multiples types of interaction. In this respect, many chromatographic matrices are in part mixed-mode materials[176], where e.g. the spacer arm can promote secondary interactions[177]. However, those secondary interactions have often been considered as the main cause of peak tailing, which scientists have tried to eliminate or minimize[178].

Many chromatographic approaches for biomolecule purification have been performed in two or more chromatographic steps, leading to a time-consuming issue.

Multimodal approaches can be useful and minimize this limitation. Indeed, it can lead to a higher selectivity, efficiency and higher capacities for the purification of many biomolecules[179–183]. In most cases, the multiple interactions will simultaneously influence the adsorption of the molecules[175]. However, this phenomenon can be controlled, for example by eliminating the electrostatic interactions. By controlling pH, the degree of charges on the target biomolecule can be utilized to achieve either pure hydrophobic interactions or a mixture including also electrostatic forces. Using this approach, elution can be achieved by electrostatic repulsion and/or by reducing hydrophobic interactions. The multimodal mechanism is illustrated schematically in Figure 13.

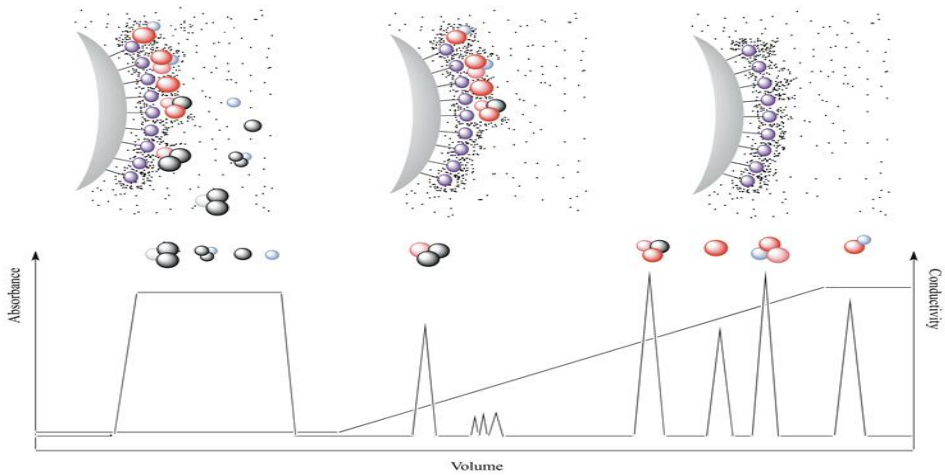

Figure 13 - Schematic model for MMC. Elution of different molecules by allowing charged molecules to elute before the ones that not only are charged, but also are hydrophobic. By increasing salt, the hydrophobic groups tend to interact while the ion-exchange mechanism is becoming eliminated. This promotes retention of these kinds of molecules compared with the ones with no hydrophobic groups but with similar charges. Blue represents the hydrophobic groups. Red represents the charged groups. Grey represents the neutral groups.

The hydrocarbyl amine is one of the most frequently used families of mixed-mode ligands (Figure 14). The amino group can serve as an active site for immobilization and also as a positively charged group for electrostatic interaction. Compared with single-mode analogues, these ligands are advantageous in their unique selectivity and their elution by charge variation[184]. In 2003, Johansson et al. found that introduction of

hydrogen bonding groups in proximity of the ionic groups in mixed-mode ligands can be beneficial for protein binding at high salt concentrations[185].

Based on this improvement, *Capto™* MMC resins appeared recently as successful adsorbents[64]. The *Capto™ Adhere* (Figure 13), used on **paper IV**, **V** and **VII**, is a strong anion-exchanger presenting an amide group to permit hydrogen bonds and a phenyl group, which allows hydrophobic interactions. These multiple interactions lead to high dynamic capacities in a wide range of salt concentrations and are thus denoted as "salt-tolerant adsorbents"[184].

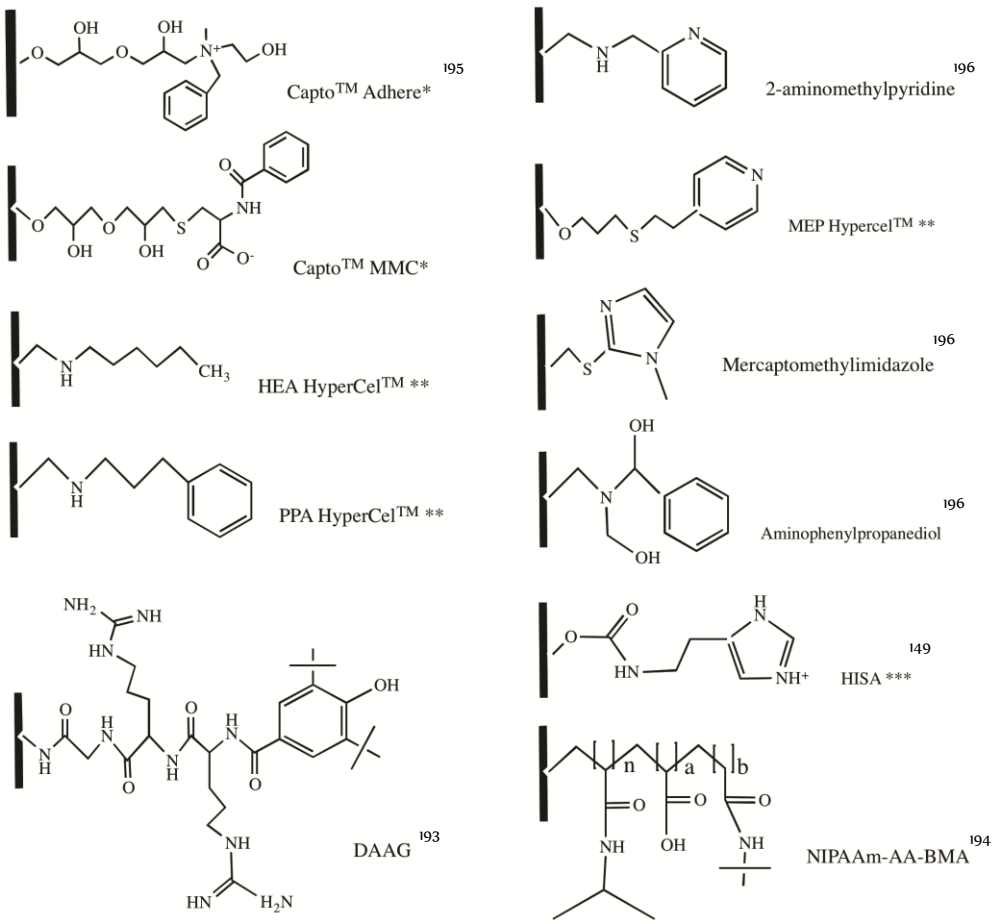

Figure 14 – A selection of multimodal ligands from literature and commercial sources. *GE Healthcare, **Pall Life Sciences, ***BIA separations.

The *Capto™ Adhere* resin was designed for intermediate purification and polishing of monoclonal antibodies after a Protein A capture step, in a two-step chromatographic downstream process[186,187]. However, this support is used in a flow-through mode for these molecules[188], binding strongly the contaminants. Its aptitude for binding contaminants in the process, such as DNA, raised interest from us.

In **Paper IV,** the characteristics of the ligand for binding different nucleic acids were examined. The pDNA molecules can be purified from crude cell lysates, and contaminants such as endotoxins and proteins are eliminated from the pDNA fraction. However, the selectivity of the ligand for different isoforms of pDNA is limited. In **Paper V**, the ss- and dsDNA can be separated based on their different degrees of hydrophobic exposure. The elution of negatively charged DNA is promoted by increasing the ionic strength. However, the ssDNA exposes hydrophobic regions. Moreover, the more elastic characteristics of ssDNA can also explain the longer retention and later elution[189]. Also pDNA adsorption is limited in presence of small DNA fragments, since the diffusion and mass transfer of small DNA fragments are faster.

In order to identify any sequence specificity in the elutions, polydeoxyadenylic acid, poly(dA), has been compared with the other homologous oligodeoxynucleotides, poly(dT), poly(dG) and poly(dC), of the same size (26-mers). Each ssDNA sample can be completely recovered (> 95%) and results in a specific elution volume at a given salt concentration in the gradient, clearly indicating that homoplymeric ssDNA molecules form secondary structures that are dependent on the nucleotide given. Also, the longer ssDNA molecules elute later in the salt gradient, indicating a possible size-dependent elution behaviour operating together with the involvement of different secondary structures. Longer ssDNA molecules thus have enhanced possibilities for interactions between the phosphate groups of the DNA and the chromatographic ligand.

This superior behaviour of the *Capto™ Adhere* resin for DNA separation and purification is justified in **Paper VI**, where the binding and elution of DNA molecules were tested on an AEX ligand, *Capto™ Q ImpRes*. This ligand is similar to the *Capto™ Adhere* ligand, except for the phenyl group that is not present. The phenyl group promotes the stability of the *Capto™ Adhere* ligand as well as provide the hydrophobic characteristics of the resin. When the phenyl group is not present, the ligand behaviour

is based on AEX only. The elution of ssDNA and dsDNA was inverted with the *Capto™ ImpRes resin*, as compared with *Capto™ Adhere*, which is a result of single-mode ionic interactions.

Based on the previous results from **Paper IV and V**, these multimodal interactions can be used for DNA fragments purification from complex mixtures, such as the ones obtained after PCR. This final mixture contains several contaminants that influence the final product purity, e.g. DNA template (normally large DNA fragments or pDNA), primers, nucleotides, enzymes and support proteins. The common commercial processes for the PCR fragment purification is based on separation by agarose gel electrophoresis, gel melting and separation by chromatographic mini-columns. In **Paper VII** a new, faster and efficient method is presented for the PCR fragments purification, using a unique chromatographic step with the *Capto™ Adhere* column. This method can separate and purify the amplified fragments with a high linearity, removing all contaminants from the PCR reaction and giving a final endotoxin free sample.

5. Concluding remarks

Several methods have been described for recovery, purification and isolation of nucleic acids. Several steps of the downstream processing are critical, in both preparative and analytical approaches. For nucleic acids, lysis, clarification and purification are the main challenging steps, and those are the ones explored and developed in this thesis.

The innovative nano-technique approach based on electropermeability explores an extraction of nucleic acids in a cleaner way, avoiding most of the contaminants present in traditional lysis mixtures that end up in total cell disruption. By using a smaller scale, the approach done by a microfluidic chip allowed the extraction of smaller nucleic acids, turning the following steps simpler.

A clarification step was presented using an aqueous two phase system, based on poly(ethyleneglycol)/poly(acrylate)/salt. This system is much less expensive than the common dextran systems available and which have been described in literature. This system can be used for smaller DNA fragments resulting in a simple process for PCR product purification, which easily can be scaled up or down.

A final purification step in biomolecule downstream processing is almost always needed. Chromatography is the main technique explored for nucleic acids and there is a need to develop alternative and better purification systems for these biomolecules. In this thesis, a series of studies using a mixed-mode chromatographic system, *Capto ™ Adhere,* has been presented. This matrix resulted in a highly efficient chromatographic support for the first step purification of pDNA, removing all kind of contaminants present in the crude cell lysate. Moreover, the column was tested with small DNA fragments and here offered the possibility of separating double- and single-stranded DNA. It was discovered that some of the limitations presented with pDNA, especially due to the large size of the molecules, were enhanced in the presence of smaller DNA fragments, leading to a lower capacity to the larger pDNA. The superiority of *Capto ™ Adhere* as a resin for DNA

separations was confirmed by comparing with the related resin *Capto Q ImpRes*. The major difference of this ligand is the absence of a phenyl group. This structural difference generates a loss of hydrophobic binding capacity of the ligand, which in turn results in a different behaviour of the ligand to the same type of nucleotide and DNA molecule. This fundamental aspect also justifies the favourable results of the PCR product purification using the *Capto* ™ *Adhere* ligand. With this system it was possible to purify the amplified fragments from other contaminants, such as primers, templates and proteins. The use of this resin turns out to be a valuable alternative to reduce the limitations that exist nowadays for PCR products purifications. The standard methods thus normally explore gel agarose separation followed by melting and purification, which often result in time-consuming processes, high costs and lower yields.

In this study, only two different ligands have been examined. However, it is obvious that a more thorough screening of a ligand library can be useful to identify specific binders for ssDNA, RNA and different dsDNA forms.

Future perspectives

For future developments it would be valuable to adapt the electropermeabilization method especially for small proteins, such as insulin, a protein with a significant importance in pharmacy. With a different type of approach, i.e. continuous extraction followed by separation of the extracted products from live cells, an on-line continuous process can be envisaged which would allow for an interesting process improvement.

Moreover, it has become clear that epigenetic[190–192] changes are involved in many human diseases as well as during normal development. The development of analytical techniques for examining the degree of DNA methylation is therefore extremely important. Liquid chromatography could be useful in these cases, since the methylation may affect not only the structure of the DNA strands, but also the charge densities. This may allow separation of methylated DNA from non-methylated in an analytical mode. The multimodal chromatography shown in this thesis presents a high selectivity and sensitivity which may be useful in this case.

MMC may also be helpful for studying pDNA stability. This is especially valuable since the influence of stability on transfection is unclear. Additional studies may lead to an increase of sc pDNA yields, which can lead to higher productivity and transfection rates. Furthermore, the analysis of interaction of sc pDNA and proteins, lipids and gene carriers should be taken into account in order to study the stability of the supercoiled structure and the corresponding efficiency of transfection in a gene therapy process.

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Sara, porque não existiam folhas de papel neste mundo que chegassem para descrever o quanto importante tu és na minha vida. És o momento, a calma, o abrigo, o que preciso no extacto momento, a vírgula e o ponto final. És o respirar fundo no meio da trovoada, sorriso no dia de chuva, a lágrima da alegria. És a razão de nunca desistir. És aquilo que em mim não existia. Hoje amanhã e sempre estarei em dívida contigo.

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Papers I - VII

Paper I

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Nucleic acid and protein extraction from electropermeabilized *E. coli* cells on a microfluidic chip

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Due to the extensive use of nucleic acid and protein analysis of bacterial samples, there is a need for simple and rapid extraction protocols for both plasmid DNA and RNA molecules as well as reporter proteins like the green fluorescent protein (GFP). In this report, an electropermeability technique has been developed which is based on exposing *E. coli* cells to low voltages to allow extraction of nucleic acids and proteins. The flow-through electropermeability chip used consists of a microfluidic channel with integrated gold electrodes that promote cell envelope channel formation at low applied voltages. This will allow small biomolecules with diameters less than 30 A to rapidly diffuse from the permeabilized cells to the surrounding solution. By controlling the applied voltage, partial and transient to complete cell opening can be obtained. By using DC voltages below 0.5 V, cell lysis can be avoided and the transiently formed pores can be closed again and the cells survive. This method has been used to extract RNA and GFP molecules under conditions of electropermeability. Plasmid DNA could be recovered when the applied voltage was increased to 2 V, thus causing complete cell lysis.

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Introduction

The most commonly used bacterial host for recombinant production of nucleic acids and proteins is *E. coli*.[1–3] Over the years, several routine procedures have been developed for this bacterium for cell cultivation, harvest, lysis and target biomolecule purification. However, particularly the cell lysis step has often been neglected and the method used for cell opening largely influences the following purification steps to generate a pure end-product.[4,5] Frequently explored approaches involve externally applied factors based on physical, chemical, mechanical, electrical and/or enzymatic methods. These processes, which often include use of detergents, extreme pH values or temperature shifts, can be detrimental to the structural maintenance and integrity of sensitive molecules such as plasmid DNA (pDNA), RNA or proteins. The method used for lysis also influences the starting level of contaminating compounds originating from the host cells.

Purified bacterial pDNA has several applications, notably as DNA vaccines, in which the vector expresses a specific structural gene.[6] This clinically oriented approach requires access to substantial amounts of highly purified pDNA preparations.[7] Beside DNA, small RNAs have also become important targets for scientific investigation particularly due to their regulatory roles in cell development.[8] Additionally, messenger RNAs are important carriers of genetic information that often need to be isolated for further characterization of cellular expression patterns.[9] Taken together, the further exploitation of these areas would benefit from the development of a holistic strategy for isolation, extraction,[10] and chromatographic purification[11] of nucleic acids. Furthermore, proteins represent an important target group of biomolecules in studies related to research matters within molecular biology and biochemistry. Particularly, some proteins have attracted a more general interest, *e.g.*, green fluorescent proteins (GFPs), which are being extensively used as reporters in characterization of protein complexes,[12] expression profiles and cell imaging.[13]

Due to the intrinsic characteristics of the bacterial envelope, the modified alkaline procedure has become the most frequently explored method for cell lysis in order to extract pDNA.[14] This method involves the use of highly alkaline conditions and a detergent to dissolve the lipid cell membranes and release the cellular contents of nucleic acids from the cytoplasm. However, RNA cannot be prepared and isolated by the same method. Extraction of RNA molecules is thus more complex and often includes an acid guanidinium thiocyanate–phenol–chloroform step, where the DNA fraction is removed after multiple centrifugations.[10] In the case of protein extractions, there are several technical hurdles associated with the cell lysis that need to be considered for improving the recovery of the product. Such concerns include for instance methods to refold proteins from inclusion bodies[15,16] or remove protein aggregates.[17] These issues must be considered during planning

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of the extraction process. In most instances, additional purification steps, using aqueous two-phase systems or different chromatography based methods are required to obtain acceptable purities of DNA,[18–20] RNA[11,21] and GFP.[22,23]

During recent years, the advances in micro- and nano-scale techniques for various analytical and preparative procedures have been remarkably rapid. Different technologies have thus been developed for separation and purification of nucleic acids.[24] In addition, microfluidic chip electrolysis to achieve cell lysis has been proven to be useful for characterization of DNA[25,26] and low molecular weight RNAs.[8] Such electrolysis is initiated by the destabilization of the lipid bilayer of the bacterial cells, and several studies have been performed to characterize the lipid cell membrane behavior. For instance, Pogodin *et al.* have examined the permeation of small molecules through lipid bilayers,[27] where permeability proved to be particularly dependent on the size of the target molecule.

In this study, we have extended the use of microfluidics to control the degree of cell opening to obtain a targeted release of biomolecules from the cells. The approach is based on electroporation, which is a simple and fast procedure well-known since the 1980's.[28–31] Electroporation has mainly been explored for cell transformation and studies of pore formation,[30-32] but lately it has been adopted in other fields, notably for tumor ablation.[33] The occurring membrane destabilization phenomenon can be reversible and transient or irreversible resulting in cell lysis.[34] This phenomenon has been described as Maxwell deformation,[31] where the lipid bilayers are reorganized as a response to an applied voltage. A channel is formed due to the presence of water, forcing the lipids to reorganize in order to minimize exposure of the hydrophobic sites.[35] A transient channel is thereby created allowing migration and diffusion of cytosolic molecules to the surrounding medium. This temporary permeability allows for the migration of molecules such as pDNA, RNA and proteins. In our study, we induced electropermeability on the bacterial envelopes using a simple microfluidic channel with integrated gold electrodes, which promoted destabilization of the membrane bilayers using a very low applied voltage (Fig. 1). Our approach intends to promote a reversible electroporation[34] in order to avoid complete cell disruption or lysis, even in a nano-scale approach,[26] and thereby minimize the level of contaminants in the starting samples used for characterization of the target molecule. By exploiting different voltage settings, we could thus control the degree of cell opening, which in turn can greatly facilitate subsequent purification steps.

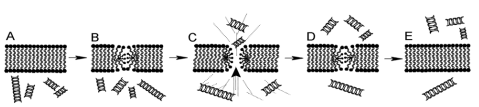

Fig. 1 State transition scheme for the molecular rearrangements of the lipids in the pore edges of the lipid vesicle membrane. (A) The closed bilayer state. The external electric field causes ionic interfacial polarization of the membrane dielectrics, analogous to a parallel plate capacitor. (B) The induced membrane field, leading to water entrance in the membrane to produce pores. In the pore, the lipid molecules are turned to minimize the hydrophobic contact with water. (C) The open pore will allow the transitions of molecules and ions from both sides of the membrane. (D) The membrane stabilizes slowly after moving out from the electrical field. (E) The membrane returns to the initial conformation.

Experimental

Chip design

The microfluidic chip consists of a microfluidic channel with four arrays of interdigitated gold electrodes on the top and bottom of the channel. Each array contains a set of ten electrodes with an electrode width of 560 μm and a pitch of 1120 μm. The electrode length is defined by the microfluidic channel width, *i.e.*, 500 μm. The gold electrodes were fabricated by standard photolithography, e-beam deposition and subsequent lift-off, as described by Illa *et al.*[36] Briefly, cyclic olefin copolymer (COC) foil (Topas 5013L, 254 μm thick, Topas Advanced Polymers Inc., Florence, KY, USA) was manually cut in 4 inch wafer size, spin-coated (RC 8 spincoater, SÜSS MicroTech AG, Munich, Germany) with a 1.5 μm thick AZ5214E image reversal photoresist (MicroChemicals GmbH, Ulm, Germany) and patterned by UV exposure (MA6 mask aligner, SÜSS, MicroTech AG, Munich, Germany). After reversal baking in a convective oven at 120 °C for 25 min and 30 s flood exposure, the developed negative resist pattern was deposited with a 200 nm gold layer by e-beam deposition (SCM 600, Alcatel, France). The excellent chemical inertness of COC allowed an acetone lift-off, to release the gold electrode structures.

The microfluidic chip was built of three layers, where the top and bottom electrode foil not only provide the electrodes but also seal the microfluidic channel that was cut into the middle layer. A 62 mm long and 500 μm wide channel was cut by micromilling in COC foil; additionally two windows were cut into the foil that allow the electric connection to the electrodes. Holes for microfluidic connection were drilled into the top electrode layer. All three layers were cleaned in a 10 min isopropanol ultrasonic bath, flushed with deionized water and dry blown with nitrogen. Before lamination, the bonding surfaces were exposed to UV light (5000-EC, Dymax Europe GmbH, Wiesbaden, Germany, measured intensity: 40 mW cm^{-2} at $\lambda =$ 365 nm) for 30 s to photolytically degrade the surface and enhance the bonding strength of the mating parts.[37,38] After surface activation, the three layers were manually aligned, so that the electrodes on the bottom and top layers faced each other and the stack was bonded at 120 °C with a bonding force of 10 kN for 10 min, using a manual hydraulic laboratory press (P/O Weber, Remshaldern, Germany).[39,40]

The thickness of the channel was defined by the foil thickness. For this series of chips, a 254 μm thick foil was used, leading to a channel thickness of 254 μm and a channel volume of 7.87 mm^3 (7.87 μL). The residence time of the cells inside the chip was approximately 2.4 to 4.7 s, depending on the flow rate.

A poly(methyl methacrylate) (PMMA) holder was fabricated in-house by micromilling, as represented in Fig. 2. The PMMA holder seals the microfluidic inlet and outlet with rubber o-rings and connects the chip *via* teflon tubings to a HPLC

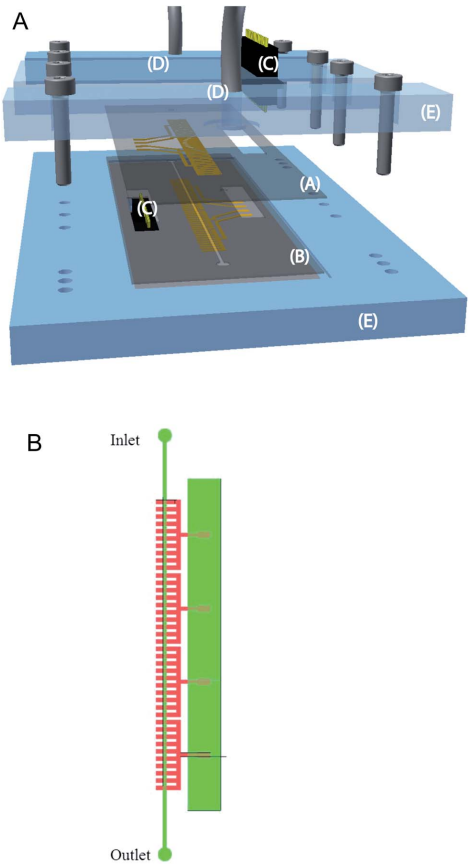

Fig. 2 (A) – Schematic drawing of the electropermeabilization setup. The exploded view shows the top electrode layer (A), and the middle channel with bottom electrode array (B). Spring loaded connectors are pressed against the contact pads of bottom and top electrodes (C). The fluidic connection (D) is sealed *via* o-rings (not shown). (E) PMMA holder. (B) – Two-dimensional design of the bottom layer of the chip; red represents the electrode array and green the chip structure and channel.

pump (2150, LKB Bromma, Sweden) and collection vessel, respectively. Spring loaded connectors (8PD series, Preci-Dip SA, Delémont, Switzerland) were slightly pressed against the electrode contact pads to ensure a good electrical contact of both, bottom and top electrode arrays. The electrical field between the facing electrodes was applied *via* a DC power supply (HQ Power PS1502A, Gavere, Belgium) that was connected to the contact pins of the spring-loaded connectors.

E. coli cells examined

E. coli DH5α harboring the pUC18 (2.686 bp) plasmid was used in the electropermeabilization tests. The cells were grown in a Terrific Broth (TB) medium (20 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 4 mL L^{-1} glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 100 μg mL^{-1} of ampicillin while being cultivated by shaking at 250 rpm. The process was terminated at the late exponential phase of bacterial growth. For reference purposes, pure plasmid DNA was also prepared using conventional alkaline lysis with the Qiagen plasmid maxi kit (Hilden, Germany).

E. coli TG1 cells expressing a green fluorescent protein (GFPuv) were also used as a marker protein to examine the degree of cell opening in the microchip electropermeability experiments. The growth was carried out in 1 L flasks at 250 rpm shaking and 37 °C, using a TB medium supplemented with 100 μg mL^{-1} of ampicillin and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at an $OD_{600} \approx 0.2$ as described by Becker *et al.*[41] This strain simultaneously produces two GFPs with different sizes, which allows for monitoring of different protein release levels from the electropermeabilized cells. The cultivation was terminated in the late exponential growth phase. For reference purposes, the GFPuv protein was extracted using cell lysis on a Q Sonica Q500 sonicator (Sonics & Materials Inc., Newtown, CT, USA) with a microtip 4420 for 10 min with 3 s pulses (25% amplitude).[37]

Voltage effects on cell opening

In order to screen for the sensitivity of the bacterial cells to exposure to increasing voltages, different voltages, up to 2.5 V, were applied perpendicularly over the channel. The bacteria were suspended in a 10 mM citrate buffer (10 mM NaCl, 30 mM glucose, pH 5.8) and pumped through the chip at a constant flow-rate of 0.2 mL min^{-1}. Fractions were collected and cells were recovered by centrifugation. The supernatant was used for further spectrophotometric and electrophoresis analyses. The pellet was collected for cell viability measurements.

The microfluidic channel was rinsed between runs with MilliQ water to remove possible contaminants.

Qualitative analyses of nucleic acids and GFP

Nucleic acids from permeabilized and lysed *E. coli* cells were examined using 1% agarose gels stained with Gel Red (0.6 μg mL^{-1}). Electrophoresis was carried out at 100 V with a TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). The gels were observed under UV light and *Quantity One* software (BioRad, CA, USA) was used to quantify DNA and RNA concentrations. DNA and RNA samples were also quantified by a Nanophotometer Pearl (Implen GmbH, Munich, Germany) at 260 nm. The ratio of absorbance at 260 nm and 280 nm was used as a first step to screen sample purity.

The quantification of GFPuv was made by fluorescence measurements using a PTI *quantamaster 40* (Photon Technology International, NJ, USA). Excitation and emission wavelengths were 390 nm and 600 nm, respectively. Additionally, protein purities and expression levels were also monitored by 22% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a Page Ruler Unstained Protein Ladder from

Thermo Scientific (Rockford, IL, USA) as molecular weight standards.

Cell viability analysis

To quantify the number of viable cells in the cultures, the cells were diluted and plated on petri dishes after the experiments. The colony numbers were counted after 12 hours and cell concentrations were determined.

Results and discussion

Electropermeabilization for isolation of nucleic acids

The degree of cell opening was directly correlated with the applied voltage. When a low voltage was used, *i.e.*, equal to or below 0.5 V, no harmful effects were observed on the cells and the viability was close to 100% after the cells had passed the channel. However, when the running buffer was analyzed, high levels of nucleic acids could be detected. When investigating the contents of the cell suspension using agarose gel electrophoresis, substantial concentrations of low molecular weight RNA molecules were detected (Fig. 3). Metabolically active *E. coli* cells thus contain high levels of transfer RNA (tRNA), which constitutes approximately 80% of the total cellular RNA,[42] and these molecules could be observed in the supernatant after passage through the channel. Even though a major fraction of total cellular RNA could be extracted from the cells at 0.5 V, the cells remained viable after electropermeabilization (Fig. 4). When the voltage was increased further, substantial lysis occurred, however. The extraction of RNA increased up to an applied voltage of 2 V, but decreased again thereafter. When these higher voltages were applied over the channel, several other detrimental effects were observed in the system, such as attachment of cell debris to the electrodes as well as partial degradation of the electrodes themselves. The maximal applied voltage was therefore generally limited to 2 V for the remainder of the experiments. 2 V corresponds to a field strength of 7.9 mV μm^{-1}.

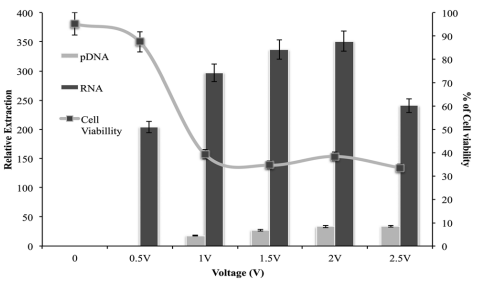

Fig. 3 Extraction of RNA and pDNA after passage of bacteria through the microchannel. Cell viability and nucleic acid extraction, as determined after agarose electrophoresis, were measured in relation to the applied voltage. 10 mM citrate containing 10 mM NaCl, 30 mM glucose, pH 5.8, was used as a carrier buffer. The extraction results are expressed in relative terms and are corrected for the low background levels at 0 V. The error bars indicate standard deviations based on three independent extraction tests and five viability determinations.

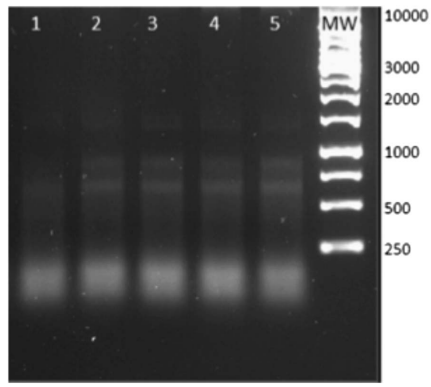

Fig. 4 Agarose gel electrophoretic analysis of nucleic acids extracted at different voltages. 1 – 0.5 V; 2 – 1.0 V; 3 – 1.5 V; 4 – 2.0 V; and 5 – 2.5 V; MW – molecular weight marker.

The results clearly indicate that the cells become permeable upon application of low voltages. A temporary rearrangement of the cell lipid bilayers allows the formation of pores, which in turn allows smaller molecules like tRNA to diffuse out from the cytoplasm. The pores will be closed when the applied voltage is removed again, and the cells can recover. This temporary cell opening does not significantly reduce the viability of the cells[43] and we could generally recover at least 90% of the original cell numbers after electropermeabilization with an applied voltage of 0.5 V. For complete recovery the cells need to be exposed to a fresh growth medium for about 30 minutes.

Transfer RNA molecules are small with an average diameter of 20 nm. For larger nucleic acids such as plasmid DNA, the formed pores need to be larger and open for extended time periods. However, this became technically impossible to generate without compromising the viability of the cells. Extraction of pDNA was thus only possible at higher applied voltages, *i.e.* at 1–2 V, where partial to complete cell lysis occurred.

When comparing the quantitative extraction efficiencies in our system with other common techniques such as alkaline lysis, we were able to obtain 10 to 15% of the total cellular pDNA in a single run through the channel. By running the same sample multiple times through the channel, higher efficiencies could be obtained, but deposition of cellular debris caused clogging of the channel and extensive rinsing with MilliQ water was required. However, when employing the chip in analytical mode based on a single passage of cells, no cleaning or rinsing was required. Our goal was to achieve very rapid extractions minimizing the time needed for analysis. Additionally, another frequently used method is based on extraction with lysozyme, which often includes a heating step.[44] Such harsh procedures may harm nucleic acid molecule structure, particularly RNA

molecules, which limit its practical usefulness. The extraction in the chip is very rapid and no heating of the cell solution was registered after passage of the channel.

Electropermeabilization of *E. coli* expressing GFPuv

The same screening procedure as described above was used for GFPuv extraction from *E. coli* TG1 cells. GFP is a common reporter protein often used to monitor cellular or metabolic activities. It has a very well-defined β-can structure where the cylinders have a diameter of 30 Å and a length of 40 Å. If GFP is released from the cells we can thereby also estimate the minimum size of the pores that were generated after exposure to the electric field. In addition, due to the well-defined fluorescent properties of GFPuv, the fluorescence signal can be used to quantify the total amounts of protein extracted from the cells. When low voltages of up to 0.5 V were applied between the electrodes, no or very limited cell lysis occurred, in agreement with the results obtained previously for RNA. When the voltage was increased further, cell lysis occurred exactly in the same way as for *E. coli* DH5α. Upon permeabilization at 0.5 V, 70% of the amounts of GFP were released to the supernatant as compared with those under lysis conditions at higher voltages (Fig. 5). In order to further delineate the release of proteins from *E. coli* cells upon exposure to voltages over the channel, the supernatant was examined by SDS-PAGE (Fig. 6). In the used expression system, GFP represents approximately 15% of the total cellular protein. At 1.5 V, the GFP level was 42% of the total protein extracted, clearly indicating a preference for release of smaller proteins. The extraction thereby allowed for a 2.8-fold enrichment of GFP simply by passing the cells through the microfluidic channel. In the case of small proteins such as GFPuv (26.9 kDa), the extraction can therefore preferably be done by electropermeabilization, which allows for an initial selection of protein size. In addition, this process is very rapid compared with the more time-consuming size fractionation processes based on, *e.g.*, chromatography. This size exclusion effect can therefore be utilized as a first elimination step to remove larger host cell proteins or other cellular components.

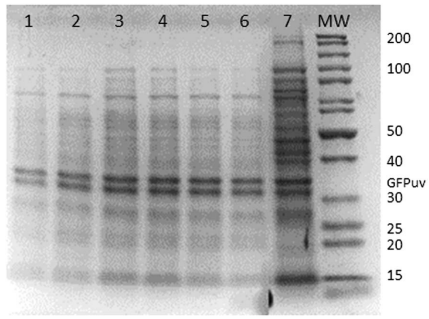

Fig. 6 SDS-PAGE for GFP extraction from *E. coli* TG1. 1 – 0.3 V; 2 – 0.5 V; 3 – 1.0 V; 4 – 1.5 V; 5 – 2.0 V; 6 – 2.5 V; and 7 – sample control from sonication lysis; MW – molecular weight marker.

Other parameters important for cell opening

Besides voltage effects, there are several other parameters for electropermeabilization of cells and lysis that need to be considered, including cell concentration injected into the channel, buffer composition and flow-rate. When these different conditions were examined, the potential was kept constant at 2 V.

Flow-rate. The size of the channel was chosen to optimize cell permeabilization, but the dimensions and fluidic resistance of the channel limited the useful flow-rate to a maximum value of 0.2 mL min^{-1}. When lower flow-rates were examined, the extraction was not improved but the risk of cell debris attachment to the electrodes and subsequent electrode passivation that could perturb the continuous electrical field was increased. The highest extraction values were generally achieved using a flow-rate of 0.2 mL min^{-1}, and this value was therefore kept constant throughout the study.

Buffer composition. When using electropermeabilization for isolating intracellular biomolecules, it is important to characterize the behavior of the cellular envelope upon exposure to differences in osmotic pressure. In addition, osmotic stress

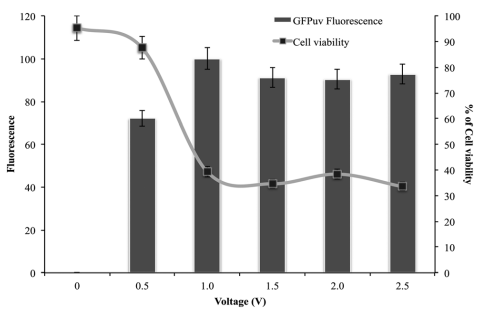

Fig. 5 Voltage effects on the extraction procedure of the reporter protein GFPuv using a 10 mM citrate buffer containing 10 mM NaCl, 30 mM glucose, pH 5.8. GFP fluorescence of the supernatants and cell viabilities are presented on the *y*-axis. The error bars indicate standard deviations based on three independent extraction tests and five viability determinations.

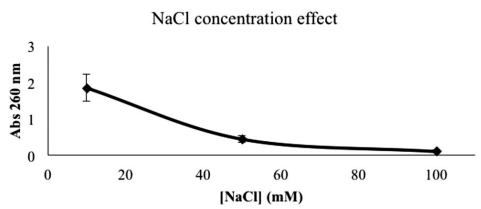

Fig. 7 Nucleic acid extraction as a function of salt concentration in the lysis process. Salt concentrations used were 10, 50 and 100 mM NaCl. The process was operated at a constant flow rate of 0.2 mL min^{-1} using an applied voltage of 2 V. The results represent an average of three independent measurements.

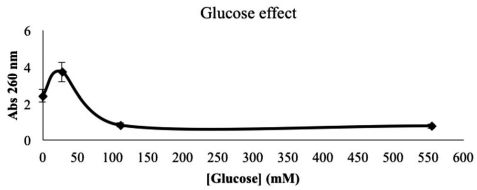

Fig. 8 Effects of glucose concentration on extraction efficiency of nucleic acids. The process was operated at a constant flow rate of 0.2 mL min^{-1} using an applied voltage of 2 V. The results represent an average of three independent measurements.

influences the intracellular mobility of particularly larger biomolecules such as GFP and nucleic acids. The internal diffusion coefficient, D_{median}, of proteins like GFP has been estimated to be 3 μm^2 s^{-1} under isosmotic conditions.[38] The translational diffusion of proteins in the cytoplasm of *E. coli* is thus still high in relation to residence time of the cells in the channel. However, different buffer compositions need to be examined focusing on ionic strength and possible osmotic protection by particularly carbohydrates. The influence of salt (NaCl) concentrations between 10 and 100 mM was determined. The optimal NaCl concentration in relation to yields of nucleic acids in the tested range, 10–100 mM, was identified to be 10 mM (Fig. 7). High salt concentrations appeared to shield the cells and protect the lipid bilayers against lysis. Similarly, the effects of glucose addition to the media were examined and addition of 30 mM glucose improved extraction (Fig. 8). Glucose additions will stabilize the cells and indirectly influence the pore size and pore stability upon exposure to an electric field. If the glucose concentration used is optimized, it is easier to control the entire permeabilization. Such a control also leads to a faster and homogeneous cell recovery.

Cell concentration. The concentration of bacteria in solution is a critical factor that needs to be optimized. At higher cell concentrations, cell suspensions will be highly viscous generating higher back pressures in the system, which in turn may lead to partial cell lysis due to mechanical shearing. In addition, a higher concentration of cells could promote a shielding effect of adjacent cells during the passage through the channel, also leading to lower extraction rates. This has often been described as a neighboring effect. On the other hand, lower cell concentrations may be favorable for the system in terms of shielding and viscosity, but the absolute yields of obtained nucleic acids will then be limited. Different cell concentrations were therefore examined and under the conditions examined we observed that 2.40×10^9 cells per mL gave the highest yields of nucleic acids as determined by absorbance measurements at 260 nm (Fig. 9).

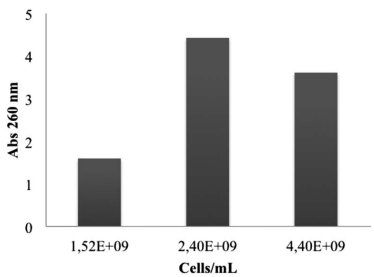

Fig. 9 Influence of cell concentration on the extraction of nucleic acid as determined by measurements of absorbance at 260 nm.

Conclusions

We here report a simple protocol for the extraction of nucleic acids and proteins by electropermeabilizing *E. coli* cells in a microfluidic chip with integrated gold electrode arrays. By controlling the applied voltage across the microchannel, the degree of cell opening for two commonly utilized *E. coli* host cell strains, TG1 and DH5α, could be controlled. Both strains behaved identically in terms of permeability and lysis. The represented system has a number of key advantages compared to the available technologies, which are either commercially available or described in the scientific literature. The presented approach is extremely fast for nucleic acid and protein extractions, especially compared to the traditional lysis methods for such molecules. Since no total cell disruption occurs, the method described represents a cleaner extraction way, leading to a simple final sample to be purified using chromatography or any other purification methods available. In addition, the process is less aggressive for extracting DNA, RNA and proteins, since the procedure uses no detergents, pH variations or high temperatures.

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Paper II

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Plasmid DNA partitioning and separation using poly(ethylene glycol)/poly(acrylate)/salt aqueous two-phase systems☆

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ABSTRACT

Phase diagrams of poly(ethylene glycol)/polyacrylate/Na_2SO_4 systems have been investigated with respect to polymer size and pH. Plasmid DNA from *Escherichia coli* can depending on pH and polymer molecular weight be directed to a poly(ethylene glycol) or to a polyacrylate-rich phase in an aqueous two-phase system formed by these polymers. Bovine serum albumin (BSA) and *E. coli* homogenate proteins can be directed opposite to the plasmid partitioning in these systems. Two bioseparation processes have been developed where in the final step the pDNA is partitioned to a salt-rich phase giving a total process yield of 60–70%. In one of them the pDNA is partitioned between the polyacrylate and PEG-phases in order to remove proteins. In a more simplified process the plasmid is partitioned to a PEG-phase and back-extracted into a Na_2SO_4-rich phase. The novel polyacrylate/PEG system allows a strong change of the partitioning between the phases with relatively small changes in composition or pH.

1. Introduction

Aqueous two-phase systems (ATPS) have been used extensively as a bioseparation tool, particularly in protein research and studies of larger biomolecular structures such as organelles and virus particles [1–15]. The most common system, discovered by Albertsson [1] is composed of poly(ethylene glycol) (PEG) and dextran. Its separation property is attractive from several points of view, namely the phase diagram is almost independent on pH, salt content and temperature of the system. However, the cost of dextran limits its use in large scale. The PEG/salt systems are less expensive, but rather low molecular weight PEGs (typically less than 600 g/mol) must be used for pDNA partitioning to the PEG-rich phase [9]. Recently, a two-phase system composed of poly(ethylene glycol), sodium polyacrylate (Na-PAA) and salt was developed and used as a separation tool for proteins [16–20]. Contrary to the PEG–dextran system it is dependent on pH and salt content and relatively insensitive to temperature. The driving force for separation seems to be an enthalpic effective repulsion between the charged carboxylate groups of polyacrylate and ethylene oxide units of PEG. The addition of salt is necessary to allow compartmentalization of the polyelectrolyte into one of the phases. Although this system contains more complex driving forces of enthalpic and entropic nature, than the PEG–dextran system, it has the advantage of being less expensive and more practical, for instance by the relatively low polymer content necessary to induce two-phase formation. In spite of the strong electrostatic interactions of the polyelectrolyte, the dominant force for protein partitioning is, under high salt concentration conditions, hydrophobic, as shown for lysozyme [18]. Herein we have studied the partition of plasmid DNA (pDNA), where the electrostatic repulsion to the polyelectrolyte is relatively strong although the salt concentration is relatively high. Since almost all ATPS-partitioning studies are performed with PEG/dextran or PEG/salt systems, this new PEG/polyacrylate system is an important addition to those methods of easily available and well defined systems for pDNA partitioning and separation. The aim of this investigation is to find conditions in which pDNA is partitioned to the polyelectrolyte rich phase or the PEG-rich phase. Recently, the interest in large-scale production of pure plasmids has increased due to the possibility to use plasmids in gene therapy [21]. However, the plasmid purification in large scale is a challenge as reviewed by Prather

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et al. [21]. The removal of genomic DNA (gDNA), RNA, proteins and endotoxins has been successful with different types of chromatography [21,22]. However, since aqueous two-phase extraction systems have several attractive advantages in terms of scalability and high loading capacity [23], we investigated here the potential of PEG–PAA two-phase system for pDNA purification. A semi-pure plasmid has been used for studies of the pH-dependent partitioning. At pH close to 5 relatively many monomer units of polyacrylate become protonated and uncharged. Their electrostatic repulsion to DNA is turned off and may be turned into attractive hydrogen bonding interactions to different groups of DNA (possibly phosphate groups [24,25]). In this work pDNA recovery from the polymer-phases has been studied using a low protein content homogenate as starting material. In order to investigate potential protein contamination in a developed process, partitioning studies were performed using *Escherichia coli* protein rich homogenate. Since these proteins are easily precipitated in the presence of SDS, partitioning studies were also performed with BSA as a highly soluble model protein.

2. Materials and methods

2.1. Materials

Polymers: sodium polyacrylate having a molecular weight of 240,000 g/mol and poly(ethylene glycol) 8000 g/mol were obtained from Sigma–Aldrich. Poly(ethylene glycol) 4000 g/mol was obtained from Merck. All other reagents were of analytical grade.

2.2. Methods

2.2.1. Two-phase systems

The two phase systems were made by mixing stock solutions of sodium polyacrylate (Na-PAA), PEG, Na_2SO_4, and water. Sodium dodecyl sulfate (SDS) was added in some cases. Semi-pure stock solutions or homogenate with pDNA was added lastly and always after the previous solutions had been mixed. The polyacrylate stock solution concentration was 14–15% and the pH was adjusted to the desired pH with NaOH (50%). The final pH of the two-phase system was adjusted by adding small quantities of HCl (37%) or NaOH (50%). The mass of the systems was 5–10 g and mixed thoroughly in a graded glass tube, by gently mixing the system by turning the tube several times up-side down. The homogenate-free systems separated into clear two-phase systems by gravity within 15 min. Systems with homogenate were left standing for 1 h or centrifuged 5 min with a table centrifuge. All separations were performed at 22 °C.

2.2.2. Phase diagram

The binodal curve that separates the single phase from the two-phase region was determined as described by Albertsson [1], by stepwise dilution of a point in the two-phase region (indicated by turbidity upon mixing), with a 4 wt% Na_2SO_4 solution. However, at some points close to the binodal curve, the difference in refractive index is very small and turbidity may not be observed in the two-phase region. Therefore, all non-turbid systems were centrifuged and inspected for two-phase formation.

2.2.3. Tie-lines

Tie-lines were determined by analyzing the PEG concentration in the top and bottom phases and fitting the composition line to the binodal curve. The PEG concentration in the top- and bottom-phases was determined by taking known amounts of the phases, which were dried in an oven at 50 °C, and then the PEG content was extracted with warm (40–50 °C) acetone. The acetone with PEG was placed in beakers and left standing for evaporation and drying. The PEG content was then determined gravimetrically.

2.2.4. Plasmid DNA and protein analysis

The pDNA concentration was determined with the Picogreen fluorescence reagent and was quantified by using a pure lambda phage standard of known concentrations using the Quant-iT PicoGreen kit (Invitrogen Life Technologies, Grand Island (NY), USA). The partition coefficient of the pDNA, K_p is the ratio of the pDNA concentration in the top and bottom phases, respectively. The pDNA yield in the phases are given in percentage of added pDNA to the total system. The pDNA concentration in the (low protein) homogenate was between 60 and 100 μg/g. The concentration of soluble pDNA in the phases was 3–20 μg/g. At least a 20-time dilution of the phase was performed before analyzing with the fluorescence probe. The BSA content was determined by absorbance at 280 nm and the presence of *E. coli* proteins was visualized by coomassie or silver staining in SDS-PAGE gels.

2.2.5. Plasmid DNA preparation and treatment of homogenate

E. coli strain TG1 was used as host in all experiments for production of plasmid pUC18 with 2686 bp (GE Healthcare, Uppsala, Sweden). Cell growth was carried out in a shake-flask at 37 °C using a Terrific Broth medium (20 g/l tryptone, 24 g/l yeast extract, 4 ml/l glycerol, 0.017 M KH_2PO_4, 0.072 K_2HPO_4) supplemented with 30 μg/ml of ampicillin. After fermentation process, cells were centrifuged at 5400 × *g* (20 min, 4 °C) and pellet conserved at −20 °C. Cells were lysed using a modification of the alkaline method. Cells pellets were resuspended in 20 ml of P1 Buffer (50 mM glucose, 25 mM Tris–HCl, 10 mM EDTA, pH 8.0). Alkaline lysis, based on the Birnboim and Doly procedure [26] was performed by adding 20 ml of P2 Buffer (200 mM NaOH and 1% (w/v) SDS) and incubated for 5 min at room temperature. Cellular debris, gDNA and proteins were precipitated by adding 16 ml of P3 (3 M potassium acetate, pH 5.0) and incubated 15 min on ice. The precipitate was removed by centrifugation at 20,000 × *g* (30 min, 4 °C). A second centrifugation step was carried out under same conditions. Briefly, the pDNA in the supernatant was precipitated by adding isopropanol (0.7 times the volume of the supernatant) and incubation on ice for 30 min. The pDNA was recovered by centrifugation at 16,000 × *g* (20 min, 4 °C). The pellets were then re-dissolved in 2 ml of 10 mM Tris–HCl (pH 8.0).

2.2.6. E. coli homogenate protein preparation

The homogenate prepared for pDNA purification had a low protein concentration. In order to observe protein partitioning an additional (protein rich) homogenate from *E. coli* cells was prepared as follows. Wet *E. coli* cells were dispersed in TE-buffer (Tris–HCl, 10 mM, EDTA, 1 mM, pH 8.0). The concentration was 13.5 wt%. The cells were sonicated with a 6 mm wide sonicator tip 600 s on ice. The energy output was a total of 9000 J on a 21 g *E. coli* mixture.

2.2.7. Plasmid DNA partitioning processes

In one process (I) pDNA was partitioned to the bottom phase in a PEG 8000/polyacrylate two-phase system. In step A pDNA was partitioned in a PEG 8000 (3.6%)/polyacrylate (1.8%)/Na_2SO_4 (4%) aqueous two-phase system. In the initial step (A) the pDNA concentration in the system was 5.7 μg/g and the SDS concentration was 0.18%. The pH was set to 5.2–5.3 by adding HCl (37%), ca. 90 mg/10 g system. The top-phase was discarded and the bottom phase was used for step B. In step B a new two-phase system was made, where the content of PEG was changed into PEG 4000. Thus the system in step B was composed by a bottom phase a solution (37.45% of the mass of system B) and a solution (62.55% of mass of system B) containing PEG 4000 and polyacrylate to compensate the discharged

top-phase in step A. The added PEG solution had the following composition: PEG 4000 (4.8 wt%), polyacrylate (1.13 wt%, Na_2SO_4 (4.0 wt%), glycine–NaOH buffer (0.33 mol/kg, pH 9.0), NaOH (0.8%). The pH in step B was set between 10 and 11 to assure all polyacrylate was completely deprotonated. The pDNA was now partitioned strongly to the PEG-rich top-phase and this phase was collected and used to form the system in step C. This phase was formed by adding 0.86 g of a 15.0 wt% solution of Na_2SO_4 per 1 g top-phase of system B. This induced a PEG/Na_2SO_4 two-phase system where practically all pDNA was partitioned to the salt-rich bottom-phase. In a more simplified process (II) the pDNA is first partitioned to a PEG 4000 rich top-phase and then extracted to a salt phase as above, corresponding to steps A and B (process II), however without addition of SDS. In this process II, the system in step A was composed of 3.0 wt% PEG 4000, 3.0 wt% polyacrylate 240,000, 4.0 wt% Na_2SO_4, and pH 5.6 ± 0.4 adjusted with HCl (37%). The pDNA concentration was 8 μg/g system. In step B of process II, the top-phase was transferred into a separate tube and solution of 15.0 wt% Na_2SO_4 was added to the system. The added salt was 0.82 g salt solution/g top-phase, which induced a PEG/Na_2SO_4 aqueous two-phase system at room temperature.

2.2.8. Removal of polymers before agarose gel electrophoresis analysis

The polyacrylate present in the bottom-phases of the samples was removed as follows: 0.4 g of bottom phase was mixed with 0.4 g of a solution containing PEG 8000 5 wt%, Glycine–SO_4 buffer 0.8 mol/kg, pH 2.0. Upon mixing polyacrylate and acidic PEG a complex was formed and precipitated from the solution (pH 2.3). The mixture was centrifuged immediately in an Eppendorf centrifuge (14,000 rpm, 2 min). The supernatant, which was practically polymer-free was transferred to another Eppendorf tube and 0.060 g of a buffer (Gly–NaOH, 2.0 mol/kg, pH 9.5) was added to adjust the pH to ca. 8.5, in order to avoid acid catalyzed degradation of DNA.

3. Results

3.1. Phase diagram

The phase behavior of solutions containing PEG 8000 and polyacrylate were tested at different pH values. In Fig. 1a and b the phase diagrams of various PEG–polyacrylate systems are shown. The diagrams are displayed as a (pseudo-)ternary system since the systems are quaternary (two polymer, water and salt). The binodal shape of the curve is typical of similar two-polymer segregating systems. Two tie lines based on determination of PEG-concentration in the phases are also shown. As expected there is a substantial decrease in size of the two-phase region when the PEG molecular weight is halved. The position of the binodal curves shows strong pH dependence between pH 4.8 and 5.5, while between 5.5 and 7.3 there is a relatively small change. The positions of the binodal curve and tie-lines indicate that there is relatively much polyacrylate in the PEG-rich phase.

3.2. Plasmid DNA partitioning in PEG–polyacrylate system

The pDNA (low protein content) was partitioned in PEG–polyacrylate at different pH values. In Fig. 2 the effect of pH is shown for pDNA partitioning in the system PEG 8000-sodium polyacrylate. The partition is expressed as percentage pDNA in the different phases. At pH 7.3 pDNA is preferentially partitioned into the top PEG-rich phase whereas at pHs 5.4 and 5.8 a more even distribution between the phases is observed. At pH 6.2 partitioning was not reproducible, but a trend can be seen where pDNA is more partitioned to the top-phase at higher pH. Interfacial partition occurs in the whole pH interval. The mass balance does not add to 100% and that could be due to inhibition in the fluorescence analysis used in pDNA detection, or to undissolved precipitated pDNA.

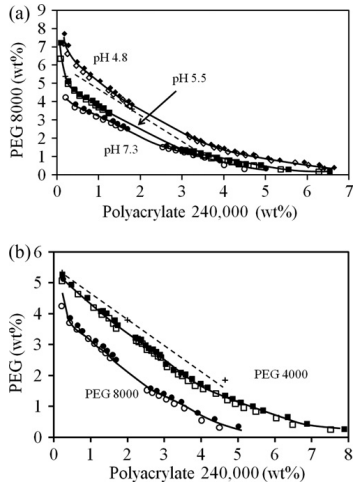

Fig. 1. (a) Phase diagram of the PEG 8000/polyacrylate 240,000/Na_2SO_4 system. Effect of pH. Concentration of Na_2SO_4 is 4 wt%. Systems with pH 5.5 and 4.8 contained sodium acetate buffer 2–4 mmol/kg. The two-phase region is above the corresponding binodal curve. The dashed line is tie-line for the system at pH 5.5. Temperature 22 °C. (b) Phase diagram of the PEG/polyacrylate 240,000/Na_2SO_4 system. Effect of molecular weight. Concentration of Na_2SO_4 is 4 wt% and pH is 7.3. The two-phase region is above the corresponding binodal curve. The dashed line is tie-line for the system with PEG 4000. Temperature 22 °C.

3.3. Partitioning of a model protein (BSA) in PEG–polyacrylate two-phase system: effect of SDS and pH

In Fig. 3 comparisons between systems with or without SDS, and systems with low or high pH are shown. It can be seen that the presence of SDS in a system at low pH (5.25) causes a strong partitioning of BSA to the PEG-rich phase, and without SDS the protein is partitioned to the bottom phase. Changing pH from 6.0 to 12, causes a dramatic increase in the BSA partition coefficient K (defined as the concentration ratio of target molecule in the phases).

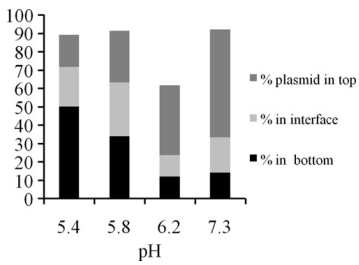

Fig. 2. Plasmid DNA partitioning in PEG 8000 (3.6%)/polyacrylate 240,000 (1.8%)/Na_2SO_4 (4%) two-phase system. pDNA concentration: 16–20 μg/g system. The percentage of pDNA in the different phases versus pH is shown.

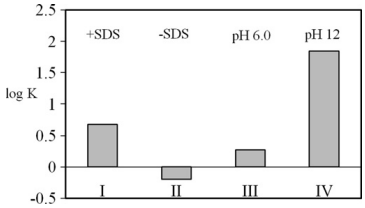

Fig. 3. Partitioning of BSA in PEG–polyacrylate two-phase systems. Effect of SDS and pH. All systems contained Na_2SO_4 (4 wt%) and BSA (0.2 wt%). Polymer composition as in Table 1 (systems I–IV): pH 5.25 (I, II), pH 6.0 (III), pH 12 (IV). The difference between systems I and II is 0.18 (wt%) SDS in system I.

3.4. Partitioning of E. coli homogenate proteins in PEG–polyacrylate systems: effect of SDS and pH

A protein-rich *E. coli* crude homogenate, free from cell-debris was mixed in four different systems (I–IV) in order to study effects of SDS or pH on the protein partitioning. The composition of the systems and protein concentration in the phases are shown in Table 1. All systems contained 40% *E. coli* sonicated homogenate. The cell debris was removed by centrifugation before mixing the homogenate with the system. The systems were mixed and left standing 10 min before centrifuging for 5 min using a table centrifuge. Samples taken from the phases were analyzed using a SDS-PAGE gel (not shown) and protein concentration determined in clear centrifuged phases. Precipitation of proteins occurred in all systems particularly in system I (with PEG 8000) which contained SDS. This explains the low value on mass-balance (assuming the rest is the precipitated protein, ca. 60%). Partial precipitation on systems II–IV was relatively low, but clearly visible.

3.5. Plasmid DNA partitioning processes

Based on the results above two cross-partitioning processes were developed as shown schematically in Fig. 4. In these experiments a homogenate containing 60–100 μg/g pDNA with low protein content was used and no extra protein-rich homogenate was added to the systems. In process I the pDNA preference for polyacrylate at low pH is utilized in the step A. In step B the pH is raised and the PEG 8000 is partially replaced with PEG 4000, which causes the pDNA to partition to the PEG phase in step B. In process II pDNA is directly partitioned to a PEG phase in step A. In both processes the pDNA is separated from PEG by inducing a PEG/salt system where pDNA is partitioned almost exclusively to the salt-rich phase. Table 2 contains the partitioning data for the processes. The total yields as pDNA obtained in the final salt rich phases compared to the added pDNA in steps A were 60% in process I and 70% in process II. As the result of protein precipitation after obtaining the pDNA homogenate, no protein bands were observed in the final phases of the two processes upon analysis through SDS-PAGE (coomassie and silver staining).

Table 1
Systems for *E. coli* homogenate protein partition. Systems I and II contained PEG 8000, and systems III and IV contained PEG 4000. All systems contained 4.0 wt% Na_2SO_4 and 40% homogenate and Na-PAA 240,000. Polymer and SDS concentrations below are given in wt%. The polyacrylate concentration is given as polyacrylate excluding the sodium counterion. Protein concentrations in mg/g. Mass balance in %. T- and B-phase mean top (PEG-rich) and bottom (PAA-rich) phase, respectively. Average total protein concentration in all systems: 3.4 mg/g (0.34 wt%).

Component	System I	System II	System III	System IV
PEG	3.6	3.6	3.0	3.0
Polyacrylate	1.8	1.8	3.0	3.0
SDS	0.18	–	–	–
pH	5.4	5.5	5.8	9.6
Protein partitioning				
Conc. in T-phase	1.2 ± 0.2	2.6 ± 0.6	5.1 ± 0.8	7.0 ± 0.3
Conc. in B-phase	1.1 ± 0.2	3.6 ± 0.2	2.7 ± 0.5	1.1 ± 0.1
Mass balance	34± 5	91± 8	100± 10	110± 10
K-value	1.2 ± 0.1	0.7 ± 0.1	1.9 ± 0.1	6.4 ± 0.9

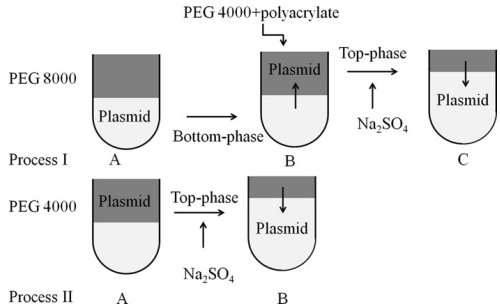

Fig. 4. Partitioning of plasmid DNA in PEG/polyacrylate aqueous two-phase systems. In process I pDNA is cross-partitioned between the PAA and PEG phase and then finally back-extracted to a salt phase. In process II the pDNA is back-extracted directly after the primary extraction to the PEG-rich phase. See Section 2.2.7 for composition details.

3.6. Plasmid DNA conformation

The analysis of pDNA conformations (supercoiled and open circle) was determined using agarose gel as shown in Fig. 5. Samples from different conditions were analyzed: the bottom phases at pH 5.4 and 5.8 from the partitioning studies in Fig. 2 above, lanes 1 and 2, respectively. Furthermore, the final Na_2SO_4-containing phases in the separation processes I and II (shown in Fig. 4 above), lanes 4 and 5, respectively. The concentrated pDNA fraction in lane 6 is a reference. The three bands furthest down in lane 6 are (starting from the furthest down) RNA, supercoiled pDNA and open circle pDNA. It can be seen in Fig. 5 that there is no significant change in pDNA conformation for all the different cases.

Table 2
Plasmid DNA partitioning in phases of bioseparation processes with PEG/polyacrylate/salt two-phase systems. Top/bottom volume ratios are: in process I, step A (1.1), B (1.55), C (0.08), in process II: step A (0.5 ± 0.1), B (0.1). Values are averages of 4 experiments (process I) or 2–5 experiments (process II). Standard deviations are given. Process I: initial pDNA concentration in step A: 5.7 μg/g. Process II: initial pDNA concentration in step A: 8 μg/g.

		Yields in the phases (%)			
		Top-phase	Bottom phase	Interface	pH
Process I					
Step	*K*-value				
A	0.33 ± 0.19	16 ± 6.5	49 ± 14	6.2 ± 4.6	5.2
B	12 ± 5.0	110 ± 6.8	7.5 ± 3.4	15 ± 14	9.5
C	0.06 ± 0.03	0.8 ± 0.8	110 ± 10	0.2 ± 0.3	9.5
Total Process[a] I yield: 60%					
Process II					
Step	*K*-value				
A	6.1 ± 2.2	57 ± 31	16 ± 11	3.3 ± 2.7	5.7
B	<0.1	0 ± 0	130 ± 0	0 ± 0.0	5.5 ± 0.5
Total Process[a] II yield: 70%					

[a] The total process yield refers to the yield when starting with step A and consider the final quantity in the salt-rich bottom phase in steps C and B, for processes I and II, respectively.

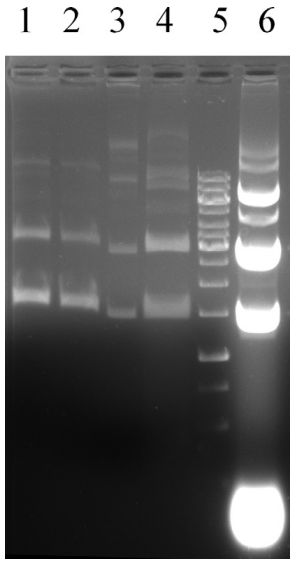

Fig. 5. Plasmid DNA analysis in different phases. Lane 1: polyacrylate rich bottom phase of a system with pH 5.4; lane 2: polyacrylate rich bottom phase of a system at pH 5.8; lane 3: salt rich bottom phase from process I; lane 4: salt rich bottom phase from process II; lane 5: Mw-ladder; lane 6: pDNA extracted from *E. coli* by alkaline lysis.

4. Discussion

4.1. Phase diagram of PEG–polyacrylate–salt two-phase systems

The shape of the binodals in the PEG–polyacrylate phase diagrams are similar to the corresponding systems of PEG/dextran, with exception that the content of polyacrylate in the PEG-rich phase is relatively high for its molecular weight. This can be understood from the stronger mixing entropy of the polyelectrolyte relatively to the corresponding non-charged hydrophilic polymer. An extensive experimental and theoretical study on similar PEG–PAA systems is found in reference [20]. The pK_a of polyacrylate is reported to be between 4 and 4.5 [27]. The increased compatibility of polyacrylate with PEG at lower pH is counter-intuitive according to the entropic driving forces, since, the lower the charged is on the PAA, the lower is the entropic cost of compartmentalization, and thus the easier is the phase separation. However, the PEG–PAA compatibility can be understood by an increased hydrophobic attraction between parts of uncharged polyacrylate with the PEG molecules or as frequently reported in the literature, increased hydrogen bonding between uncharged carboxylic groups with the ethylene oxide groups [28]. The tie-lines are not closing into the binodals which may be due to the fact that the system is quarternary, an indication that the phase boundary determined by turbidity titration, may be different from the binodals determined by composition points given by tie-lines.

4.2. Plasmid DNA partition

The strong influence of pH on pDNA partitioning is at first surprising. Polyacrylate and DNA repel each other electrostatically and DNA is expected to partition to the PEG-phase. However, since the hydrophobic groups of double stranded super coiled DNA are mostly non-exposed to the solution, the chemical character of DNA is strongly hydrophilic, which in the high salt concentration of the system may facilitate partition to the polyacrylate phase.

Generally, the driving force of entropic repulsion favours biomolecule partitioning to the polymeric phase with the smallest polymer size [29,30]. At lower pH there is an increased polyacrylate content in the PEG-phase, making the PEG-phase less entropically attractive. An alternative explanation could be hydrogen bonding between the non-charged carboxylic groups of polyacrylate at pH 5.2 with the hydrogen bond acceptor groups of the diester-phosphate groups as has been suggested in the literature [24,25]. It should be noted that the presence of the Na_2SO_4 favours partitioning of anions to the PEG phase by the well known electro-chemical driving force acting in aqueous two-phase systems containing a dominant salt [31]. Since, there are both entropic and enthalpic driving forces for DNA partitioning to the PEG-rich phase, another stronger enthalpic force favours partitioning to the polyacrylate phase. The partitioning of pDNA is complicated by the possibility of pDNA to adopt different structures, for instance a more compact form as discussed by Frerix et al. [8]. Polymers may induce compaction of DNA, which may also explain the occurrence of precipitation of a fraction of the pDNA [32].

4.3. Protein partitioning

The strong difference in BSA partitioning upon adding SDS to the system at pH 5.3 can be explained as follows: SDS associates with and denature BSA, creating a complex that is highly charged, and therefore repelling polyacrylate, and at the same time increasing the hydrophobicity of BSA, all this favouring a strong partitioning to the PEG-phase. In systems without SDS, the hydrophilic nature of BSA drives it to the polyacrylate phase. At pH 5.3 the charge of BSA is close to zero and no significant repulsion to the polyelectrolyte is expected. When BSA is completely charged at pH 12 it turns into a strong polyelectrolyte with a strong repulsion to the polyacrylate phase. Since BSA unfolds at pH between 9.5 and 10.9 [33,34] the exposed hydrophobic residues may have a strong influence on the increase of the partitioning to the PEG-rich phase. Using these findings, SDS was included in a system with *E. coli* proteins (Table 1). The presence of moderately high SDS concentration in a system with *E. coli* homogenate causes extensive aggregation and precipitation of most of the proteins, which in a bioseparation process for pDNAs is advantageous. Furthermore, hydrophobic endotoxins may be removed by the SDS addition and by partitioning to the PEG-rich phase. Increasing pH close to 10 in systems with *E. coli* homogenate proteins leads to the same effect explained above for BSA. However, since pDNA also partitions to the PEG-phase at high pH the process with SDS at pH 5.3 is expected to be more efficient in removing proteins from pDNA.

4.4. Bioseparation process

The partitioning driving forces (enthalpic and entropic) of flexible polymeric macromolecules are very large since they scale with molar volume [30]. Insolubility or precipitation into a separate interfacial phase is very common [6]. One way to overcome this is to choose aqueous two-phase system composition close to the critical point at the binodal curve. At this point phase differences are relatively small and partitioning becomes less extreme and precipitation can be avoided. However, if the starting material is a homogenate, whose composition may vary substantially due to changes in protein composition, the risk of strong changes in phase volume ratios and even prevention of two-phase formation is high, if the system composition is close to the critical point. If the composition point is far from the critical point the system becomes more

stable, separates faster, but more proteins and nucleic acids precipitate. The systems chosen here have been a compromise between these effects. In process II the pDNA is directly partitioned to the PEG-phase and then back-extracted to a salt rich phase. Proteins seems to partition also to the top-phase and it is therefore advantageous to add SDS to the homogenate or to the system as in process I, to remove most of the contaminating proteins by precipitation. The determined yield values and their total sum deviate strongly from 100% (complete mass balance). A trend can be seen, in the systems of process I step A, that they are lower than 100% and in the other steps they are higher. This may be due to an underestimation of pDNA content in step A, due to the presence of SDS, which may interfere with the fluorescence measurements. The total recovery in the presented system is in the same range as reported for other aqueous two-phase systems [13]. The novel polyacrylate/PEG system allows a strong change of the partitioning between the phases with relatively small changes in composition or pH.

4.5. Plasmid DNA conformation

As presented above (Fig. 5) there is no dramatic change in fraction of pDNA that is supercoiled. Since a supercoiled pDNA is relatively hydrophilic due to the high fraction of buried nucleotides, the partition force to the bottom-phase is probably of hydrophilic nature as discussed above. The high salt concentration and the presence of hydrophilic polymer may favor the more compact supercoiled structure of pDNA. The treatment of pDNA in a polyacrylate-rich bottom phase with a low pH buffer and PEG did not significantly change its conformation. This process of removing the polyacrylate polymer by co-precipitation with PEG at pH 2, could in principle be used in a recovery process for pDNA. However, since the pDNA is exposed to acidic conditions, the possibility of degradation of purines [35,36] should be evaluated. The pDNA can more safely be recovered from the polymer by cross-partitioning to the PEG and then back-extracted into a salt-phase as shown in process I.

5. Conclusion

A bioseparation process in which pDNA can be directed to PEG or to polyacrylate in an aqueous two-phase system has been found. In one process the pDNA is partitioned first to a polyacrylate-rich phase and then back extracted to a PEG-phase by replacing the PEG with one having a lower molecular weight. In another process the pDNA is directly partitioned to a PEG-phase. In both processes the pDNA is separated from the PEG by addition of salt which induces the formation of a PEG/salt system. The yield of pDNA obtained in the final salt phases is 60–70%. pDNA seems to have an attractive interaction with polyacrylic polymer at pH 5–6, and a repulsive interaction above pH 6. It is possible to enhance partitioning by lowering the molecular size of PEG thus favoring partitioning of pDNA to the PEG-rich phase. The recovery of pDNA from the PEG–polymer is performed by addition of salt (e.g. Na_2SO_4) to the PEG-rich phase which thereby induces a PEG/salt two-phase system, where pDNA is obtained in the salt phase. The supercoiled conformation of pDNA is retained in the separation processes.

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Paper III

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Isolation of PCR DNA fragments using aqueous two-phase systems

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ABSTRACT

Aqueous two-phase systems have frequently been utilized as an efficient bioseparation tool in the downstream processing of different biomolecules. The poly(ethyleneglycol)/poly(acrylate)/salt system, already explored successfully for purification of proteins and plasmid DNA, is here presented as an alternative approach for the isolation of small DNA fragments generated during *in vitro* DNA polymerase chain reactions. The polymerase chain reaction (PCR) is one of the most versatile laboratory techniques, but the purification of the amplified fragments often represents a major bottleneck. In this work we describe a simple and cost-effective method for isolation of DNA fragments obtained from PCR mixtures. The composition of the aqueous two-phase system in this work has been chosen to precipitate DNA molecules larger than 5000–7000 bp in the interphase, while the PCR products are partitioned very strongly between the phases in a two-step extraction process. In the first step, the DNA is partitioned quantitatively to the poly(ethyleneglycol)-phase. In the second, i.e. the back-extraction step, the DNA is strongly partitioned to a salt-rich phase that contains only low amounts of polymer. This system promotes rapid and high yields of purified small DNA products, less than 4000 bp, without contamination of proteins or large DNA templates present in the reaction mixture.

1. Introduction

Since 1956, when Albertsson first discovered that molecules could be partitioned between two aqueous phases [1], the aqueous two-phase systems (ATPS) have been widely explored as a powerful bioseparation tool. Particularly the ability to easily scale-up the process has been attractive for pharmaceutical production systems. The possibilities to fractionate different molecules such as native and tagged proteins [2–5] as well as DNA [6–8] using ATPS have all been described previously. The isolation of nucleic acids represents the most recent advance of the technique. In this work we present a new alternative approach for isolation of small DNA fragments based on a poly(ethyleneglycol)/poly(acrylate)/salt ATPS, which is a cost-effective option compared with other frequently used polymer systems.

The PEG/poly(acrylate) two-phase system has recently been examined for evaluating its potential for phase separation and use for isolation of biomaterials [9], as well as in partitioning of various proteins such as green fluorescent protein (GFP) [10], hemoglobin and lysozyme [11], and plasmid DNA (pDNA) [12]. In our earlier studies, pDNA could be partitioned between the PEG-rich and the poly(acrylate)-rich phases when operating close to the critical point of the binodal curve. For systems containing high polymer concentration, the pDNA tends to precipitate at the interface. This is a general effect, namely that partitioning becomes more extreme for larger molecules and for systems with larger tie-line lengths, i.e. larger differences in polymer composition between the phases [13–15].

In our previous work [12], sodium dodecyl sulfate (SDS) was added to the system to enhance protein precipitation of a homogenate sample obtained from lysis of *Escherichia coli* cells. This operational principle was maintained in this work, although the DNA solution sample contained much lower initial protein concentrations. The strategy for purifying a biomolecule in PEG-containing systems is to direct the target molecule to the PEG-rich phase in a primary two-phase system, which is followed by a back-extraction of the target molecule by inducing a PEG/salt two-phase system using the PEG-rich phase from the primary two-phase system. The impurities should ideally be partitioned to the opposite phase, in the present case to the poly(acrylate)-rich phase, in the primary two-phase system or be precipitated at the interface. The purified target molecule is then obtained in a salt-rich and

Abbreviations: ATPS, aqueous two-phase system; GFP, green fluorescent protein; PCR, polymerase chain reaction; pDNA, plasmid DNA; PEG, poly(ethyleneglycol); SDS, sodium dodecyl sulfate.

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almost polymer-free phase. The final polymer-free phase in a downstream process is more attractive since it is often necessary to further purify the product by filtration or chromatographic separations. A number of different PEG/poly(acrylate) phase diagrams have been investigated previously, where the PEG and poly(acrylate) sizes have been varied [9,12]. An efficient way to favor large molecules to partition to the PEG-phase in the first extraction step is to choose a system with high molecular weight bottom phase polymer and low molecular weight PEG [13]. The size of PEG must not be much lower than 1000 g/mol since nucleic acids may have a strong partitioning to the PEG-phase in a PEG/salt system for PEGs less than 1000 g/mol, but partitioning behavior can be complex under these conditions [16–20]. Furthermore, in order to find a suitable system for small DNA fragments, a number of parameters can be varied. One way of changing the partitioning of a substance in an ATPS is to increase the polymer concentration, leading to an increase of the tie-line length. This will lead to a more extreme partitioning of the biomolecules towards one of the phases, mainly due to the fact that polymer phases differ more in composition at higher concentrations than close to the binodal curve [21]. The differences in phase concentrations allow the separation of small DNA fragments while the pDNA or larger DNA templates are excluded from both the polymer-rich phases and precipitates at the interface [19]. This technique is sometimes used in protein purification processes as shown, for instance in the factorial design study by Persson et al. [13], where the target molecule is more enriched because of the large precipitation of contaminating proteins at the large tie-line length. However, at very large tie-line lengths both yields and purification factors may decrease [13]. The same principle is also used for back-extraction of target molecules from the PEG-rich phase in a PEG/salt system. As mentioned above the PEG size should be high in order to efficient exclusion from the PEG-phase.

For smaller DNA fragments, less than 4000 bp and generated e.g. by a polymerase chain reaction (PCR), less precipitation can be expected. PCR is an *in vitro* DNA amplification method where the fragments are originating from polymerization cycles based on temperature-dependent steps [22]. This technique is used in multiple fields including e.g. molecular evolution, molecular biology, forensic biology, diagnosis of hereditary diseases, genome mapping and sequencing projects [23–25]. The main bottle-neck of PCR is often to obtain an acceptable purity of these fragments, since contaminants such as enzymes, support proteins and other larger DNA molecules often are present together with the final product. Some commercial kits allow the purification of the amplified products, using particularly extraction after agarose gel electrophoresis [26,27]. These methods frequently result in low recoveries, which can be an issue in subsequent steps. In the presented work, we have achieved PCR fragments purification yields above 95% for the small amplified DNA fragments. The system is particular useful since it can be scaled down to allow use of conventional laboratory work volumes, i.e. milliliters or less, as represented in Fig. 1.

2. Materials and methods

2.1. Materials

2.1.1. Polymers

Sodium poly(acrylate) (25 wt% solution) having a molecular weight of 240,000 and poly(ethylene glycol) 8000 were obtained from Sigma–Aldrich. Poly(ethylene glycol) 4000 was purchased from Merck. All other chemicals were of analytical grade.

2.1.2. Stock solutions

A poly(acrylate) stock solution (1) of 15.0% (the concentration refers to the polyacrylate anion, not to the neutral sodium-polyacrylate), pH 7.3 was made by titrating the commercial poly(acrylate) solution with NaOH. The other solutions were: PEG 4000 (40.0 wt%) stock solution (2) and a Na_2SO_4 (3) (15.0 wt%). Buffer solutions: (4) Gly-H_2SO_4 (2.0 mol/kg solution, pH 2.0), acidic PEG 8000, solution (5), prepared by mixing 3.13 g of PEG 8000 (40 wt%) with 6.24 g of buffer (4), and 15.64 g water. The final concentrations were: PEG 8000 5.01 wt% and Gly-H_2SO_4 0.501 mol/kg solution). High pH buffer (solution 6) was composed of Gly-NaOH (2.02 mol/kg solution) and the SDS solution (7) was 2.0 wt%. Basic (underlying) aqueous two phase systems, b-ATPS, were made by mixing: 3.92 g solution (1), 1.92 g solution (2), 5.22 g solution (3) and water until the final mass of 14.0 g. The final concentrations were PEG 4000 (5.46 wt%), poly(acrylate) (4.20 wt%), Na_2SO_4 (5.58 wt%). HCl 0.18 g (37%)/14 g system or SDS 1.82 g (solution 7)/14 g b-ATPS system was added to some basic systems and thereby replacing some of the added water. The b-ATPS solutions could be stored for months at room temperature.

2.1.3. Two-phase systems

An efficient way to obtain reproducible two-phase extraction systems is to first prepare a b-ATPS and then mix it with the target-containing solution, in this work the DNA-containing solution. In our case we mixed 1.40 g b-ATPS with 0.6 g DNA-solution (containing PCR-fragments and proteins). The mixture formed is the primary ATPS. The basic-ATPS must be thoroughly mixed before mixed with the DNA-solution. The mixture is then centrifuged at 2×10 min at 10,000 rpm in an Eppendorf centrifuge. The

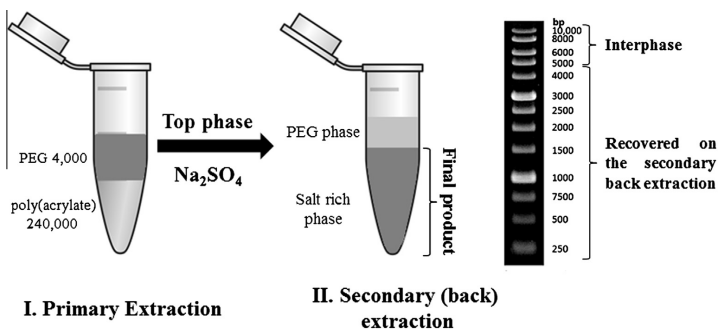

Fig. 1. Principle of the aqueous two-phase system used for isolation of small DNA fragments from a mixture containing several different sizes (a DNA ladder). DNA fragments larger than 5000 bp will accumulate in the interphase.

top-phase is transferred to another tube and Na_2SO_4 solution is added, i.e. solution 3, to induce the formation of a PEG/salt two-phase system. The mass ratio of added Na_2SO_4-solution/top-phase is 0.97, i.e. 0.485 g of solution 3 was added per 0.5 g top-phase. The PEG/salt solution is left standing on a cooling/heating block at 22–23 °C for 10 min. and then centrifuged for 2×10 min at 10,000 rpm. The bottom phase of this second two-phase system contains the PCR-product (see Fig. 1).

2.1.4. Precipitation of poly(acrylate)

The bottom phase in the primary ATPS is enriched in poly(acrylate). In order to avoid problems of interfering poly(acrylate) in the subsequent agarose gel electrophoresis, most of the poly(acrylate) can be removed by acid precipitation. This is performed as follows: 0.4 ml (4.1 g) bottom phase is mixed with 0.4 ml solution 5. A precipitate of PEG 8000 and poly(acrylate) is immediately formed and the total system is centrifuged for 2×10 min at 10,000 rpm. The supernatant is transferred to another tube. The pH is made alkaline by mixing 0.6 ml supernatant with 0.4 ml solution 6. The final solution is poly(acrylate)-removed bottom phase which can be analyzed by agarose gel electrophoresis.

2.2. PCR samples

PCR samples were prepared according to conventional protocols. The purified pDNA 5420 bp – pETDuetM-1 carrying the sugar beet hemoglobin class II GLB2 gene (426 bp), was used as an example of a typical template DNA and isolated from *E. coli* DH5α using QIAGEN® Plasmid Purification Maxi Kit (Hilden, Germany) [28]. The desired sequence was amplified by PCR in 100 μL reaction volumes, containing 1 μL of plasmid pETDuetTM-1 GLB2, 1 μL of both forward and reverse primers, 1 μL of 25 mM dNTP mixture (obtained by mixing equal volumes of 100 mM of dATP, dGTP, dCTP and dTTP, respectively), 10 μL of buffer, 16 μL of 25 mM $MgCl_2$, 59 μL of deionised water miliQ and 1 μL of Taq DNA polymerase (5U/μL) obtained from Fermentas (Helsingborg, Sweden). Due to the characteristics of the template used, the end-product could be obtained with different sizes depending on the primers used. Four different primers were utilized: pET Upstream-1 – CTACGCAGGCCGGCATCT, DuetDOWN1– CCGGCACATGTTCTAATACG, DuetUP2 - GCATAATCTTGTACACGGCC and T7 terminator– CGCTGAGCAATAACTAGC. The combination of pET Upstream with T7 terminator resulted in a DNA fragment of 956 bp (46.86% GC content), the combination of pET Upstream with DuetDOWN1 in a 704 bp fragment (47.73% GC content) and the combination of DuetUP2 with T7 terminator in 272 bp fragment (48.16% GC content).

2.3. Qualitative analysis

Resulting DNA fragments from the ATPS fractionations were analyzed on 15 cm long agarose gel electrophoresis (1%), stained with Gel Red (0.6 μg/mL). Electrophoresis was carried out at 100 V for 40 min, with TAE (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) as running buffer.

The quantitative measurements of protein concentrations in the final samples were made by Bio-Rad Protein Microassay (Bio-Rad, California, USA).

3. Results

3.1. Extraction process

After completing the PCR reaction, the mixture is composed of several components including Taq DNA polymerase and other support proteins, template DNA and the amplified DNA fragments. The PCR samples are prepared and mixed together with the basic ATPS. The primary extraction system (I) is a PEG (top-phase)/poly(acrylate) (bottom phase) two-phase system. The secondary (II) is a PEG/Na_2SO_4 two-phase system. The amplified PCR product is partitioned to the PEG rich top phase in system I and then back-extracted to the salt-rich phase in system II. Proteins are partitioned to the poly(acrylate) phase in system I and the template plasmid DNA is precipitated at the interface of system I.

3.2. Determining DNA-cut-off in the extraction process

Since high molecular weight DNA tends to precipitate at the interface, experiments were initially performed to determine which size of DNA fragments that can be maintained in solution. This was initially made by partitioning DNA of different sizes ranging from 250 to 10,000 bp in the extraction process. In Fig. 2, the size distribution of DNA in the two phases is shown. The starting material, the DNA ladder, contains high molecular weight DNA fragments (lane1), but in the first extraction a significant portion of this fraction is removed and precipitated at the interface (lane 3). However, the low molecular weight DNAs are partitioned quantitatively to the PEG phase. In the back extraction to the salt-rich phase all DNA is transferred to the salt-rich phase. There are some high molecular weight DNA (i.e. >5–6 kb) in the final extraction phase, but the ratio of high/low molecular weight DNA fragments is less than 1 as judged by the gel electrophoresis. The experiment indicates that the "cut-off" weight in this extraction process is around 6 kb. The yield of DNA partitioning was estimated by using 1 kb DNA ladder (Invitrogen Life Technologies Ltd., Paisley, UK)

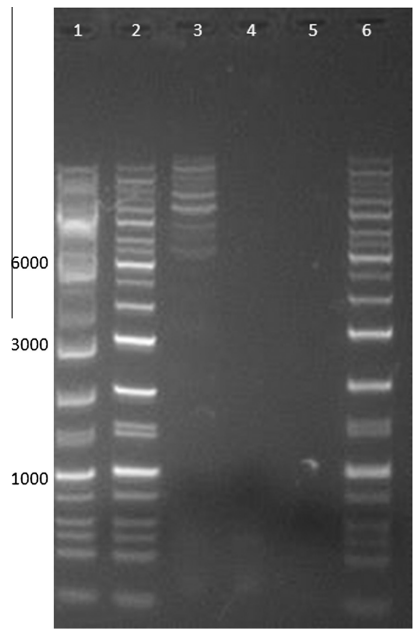

Fig. 2. Agarose gel electrophoresis after partitioning of a DNA molecular weight marker. Captions: 1 – initial sample: molecular weight marker 1 kb; 2 – top phase; 3 – interphase; 4 – bottom phase; 5 – top phase secondary extraction; 6 – bottom phase secondary extraction.

where the system limited the migration of DNA fragments larger than 6000 bp to the top phase. From the first step, 60% of the total DNA sample migrated to the top phase, largely corresponding to the smaller DNA fragments (⩽6000 bp). The larger DNA fragments were precipitated in the interphase. In the secondary extraction, the yield achieved was above 95%.

3.3. Partitioning of different PCR-product sizes

Three different PCR fragments were partitioned under the same conditions and analyzed by agarose gel electrophoresis. In all cases examined, the amplified fragments were enriched from the other contaminants of the PCR reaction. Fig. 3 shows the results for the fragments with the sizes 956, 704, and 272 bp, respectively. The partitioning is very strong in all cases, both to the PEG-rich top phase in the primary extraction system, and then to the Na_2SO_4-rich phase in the back-extraction system.

When analyzing the protein concentrations in the phases, the results clearly showed that proteins were not detectable in the secondary phase of the extraction (Table 1). The SDS in the primary system partitions strongly to the PEG-rich phase in the back-extraction system. The bottom phase of the secondary extraction resulted in a pure amplified DNA fragment sample without template and protein contaminations.

4. Discussion

The isolation of an amplified PCR product requires that the DNA molecules can be separated from proteins and that the nucleic acids can be fractionated according to size. The PEG/poly(acrylate) system can be modified to fulfill these prerequisites. The properties of PEG, an uncharged polymer, and sodium-poly(acrylate), a strongly negatively charged polymer, hence lead to an effective repulsion between these two polymers in an aqueous solution. The polyelectrolyte is thus not easily miscible with relatively low-polar components such as PEG. In spite of the effective strong repulsion between the two polymers, they will not separate into two phases at moderate concentrations (2–10%), unless a critical amount of salt is added to the system. The reason is that such separation will cause a strong compartmentalization of the counter

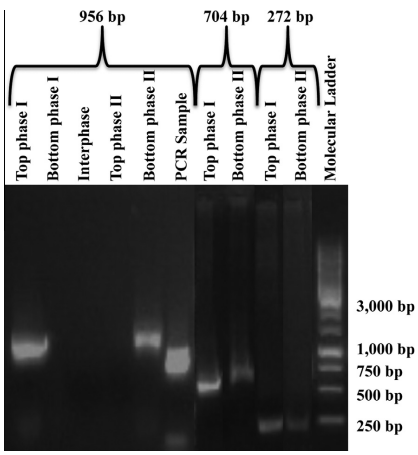

Fig. 3. Agarose gel electrophoresis after partitioning of different PCR samples: 956, 704 and 272 bp.

Table 1
Protein and DNA concentrations in the initial and final samples.

Samples	Abs 260/280	Protein concentration (Bradford method, μg/mL)	DNA concentration (μg/mL)
PCR initial sample	1.210	150	~1700
Purified fragment	1.800	Not detectable	~300

ions of the polyelectrolyte, which is entropically unfavorable. With the addition of salt to the system, there is no strong difference in ion concentration between the phases, and therefore not so strong loss in entropy upon phase separation. The dominant (majority electrolyte) salt in the system is Na_2SO_4, which is known to favor the partitioning of negatively charged biomolecules to the PEG-phase [11]. This is an effect of the stronger preference of the SO_4^{2-}-ion for the hydrophilic phase than the Na^+-ion. The negative charges from sodium-poly(acrylate) may also promote a strong repulsion on negatively charged biomolecules, such as DNA, however it is probably not as the major force in the system due to the screening effects of the salt. This partitioning is also dependent on the pH of the system [12]. In systems with pH close to 5.5 a significant degree of the poly(acrylate) is protonated and can accommodate partially hydrophobic molecules as well as forming hydrogen bonds to phosphate groups on the DNA backbone. The system presented here leads to the direct partitioning of the DNA molecules to the PEG-phase and then followed by back-extraction to a salt-rich phase. In the latter step, the concentrated PEG-phase formed in the salting-out process, excludes the DNA molecules strongly, presumably because the partial molar entropy of the DNA is much lower in the PEG-phase than in the salt-rich phase. This is an effect of lower number of molecules per volume unit in the PEG-rich phase compared to the salt-rich phase.

Due to the presence of different DNA molecules, the smaller amplified fragments products and the larger template pDNA, there is a need to modify the system to avoid common partitioning of both DNA molecules. This can be achieved by increasing the polymer concentration, i.e. increasing the tie-line length. The polymer phases differ more in higher concentrations compared to the ones close to the binodal curve, leading to more extreme partitioning [21]. Indeed the pDNA could be precipitated in the interface while the formed PCR fragments were quantitatively partitioned to the PEG-phase. The DNA recovery from the PEG-phase is performed by adding salt (Na_2SO_4), which induces a PEG/salt two-phase system, where the DNA is obtained in the salt-rich phase.

5. Conclusions

The PEG/polyacrylate ATPS is an effective and rapid system for isolating PCR products. The yield of DNA fragments obtained in the final salt phase was higher than 95% with no contamination of proteins or larger DNA molecules. The size limit of the DNA fragments that migrate to the top phase can be modified by changing the polymer concentrations, leading to an increase of the selectivity to smaller fragments with higher polymer concentrations. The pure small DNA molecules in the PEG-phase can be obtained in pure form by adding salt, creating a PEG/salt two-phase system, where DNA will partition exclusively to the salt-rich phase. The described system therefore offers an attractive alternative to the commonly used techniques based on agarose gel electrophoresis followed by extraction of the desired DNA fragments.

Acknowledgements

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Paper IV

Plasmid DNA Purification Using a Multimodal Chromatography Resin

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Abstract

Multimodal chromatography (MMC) is widely used for isolation of proteins since it often results in improved selectivity compared to conventional separation resins. The binding potential and chromatographic behavior of plasmid DNA have here been examined on a *Capto Adhere* resin. *Capto Adhere* is a recent MMC material allowing molecular recognition between the ligand and target molecule which is based on combined ionic and aromatic interactions. *Capto Adhere* proved to offer a very strong binding of nucleic acids. This property could be used to isolate plasmid DNA from a crude *E. coli* extract. Using a stepwise NaCl gradient, pure plasmid DNA could be obtained without protein and endotoxin contaminations. The RNA fraction bound most strongly to the resin and could be eluted only at very high salt concentrations (2.0 M NaCl). The chromatographic separation behavior was very robust between pH values 6 to 9, and the dynamic binding capacity was estimated to 60 µg/ml resin.

Key Words: plasmid DNA, RNA, endotoxin, multimodal chromatography, hydrophobic interaction, ionic recognition.

Introduction

The recent advances in gene therapy (GT) and DNA based vaccination have led to increasing demands for identifying and characterizing robust separation materials for purification of DNA molecules. Such chromatography resins should allow purification of nucleic acids, particularly plasmid DNA (pDNA) in high amounts, often ranging up to milligrams of product (Ghanem et al., 2013; Listner et al., 2006). This challenge of purifying pDNA in large-scale needs to be combined with careful monitoring of contaminants, where specifications mostly are given by regulatory agencies, such as FDA or EMEA (Paril et al., 2009; Stadler et al., 2004). Besides such clinically oriented applications of pDNA, there are several other areas in molecular biology where nucleic acids need to be purified effectively. This includes screening of biological libraries and enrichment and purification of nucleic acids to allow further enzymatic processes such as DNA labelling and PCR.

The chemical and physical properties of pDNA with a negatively charged backbone combined with a hydrophobic interior caused by the purine-pyrimidine base pairs, the large size of the molecules and the different conformations of pDNA gives the molecule a complex behaviour. The molecular recognition between nucleic acids and surrounding molecules therefore involves several different modalities which need to be taken into consideration when designing a purification process. Several alternative strategies are available and purification of DNA can be based on a range of different technologies including various forms of chromatography, but other methods such as precipitation (Freitas et al., 2006), filtration (Guerrero-German et al., 2009), aqueous two-phase systems (Barbosa et al., 2010; Johansson et al., 2012; Wiendahl et al., 2012) have all been explored. Particularly liquid chromatography has proved effective and has become an essential operational process for the large-scale purification of pDNA products, both as a preparative step and/or as an analytical tool in the process (Guerrero-German et al., 2011; Mahut et al., 2013; Mota et al., 2013; Sousa et al., 2012).

Due to the biophysical similarities of frequently occurring impurities, such as RNA, genomic DNA (gDNA) and endotoxins to pDNA, it is important to develop alternative chromatographic supports that improve the selectivity of the pDNA to the resin. An attractive option is to utilize anion-exchange chromatography (AEX) that has been used particularly as a first purification step to capture and concentrate pDNA and other DNA molecules (Carbone et al., 2012; Yang et al., 2008). Here the strong ionic interactions between the ligands and the

phosphate groups of DNA are explored to generate specificity in the binding between the resin and the target nucleic acid. Similarly, hydrophobic interaction chromatography (HIC) has proved to be useful a modality for pDNA purifications. For instance, the differences in hydrophobicity between single stranded nucleic acids, which show a higher exposure of the hydrophobic aromatic bases compared with different conformations of double stranded nucleic acid molecules, often provide for a high specificity in recognition (Matos et al., 2013). HIC has therefore also been utilized both as an analytical (Diogo et al., 2003) and preparative (Freitas et al., 2009; Pereira et al., 2010) method for pDNA purification. Besides developing new chromatographic ligands, much effort has been put on designing alternative support materials that can facilitate the diffusion and binding between pDNA and resin. Consequently, supports with larger pore sizes (Tarmann and Jungbauer, 2008), monoliths (Yamamoto et al., 2007, 2009), use of different ligand densities (Chen et al., 2011) and membranes (Zhong et al., 2011) have all been examined to promote higher pDNA yields. Multimodal chromatography (MMC) has been developed into a versatile and general purification approach and has frequently been used for protein and antibody purifications (Becker et al., 2008; Chung et al., 2010; Freed et al., 2011; Hou and Cramer, 2011; Kallberg et al., 2012). This modality utilizes more than one form of physical interaction between the stationary phase and the target molecules, for example, ion-exchange (IEX) and hydrophobic interactions (HIC) (Becker et al., 2008; Chung et al., 2010; Hou and Cramer, 2011) or reversed-phase and hydrophilic interactions (Lammerhofer et al., 2008; Wu et al., 2008). Compared with the traditional mono-modal ligands, a higher selectivity can often be achieved with these ligands. Several amino acid based ligands have been utilized for pDNA separations and these resins have MMC-like behaviour (e.g. Černigoj et al., 2013). In this study we have examined Capto Adhere, which is a recent MMC support material (Figure 1) based on HIC and AEX type of interactions (Chen et al., 2010).

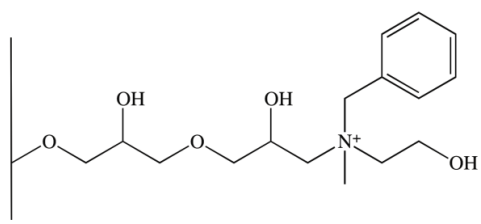

Figure 1: The *Capto Adhere* ligand: N-benzyl ethanolamine. The ligand harbours a high ion-exchange capacity linked with an aromatic moiety. This hydrophobic group also promotes its stability.

Capto Adhere exhibits very strong binding of nucleic acids and it was initially designed as a polishing material for purification of monoclonal antibodies to remove nucleic acids and other contaminants after a protein A column. Removal of nucleic acids and viruses from monoclonal antibodies is performed in flow-through mode in which the antibodies pass directly through the column while the contaminants are adsorbed (Gagnon, 2009). However, several other applications have been described. Due to the high selectivity of this support for nucleic acids, we have in this study used the resin to purify pDNA directly from a crude *E. coli* lysate. The binding capacity, the linearity when applying increasing amounts of pDNA to the column and the sensitivity of pH on the separation, have been examined in order to optimise the recognition and the purification process.

Materials and Methods

Growth of bacteria and plasmid production

Escherichia coli strain DHα5 was used as host in all experiments for production of pUC18 (2,686 bp). Growth was carried out overnight in shake-flasks (250 rpm) at 37°C using a Terrific Broth medium supplemented with 100 µg/ml of ampicillin (Montgomery and Prather, 2006). After fermentation, cells were harvested at the late log phase by centrifugation, 5,445g (20 minutes, 4°C), and the pellet was stored at -20°C before further use (Montgomery and Prather, 2006).

Alkaline lysis and preparation of a crude bacterial extract containing pDNA

Cells were lysed using a modification of the alkaline method (Clemson and Kelly, 2003). The final pellets containing the pDNA samples were dissolved in 10 mM Tris-HCl (pH 8.0) buffer. All samples were stored at -80ºC until use.

Purification of control pDNA

Control pDNA was purified using the Qiagen (Hilde, Germany) plasmid maxi kit according to the manufacturer's instructions. The protocol is based on a modified alkaline lysis procedure followed by an anion exchange resin with appropriate low-salt and pH washing and elution conditions. The resulted pDNA preparations were dissolved in 10 mM Tris-HCl (pH 8.0) buffer and stored at -80ºC until use. These samples were used for initial

evaluation of chromatographic behaviour and for determination of the dynamic binding capacity of the *Capto Adhere* column used.

Chromatography

All preparative and analytical chromatographic experiments were performed using the Äkta purifier system (GE Healthcare, Uppsala, Sweden) controlled by UNICORN software, version 4.11. A 2.5mL *Capto Adhere* column was packed according with manufacturer's protocol (GE Healthcare). The column was equilibrated for 12 column volumes (CV) with binding buffer, 10 mM Tris-HCl (pH 8.0), at a flow-rate of 1.0 $ml.min^{-1}$, prior to 100 µl sample injections. After injection, the column was washed with five CVs using the same buffer. A step gradient was then applied using firstly 1.0 M of NaCl (10 mM Tris-HCl, pH 8.0) for five CVs followed by 2.0 M NaCl (10 mM Tris-HCl, pH 8.0) for another five CVs. Peak absorbance was monitored at 260 nm. All buffer solutions for the chromatographic experiments were freshly prepared using ultra-pure grade deionized water and the solutions were filtered through a 0.45 µm filter (Whatman, Dassel, Germany) and degassed before use.

Fractions were also collected and analyzed for the presence of DNA using agarose gel electrophoresis. Before analysis, DNA samples were concentrated and desalted with Vivaspin concentrators (Sartorius Stedim Biotech, France). The electrophoresis was carried out using 1% agarose gels at 100V, for 40 minutes, using a TAE running buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). DNA concentrations in the gels were determined by a gel documentation system with Quantity One software (version 4.6.8) from Bio-Rad (California, USA).

Dynamic Binding Capacity

The dynamic binding capacity of pDNA was determined at >10% breakthrough for pUC18. The pDNA was diluted in the running buffer (10 mM Tris-HCl, pH 8.0) to a final concentration of 0.045 mg/mL. The solution was pumped with a flow-rate of 0.5 $ml.min^{-1}$. The same test was then done under non-binding conditions (2.0 M NaCl in 10 mM Tris-HCl, pH 8.0).

Protein and endotoxin determinations

Possible protein contamination in the DNA containing samples was initially screened by determining the ratio of absorbance at 260 and 280 nm. The specific levels of protein and endotoxin in the samples and fractions collected were subsequently determined using the ToxiSensor kit from GeneScript, USA and the Bio-Rad Protein Microassay (Bio-Rad, California, USA), respectively, according to the manufacturers' protocols.

Results and Discussion

Preparative chromatography of pDNA

An initial screen of pDNA behavior on a *Capto Adhere* column was first performed using pure nucleic acid samples prepared by conventional protocols. Different conditions for elution were examined, using both linear and stepwise gradients with increasing salt levels. A linear gradient was the first choice, but RNA was then partly co-eluted with DNA. Using a linear gradient, with increasing NaCl concentrations, pDNA and RNA were thus eluted at approximately 0.9 M and 1.2 M, respectively. However, based on these initially obtained elution data, suitable conditions for a stepwise NaCl gradient could be determined. This latter approach proved to be a simple and efficient method for eluting pDNA, and in addition, lower overall salt concentrations were obtained in the eluted DNA fractions. The possibilities of separating between different pDNA conformations, particularly the open circular (oc) and supercoiled (sc) forms, were also examined. However, only partial separation could be achieved and when a linear NaCl gradient was explored, a sc pDNA purity of 70% was obtained in an optimized system. The *Capto Adhere* column can in this aspect therefore not compete with other more specific resins, such as MMC Histamine (e.g. Černigoj et al., 2013). More crude and complex samples were subsequently tested and the chromatographic profile of pDNA elution from a clarified *E. coli* lysate is presented in Figure 2. 100 µL of a clarified *E. coli* lysate sample was injected to the 2.5 ml column previously equilibrated with 10 mM Tris-HCl buffer, pH 8.0. A first peak appeared in the void, resulting mostly from a flow-through of proteins. There was also a small portion of unbound nucleic acids. When concentrated five-fold and analyzed by agarose gel electrophoresis, this fraction proved to be enriched in higher molecular weight DNAs together with a smaller portion of pDNA (Figure 2). However, this fraction was completely devoid of RNA. In the first step of the gradient, application of 1.0 M NaCl resulted in the elution of a well-defined nucleic acid containing

peak. When analyzed by agarose electrophoresis, only pDNA was observed in this fraction. When increasing the salt level further to 2.0 M NaCl, the bound RNA molecules could be eluted as a sharp peak, which could be verified by agarose gels. When using the *Capto Adhere* material, it is obvious that ionic interactions dominate. However, the aromatic portion of the ligand, the phenyl group, gives additional specificity in recognition, since RNA and DNA molecules behave similarly on a conventional AEX column. The binding between nucleic acids and the *Capto Adhere* resin is also stronger and only at higher ionic strengths, the breakage of electrostatic interactions between the negative pDNA phosphate groups and the positively charged ligands occur.

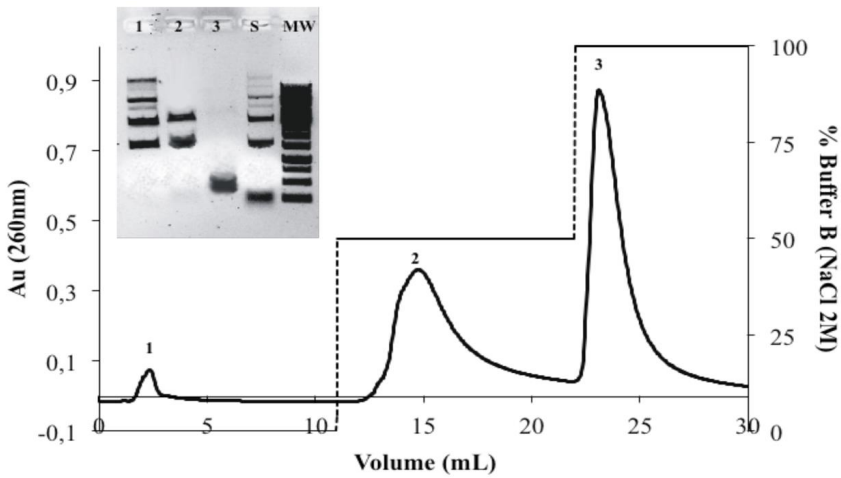

Figure 2: Chromatogram of an *E. coli* cell crude extract injected onto the *Capto Adhere* column. The sample concentration was around 100µg/mL using a volume of 100µL and flow rate of 1.0 mL.min^{-1}. The collected fractions were analyzed by agarose gel electrophoresis as presented in the figure; 1, peak 1 concentrated 5-fold before application on the gel to visualize presence of different nucleic acids; 2, peak 2 showing pDNA without concentrating the sample; 3, peak 3 containing the RNA fraction of the sample. This fraction contains 2.0 M NaCl which partly influence the migration of the RNA molecules; S, original sample; MW, molecular weight marker.

The RNA molecules, with even tighter binding to the ligand, thus eluted first when the ionic strength was increased to 2.0 M NaCl. During handling of crude bacterial extracts, the RNA fraction of the sample can easily be degraded by ribonucleases from the host cells. The degree of this degradation can be difficult to control, but when using *Capto Adhere*, this does

not influence the separation, since the RNA molecules in whatever sizes bind stronger to the resin than pDNA. The agarose gel results also showed that pDNA was eluted with no other nucleic acids contamination, such as linear, denatured, gDNA or RNA. Even if high NaCl levels were used for eluting pDNA, no change in the ratio of supercoiled to open circular pDNA could be detected during handling of the samples.

For each fraction, the levels of protein and endotoxin were also determined (Table 1). The results of the protein assay could conclude that no proteins were present in the pDNA containing fraction (peak 2). Additionally, the results of the endotoxin analysis indicated lower levels than the maximal allowed by regulatory agencies, 0.1 EU (µg plasmid)$^{-1}$.

Table 1 – Determination of protein and endotoxin levels in the eluted fraction after separation of a crude *E. coli* sample. The results are averages obtained from three independent samples.

	Peak 1	***Peak 2*** **(pDNA fraction)**	**Peak 3**
Endotoxins (EU/µg pDNA)	0.105	***0.094***	0.123
Proteins (µg/mL)	0.118	***not detectable***	0.006

Dynamic Binding Capacity of *Capto Adhere* Column for pUC18

Agarose supports can be considered as non-porous for most of pDNA molecules. The average pore size is thus around 70 nm and the pDNA molecules have a similar size under the conditions used (Tiainen et al., 2007a). Determination of the dynamic binding capacity (DBC) is therefore needed to understand the limitations of the column for pDNA purifications. The binding of pDNA will mainly occur on the pores' surface and it will depend upon how the DNA molecules are shaped and oriented, which in turn is a function of the pDNA conformation, concentration and ionic strength (Chen et al., 2011; Ferreira, 2005; Teeters et al., 2004).

The DBC analysis for pDNA on the *Capto Adhere* column was performed using pre-purified pUC18 by saturating the column using binding and unbinding buffer conditions, 0 M and 2.0 M NaCl (10 mM Tris-HCl, pH 8.0), respectively (Figure 3). The initial sample used had a final concentration of pUC18 of 0.045 mg/mL. Under binding conditions employed, the column had a 50% capacity factor ($C_{50\%}$) of 0.06 mg pDNA/mL gel, which is lower than comparable conventional AEX materials (Eon-Duval and Burke, 2004; Tiainen et al., 2007b), However, the present matrix has not been developed for such large molecular entities as pDNA and a monolith format would enhance the binding capacity of the ligand.

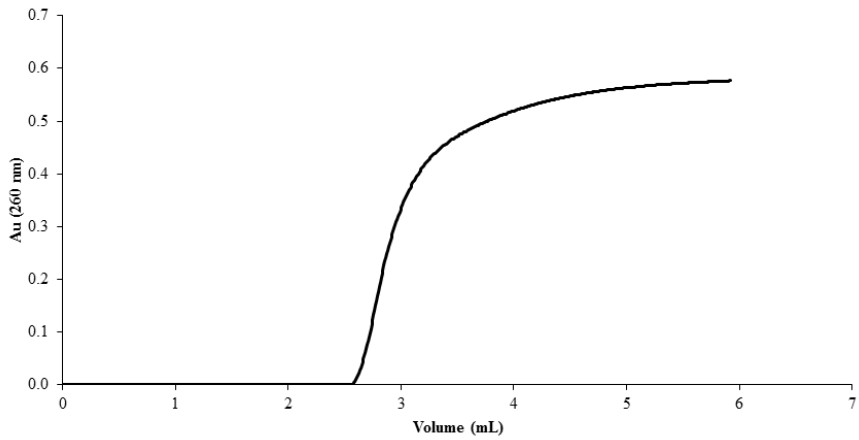

Figure 3: Plasmid DNA breakthrough curves for the *Capto Adhere* column (2.5 ml) under binding conditions (10 mM Tris-HCl, pH 8.0). The initial pDNA sample was prepared in the running buffer with a final concentration of 0.045 µg/mL. The solution was pumped with a flow-rate of 0.5 mL.min^{-1}.

pH dependence on pDNA separation

The chromatographic separation of pDNA can often be influenced by the pH value of the buffer used. The potential pH effects on the chromatographic behavior of *Capto Adhere* was therefore examined over a wide range, pH 6-9, using the same stepwise NaCl gradient as previously described (Figure 4). The results indicated no or very limited effects on the chromatographic behavior over the entire pH range tested. The only different significant behavior occurred at pH 9.0, where the peak changed shape and partially was split into two. At pH 9.0, there is a tendency for DNA strand separation which easily could be observed in

the chromatograms. Besides this intrinsic behavior of the DNA molecule, the peak shapes were identical over the pH values tested and appeared very stable in this range.

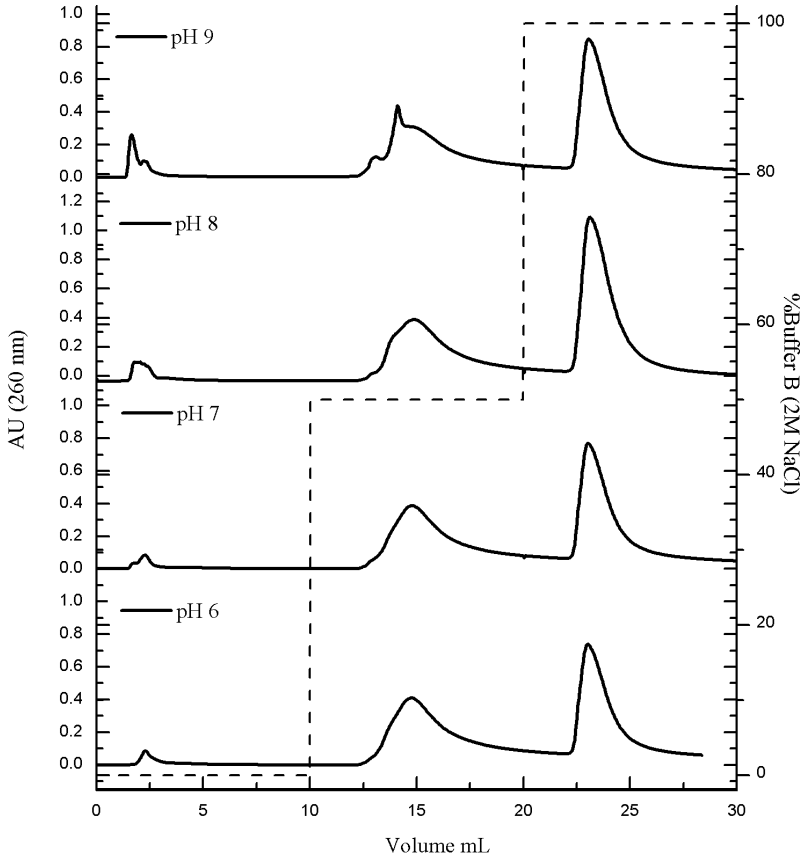

Figure 4: pH dependency on chromatographic behaviour. In the separation 10 mM Tris-HCl, pH 8.0, was used as running buffer with a flow-rate of 1.0 mL.min^{-1}.

Linearity studies

The binding of pDNA to a chromatographic material like *Capto Adhere*, largely takes place on the surface of the resin. In addition, the inherent high viscosity of DNA samples can cause diffusional restrictions which may limit the binding efficiency. Several different pDNA concentrations were therefore applied on the column and the behavior of separation was analyzed using the same stepwise elution regime as previously used. Possible effects of

sample concentration on the chromatographic process were examined by linearity studies. In these separations no effects side-effects were observed and the recovery of pDNA was identical (Figure 5).

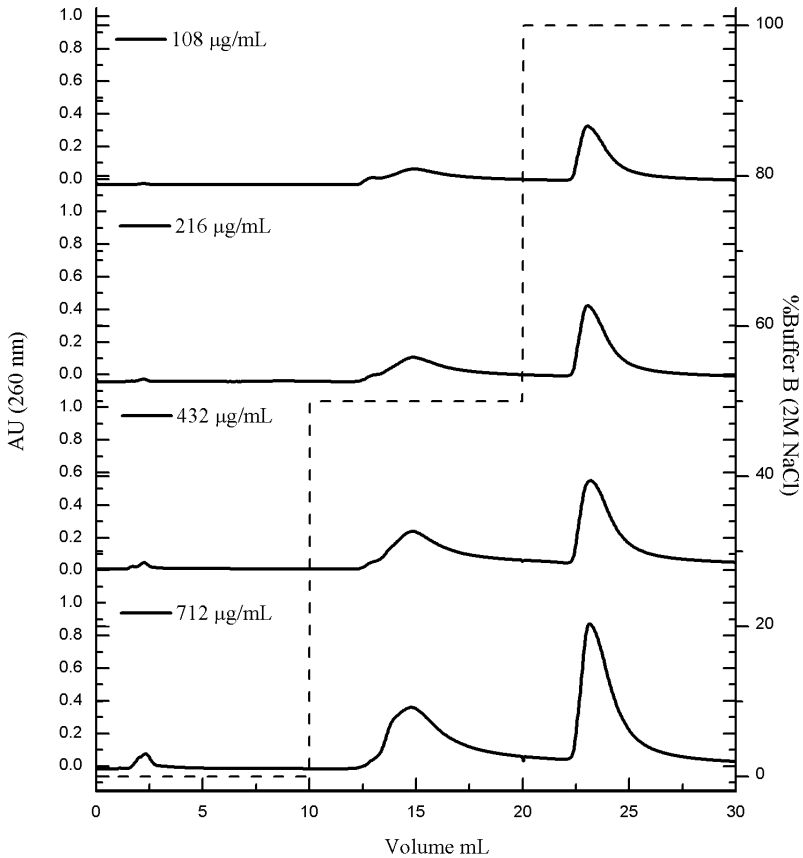

Figure 5: Linearity studies using four different sample concentrations within the range of 100-750 µg pDNA/mL. The chromatographic conditions were maintained as in the previous tests.

Conclusions

The use of *Capto Adhere* for pDNA isolation from crude *E. coli* extracts is very effective and robust. The chromatographic separation can thus be achieved over a range of different pH values and sample concentrations. The recognition of nucleic acids to the ligands is exceptionally strong, largely due to the hydrophobic phenyl group of the ligand. However, this moiety partly prevents the separation of the supercoiled and open circular forms of pDNA. The resin is also very effective for removal of several contaminants present in bacterial homogenates including RNA, genomic DNA, proteins and endotoxins. This MMC approach therefore turns highly applicable for DNA vaccine and gene therapy oriented products.

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Paper V

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Binding and elution behavior of small deoxyribonucleic acid fragments on a strong anion-exchanger multimodal chromatography resin

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ABSTRACT

The separation behavior of small single-stranded from double-stranded DNA molecules has been determined on a multimodal (mixed-mode) chromatography system. The resin used is a strong anion exchanger which also modulates hydrophobic recognition. The intrinsic differences between single- and double-stranded DNAs concerning charge, hydrophobicity and three-dimensional structure render this form of MMC suitable for separation of the different nucleic acid molecules. All DNAs tested bound strongly to the resin and they could be eluted with increasing NaCl concentrations. Each homopolymeric ssDNA sample resulted in a base-specific elution pattern when using a linear NaCl gradient. The elution order was poly(dA) < poly(dC) < poly(dG) < poly(dT) and this order was dependent on the secondary structure of the molecule. Such differences were not observed for small double-stranded DNAs. Due to the more hydrophobic nature of single-stranded DNA molecules they could be separated from double-stranded DNAs.

1. Introduction

Multimodal (mixed-mode) chromatography (MMC) methods first appeared already in the 1950s [1] and hydroxyapatite is the archetype of multimodal chromatography methods, combining cation exchange and metal affinity [2]. Several alternative MMC media, which allow for multiple binding interactions, have recently been developed and they are today extensively used in a variety of applications including separations of post-translationally modified proteins [3], mutant proteins [4,5], oligosaccharides [6] and antibodies [7,8]. Particularly in the downstream processing of IgG, the frequently used protein A step, which allows an efficient adsorption of the antibodies, is followed by an MMC flow-through of the product to remove possible IgG aggregates or remaining DNA. Additionally, these MMC matrices have been explored for host cell protein (HCP) removal from the target monoclonals and they may thereby reduce the number of conventionally used chromatographic operations [9–11].

In MMC, multiple and different physical interactions can take place between the target molecule and the affinity ligand coupled to the stationary phase. For instance, ionic and hydrophobic interactions may occur simultaneously. Such cooperativity can often increase the selectivity and specificity of the chromatographic process. However, it is feasible to use these multimodal media in a "single-mode" fashion by choosing the binding and eluting conditions such that only a single type of interaction occurs [12]. This possibility renders MMC media very versatile for any separation of biomolecules.

The chromatographic behavior of small DNA molecules as well as plasmid DNA (pDNA) has been investigated by several researchers. Due to the charged characteristics of nucleic acids at neutral pH, anion-exchange chromatography (AEX) has proved very useful and is commonly explored as a first step in the purification of plasmid DNA (pDNA) [13–15]. However, the mechanisms behind the binding and elution of DNA on a chromatographic resin are complex and several conditions need to be assessed including choice of ligand, ligand density, matrix and size of target molecules [16–19]. Particularly the large volume of pDNA is an operational hurdle for most chromatographic matrices available, since the size of the nucleic acid affects the access and diffusion through the pores of the chromatographic particles [20]. Convection-aided monolithic column chromatography or the use of macro-porous gel particles can in some instances be very effective and efficient for separating large DNAs, such as pDNA [15,21]. In addition, particularly large nucleic acid molecules may influence the viscosity of the sample substantially, meaning that small fragments of DNA can diffuse easier during a chromatographic separation.

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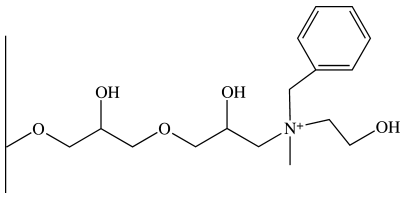

Fig. 1. The Capto adhere ligand: N-benzyl-N-methyl ethanol amine. The ligand has a high ion-exchange capacity mixed with hydrophobic moieties, which also promote its stability. The total ionic capacity is 0.09–0.12 mmol Cl^-/ml and the mean particle size is 75 μm.

Beside electrostatic interactions, the hydrophobic nature of nucleic acids may be explored for developing a chromatographic system. This has been demonstrated in several independent studies [22–24]. Due to the hydrophobic properties of the bases in nucleic acids, a mixed-mode ligand composed of hydrophobic and ion-exchange properties may therefore be favorable for separating different conformations of DNA molecules. The bases in single-stranded DNA molecules are more exposed and the hydrophobic property of the ligand can be used for ameliorating a separation between ss- and dsDNA.

In this study, the chromatographic behavior of small DNA molecules in MMC was investigated systematically. The binding and elution of ss- and dsDNA molecules in the size range 19–33 mers were determined on a strong anion-exchanger mixed-mode ligand–Capto adhere (Fig. 1). The differences in charge and hydrophobicity of these different DNA molecules render this form of MMC particularly suitable. All DNAs tested bound strongly to the resin and they could be eluted with increasing NaCl concentrations. We particularly focused on separating small ss- from dsDNA molecules since this is an essential separation issue in molecular biotechnology. Such separations are thus often needed, e.g. in the preparation of phage DNA, studies of single nucleotide polymorphism and in the preparation of an amplified DNA product after a polymerase chain reaction. Chromatographic approaches are then attractive alternatives to the frequently used electrophoretic methods, which often are time-consuming and offer less resolution.

2. Materials and methods

2.1. Materials

Poly(dA), poly(dC), poly(dG), poly(dT), which are single-stranded DNA (ssDNA) molecules of equal length (26-mers), were obtained from Sigma–Aldrich (Stockholm, Sweden). Sal-3 and HAXpETforw are two ssDNA molecules used as primers for amplifying DNA by a polymerase chain reaction, and theywere purchased from Eurofins (Ebersberg, Germany). The dNTP samples were obtained from Thermo Scientific (Thermo Fisher Scientific, Sweden). The dsDNA samples were formed by mixing equal amounts of complementary ssDNA, poly(dG) and poly(dC) as well as poly(dA) and poly(dT), respectively, at 90 °C for 10 min and then let them hybridize by slowly decreasing the temperature to ambient conditions. The integrity of the dsDNA preparations was verified by 1% agarose gel electrophorsesis using GelRed (0.6 μg/ml) staining. A pure plasmid DNA preparation was obtained by cultivating *Escherichia coli* DH5α cells harboring pUC18 in LB medium [25] followed by purification using the Qiagen plasmid purification maxi kit (Hilde, Germany). All nucleotide and DNA samples studied are accumulated in Table 1.

All solutions were freshly prepared using ultra-pure grade deionized water (PureLab ultra, ELGA Veolia Water Solutions and Technologies, Väsby, Sweden) and analytical grade reagents. The buffer solutions were passed through 0.45 μm filters from Whatman (Dassel, Germany) and degassed in a vacuum system before the chromatographic separations.

Table 1
DNA and nucleotide samples used in this study including their length and M_w values.

Sample	Sequence (5′ → 3′)	Length (nt)	M_W (g/mol)
dATP			557
dCTP			533
dGTP			573
dTTP			548
Sal-3	TATCGCCACGTTCGGGCAA	19	5789
Poly (dC)	dC_{26}	26	7457
Poly (dT)	dT_{26}	26	7847
Poly (dA)	dA_{26}	26	8082
Poly (dG)	dG_{26}	26	8498
HAXpETforw	GTGTCCAGCGCATATGCCGTTACCGCCACACCC	33	9995
pUC18		2686	1.74×10^6

2.2. Chromatography

Capto adhere, based on the ligand N-Benzyl-N-methyl ethanol amine, which is coupled to a cross-linked agarose matrix, was obtained from GE Healthcare Biosciences (Uppsala, Sweden). All experiments were carried out on a 2.5 mL column (0.7 cm diameter and 6.6 cm height) using ÄKTA explorer, a liquid chromatography system from GE Healthcare (GE Healthcare Biosciences, Uppsala, Sweden). The column was equilibrated with a starting buffer, buffer A composed of 10 mM Tris-HCl, pH 8.0. Elution of the samples was achieved by a linear gradient of NaCl until a final concentration of 2 M NaCl was reached, using buffer B, i.e. buffer A supplemented with 2 M NaCl. The flow rate was kept constant at 1 mL/min. After the chromatographic separations, the nucleic acid samples were identified by agarose gel electrophoresis.

3. Results

3.1. Elution behavior of dNTPs

Firstly, the chromatographic behavior of single deoxynucleotides, dNTPs, at a concentration of 100–200 μM, was examined on the Capto adhere matrix. The samples were all strongly adsorbed to the column at the pH used (8.0), and they could all be completely recovered as a single peak at 0.6–0.8 M NaCl (Fig. 2). Small but significant differences in the elution characteristics between the four dNTPs were observed. The elution values are presented in

Table 2
Elution volumes, as determined from the start of the linear gradient, and the corresponding salt concentrations needed for elution of the different nucleic acid and nucleotide samples.

Sample	Elution volume (mL)	[NaCl] M
pUC 18	2.8	0.28
dCTP	6.27	0.63
dTTP	6.36	0.64
dATP	7.10	0.71
dGTP	7.39	0.74
Poly(dA/dT) (dsDNA)	11.5	1.15
Poly(dG/dC) (dsDNA)	11.8	1.19
Sal-3 (ssDNA)	13.5	1.35
Poly(dA)	14.0	1.41
HAXpETforw (ssDNA)	16.8	1.68
PolydC)	31.4	2.14
Poly(dG)	36.3	2.63
Poly(dT)	38.9	2.88

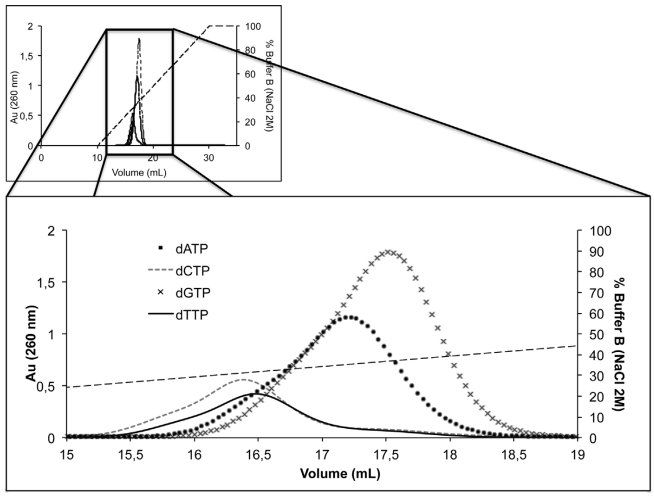

Fig. 2. Chromatograms of the single deoxynucleotides injected onto the Capto adhere column. The sample concentration was varied between 100–200 μM using a volume of 100 μL.

Table 2 and as shown, particularly dATP and dGTP, the pyrimidines, exhibited the strongest interaction to the matrix.

3.2. Elution behavior of single- and double-stranded DNA

Oligodeoxynucleotides, in single- and double-stranded forms, were subsequently examined on the Capto adhere column. The ssDNA molecules generally exhibited stronger binding to this matrix compared with the double-stranded nucleic acids of the same size. In order to identify any sequence specificity in the elutions, polydeoxyadenylic acid, poly (dA), was compared with the other homologous oligonucleotides, poly (dT), poly (dG) and poly (dC), of the same size (26-mers). Each ssDNA sample could be completely recovered (> 95%) and resulted in a specific elution volume at a given salt concentration in the gradient. The elution order was poly (dA) < poly (dC) <poly (dG) <polydT, clearly indicating thathomoplymeric ssDNA molecules form secondary structures that are dependent on the nucleotide given. For instance, poly(dA) spontaneously forms a helix while poly(dG) generates a quadruplex structure [26]. Similarly, poly(dC) molecules are folded into i-tetraplexes while poly(dT) shows extensive regions of hairpins by intra-strand base-paring exposing the negative charged phosphates of the DNA backbone [27]. These structures influence the charged and hydrophobic characteristics of the molecules and thereby their behavior on the chromatographic material. The strongest binding was thus observed for poly (dT), which was eluted at 2.88 M NaCl. Two other ssDNA molecules, Sal-3 and HAXpET-forw, with a mixed base composition were also examined. Here, the longer ssDNA molecules appeared to elute later in the NaCl gradient (see Table 2) indicating a possible size-dependent elution behavior operating together with the involvement of different secondary structures. Longer ssDNA molecules thus have enhanced possibilities for interactions between the phosphate groups of the DNA and the chromatography ligand. Such interactions have a complex

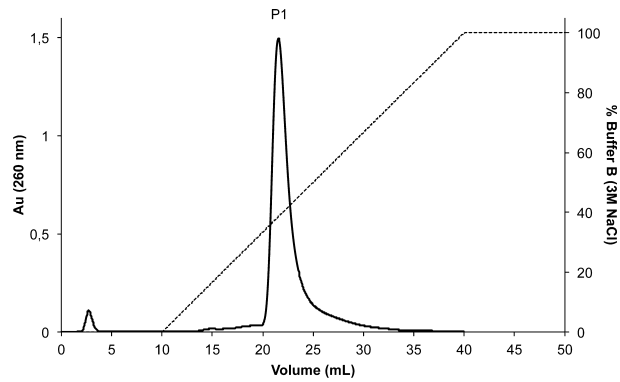

Fig. 3. 200 μL injection of ds poly(dA/dT) at a concentration of 500 μg/mL; P1, peak 1, represents the dsDNA molecule.

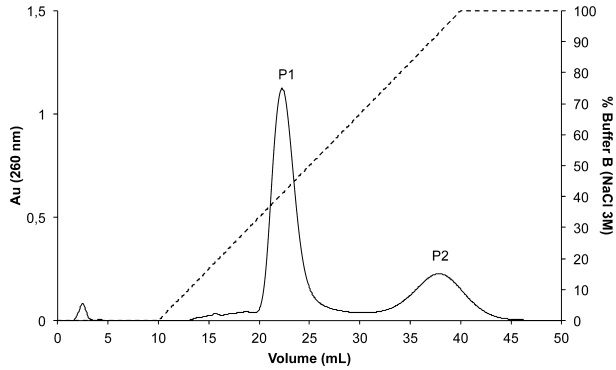

Fig. 4. 200 μL injection of a 1:1 mixture of ds poly(dA/dT) (100 μL) and ss poly(dA) (100 μL); concentrations used: 500 μg/mL. P1- poly(dA/dT); P2- poly(dA).

nature and ionic, hydrogen bonding and hydrophobic interactions are all involved [12]. When compared with dsDNA, the ssDNA molecules generally eluted later in the gradient, most probably due to stronger hydrophobic interactions with the MMC ligand. A typical elution curve for dsDNA is shown in Fig. 3. There were significant differences in elution behavior between dsDNA and ssDNA which could generate effective separations between small dsDNA and ssDNA fragments (Fig. 4). By determining the area under each peak it was possible to quantify the amounts of dsDNA and ssDNA, respectively. Different forms of gradient elutions were examined but a linear gradient proved to give the best resolution. In these chromatographic tests, the peak shape was generally not affected by the flow-rate, and 1.0 ml/min could be used to obtain fast separations (data not shown). A chromatogram could thereby be finished within 30 min. However, by using lower flow-rates, 0.1 ml/min, resolution could partly be improved and resulting in base-line separation between ss- and dsDNA molecules.

3.3. *pDNA vs ssDNA/dsDNA*

For reference purposes, we also included supercoiled plasmid DNA (pDNA) to our test mixtures. Plasmid pUC18 was hence used as a control sample and this DNA is approximately 100-fold larger than the previously studied dsDNA samples (Table 1). The removal of small DNA fragments is an important separation issue in many experiments in molecular biology. When applied alone, the pDNA bound strongly to the column and could be eluted at 0.28 M NaCl, which is much earlier in the gradient compared with the other smaller ss or dsDNA samples. Since nucleic acid samples often contain mixtures of different molecular sizes, the pDNA was mixed with either single- and or double-stranded DNA separately. The mixtures with pDNA were prepared in a proportion 1:1 (w/w) with pUC18 (400 μg/mL) and ssDNA (poly (dA), (dT), (dG) or (dC)). The separation of ssDNA and dsDNA, respectively, from pDNA was examined using the same conditions as previously. We could thereby dissect the competitive binding between the larger pDNA molecules with the smaller ones (Fig. 5). Under the conditions used, most of the pDNA sample did not bind to the ligand since the smaller fragments were able to attach the ligands faster and more effectively. The diffusion of the pDNA in the system is much lower, and the binding process will be limited to the restricted amount of ligands still available on the bead surface. In addition, the elasticity of pDNA is also limited, which further promotes a reduced accessibility to the free ligands. The column can thereby be utilized in a flow through fashion, where the pDNA can be removed from small DNA molecules by very simple means.

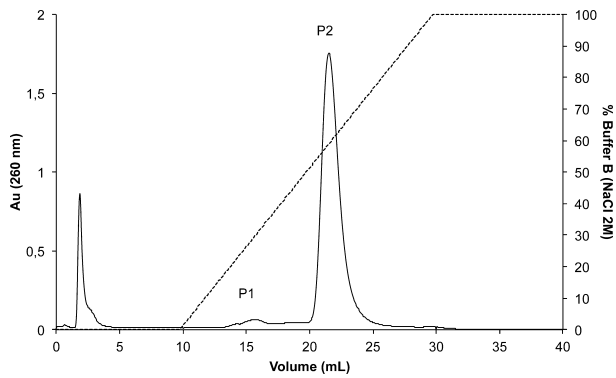

Fig. 5. 200 μL injection of a 1:1 mixture of poly(dA/dT) (100 μL) and pUC18 (100 μL). Concentration used 500 μg/mL. P1 – pUC18; P2 – poly(dA/dT).

4. Discussion

MMC has previously almost exclusively been explored for protein separations. We and others have analyzed the molecular recognition behavior of protein variants and developed mathematical models for predicting binding and elution performances. The different characters of the binding sites involved in MMC provide very important information on the recognition behavior [12]. However, the individual recognition elements involved in chromatography of nucleic acids are not as well understood as those of other biomolecules, particularly those of proteins. As DNA molecules easily may change secondary and tertiary structures [28], the separation mechanism becomes complex. In the ssDNA samples analyzed, the number of charges of the DNA backbone is identical and the separation differences observed can therefore be related to differences in folding, and consequently, the possibilities for interactions between the ligand of the support material and the bases of the nucleic acids. Similarly, the dsDNA samples have more charges available compared with ssDNA of equal size. By making such distinctions, particularly the involvement of ionic vs hydrophobic interactions can be distinguished.

In this study, we have treated the MMC material largely as an AEX resin. AEX separations of DNA fragments have been investigated earlier by several researchers [29,30] and for review cf [31]. From these studies it is evident that it is cumbersome to separate small ss- and dsDNA molecules in the range 10–50 nucleotides. Separations therefore need to be accomplished with strict control over elution by using a very shallow gradient slope and high initial salt concentrations. In conventional AEX, small or medium-sized DNAs, a dsDNA conformation show higher retention values compared to ssDNA of the same size. This is due to the fact that the dsDNA has more charges per contact area with the AEX ligand. However, in our study we showed the opposite behavior with a stronger binding of ssDNA compared with dsDNA. Similarly, Murphy et al. [32] demonstrated that the affinity of ss-poly (dA) (20mer) to an immobilized metal affinity chromatography (IMAC) resin proved to be much higher than that of the corresponding ds-poly dAdT 20mer. These results clearly indicate that a dsDNA molecule shields the bases which are easily accessible for the ligand on ssDNA. The additional interactions and change of the available area for the ligand of the resin therefore contribute to give ssDNA strong retention in this case. Different interactions including electrostatic interactions with the phosphate backbone combined with hydrophobic binding can therefore be utilized for achieving an optimized separation of nucleic acids.

The hydrophobic moiety of the MMC ligand, the benzyl group, is thus essential for achieving the observed separation between the ssDNA and dsDNA samples (Fig. 1). The use of NaCl for elution from the MMC resin represents a mixture of two different interactions. Increasing NaCl concentrations promote hydrophobic interactions, but on the other can reduce electrostatic interactions.

Even though Capto adhere originally has been developed for protein purification purposes, it is essential to characterize its binding behavior also for other biomolecules, particularly different classes of biomolecules which may be present as contaminants in a protein sample. In most instances, Capto adhere is explored in a flow through mode, in which the protein is quickly passed through the column while contaminating DNA should be adsorbed. It is evident from the present investigation that particularly longer dsDNA molecules may interfere in such a purification strategy. Such DNA may originate from genomic DNA or pDNA present in the protein sample. If the contaminating DNA has been partly degraded, the longer DNAs still present, may easily follow the flow through fraction. It is therefore essential both to monitor the amounts and composition of contaminating nucleic acids in these instances in order to fully explore the MMC system in the desired mode.

5. Conclusions

In this study we have demonstrated that it is possible to achieve separations between small ss and dsDNA samples using Capto adhere MMC columns. Since this material is a very strong anion exchanger largely insensitive to pH changes and the isolectric point of DNA is low, around 4.0, and instead of controlling elution by pH, we have instead focused on electrostatic and hydrophobic interactions using NaCl for elution. Base-specific elution characteristics have been obtained for single-stranded homo-oligonucleotides, showing the strongest binding for polydT, but it still remains to be tested whether this property can be explored more generally, for instance to separate different oligonucleotides with random sequences. Such base-specific separations were not observed for dsDNAs since the ligand not is able to interact with the base pairs. The development of MMC methods is more complex than conventional chromatographic methods, but mixed modes can often extend purification capabilities into dimensions beyond the scope of conventionally used AEX or HIC methods.

Acknowledgments

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Paper VI

Capto™ Resins for DNA Binding and Elution: A tiny Difference in Ligand Composition Makes a Big Impact in Separation of Guanidyl Containing Fragments

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Abstract

The difference in chromatographic behaviour of individual deoxynucleotides as well as small single-stranded and double-stranded DNA molecules has been examined for two different resins from the *Capto* family: *Capto Adhere* and *Capto Q ImpRes*. *Capto Adhere* is a strong anion exchanger which also modulates aromatic recognition, while *Capto Q ImpRes* is a strong anion exchanger with a similar ligand, but without a phenyl group. The intrinsic differences between single- and double-stranded DNAs are dependent on charge, hydrophobicity and three-dimensional structure. These variations in biophysical properties have been utilized for comparative separations on these resins. All deoxynucleotides and DNAs tested bound strongly to the chromatographic materials and could be eluted by a linear gradient of increasing NaCl concentration. *Capto Q ImpRes* provided a recognition for guanylate bases when samples of deoxynucleotides or poly(dG) were examined. This recognition was not observed for *Capto Adhere*. Another pronounced difference between the resins was observed in the inverted elution of ss- and dsDNA, where ssDNA elutes at 2.88 M NaCl on *Capto Adhere*, while on *Capto Q ImpRes* ssDNA elutes already at 1.47 M NaCl. This behaviour can be linked to the presence of the more hydrophobic phenyl group in *Capto Adhere*, leading to stronger retention of ssDNA molecules, which have a more hydrophobic character due to higher degree of base exposure.

Keywords: ion-exchange chromatography; plasmid; nucleotide; single-stranded DNA; double-stranded DNA; oligodeoxynucleotide.

Abbreviations: AEX, anion exchange; dNTP, deoxynucleotide; dsDNA, double-stranded DNA; HIC, hydrophobic interaction chromatography; IgG, immunoglobulin; MMC, multimodal chromatography; nt, nucleotide; pDNA, plasmid DNA; poly(dA), polydeoxyadenylate; poly(dC), polydeoxycytidylate; poly(dG), polydeoxyguanylate; poly(dT), polythymidylate; ssDNA, single-stranded DNA

Introduction

In the last decades, liquid chromatography has frequently been explored both as an analytical and preparative tool for nucleic acid separation and purification [1]. The application of different chromatographic modalities has thus been utilized for purification of DNA fragments and plasmid DNA (pDNA), which largely have been intended for uses in gene therapy (GT) or DNA vaccination [2–4]. In addition, there is an increasing need for rapid and quantitative analyses of the composition and conformation of DNA and related molecules by chromatography.

Several studies have been undertaken to generate an understanding of the nucleic acid behaviour on different chromatographic matrices [5–7]. At neutral pH, nucleic acid molecules harbour negative charges on their backbone structure. Due to this, anion-exchange chromatography (AEX) has proved most versatile and is the most used chromatographic modality for this group of molecules [8–11]. However, the mechanism of binding and elution of DNA depends on several experimental conditions, e.g. including ligand type, ligand density, matrix, size as well as conformation and structure of the target nucleic acid molecules [12–14]. In addition, for large entities such as pDNA, the size of the molecules influences the access and diffusion through the pores of the chromatographic bead particles [6,7].

Besides the use of electrostatic interactions, hydrophobic interaction chromatography (HIC) which exploits the hydrophobic properties of nucleic acids, has also proved to be most effective [2,15]. However, it has been particularly valuable for achieving a high selectivity in the system to combine ionic and hydrophobic interactions in the separation. Several such mixed-mode

chromatography (MMC) resins have recently been developed, which combine more than one kind of modality for binding between the target molecule and the chromatographic ligand [16,17]. These multiple interactions, based on e.g. ionic and hydrophobic binding, may occur simultaneously, resulting in a cooperativity that increases the selectivity and specificity of the chromatographic process [18]. This results in very versatile media that have been explored in a variety of applications including separation of post-translationally modified proteins [19], mutant proteins [20,21], oligosaccharides [22] and antibodies [23,24]. Recently, we have shown that MMC, with hydrophobic and ion-exchange properties, can be particularly favourable for separation of different DNA conformations [7]. The different degrees of exposure of charges and hydrophobicity in single- and double-stranded DNA (ssDNA and dsDNA), respectively, have been explored in MMC *Capto Adhere* and selective separations have been achieved [7].

In this work, a comparative study between two different ligands from the ***Capto*** family (GE Healthcare Biosciences, Uppsala, Sweden), the ***Capto Adhere*** and ***Capto Q ImpRes***, has been performed. The difference between these two ligands is the presence of a phenyl group in ***Capto Adhere***, which results in a recognition for base exposure in nucleic acids (Figure 1).

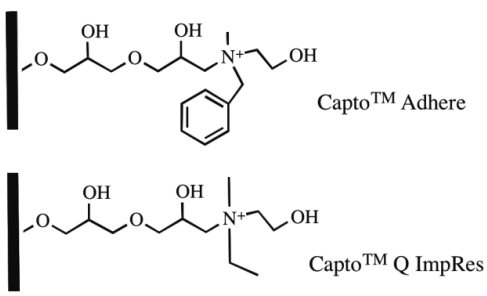

Figure 1 – The *Capto Adhere* and *Capto Q ImpRes* ligands. The *Capto Adhere* ligand, N-benzyl-N-methyl ethanol amine, has a high ion-exchange capacity mixed with an aromatic moiety, which also promotes its stability. The *Capto Q ImpRes* ligand has a high ion-exchange capacity due to a quaternary amine.

The behaviour of small DNA molecules on Capto Q ImpRes was investigated systematically. The binding and elution of ss- and ds-DNA molecules in the size of 26 bp were determined and

compared with our previous work [7]. All DNAs tested bound strongly to the resin and they could be eluted with a linear gradient of increasing NaCl.

2. Materials and Methods

2.1. Materials

Single-stranded DNA (ssDNA) molecules, poly(dA), poly(dT), poly(dG) and poly(dC), of equal length (26-mers), and the dNTP samples were obtained from Thermo Scientific (Thermo Fisher Scientific, Sweden). The dsDNA samples were prepared by mixing equal amounts of complementary ssDNA [poly(dA) and poly(dT); poly(dG) and poly(dC)] at 90°C for 10 minutes and let them hybridize by slowly decreasing temperature until ambient conditions were reached. The integrity of dsDNA preparations were verified by 1% agarose gel electrophoresis stained with 0.6 μg/ml GelRed. A pure plasmid DNA sample was obtained by a Qiagen plasmid purification Maxi kit (Hilde, Germany) after cultivation of *Escherichia coli* DH5α cells harbouring pUC18 in LB medium [25]. All DNA and nucleotide samples studied are accumulated in Table 1.

Table 1 - DNA and nucleotide samples used in this study including their length and M_w values.

Sample	Sequence (5'->3')	Length (nt)	M_W (g/mol)
dATP			557
dCTP			533
dGTP			573
dTTP			548
Poly (dC)	dC_{26}	26	7,457
Poly (dT)	dT_{26}	26	7,847
Poly (dA)	dA_{26}	26	8,082
Poly (dG)	dG_{26}	26	8,498
pUC18		2686	$1.74x10^6$

All solutions were freshly prepared using ultra-pure grade deionized water (PureLab ultra, ELGA Veolia Water Solutions and Technologies, Väsby, Sweden) and analytical grade reagents. The buffer solutions were filtered through 0.45µm Whatman filters (Dassel, Germany) and vacuum degassed before the chromatographic runs.

2.2. Chromatography

Capto Q ImpRes, which carries a quaternary amine group ligand coupled to a high flow agarose, was obtained from GE Healthcare Biosciences (Uppsala, Sweden). All experiments were carried out on a 4.5 mL column (0.77 cm diameter and 10 cm height) using ÄKTA Explorer, a liquid chromatography system from GE Healthcare (GE Healthcare Biosciences, Uppsala, Sweden).

The column was equilibrated with a starting buffer, buffer A composed of 10 mM Tris-HCl, pH 8.0. Elution of the samples were achieved by a linear gradient of NaCl until a final concentration of 3.0 M NaCl was reached, using buffer B, i.e. buffer A supplemented with 3 M NaCl. The flow rate was kept constant at 1 mL/min. After the chromatographic separations, the nucleic acid samples were identified by agarose gel electrophoresis.

3. Results

3.1. Elution behaviour of dNTPs

The single deoxynucleotides, dNTPs, were examined on the *Capto Q ImpRes* resin at concentrations in the range 100-200 mM. The samples were all strongly adsorbed to the column at pH 8.0 and could be completely recovered as a single peak using a linear gradient of NaCl (Figure 2). Only dGTP had a significant different elution pattern compared with the other nucleotides injected. This clearly indicates that dGTP aggregates in solution under these conditions. This is recognized on the resin as a compound of higher charge density compared with the other dNTPs. The elution values were also compared with previous *Capto Adhere* experiments and are presented in Table 2.

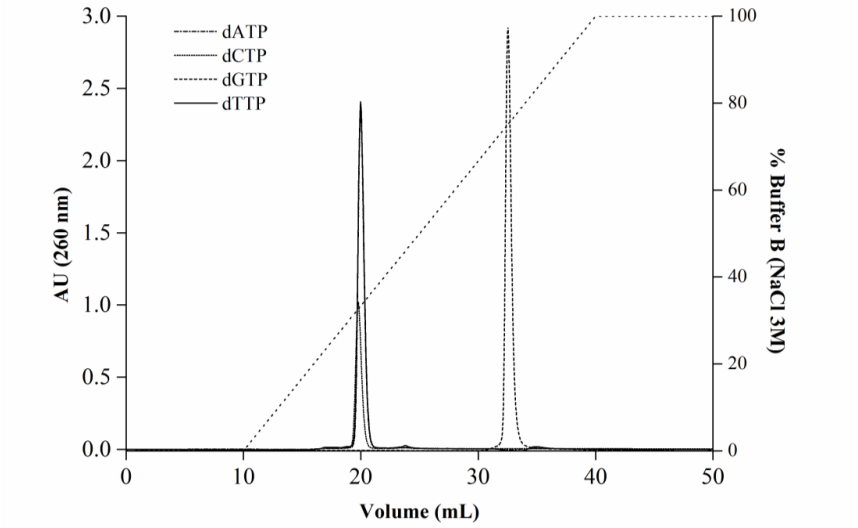

Figure 2 - Chromatograms of single deoxynucleotides injected to a *Capto Q ImpRes* column. The sample concentrations were varied between 100-200 µg/mL using a volume of 100 µL.

Table 2 - Elution volumes, as determined from the start of the linear gradient, and the corresponding salt concentrations needed for elution of the different nucleic acid and nucleotide samples.

Samples	*Capto Q ImpRes*		*Capto Adhere*[7]	
	Elution Volume	**% Buffer B**	**Elution Volume**	**% Buffer B**
dCTP	9.8	32.7	6.3	21.0
dATP	10.0	33.3	7.1	23.7
dTTP	10.0	33.3	6.4	21.3
poly(dA)	11.8	39.3	14.0	47.0
poly(dT)	12.4	41.3	38.9	96.0
poly(dC)	12.7	42.0	31.4	71.3
poly(dGdC)	12.7	42.7	11.8	39.7
poly(dAdT)	13.7	45.7	11.5	38.3
pUC 18	14.0	46.7	2.80	9.33
poly(dG)	14.7	49.0	36.3	87.7
dGTP	22.5	76.7	7.39	24.7

3.2. Elution behaviour of single- and double-stranded DNA

Single- and double-stranded oligodeoxynucleotides were subsequently examined on the *Capto Q ImpRes* column. As opposed to *Capto Adhere*, the ssDNA exhibited weaker binding to this resin compared with dsDNA. In order to identify any sequence specificity in the elutions, poly(dA), poly(dT), poly(dG) and poly(dC) of the same size (26-mers) were compared. Each ssDNA sample could be completely recovered (>95%). The small ssDNA molecules tend to have different secondary structures, dependent on the nucleotide composition. It has thus previously been described that poly(dA) spontaneously forms a helix, poly(dC) folds into i-tetraplexes, poly(dT) forms harpins by intra-strand base paring exposing the negative charges of the DNA phosphate backbone, while poly(dG) generates a quadruplex structure [26,27]. These structures influence the ionic and hydrophobic characteristics of the molecules and thereby their behaviour on the chromatographic support. The strongest interaction with *Capto Q ImpRes* was achieved with poly(dG) while the other ssDNA samples eluted at the same NaCl concentrations. This may be due to the quadruplex structure that promotes a higher density of charges for interacting with the matrix [26]. Those interactions are based on both ionic and hydrogen bonding. However, when compared with *Capto Adhere*, the hydrophobic interactions present a crucial additional effect to the retention of these molecules. A typical elution for dsDNA is shown in Figure 3A. There were significant differences in elution behaviour between ssDNA and dsDNA fragments (Figure 3B and 3C). A linear gradient proved to give the best resolution after different elution regimes were tested. In these chromatographic tests, the peak form was not affected by flow-rate, and 1.0 mL/min was kept to obtain a fast separation.

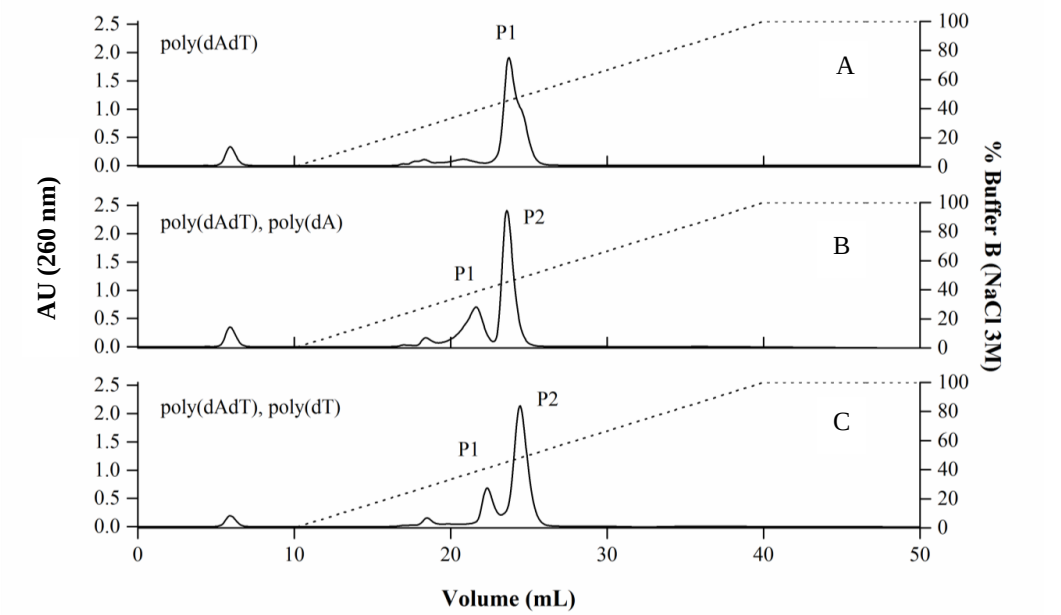

Figure 3-Chromatographic behaviour of ss and ds DNA molecules on *Capto Q ImpRes*. A - injection of ds poly(dAdT) at a concentration of 100µg/mL; P1, peak 1, represents the dsDNA molecule. B - injection of ds poly(dAdT):poly(dA); P1, peak 1, represents the ssDNA molecule and peak 2 the dsDNA. C- injection of ds poly(dAdT): poly(dT); P1, peak 1, represents the ssDNA molecule and peak 2 the dsDNA.

3.3. pDNA vs ssDNA/dsDNA

Plasmid pUC18 was used as a control sample. This molecule is approximately 100-fold larger than the previously dsDNA samples used (Table 1). In many molecular biology experiments, the removal of small DNA fragments from plasmid preparations is most important. When applied alone, the pDNA bound strongly and could be eluted at 1.4 M NaCl, with some tendencies for pDNA isoform separation, although not complete. To modulate a homogeneous mixture, the pDNA was mixed with smaller dsDNA. The mixtures were prepared in proportion 1:1 (w/w) with pUC18 (100µg/mL) and dsDNA, i.e. the smaller dsDNA molecules were present in 100-fold molar excess. The separation of dsDNA from pDNA was analysed as previously. As

opposed to the *Capto Adhere* results, the competitive binding between the different samples were not so visible with *Capto Q ImpRes*. Under the conditions used, the dsDNA eluted earlier in the gradient than pDNA, with a low separation resolution. Compared with *Capto Adhere*, in which the diffusion of pDNA in the system was much lower, the pDNA had a stronger interaction with the ligands and were not affected with the competitive binding compounds in the samples.

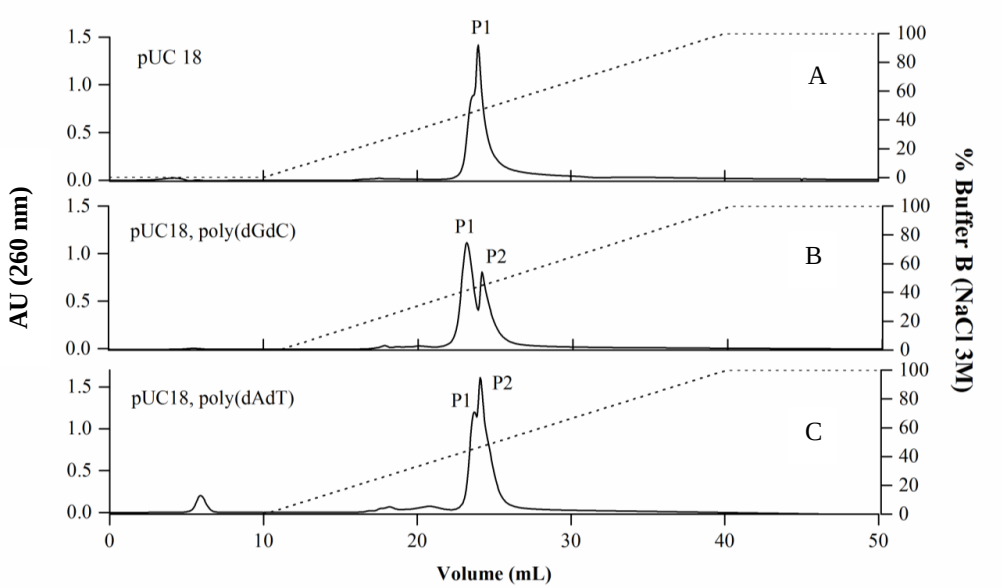

Figure 4 – Chromatographic behaviour of pDNA (pUC18) in mixtures with smaller dsDNA molecules. A - pUC18 (100µg/mL). B - 1:1 mixture of poly(dGdC) and pUC18; Peak 1 - poly(dGdC) and peak 2 - pUC18; C - 1:1 mixture of poly(dAdT) and pUC18; Peak 1 - poly(dAdT) and peak 2 - pUC18.

4. Discussion

The binding and elution characteristics of AEX in comparison with MMC provide important information on the recognition behaviour of different ligands to nucleic acids. However, this behaviour is not fully understood since DNA molecules easily change secondary and tertiary structures [28], thereby often turning the separation mechanism much more complex than first expected. The ssDNA samples utilized in this study carry an equal charge level, as a

consequence of the phosphate groups in the backbone of the DNA structure. However, the separation between poly(dG) from the others ssDNA samples originates from different folding phenomena. Similarly, the dsDNA samples present more available charges compared with the ssDNAs of equal size. Due to this, a partial to complete separation between ssDNA and dsDNA was possible to achieve.

The AEX resins have been investigated earlier for DNA fragment separations [29–31]. In this study, we have compared a strong AEX resin, *Capto Q ImpRes*, with an MMC resin from previous work [7]. In conventional AEX resins, the dsDNA fragments present a stronger interaction compared with ssDNA. This was also obtained on a *Capto Q ImpRes* resin. On this resin, the interactions are based only on ionic and hydrogen bond interactions, resulting in an elution dependent on the charges available on the DNA backbone. As expected, the dsDNA therefore resulted in stronger retention compared with ssDNA. In addition, we could observe that poly(dG) forms more complex aggregation patterns and are thereby elutes later due to higher charge densities. As opposed to the *Capto Adhere* resin, where the hydrophobic interactions play a fundamental role, the *Capto Q ImpRes* does not promote an efficient separation between different ssDNA samples besides poly(dG).

5. Conclusion

In this study we have compared the separation between ssDNA and dsDNA molecules using *Capto Q ImpRes* with our previous work on *Capto Adhere*. Both materials are strong anion exchangers. However, the phenyl group on the *Capto Adhere* ligand promotes a hydrophobic chromatographic behaviour, resulting in a multimodal performance of this matrix. When analysing the performance of these resins it is obvious that very small modifications of the ligand are instrumental for achieving separations of different forms of nucleic acids. Recognition is thus sensitive to minor changes in the ligand structure and composition, implying that screening of a ligand library would be valuable for identifying and optimising a specific nucleic acid separation issue.

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Paper VII

Preparative Isolation of PCR Products Using Mixed-mode Chromatography

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Abstract

The polymerase chain reaction (PCR) has become one of the most useful techniques in molecular biology laboratories around the world. After the PCR reaction, the resulting target amplified DNA fragments are present in a complex mixture which includes several contaminants such as proteins, different DNA fragments, primers and free nucleotides. The purification of the target DNA product therefore often becomes challenging and most users are restricted to employing available commercial kits. These materials are frequently based on multistep protocols where the DNA fragments are isolated from agarose gels after their separation by electrophoresis. Liquid chromatography, using especially resins carrying chromatographic modalities based on anion-exchange or hydrophobic interactions, has been widely exploited for DNA purifications. The recent developments in multimodal chromatography have shown higher selectivity for a variety of homogeneous samples. *Capto*^{TM} *Adhere* is multimodal chromatography resin that offers two types of interactions, anion-exchange and hydrophobic interactions. The higher selectivity of this ligand is here applied for the purification of amplified DNAs from PCR mixtures in a single step. Moreover, the purification method is simple and fast and can be completed in 10 minutes. The yields are excellent, above 95%, and the method shows a high linearity and precision for different concentrations. DNA fragments in the range of 270 to 960 bp have been examined.

***Keywords*:** Polymerase chain reaction; single-stranded DNA; double-stranded DNA; plasmid DNA; multimodal chromatography; ion-exchange chromatography; hydrophobic interaction chromatography.

Abbreviations: anion-exchange chromatography, AEX; base pair, bp.; column volume, CV; deoxynucleotides, dNTPs; double-stranded DNA, dsDNA; hydrophobic interaction chromatography, HIC; multimodal or mixed-mode chromatography (MMC); plasmid DNA, pDNA; polymerase chain reaction, PCR; single-stranded DNA, ssDNA

1. Introduction

The polymerase chain reaction (PCR)1,2 is currently the most used technique for in *vitro* amplification of DNA sequences in numerous fields including molecular evolution, molecular biology, forensic biology, diagnosis of hereditary diseases, genome mapping and sequencing[3–7]. The polymerization is composed of cycles of repeated heating and cooling to initiate DNA melting and enzymatic elongation. Many optimizations have been developed over time to achieve higher specificity and to widen the application areas[3,5,8,9].

A major hurdle that concerns the obtained PCR samples is the purity of the final DNA fragment. If possible, the amplified DNA should be pure from contaminants used in the reaction, such as primers, dNTPs, template, Taq polymerase and other associated proteins. Some available kits allow the purification of these products. However, this purification is most often based on two main steps: agarose gel electrophoresis followed by gel melting and chromatography by gravity in small scale[10]. As a consequence, this method is time-consuming and often results in low yields.

Liquid chromatography has frequently been used for DNA purification[11,12]. Due to the physical characteristics of DNA, especially the charged phosphate groups in the backbone structure, anion-exchange chromatography (AEX) has been the most employed chromatographic modality[13–15]. Besides the electrostatic and ionic interactions, the hydrophobic nature of nucleic acids has also been explored for purification purposes by hydrophobic interaction chromatography (HIC). Particularly single-stranded nucleic acid molecules where the hydrophobic aromatic bases are more exposed compared with double-stranded ones, are suitable for HIC[16,17]. Recent advances in multimodal or mixed-mode

chromatography (MMC) have shown the high selectivity of these type of resins for DNA molecules[17,18], antibodies[19,20], proteins[21,22] and oligosaccharides[23]. On MMC resins, multiple and different physical interactions may occur simultaneously between the target molecule and the ligand coupled to the stationary phase. The manipulation and use of different combinations of these interactions, e.g. ionic and hydrophobic interactions[24], often increase the selectivity, specificity and efficiency.

In this study, we have focused on the multimodal *Capto*^{TM} *Adhere* resin (GE Healthcare Biosciences, Uppsala, Sweden - Figure 1), which initially has been designed for intermediate purification and polishing of monoclonal antibodies after capture on a protein A column[25]. We have previously shown the efficiency of this resin for the isolation and separation of single-stranded (ssDNA) from double-stranded DNA (dsDNA)[17]. Based on this, we here present a simple and fast method for purification of DNA fragments from PCR mixtures based on a single chromatographic step. The resulting DNA fraction is free of nucleotides, proteins and endotoxins. Moreover, the linearity, efficiency and repeatability of this resin confirm the versatility of this method.

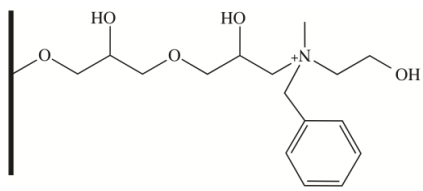

Figure 1 - The *Capto Adhere* ligand, N-benzyl-N-methyl ethanol amine, shows a high ion-exchange binding capacity which is combined with a phenyl group and thereby also promotes hydrophobic interactions.

2. Results and Discussion

2.1. Chromatographic separation of PCR products

Several different DNA fragments were generated by conventional PCR amplification reactions. The PCR samples were subsequently injected directly to a small *Capto Adhere* column previously equilibrated with the running buffer. Different conditions for elution were examined using increasing salt concentrations. When using a linear NaCl gradient, three peaks could be identified. The major peak (P2) appearing first in the gradient, contains most of the support proteins of the PCR reaction as initially indicated by an A260/A280 ratio of 1.5 (Figure 2B). The amplified fragment is well separated from this peak and is eluted at 1.0-1.2 M NaCl depending on the size of amplified sequence (P3). Fractions were collected and immediately desalted and analyzed by (1%) agarose gel electrophoresis, stained with Gel Red (0.6µg/mL) (Figure 3). It could then be demonstrated that peak 3 (P3) contains the amplified fragment. The A260/A280 ratio of this fraction was close to 1.8, thereby clearly indicating the presence of a pure DNA. The larger template DNA and oligonucleotides are eluted in the P2 fractions and are thus well separated from the PCR product. The peaks were also analysed separately for proteins and possible endotoxin contaminations and it was verified that the PCR product is also well separated from these contaminants (Table 1). A control sample containing the PCR mix before the amplification reaction was examined by running the chromatography under the same conditions. No peak was then observed at the position (P3) for the amplified fragment (Figure 4A).

The chromatographic separations of the PCR samples were indeed simple to achieve on a *Capto Adhere* column and in order to facilitate and further speed up the separations, a stepwise gradient was also exploited. By introducing a step of 0.5 M NaCl, the proteins and template DNA could be eluted in one step before application of a 2.0 M NaCl pulse (Figure 2A). In order to further reduce the use of gradients, it is possible to apply the PCR sample directly at 0.5 M NaCl to allow fast separations and then simply elute the target PCR product by a pulse of 2.0 M NaCl.

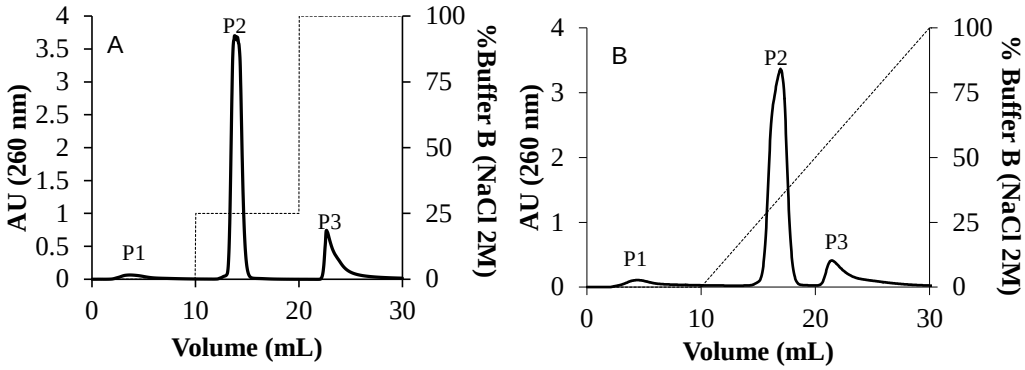

Figure 2 - Isolation of PCR products using *Capto Adhere*. A – Chromatogram for PCR product purification using a step gradient with 25% Buffer B. B – Chromatogram for PCR product purification using a linear gradient approach until 100% buffer B was reached. The sample applied was a PCR product of 272 bp; Primers used: pET upstream, DuetDOWN1.

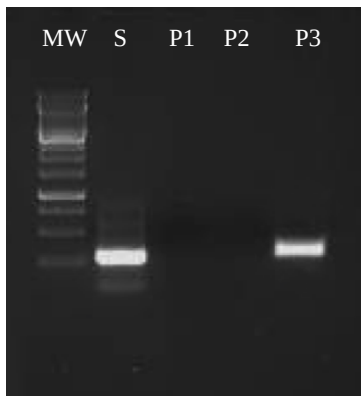

Figure 3 – Agarose gel electrophoresis of peaks in the PCR product chromatogram. The electrophoresis represents the peaks collected from the chromatogram with a 272 bp PCR sample. P1- P3 corresponds to peaks 1-3 in the chromatogram; MW - Molecular weight marker; S1 - sample.

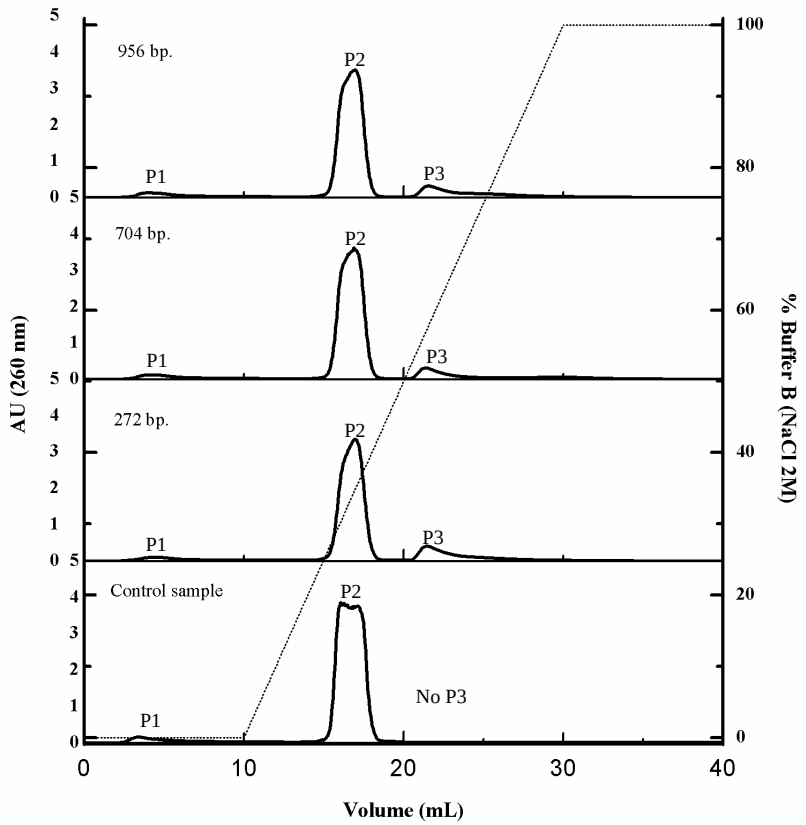

Figure 4 - Chromatograms of PCR fragment purification with different sizes and composition. P1- P3 corresponds to peaks 1-3 in each chromatogram.

To further dissect the possible relation between the size of the amplified fragment and the eluting NaCl concentration, the chromatographic behaviour of several different generated DNA fragments were examined. A clear logarithmic size dependency was observed and each fragment was eluted at a given NaCl concentration (Fig. 5). The elution could therefore be described as:

Log (size) = y + k x [NaCl]

From this equation it is important to realize that there must be a distinct difference in size between the template DNA and the amplified DNA product in order to obtain a useful chromatographic separation. In the studied case, pDNA was used as template which is substantially larger and also has a different conformation compared with the amplified DNA fragments. This means that the template is eluted at 0.5 M NaCl.

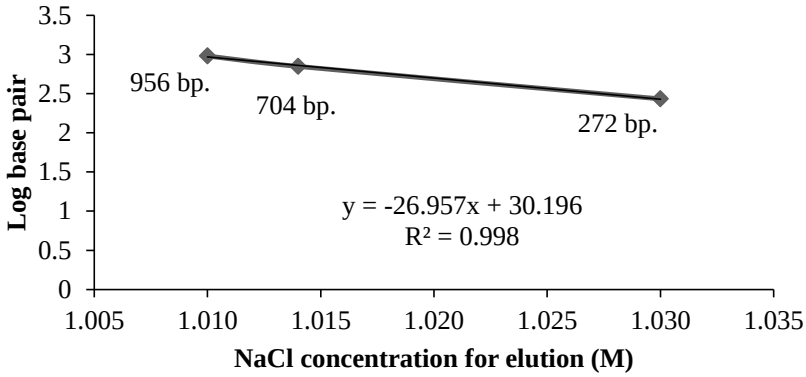

Figure 5 – Dependence of PCR product size and NaCl concentration needed for elution on *Capto Adhere*.

2.2.Linearity and precision

The effect of sample concentration on the chromatographic behavior was tested by examining the linearity of the eluted peak areas. Seven different samples in a range between 40-500 µg/mL were processed using the same conditions as before. The resulting curves were prepared by triplicate assays for each pDNA concentration. The results for the obtained chromatograms are shown in Figure 6A. The linearity was then analysed by plotting absorbance versus peak area (Figure 6B). The correlation coefficient achieved was R^2=0.9962.

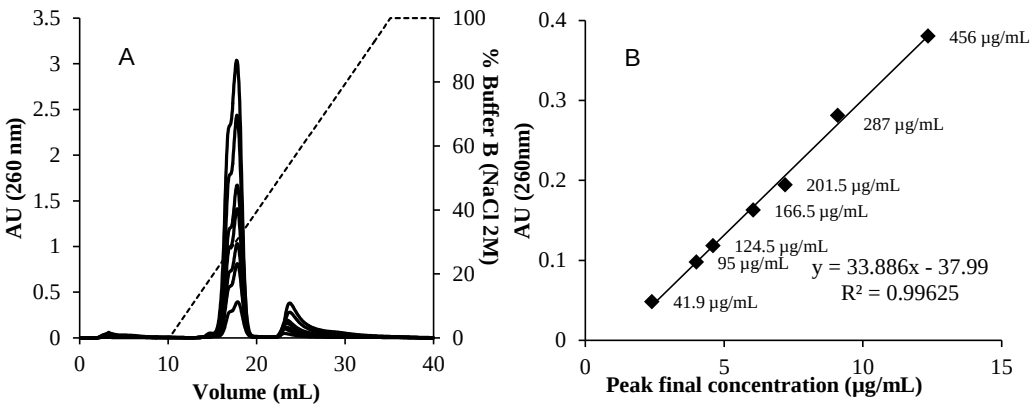

Figure 6A – Consecutive chromatograms showing different sample concentrations using the same conditions for equilibration and elution [Buffer B is 2.0 M NaCl in Buffer A (10 mM Tris-HCl, pH 8.0)]. Figure 6B – Correlation between concentration of the sample and the peak area corresponding to the pDNA fraction.

In order to be applicable for a practical PCR setting, the precision of the method needs to be determined both in terms of intra-day and inter-day precisions. For the intra-day assay, several consecutive injections of PCR samples with same concentration were injected. The repeatability was excellent showing a standard variation of 0.2 for the volume retention of the peak of interest in chromatographic separations. The inter-day precision assays were realized by injecting the PCR samples over 5 consecutive days. The method proved to be very robust and the chromatograms were stable over the days, with a standard deviation of 0.3 for volume retention relative to the peak of interest.

Table 1 – Concentrations of endotoxins and proteins in the fraction containing the PCR fragments.

Samples	DNA Concentration (Abs 254)	Endotoxins concentration EU/ng DNA (LAL)	Protein concentration (Bradford)
Initial Sample	~1750 µg/ml	>1	153 µg/ml
Purified fragment	~300 µg/ml	<1	Not detectable

3. Conclusions

The MMC resin employed in this study offers several advantages over traditional ion-exchangers supports, particularly by increasing the selectivity due to the combination of anion-exchanger and hydrophobic interactions. This enhancement in selectivity for different DNA molecules is caused by a specific recognition for small DNA fragments. The yield of DNA fragments obtained in the final fraction was higher than 95% with no contamination of proteins or other DNA fragments. In addition, the endotoxin assays of the purified fragments presented values lower than 1 EU/ng DNA. The obtained linearity and high precision should allow for further practical use of the described method for purifying amplified DNA fragments instead of the more cumbersome approaches generally used today.

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4. Methods

4.1.Materials

The PCR experiments were performed with a commercial PCR kit (Fermentas GmbH, Germany), including all compounds for the reaction, using SensoQuest, LabCycler (SensoQuest GmbH, Germany) for amplification and following conventional PCR protocols. Plasmids were purified using the Maxi Kit from Qiagen (Hilden, Germany). Sodium chloride and tris(hydroxymethyl)aminomethane (Tris) were of analytical grade and obtained from Merck (Frankfurt, Germany). The buffer solutions were filtered using 0.45 µm filters from Whatman (Dassel, Germany) and degassed in a vacuum system. The samples were desalted with Vivaspin concentrators (Sartorius Stedim, Biotech, France)

4.2.Sample preparation

The plasmid pETDuetTM-1 GLB2 (5420 bp) carrying the sugar beet hemoglobin class II gene (426 bp)[26]] was isolated from *E. coli* bacteria using the plasmid purification kit. Three different sequences from the plasmid were amplified by PCR. Each tube had a reaction volume of 100µL, which consisted of 1µL of plasmid pETDuetTM-1 GLB2 (1 µg/mL), 1µL of both forward and reverse primers (Table 1), 1µL of 25mM dNTP mixture (mixture of dATP 100mM, dGTP 100mM, dCTP 100mM and dTTP 100mM), 10µL of buffer, 16 µL of $MgCl_2$ 25mM, 59µL of deionised ultra-pure autoclaved water and 1µL of enzyme Taq Polymerase (5u/µL). The PCR was programmed for 30 cycles, which were set to 94^{o}C for 30 seconds, 62^{o}C for 30 seconds and 72^{o}C for 6 minutes. The process was proceeded by a 5-minute initialisation step at 94^{o}C and completed by a final elongation step at 72^{o}C for 7 minutes. The final products had sizes of 956 bp (%GC = 46.86), 704 bp (%GC = 47.73) or 272 bp (%GC = 48.16), due to the combination of different primers, pET Upstream-T7 Terminator, DuetDOWN1-Terminator T7 or DuetUP2-pET Upstream, respectively (Table 3).

Table 1 – Primer sequences used for amplification of different regions of the plasmid pETDuetTM-1 GLB2.

Primer	Primer type	Primer sequence (5'->3')
pET Upstream	Forward	ATGCGTCCGGCGTAGA
DuetDOWN1	Reverse	GATTATGCGGCCGTGTACAA
DuetUP2	Forward	AACATGTGCCGGCGTATTAG
T7 Terminator	Reverse	GCTAGTTATTGCTCAGCGG

4.3.Chromatography and samples analysis.

Chromatographic runs were performed on an AKTA Explorer system (*GE HealthCare Biosciences*, Uppsala, Sweden) consisting of a compact separation unit and a personal computer with Unicorn control system Version 4.11. The *Capto*^{TM} *Adhere* column was prepared according to the manufacturers' protocol (*GE HealthCare*, Uppsala, Sweden) in a column volume of 2.5 ml (0.7 mm diameter and 6.6 mm height). The equilibration was performed by washing the resin with 5 column volumes (CV) of 10 mM Tris-HCl (pH 8.0), followed by injecting the sample in the same buffer. After the injection, the column was washed with 4 CVs. The elution was achieved in 10 CVs either using a linear or specific stepwise gradient until 2.0 M NaCl (10 mM Tris-HCl, pH 8.0) was reached. The flow rate was kept constant at 1 ml/min. The absorbance was monitored at 260 nm. The peaks were collected, according to the chromatograms obtained, in 1 ml tubes and kept on ice. Electrophoretic agarose analyses were carried out at 100V, for 40 minutes, with TAE buffer (40mM Tris base, 20mM acetic acid and 1mM EDTA, pH8.0). Protein quantifications were done by Bio-Rad Protein Microassay (Bio-Rad, California, USA). Endotoxins assays were done by ToxinSensor^{TM} (Endotoxin Detection System, GeneScript, USA).