UNIVERSIDADE DA BEIRA INTERIOR
Covilhã | Portugal

The Use of Nano and Microfibers as Novel Supports for the Separation of Biomolecules

Adsorption of pDNA in Different Matrix-Based Supports

Master's Thesis in Biochemistry

By *Carolina Antunes da Costa Esteves*

Outubro, 2010

DECLARATION

I hereby declare that I take full responsibility for this thesis and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

_____

Carolina Antunes da Costa Esteves

ACKNOWLEDGEMENTS

I want to thank to:

Prof. Dra. Ana Cristina Dias-Cabral, for all the help and support that she gave to me to accomplish this work,

Dra. Angela Sousa, for the help that she gave to me in some laboratory procedures, and

Dr, Marvin Thrash, for the PVA nanofibers.

Additionally, I would like to acknowledge all the support that my family, especially, my father, my mother and my sister, and my friends, particularly, Irina Veloso and Anabela Nunes, gave to me in everything, not just in this thesis.

ABSTRACT

The use of plasmid deoxyribonucleic acid (pDNA) in the emerging gene therapy and nucleic acid based biopharmaceuticals requires pure pDNA in large quantities, so it calls for a production of safe pDNA on large scale.

The main achievement of this experimental research was to find alternative matrices to pDNA purification, regarding to economical, simple and efficacy features. The choice of cellulose is due their easy and cheap obtainment and their relatively trouble-free modification; sepharose was mainly to compare results. Were tested two types of adsorption: hydrophobic interaction (is the most common and is easy and low-cost to perform) and anion-exchange (is also easy and low-cost and, generally, is more effective). The method of choice was batch adsorption, because of possible problems such as column chromatography high back pressure and poor capacity pDNA binding, furthermore is an easier method to scale-up.

Through hydrophobic interaction was possible to adsorb up to 0,4 μg of pDNA per mg of cellulose microfibers gel, with ammonium sulfate 3,0 M, an enormous concentration of salt, so was concluded that the hydrophobic interaction is due to amount of salt. Through anion-exchange batch, the adorption was up to 2,5 μg of pDNA per mg of gel. Comparing with sepharose based-matrix (adorption was up to 0,7 μg of pDNA per mg of gel), cellulose microfibers are a better support, probably due the surface area expose to ligands. Factors that can be improved in these alternative matrices to pDNA purification experiments are: an increase of pDNA initial concentrations, a decrease of gel mass and, if possible, an increase of matrix surface area, i.e. a decrease of particle/fibers size.

Keywords: adsorption in batch, anion-exchange, cellulose microfibers, hydrophobic interaction, pDNA purification, sepharose particles and surface modification

RESUMO

O uso de ácido desoxirribonucleico plasmídico (pDNA) na emergente terapia génica e em biofármacos requer pDNA puro em grandes quantidades, portanto há uma necessidade de produção de pDNA seguro em larga escala.

O principal objectivo desta investigação experimental foi encontrar matrizes alternativas para purificação de pDNA, dando importância a recursos económicos, simples e eficazes. A escolha da celulose foi devida à sua obtenção fácil e económica e modificação sem grandes problemas; a escolha da sefarose foi, principalmente, para estabelecer termo de comparação. Foram testados dois tipos de adsorção: interacção hidrofóbica (é a mais comum e a mais fácil e económica) e troca-aniónica (também é fácil e económica e, no geral, é mais eficaz). O método de escolha foi adsorção em *batch*, devido a possíveis problemas inerentes a cromatografia em coluna, como elevada *back pressure* e baixa capacidade de ligação do pDNA, para além disso, é um método mais fácil para larga escala.

Por interacção hidrofóbica, foi possível adsorver até 0,4 μg de pDNA por mg de gel de microfibras de celulose, com sulfato de amónia 3,0 M, uma concentração enorme de sal, portanto, concluiu-se que esta interacção hidrofóbica é por causa do sal. Através de troca-aniónica, foi possível adsorver até 2,5 μg de pDNA por mg de gel. Comparando com a matriz de sefarose (adsorção até 0,7 μg de pDNA por mg de gel), a matriz de celulose é um suporte melhor, provavelmente devido à área da superfície exposta aos ligandos. Factores que podem ser melhorados nestes ensaios com este tipo de matrizes para purificação de pDNA são: um aumento da concentração inicial de pDNA, uma redução da massa do gel e, se possível, um aumento da área de superfície da matriz, ou seja, uma redução do tamanho das fibras ou das partículas.

Palavras-chave: adsorção em *batch*, interacção hidrofóbica, microfibras de celulose, modificação da superfície, troca-aniónica, partículas de sefarose e purificação de pDNA.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

AEC	Anion-exchange Chromatography
AT	Adenine-Thymine
DNA	Deoxyribonucleic Acid
dsDNA	Double-strand Deoxyribonucleic Acid
GC	Guanine-Cytosine
gDNA	Genomic Deoxyribonucleic Acid
HIC	Hydrophobic Interaction Chromatography
IEC	Ion-exchange Chromatography
Oc	Open circular
OD	Optic density
pDNA	Plasmid Deoxyribonucleic Acid
PVA	Poly(vinyl) alcohol
Rpm	Round per minute
Sc	Supercoiled
SEM	Scanning Electron Microscope/Miscroscopy
TB	Terrific Broth

INTRODUCTION

The use of plasmid deoxyribonucleic acid (pDNA) in the emerging gene therapy and nucleic acid based biopharmaceuticals requires pure pDNA in large quantities, so it calls for a production of safe pDNA on large scale.

There are a great number of kit-based DNA purification techniques and conventional chromatography media for adsorption of pDNA, but large scale effective pDNA purification remains a biotechnological challenge, as chromatographic processes normally offer low dynamic binding capacities and poor productivity. Consequently, there is an urgent need for high selectivity, high capacity and high productivity purification methods for pDNA.

Previous work reports about rapid and selective adsorption of DNA to submicron sized polymeric particles in high capacity and recovery (Kim and Rha, 1989). Based on that, there is interest in investigate polymeric structures that can be modified and applied to plasmid adsorption, in this particular case, cellulose microfibers and polyvinyl alcohol (PVA) nanofibers.

OUTLINE

Chapter one focuses in fundamental concepts and background about plasmid DNA and its major applications, gene therapy and DNA vaccination.

A general view about plasmids purification is given in Chapter two, as well as a description about the purification methods based on hydrophobic interactions and anion-exchange, which are the techniques used in this experimental project.

Notions about cellulose fibers, PVA nanofibers (especially those produced by electrospinning) and stationary phases in general are given in Chapter three.

Research aim and main questions beneath this investigation project are enumerated at Chapter four.

Chapter five describes the obtainment of plasmids that were used in the adsorptions studies, with a methodology based on a commercial kit.

Chapter six explains how the several gel matrices were produced and shows the major results.

At Chapter seven, are the adsorptions experiments procedure and the intermediate results.

In Chapter eight, the obtained results are analyzed and clarified.

At the end, the study is concluded and future research suggestions related with this project are given.

CHAPTER 1. PLASMID DNA SIGNIFICANCE AND APPLICATIONS

Plasmid earned an increasing attention as a biotherapeutic in gene therapy and DNA vaccination. However, a full treatment require milligram quantities of plasmid DNA and that's why is important to developed a cost effective large-scale process for the production of plasmid DNA, which should be capable of providing the product plasmid at required levels of product purity, potency, efficacy and safety (Deshmukh and Lali, 2005; Ferreira *et al*, 2000).

1.1. PLASMID DNA

By definition, plasmids are circular, covalently closed, double stranded DNA molecules, very large, when compared with proteins, and comprise approximately 1% of the total content of the host bacterial cell (table 1) (Prazeres *et al*, 1999).

TABLE 1 BACTERIAL CELL CONTENT (ADAPTED FROM ATKINSON AND MAVITUNA, 1991)

Proteins	15%
RNA	6%
Carbohydrates	3%
Lipopolysacharides	2%
Others	2%
Genomic DNA	1%
Plasmid DNA	1%
Water	70%

Each pDNA molecule strand is a linear polymer of deoxyribonucleotides linked by phosphodiester bonds, which are negatively charged for pH > 4. The two strands are winding in an anti-parallel way, around each other and around a common axis that gives them the typical right handed double helix structure, stabilized by hydrogen bonds

between adenine-thymine (AT) and guanine-cytosine (GC) base pairs and by stacking forces. Double-helix inside is extremely hydrophobic, because of the aromatic bases close packing. The pDNA helix axis can also coil itself, forming supercoiled pDNA, which is the plasmid configuration that matters to gene therapy and DNA vaccine applications. The other forms of pDNA molecules, besides supercoiled (sc) and open circular (oc), that could be found in cell lysate are: linear (resulting from chemical or enzymatic cleavage of the phosphodiester bonds), denatured (resulting from hydrogen bonding disruption between complementary strands) or oligomeric (resulting from homologous recombination) (Sinden, 1994; Summers, 1996).

1.2. GENE THERAPY

Gene therapy is an approach for tackling diseases at the genetic level by, for example, substitution of a defective gene with the correct nucleic acid sequence in order to restore normal cellular function, through delivery of one or more functional genes into a patient (Anderson, 1992; Crystal, 1995).

One of the major challenges in gene therapy is to get safe vectors able of transporting genes efficiently into the target cells (Legendre *et al*, 1996).

In the past, viral vectors (examples at table 2) have been used to exploit their natural ability to infect cells, but this use was limited because of safety and regulatory concerns, that include immunogenicity, toxicity and the oncogenes and tumor suppressor genes possible activation/deactivation (Anderson, 1998; Ferreira *et al*, 1998; Fox, 1999; Luo and Saltzman 2000).

TABLE 2 VIRAL SYSTEMS THAT HAVE BEEN DEVELOPED FOR GENE THERAPY (ADAPTED FROM DARBY AND HINE, 2005).

Modified Viral System	Reference
Adenoviral	Montain, 2000; Robbins *et al*, 1998; Sanda *et al*, 1999.
Retroviral	
Adeno-associated	
Poxviruses	Robinson *et al*, 1999
Herpes simplex virus	Krisky *et al*, 1999

The use of viral vectors have resulted in adverse reactions in patients, with several deaths reported (Fox, 1999). Also, since 2003, all retroviral-based gene therapy to insert genes into blood stem cells clinical experiments are suspended by Food and Drug Administration (FDA) (FDA, 2003).

Plasmids vectors are considered to be a safer option, free from specific safety concerns viruses-associated and, in general, simpler to develop (Scherman, 2001). However, they are less effective than viral vectors. It is estimated that only one in every 1000 plasmid molecules presented to the cells reaches the nucleus and is expressed, with full treatments requiring milligram quantities of plasmid DNA (Crystal, 1995; Luo and Saltzman, 2000).

1.3. DNA VACCINATION

Plasmid DNA has been also used as a new and safer generation of vaccines, expressing specific antigens on cell membranes, stimulating and enhancing the immune system's response and memory (Tighe *et al*, 1998).

It is believed that pDNA vaccination pathway is similar to the natural intracellular pathogen gene expression pathways, which leads to cellular and humoral responses, and therefore can achieve the same protection afforded by virulent or attenuated infectious microorganisms (figure 1) (Prather *et al*, 2003).

However, similarly to what happens in gene therapy, DNA vaccines are not effective enough (compared with the viral). In small animals, such as mice, strong immune responses can be induced, but in larger animals (so, the same happens in humans) multiple immunizations of high DNA doses are often required to achieve modest responses (Widera *et al*, 2000).

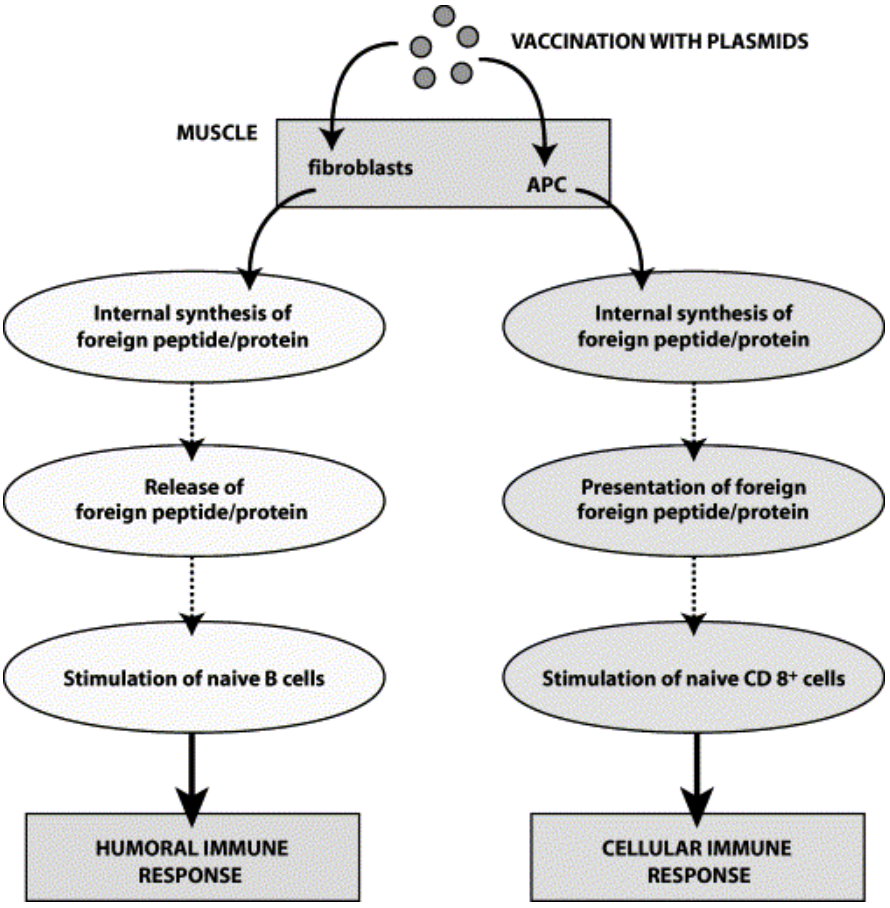


FIGURE 1 IMMUNE RESPONSE TO PLASMID DNA VACCINATION (ADAPTED FROM PRATHER *ET AL*, 2003) [APC = ANTIGEN PRESENTING CELL; CD 8^+ = LYMPHOCYTE THAT CARRIES THE CLUSTER OF DIFFERENTIATION 8]

CHAPTER 2. PLASMID DNA PURIFICATION

2.1. GENERAL PROCEDURE

Plasmid DNA production can be divide in two stages: upstream processing and downstream processing, however these stages of process development are integrated and must not be approached on an individual basis. So, pDNA production usually begins with the construction and selection of appropriate expression vectors and production microorganisms, followed by the selection and optimization of the fermentation conditions, cell growth, and finally the isolation and purification steps (Kelly and Hatton, 1991).

Upstream processing stage objectives are the design, selection and optimization of appropriate plasmid vectors, production microorganism strains and growth conditions, in order to enable the production of large quantities of stable supercoiled plasmid DNA. Safety and potency demands of gene-therapy vectors, and the inherent requirements for large-scale production and purification of the plasmid vector should be considered at these stages (Prazeres *et al*, 1999; Schleef, 1999). Fermentation is followed by a sequence of purification steps, which eliminate impurities, to obtain a final plasmid DNA preparation that fulfills the approval specifications – Table 3 (Ferreira 2000).

Within downstream stage are some steps that could be identified as: cell harvest, cell lysis, cell debris removal, affinity precipitation, adsorption, and buffer exchange/concentration (figure 2 illustrate those steps), but is not that straightforward. There are several difficulties associated with direct scale-up of some common laboratory procedures.

Current purification methods are kits marketed by commercial companies that take advantage of techniques such as alkaline lysis and the use of disposable chromatography columns. However, these purification conditions do not lend themselves to scale up because they may employ risky chemicals, such as phenol and chloroform, require the use of large amounts of chromatographic resin per milligram of plasmid purified, or necessitate the use of relatively expensive enzymes in order to degrade contaminants, for instance, RNase, and the use of some solutions at high

production scale would turn into considerable capital investment. Due to their simplicity, these purification methods are however the method of choice for the purification of small amounts of plasmid DNA (<20 mg) and are extensively used in research laboratories (Sambrook and Russell, 2001).

TABLE 3 THE PRINCIPAL APPROVAL SPECIFICATIONS AND RECOMMENDED ASSAYS FOR ASSESSING THE PURITY, SAFETY AND POTENCY OF DNA PREPARATIONS FOR GENE THERAPY AND DNA VACCINES (ADAPTED FROM FERREIRA *ET AL*, 2000)

Impurity	**Recommended assay**	**Approval Specification**
Proteins	Bicinchoninic acid (BCA) protein assay	Undetectable
RNA	Agarose-gel electrophoresis	Undetectable
gDNA	Agarose-gel electrophoresis	Undetectable
	Southern blot	< 0.01 µg (µg plasmid)$^{-1}$
Endotoxins	Lymulus amebocyte lysate (LAL) assay	< 0.01 EU (µg plasmid)$^{-1}$ (EU = endotoxin units)
Plasmid isoforms (linear, relaxed, denatured)	Agarose-gel electrophoresis	< 5%
Biological activity and identity	Restriction endonucleases	Coherent fragments with the plasmid restriction map
	Agarose-gel electrophoresis	Expected migration from size and supercoiling
	Transformation efficiency	Comparable with plasmid standards

Back to the main steps of pDNA purification, the first one that raises concern is cell lysis. In this step, all the intracellular components, including plasmid DNA, RNA, gDNA, endotoxins and proteins, are released. The shear sensitivity of plasmid and gDNA molecules, as well as the high viscosity of the lysates, is of major concern during plasmid release (Ciccolini *et al*, 1998; Levy *et al*, 1999; Prazeres *et al*, 1999). By far, the most common technique used in the production of plasmid DNA is alkaline lysis. This procedure relies on the use of sodium hydroxide and the detergent sodium dodecyl sulfate (SDS), which combined effects result in the disruption of the cell membrane,

leading to lysis and release of the intracellular components. Subsequent neutralization with sodium acetate precipitates protein and genomic DNA. Upon neutralization, supercoiled plasmid DNA stabilize from its pH-denatured state while large molecular weight (~200 kb) genomic DNA cannot diffuse and anneal properly, resulting in single-stranded genomic DNA precipitation. In addition, at high pH, RNA degrades, which benefits downstream separation steps. However, this lysis is rather delicate as plasmid DNA can be degraded at high pH if high shear conditions are produced during this procedure, and some of the supercoiled DNA could be converted to alternative forms (denatured supercoiled, multimeric, open circle, and linear).

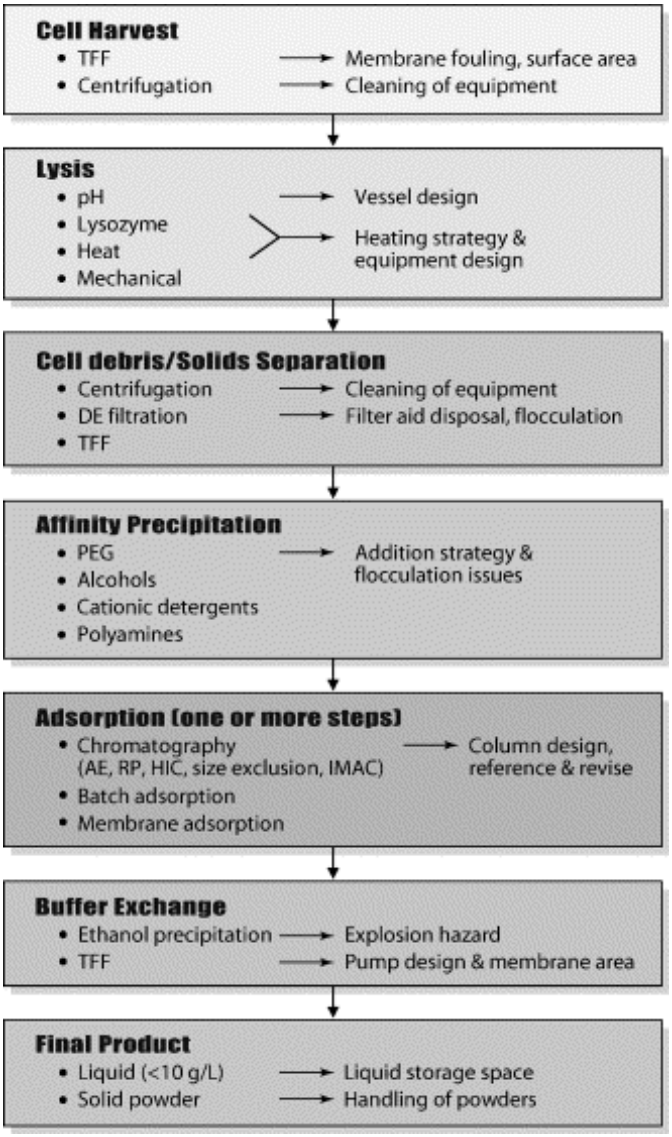


FIGURE 2 LARGE-SCALE PLASMID DNA PURIFICATION STEPS. EACH IMPORTANT STEP LISTED WITH THE CHOICES (LEFT) AND SCALE-UP ISSUES (RIGHT) FOR EACH APPLICABLE TECHNOLOGY (ADAPTED FROM PRATHER *ET AL*, 2003)

Following alkaline-lysis, a precipitate is formed that contains cell debris, denatured proteins and nucleic acids, and this must be removed. This might be accomplished by using any solid–liquid unit operation provided that low shear forces are used. Centrifugation on fixed-angle rotors is the most common operation at the laboratory and preparative scales. In large-scale production, filtration is the best option (Ferreira *et al*, 1999; Marquet *et al*, 1995; Prazeres *et al*, 1998; Theodossiou *et al*, 1997).

Subsequent steps, clarification and concentration, are designed to remove remaining proteins and host nucleic acids, which are usually removed by 'salting out' using a high concentration of chaotropic salts (Chakrabarti *et al*, 1992; Horn *et al*, 1995). Plasmid DNA is commonly concentrated using polyethylene glycol (PEG) precipitation after the clarification step in order further to remove small nucleic acids and to reduce the volume of process streams before adsorption purification. PEG precipitation also enables a buffer exchange, preparing the plasmid extracts for the next purification step (Ferreira *et al*, 2000).

To purified sc pDNA, liquid chromatography is the method of choice. The size and chemical properties of the target nucleic acid molecules (charge and hydrophobicity), the accessibility of the nucleotide bases to ligands, and the topological constraints imposed by supercoiling are exploited via the interaction of nucleic acids with solid supports, with the objective of selectively isolating and purifying plasmid DNA from impurities. Some of the methods are explained below (subchapters 2.2., 2.3. and 2.4.).

2.2. CHROMATOGRAPHIC METHODS

Generally, chromatography can be described as a separation method that is accomplished by distributing the substances between the mobile and the stationary phases.

As said before, liquid chromatography (LC) is the method of choice to purified sc pDNA. LC consists in a separation of mixture components between two phases: the

mobile phase, which is liquid and moves in a specific direction, and the stationary phase (Sewell *et al*, 2000). This separation can be based in size, charge, hydrophobicity or affinity of the component that needs to be purified relatively to the stationary phase.

Main chromatographic techniques applied to pDNA purification are: size-exclusion, ion-exchange, hydrophobic interaction, reversed-phase, thiophilic adsorption and affinity, either as an isolated step or integrated in an overall purification process (Sousa *et al*, 2008).

As said before, LC can be support by size, charge, hydrophobicity or affinity. The methods used in this project are charge and hydrophobicity-related, but a table with all types (table 4) is given below.

TABLE 4 CHROMATOGRAPHIC METHODS APPLIED TO PDNA PURIFICATION

Size-related	Size-exclusion chromatography
	Slalom chromatography
Charge-related	**Anion-exchange chromatography**
	Hydroxyapatite chromatography
Hydrophobicity-related	Reversed-phase liquid chromatography
	Reversed-phase ion-pair chromatography
	Hydrophobic interaction chromatography
	Thiophilic adsorption chromatography
Affinity-related	Triple-helix affinity chromatography
	Protein–DNA affinity chromatography
	Immobilised metal affinity chromatography
	Boronate affinity chromatography
	Polymyxin B affinity chromatography

Note: at bold are adsorptions used in this project.

Before describing hydrophobic and anion-exchange chromatographic methods, it is important to describe the limitations of these techniques when applied to pDNA purification. These limitations are due the physical and chemical similarities between impurities and pDNA that result in poor selectivity due to competition or co-elution of impurities in pDNA pools; however this can be improved by reducing impurity load prior to chromatography. Other problem is the high viscosity of feed solutions that increase pressure drop and decreased linear flow rates and throughput, but this could be circumvent by avoiding small beads, dilute feed streams or use expanded beds. At last,

chromatography purification can be limited by the large pDNA size, large size of high *M*r RNA and small pore size, leading to small diffusion coefficients (so poor internal mass transfer), large peaks (low recovery), small flow rates (long separation times) and poor capacity for pDNA due to lack of accessibility, that can be solved by pDNA compaction, bead size decrease, use of beads with "superpores" or "tentacles", use formats such as membrane layers and monoliths, pre-digestion of high *M*r RNA with RNase and use micropellicular stationary phases (Diogo *et al*, 2005). One of the most reported drawbacks of pDNA chromatography is poor capacity of stationary phases for pDNA binding, and that is why is important the development of new stationary phases, the major aim of this project (chapter 3) (Diogo *et al*, 2005).

2.3 HYDROPHOBIC INTERACTION ADSORPTION

Hydrophobic interaction chromatography (HIC) is a well-established bioseparation technique in laboratory and industrial scale protein purification. HIC explores the interaction between immobilized hydrophobic ligands and non-polar regions of, for example, a protein surface in a non-denaturing environment (Queiroz *et al*, 2001). These interactions are promoted mainly by van der Waals forces (Van Oss *et al*, 1986) and the underlying mechanisms have been described using different approaches such as the solvophobic theory (Melander *et al*, 1984; Diogo *et al*, 2002) and preferential interaction theory (Arakawa *et al*, 1982). HIC is entropy driven process, driven by the release and rearrangement of water molecules that occur during, for example, protein adsorption to the stationary phase (Janson and Rydén, 1998).

The mechanism for HIC adsorption is quite complex and depends on a number of process parameters, including the temperature, pH, salt concentration, adsorbent ligand density, and adsorbent ligand hydrophobicity. The salt type chosen is determinant for the separation success of HIC, the one usually chosen is ammonium sulfate (at 1,0M - 2,4M concentrations) due to its high salting-out ability, high solubility (barely varying in a range of 0–30 ◦C), stability up to pH 8.0 and low cost (Scopes, 1982).

HIC purifies plasmids on the basis of the differences in the hydrophobicity of pDNA and impurities. RNA and denatured gDNA, which are essentially single stranded molecules, have a high hydrophobic character due to the exposure of their hydrophobic bases, whereas pDNA molecules typically present a low hydrophobicity, since the majority of the bases are shielded inside the double helix. Consequently, pDNA can be purified with negative HIC by loading feed solutions at a high concentration of an adequate salt and subsequently performing step or gradient elution with low salt to remove bound impurities (Deshmukh and Lali, 2005; Diogo *et al*, 2000; Diogo *et al*, 2001; Li *et al*, 2005). But the retention time could be a problem in HIC and affect DNA isoforms separation; could induce a partial denaturation of the double helix at AT rich spots, leads to the exposure of the bases to the ligands, so to an increase in the hydrophobic interaction strength. This hypothesis is consistent with the observation that single-stranded oligonucleotides bind more tightly to reverse-phase and hydrophobic interaction matrices than duplex DNA fragments of the same length (Colote *et al*, 1986; Diogo *et al*, 2000; Huber, 1998).

One of the widely used stationary phases on HIC is sepharose CL-6B derivatised. Experiments carried out with Sepharose CL-6B derivatised with a mildly hydrophobic ligand (1,4-butanediol diglycidyl ether), proved that is possible to separate native sc pDNA from the more hydrophobic nucleic acid impurities (RNA, gDNA, oligonucleotides, denatured pDNA) under non-denaturing conditions and in the presence of 1,5M of ammonium sulphate (explained by the fact that pDNA molecules have the hydrophobic bases packed and shielded inside the double helix, and thus, the hydrophobic interaction with the HIC media is minimal, whereas single stranded nucleic acids impurities show a higher exposure of the hydrophobic bases, and thus, interact with the hydrophobic ligands). Sepharose CL-6B derivatised with 1,4-butanediol diglycidyl ether was successfully used for the separation of pDNA isoforms (in the presence of 1,5M of ammonium sulphate), so has also been used analytically for the monitoring and control of pDNA quality. Other cellulose and sepharose based HIC materials have also been found to promote the binding of poly A, denatured DNA or viral RNA. (Cashion *et al*, 1980; Diogo *et al*, 1999; Diogo *et al*, 2002[2]).

2.4. ANION-EXCHANGE ADSORPTION

Ion-exchange chromatography (IEC) is one of the most commonly used methods for downstream recovery of biomaterials. Although this process is based essentially on the interactions between charged biomaterials and charged resins, hydrophobic and other non-specific interactions may also be involved, with increased salt concentration the ion shield reduced the electrostatic attraction and retention volume in the column, on the other hand, as the salt concentration raises to reaches a specific threshold, retention volume increases with the salt concentration. The advantages of this technique are rapid separation, no solvent requirement, decontamination with sodium hydroxide and a wide selection of process-grade stationary phases (Eon-Duval and Burke, 2004; Melander *et al*, 1989).

Particularly, in pDNA purification, the separation is based on the interaction between DNA negatively charged phosphate groups and positively charged ligands on the stationary phase (Prazeres *et al*, 1998). The polyanionic structure of nucleic acids can be exploited by IEC, because the overall charge of nucleic acids depends on the number of bases that make up the molecule (one negative charge per base), the expected elution profiles follow the order of increasing molecular size, but it also could be sequence dependent. A salt gradient is employed to displace the different nucleic acids that should elute in order of increasing charge density, a property, which in turn is a function of chain length and conformation. In some cases, base sequence and composition affect the elution pattern of nucleic acids in anion exchangers. Several inversions in retention time as a function of chain length were attributed to a higher AT content of the more retarded molecule (as said before, hydrophobic interactions may be involved) (Huber, 1998; Prazeres *et al*, 2001; Sinden, 1994; Yamakawa, 1996).

When using AEC to separate pDNA, the clarified lysate feed should always be loaded at a sufficiently high salt concentration (typically >0.5M NaCl) to avoid an unnecessary adsorption of low charge density impurities, such as low *M*r RNA, oligonucleotides and proteins. Under these conditions, a significant amount of impurities elute in the flow through and capacity can be fully exploited for pDNA adsorption. Molecules with a high charge density, such as pDNA isoforms, high *M*r RNA and gDNA are retained and subsequently eluted by increasing the ionic strength of the mobile phase. Many AEC stationary phases display poor separation selectivity

towards pDNA and impurities due to their similar binding affinities. This lack of selectivity makes purification of pDNA very difficult to achieve in a single AEC step. In most cases a second chromatographic step is needed to meet the required purity or pre-purification steps are required (Colpan and Riesner, 1984; Eon-Duval and Burke, 2004; Ferreira *et al*, 2000; Prazeres *et al*, 1999).

Q-Sepharose has been extensively used for AEC of pDNA too. This strong anion exchanger is made of 6% highly cross-linked agarose particles derivatised with quaternary amino groups. A comparison of the performance of Mono Q- and Hi-load Q-Sepharose columns on the small and large-scale purification of pDNA showed that both columns were able to separate sc and oc isoforms (Chandra *et al*, 1992).

CHAPTER 3. MATRICES AS STATIONARY PHASE

As said before, one of the major problems in plasmid purification is the stationary phase capacity. So, there are a great interest in explore alternative matrices that could be modified and applied to plasmid adsorption. As plasmids are large molecules, is newsworthy to give special attention to micro and nano-particles based-matrices. The polymers used to produce these matrices were cellulose, a natural one, and polyvinyl alcohol, a synthetic one.

A technique of growing interest, and the one used to fabricate the PVA nanofibers used in this project, is electrospinning, whose important advantages are the production of very thin fibers to the order of few nanometers with large surface areas, simplicity of functionalization for various purposes, superior mechanical properties and ease of process. The possibility of large scale productions combined with the simplicity of the process makes this technique very attractive for many different applications (like tissue engineering, drug release, implants and biotransformation to wound healing) (Min, 2004).

3.1. NANOFIBERS

Nanofibers have very small diameters, large surface area per unit mass and small pore size, making them interesting candidates for a wide variety of applications, such as templates (Liu, 2004; Sun *et al*, 2003), reinforcement (Bergshoef and Vancso, 1999; Kim and Reneker, 1999), filtration (Yoon *et al*, 2006; Ma *et al*, 2005), catalysis (Stasiak *et al*, 2007; Chen *et al*, 2007), biomedical and pharmaceutical applications (Welle *et al*, 2007; Cui *et al*, 2006; Luu *et al*, 2003) and electronic and optical devices (Choi *et al*, 2003; Kim and Yang, 2003; Wang *et al*, 2002). Properties such as strength, weight, porosity and surface functionality can be manipulated, depending on the polymer used. Furthermore, small insoluble particles can be added to the polymer solution and encapsulated in the dry nanofibers (Frenot and Chronakis, 2003).

The major purpose of nanofibers application in purification processes is benefit from an increased surface area, because specific surface area increases as dimensions decrease, in other words, a higher proportion of atoms is on the surface.

There are a few different methods to produce nanofibers, including, for example, phase separation (Ma and Zhang, 1999) and drawing (Ondarçuhu and Joachim, 1998), but a unique synthetic method, electrospinning, has received a special attention lately.

3.1.1. ELECTROSPINNING

Electrospinning, or electrostatic spinning, is a novel process for forming fibers with submicron scale diameters through the action of electrostatic forces (Fong and Reneker, 2001). While conventional fiber spinning techniques are capable of produce polymer fibers with diameters down to micrometer range, electrospinning is capable of produce fibers in the nanometer diameter range, including diameters lower than 100nm (Frenot and Chronakis, 2003).

Electrospinning was first described as a fabrication technology by Rayleigh in 1897 (Burger *et al*., 2006), studied in more detail by Zeleny in 1914 (Zeleny, 1914) and patented by Formhals in 1934 (Formhals, 1934). However, it only has been applied in nanotechnology in 1990 and since then, an increasing number of studies have shown that a large variety of polymers can be electrospun (Reneker and Chun, 1996).

Electrospinning has substantial adaptability for processing a variety of polymers with possible control of the fiber fineness (Greiner and Wendorff, 2007; Jayaraman *et al*, 2004; Li and Xia, 2004; Reneker and Chun, 1996), orientation (Li et al, 2003), surface morphology (Casper et al, 2004) and bicomponent cross-sectional configuration (Lin *et al*, 2005; Sun *et al*, 2003).

In general, in electrospinning, a high electric potential is applied to a polymer solution (majority) or melt. The fibers are derived by charging a liquid typically to 5-30 kV *versus* a ground a short distance away, which leads to charge injection into the liquid from the electrode. The sign of the injected charge depends on the polarity of the electrode, so a negative electrode produces a negatively charged liquid. The charged

liquid is attracted to the ground electrode of opposite polarity, forming a Taylor cone at the nozzle orifice as the polymer solution is drawn out by the electric field to form a liquid jet. As the solvent of the polymer jet evaporates, the jet solidifies and a polymer fiber is formed. In the past decade, substantial attention was drawn to electrospinning with research interests focusing on the theoretical foundation of the electrospinning process, including fiber initiation, jet instability, and the structure and morphology of the electrospun fibers (Deitzel *et al*, 2001; Fong *et al*, 1999; Reneker and Chun, 1996; Shin *et al*, 2001; Yarin *et al*, 2001; Yarin *et al*, 2001[2]). A general scheme of electrospinning process is represented at figure 3.

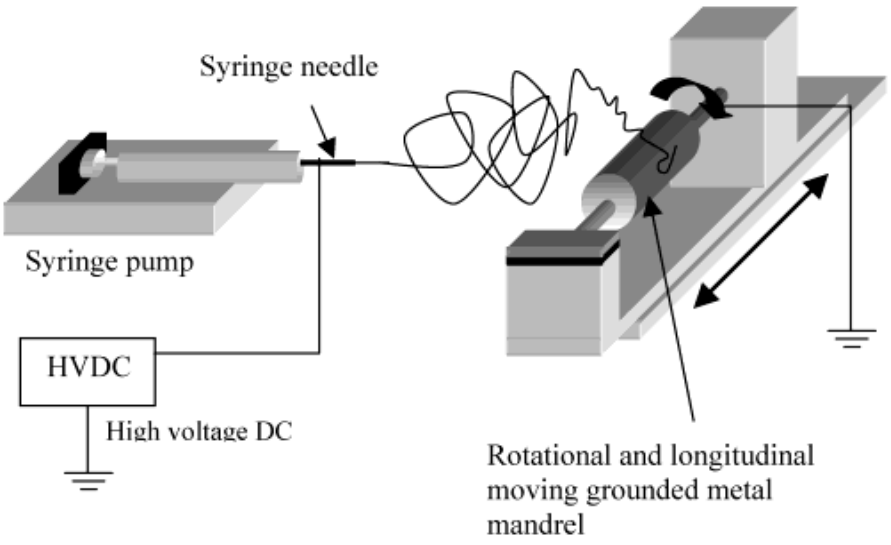


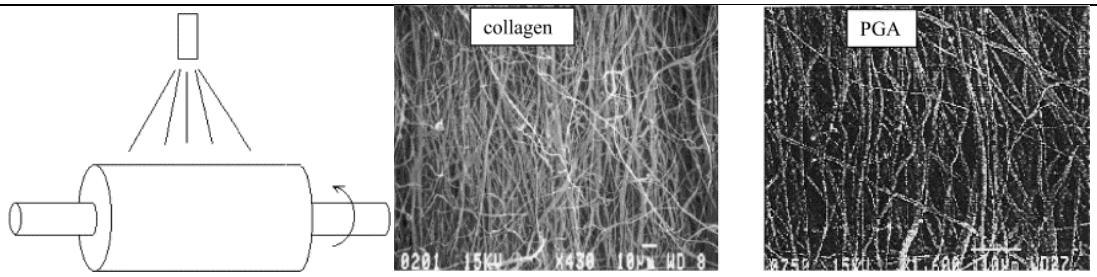
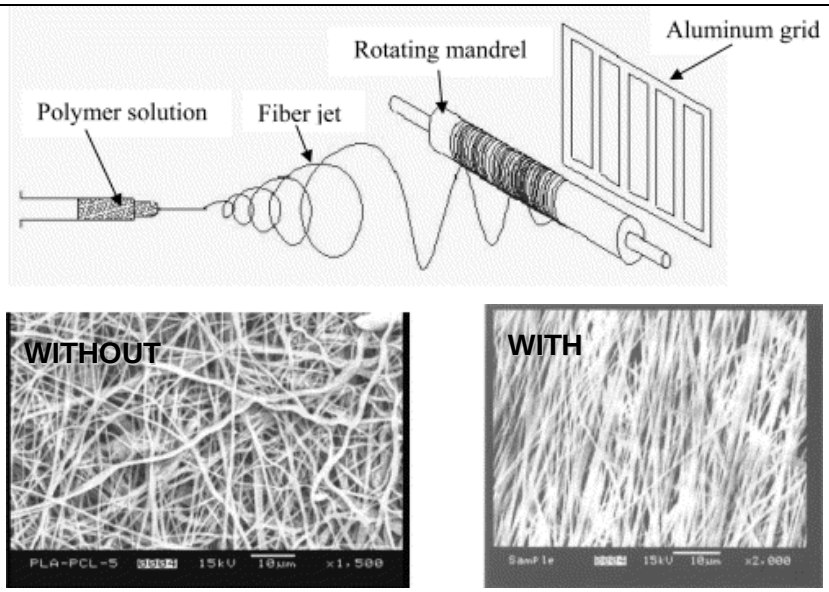
FIGURE 3 GENERAL SCHEME OF ELECTROSPINNING DEVICE [DC = DIRECT CORRENT] (ADAPTED FROM YAO *ET AL*, 2003)

Essentially, an electrospinning consists of three major components: a high-voltage power supply, a spinneret and an electrically conductive collector. For most experiments, an ordinary hypodermic metallic needle and a piece of aluminum foil work well as the spinneret and the collector, respectively (Li *et al*, 2006).

There is only one procedure to electrospinning, but there are several nanofibers collectors, so it is possible to obtain different types of nanofibers, depending on the end use of resultant nanofibers. The most commons are: a cylinder collector with high

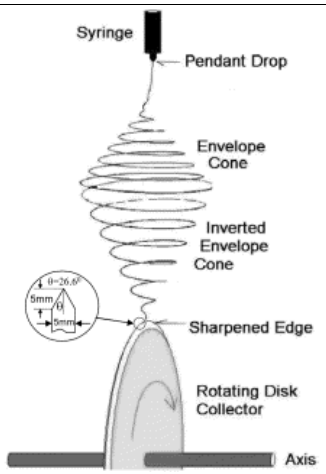
rotating speed (the one represented at figure 3), an auxiliary electrode/electrical field, a thin wheel with sharp edge and a frame collector. The collector design and the nanofibers structure obtained are represented at table 5.

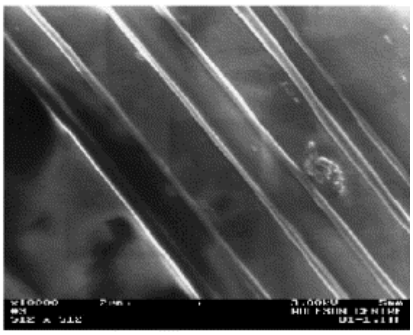
TABLE 5 TYPES OF COLLECTORS AND FIBERS ALIGNEMENT (ADAPTED FROM HUANG *ET AL*, 2003)

CYLINDER COLLECTOR WITH HIGH ROTATING SPEED
It has been suggested that by rotating a cylinder collector at a very high speed up to thousands of rpm, electrospun nanofibers could be oriented circumferentially (Boland *et al*, 2001; Matthews *et al*, 2002).

AUXILIARY ELECTRODE/ELECTRICAL FIELD
Deposited fibers can be circumferentially oriented substantially by employing an auxiliary electrical field. The alignment effect with and without the auxiliary electrical fields can be seen from the comparison shown below (Bornat, 1987).


THIN WHEEL WITH SHARP EDGE

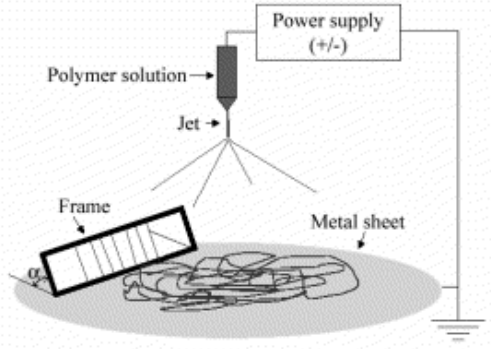
The tip-like edge substantially concentrates the electrical field so that the nanofibers are almost all attracted to and can be continuously wound on the bobbin edge of the rotating wheel. The distance between two fibers can varied from 1 to 2 μm; before reaching the electrically grounded target, the nanofibers retain sufficient residual charges to repel each other (Theron *et al*, 2001).

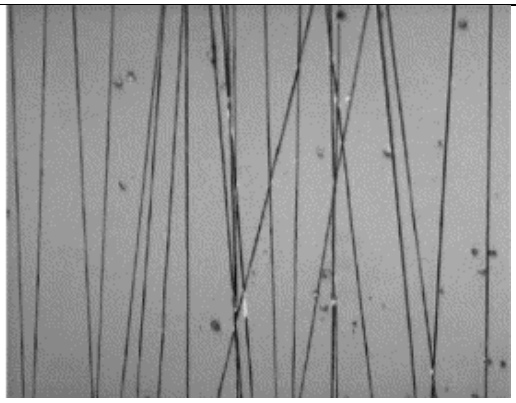




FRAME COLLECTOR

Another approach to fiber alignment can be by simply placing a rectangular frame structure under the spinning jet other (Huang *et al*, 2003).





As said before, there are a number of factors that can be controlled in electrospinning, but there are several external parameters and processing variables that affect the electrospinning process: system parameters such as molecular weight, molecular weight distribution and architecture (branched, linear etc.) of the polymer and

solution properties (viscosity, conductivity and surface tension), and process parameters such as electric potential, flow rate and concentration, distance between the capillary and collection screen, ambient parameters (temperature, humidity and air velocity in the chamber) and finally motion of target screen (Wilkes, 2001). For instance, the polymer solution must have a concentration high enough to cause polymer entanglements yet not so high that the viscosity avoids polymer motion induced by the electric field. The solution must also have a surface tension low enough, a charge density high enough, and a viscosity high enough to prevent the jet from collapsing into droplets before the solvent has evaporated. Morphological changes can occur upon decreasing the distance between the syringe needle and the substrate. Increasing the distance or decreasing the electrical field decreases the bead density, regardless of the concentration of the polymer in the solution (Deitzel *et al*, 2001).

3.2. CELLULOSE FIBERS

Cellulose is a naturally occurring polymer of great interest, because of its abundant availability, biodegradability, compatibility with biological systems and low non-specific binding when used during purification. It consists of a linear polymer of 1,4 β-D-glucose (fig. 4). Cellulose fibers are useful in wide range areas, such as filtration, biomedical applications and protective clothing (Zhang *et al*, 2008, Cuculo *et al*, 2001). There has been growing interest in the use of cellulose in chromatographic techniques, because it provides an interesting spectrum of geometrical and morphological forms, the support is relatively inexpensive and the raw material is renewable (Gemeiner *et al*, 1998).

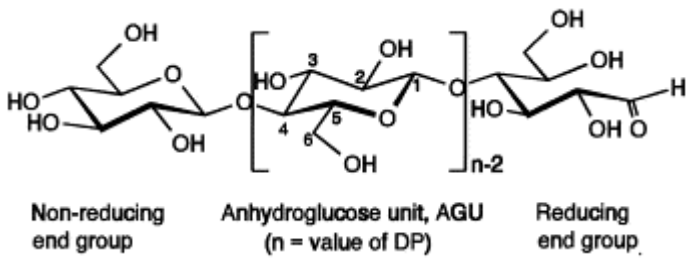


FIGURE 4 MOLECULAR STRUCTURE OF CELLULOSE

Cellulose-based media displays the characteristics for an ideal matrix: a high specificity, the absence of hydrophobic binding sites (hydrophilic), good chemical stability (neutral and stable under storage and operational conditions), good mechanical rigidity, high binding capacity (contain functional groups to allow attachment of the affinity ligands), good recoverability, high reproducibility and low cost. However, there is main objection against cellulose-based affinity adsorbents; they may exhibit nonspecific adsorption (Aniulyte *et al*, 2006; Clonis, 1987; Groman *et al*, 1987; Liu *et al*, 1994; Narayanan *et al*, 1990).

3.3. POLYVINYL ALCOHOL FIBERS

Polyvinyl alcohols (PVA) are synthetic polymers used since the early 1930s in a wide range of industrial, commercial, medical and food applications including resins, lacquers, surgical threads and food-contact applications (DeMerlis and Schoneker, 2003).

PVA empirical formula is $(C_2H_4O)_n(C_4H_6O_2)_m$ and has a molecular weight between 30,000 and 200,000 g/mol, is water soluble and is insoluble in aliphatic and aromatic hydrocarbons, esters, ketones and oils. Physically, in general, PVA is an odorless white to cream-colored granular powder (Handbook Pharm. Excip., 1994; Japan. Pharm. Excip. Dir., 1996).

The physical characteristics of PVA are dependent on its method of preparation from the hydrolysis, or partial hydrolysis, of polyvinyl acetate (fig. 5), being classified as partially hydrolyzed or fully hydrolyzed (DeMerlis and Schoneker, 2003).

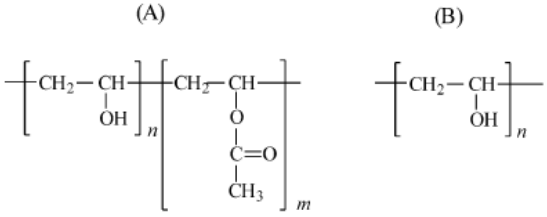


FIGURE 5 STRUCTURAL FORMULA FOR PVA: (A) PARTIALLY HYDROLYZED; (B) FULLY HYDROLYZED.

PVA has already been used to make anion-exchange membranes, prepared through a reaction with quaternary ammonium groups, but this was meant to be applied in a completely different area of purification processes; was made for alkaline direct methanol fuel cell applications (Xiong *et al*, 2008). So, its application to pDNA adsorption is completely new.

CHAPTER 4. PURPOSE AND FUNDAMENTAL RESEARCH QUESTIONS

As said before, one of the major problems in plasmid purification is the stationary phase capacity. So, the main achievement of this experimental research is to find alternative matrices to pDNA purification, regarding to economical, simple and efficacy features.

The choice of cellulose is due their easy and cheap obtainment and their relatively trouble-free modification; sepharose was mainly to compare results.

At the beginning, the aim was to produce cellulose nanofibers from cellulose pulp solution by electrospinning, but that was not possible to accomplish. Since good results about sepharose derivatized are known and cellulose has a similar structure, cellulose derivatized microfibers were test with the possibility to be a better matrix than sepharose, with two types of adsorption: hydrophobic interaction (is the most common and is easy and low-cost to perform) and anion-exchange (is also easy and low-cost and, generally, is more effective).

Back to the major intend, due the higher surface capacity, the study of nanofibers is indispensable. The electrospun nanofibers sample was send from Cincinnati, Ohio, USA, but it was a small sample and the polymer used was PVA. Due the polymer structure and small amount, PVA nanofibers are going to be modified only to carry out anion-exchange adsorption.

The method of choice is batch adsorption, because of possible problems like column chromatography high back pressure (due the small particles sized stationary phase) and poor capacity pDNA binding (due pDNA large size), furthermore is an easier method to scale-up.

So, the fundamental research question is: how those matrices behave in plasmid DNA purification? The approaches how to answer this question are represented schematically at figures 6 and 7.

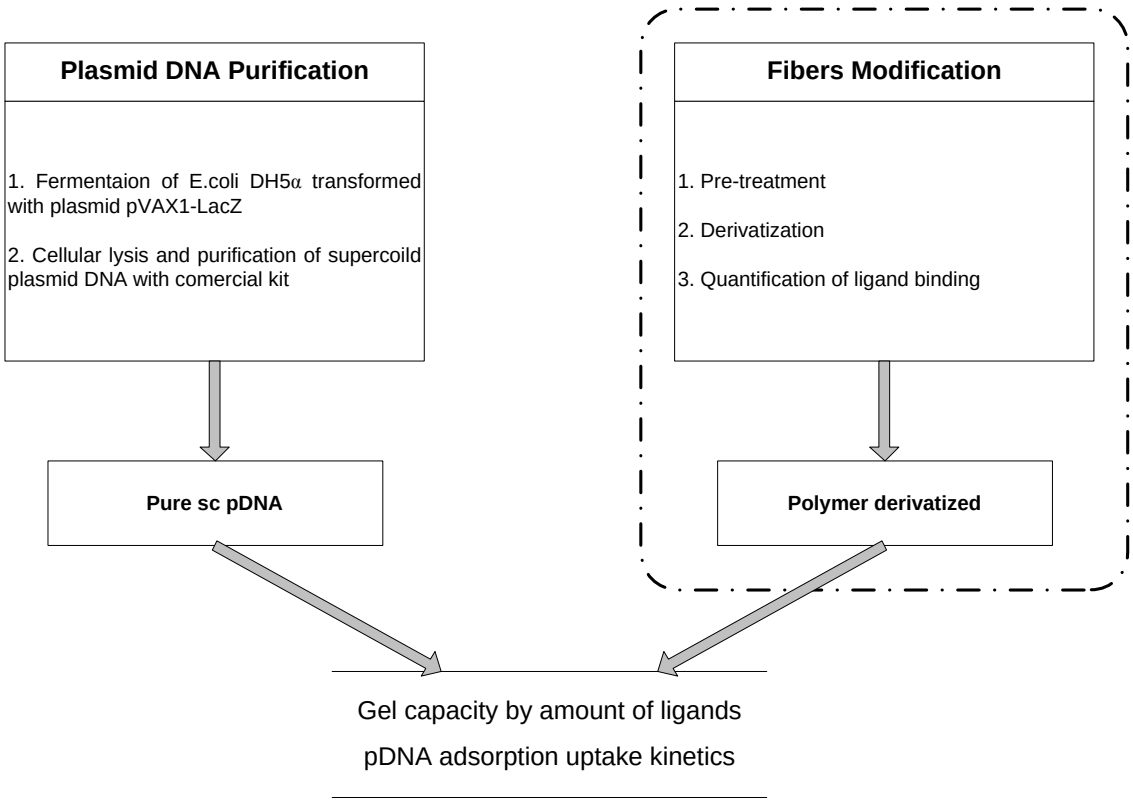


FIGURE 6 GENERAL RESEARCH PLAN. ACCORDING TO THE TYPES OF POLYMER AND INTERACTION, MODIFICATION PROCEDURE DIVERGE.

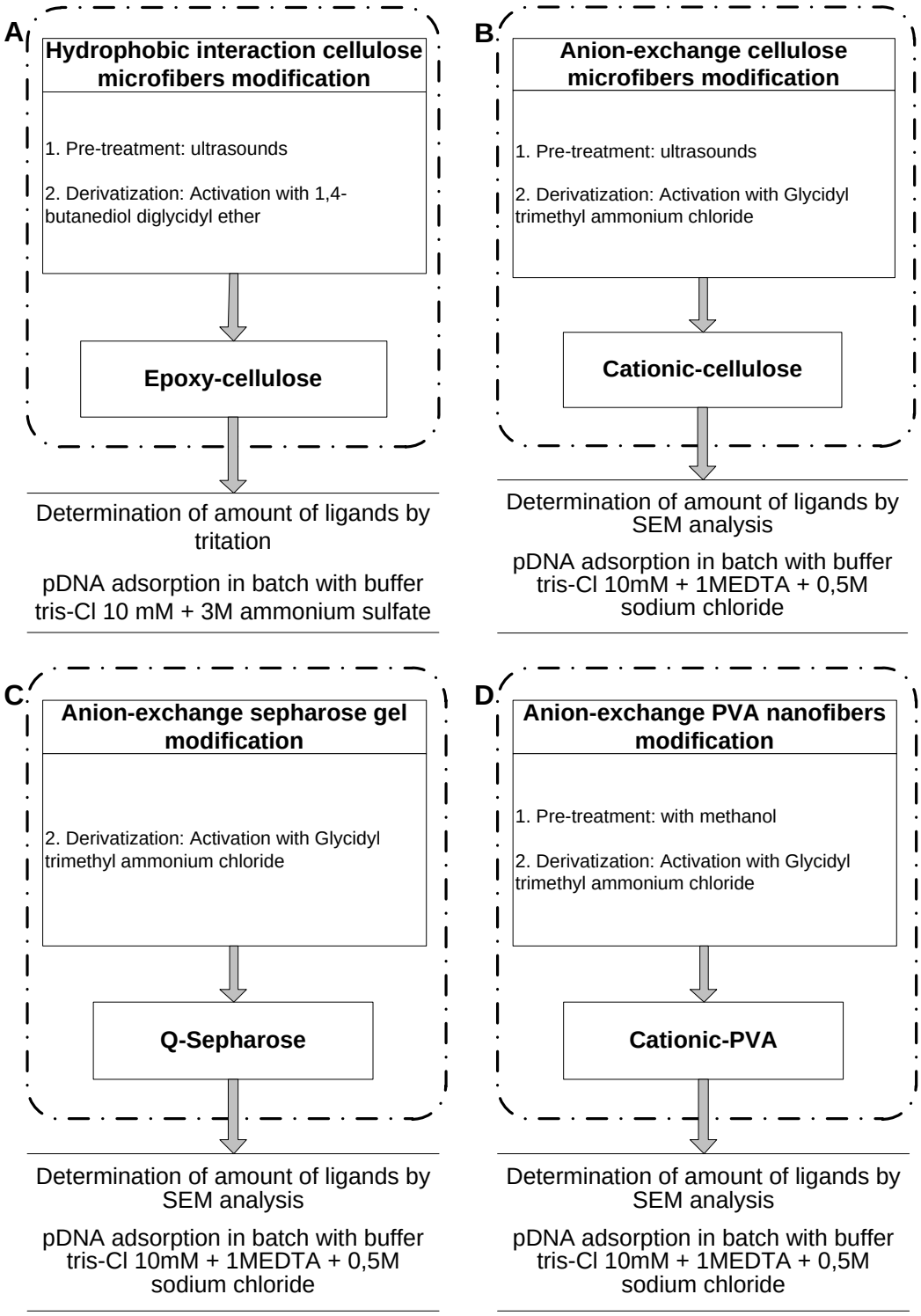


FIGURE 7 MODIFICATIONS GENERAL PROCEDURE SKETCH OF: **A**. CELLULOSE MICROFIBERS TO HYDROPHOBIC INTERACTION; **B**. CELLULOSE MICROFIBERS TO ANION-EXCHANGE; **C**. CL-6B-SEPHAROSE GEL TO ANION-EXCHANGE AND **D**. PVA NANOFIBERS TO ANION-EXCHANGE.

CHAPTER 5. EXPERIMENTAL PLASMID DNA PRODUCTION

5.1. *E. COLI* FERMENTATION

The 6,05 kpb plasmid pVAX1-*LacZ* (Invitrogen, Carlsband, CA, USA) used in the experiments was produced by a cell culture of *Escherichia coli* DH5α.

Globally, growth was carried out at 37°C in a bioreactor with 250 mL of Terrific Broth (table 6) supplemented with 30 μg/mL kanamycin. The dissolved oxygen concentration was kept at 30% of saturation, and growth was suspended at the late log phase ($OD_{600} \approx 8$).

Cells were recovered by centrifugation and stored at −20°C.

TABLE 6 REAGENTS USED TO PRODUCE TERRIFIC BROTH (TB)

Reagents	**Information available**	**Concentration required**
Tryptone	-	12 g/L
Yeast extract	-	24 g/L
Glycerol	M=92,10g/mol	4 mL/L
KH_2PO_4 and K_2HPO_4 solution[1]	M=136,09g/mol and M=174,18g/mol	0,017 M and 0,072 M

[1] solution 10 times more concentrated that the one is needed is prepared and stored, previously.

5.1.1. *E.COLI* INOCULATION

The solutions, LB-Agar and TB media, and all the required material were autoclaved previously.

Petri dishes with LB-Agar with kanamycine were prepared to inoculate the cells. *Escherichia coli* DH5α transformed with plasmid pVAX1-*LacZ* was inoculated and grew overnight, at 37ºC.

5.1.2. PRE-FERMENTATION

A few bacterial colonies that grew overnight were inoculated in 62,5 mL of TB media with 30 μg/mL kanamycin. The suspension were kept under rotation (250 rpm), at 37ºC, until a $OD_{600} \approx 2{,}6$ was reached.

TABLE 7 SAMPLE ABSORBENCIES DURING PRE-FERMENTATION

Time (h)	Absorbency'	Dilution	Absorbency
1,0	0,457	None	0,457
2,5	0,939	1:2	1,878
3,0	0,638	1:4	2,552
Final (3,1)	0,684	1:4	2,736

The volume used to carry out the fermentation was calculated through the following formula:

$$OD_{pre-ferment} \times V_{pre-ferment} = OD_{ferment} \times V_{ferment+\ pre-ferment}$$ [2]

So, the pre-fermentation suspension volume was 9,842 mL to each 125 mL of fermentation broth.

[2] $OD_{pre\text{-}ferment}$ = pre-fermentation suspension optic density;

$V_{pre\text{-}ferment}$ = pre-fermentation suspension volume;

$OD_{ferment}$ = fermentation suspension optic density (0,2);

$V_{ferment\ +\ pre\text{-}ferment}$ = fermentation suspension volume with the pre-fermentation suspension volume.

5.1.3. FERMENTATION

Fermentation suspensions (already with kanamycin and pre-fermentation suspension) were kept under rotation (250 rpm), at 37ºC, until a $OD_{600} \approx 8,0$ was reached. The absorbency was measured hour by hour, in the beginning, just to see how *E.coli* population grows (tables 8 and 9).

The growth was stopped with centrifugation. The suspensions were transferred to falcons that were centrifuged 15 min, at 4ºC and 5000g. Then, supernatant was discarded and cells pellet was frozen at -20ºC.

TABLE 8 SAMPLE A ABSORBENCIES DURING FERMENTATION

Time (h)	Absorbency'	Dilution	Absorbency
1,0	0,555	None	0,555
2,0	0,701	1:2	1,402
3,0	0,433	1:5	2,165
4,0	0,536	1:5	2,680
Overnight			
19,0	0,765	1:10	7,65
20,0	0,770	1:10	7,70

TABLE 9 SAMPLE B ABSORBENCIES DURING FERMENTATION

Time (h)	Absorbency'	Dilution	Absorbency
1,0	0,549	None	0,549
2,0	0,698	1:2	1,396
3,0	0,427	1:5	2,135
4,0	0,562	1:5	2,810
Overnight			
19,0	0,768	1:10	7,68
20,0	0,786	1:10	7,86

The procedure was repeated, several times, with a few changes: the tryptone mass measured was 6,25g (instead of 3,858g) – but only samples C and D –, the

centrifuge velocity used, at the end, to precipitate de pellet was 4500 rot/min (instead of 5000 rot/min) and the absorbency measure hour by hour was no long performed – all. The tryptone mass used at the first time is the one that is currently used at the laboratory (concentration of 12g/L) and the second is the one that is indicated at the papers (concentration of 20g/L). The centrifuge velocity alteration was due the finding that 4500 rot/min is sufficient to precipitate the cells. The absorbencies after pre-fermentation and fermentation as the volume needed to carry out fermentations are at annex A.

5.2. CELLULAR LYSIS AND PLASMID DNA PURIFICATION WITH QIAGEN® KIT

QIAGEN Plasmid Purification Kits are based on the selectivity of patended QIAGEN Resin, allowing purification of ultrapure supercoiled plasmid DNA with high yields.

QIAGEN plasmid purification protocols are based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low-salt and pH conditions. RNA, proteins, dyes, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation (QIAGEN Plasmid Purification Handbook 11/2005).

The kit buffers composition and the procedure are described at annex B.

5.3. ELECTROPHORESIS

Agarose gel electrophoresis was performed to analyzed pDNA yield and quality. To do that, were saved four aliquots from different steps of pDNA purification procedure that are summarized at table 10.

TABLE 10 FRACTIONS SAVED FOR AGAROSE GEL ELECTROPHORESIS

Sample id.	Fraction	Volume (μL)	Contain
1	Cleared lysate	120	sc and oc pDNA and degraded RNA
2	Flow-through	120	only degraded RNA
3	Buffer QC wash fractions	240	completely clear
4	Buffer QF/QN elute	60	Pure pDNA

Figure 8 shows the analytical gel of the different steps fractions from two performances of QIAGEN kit pDNA purification (A and B). The lanes identification and results analysis are below at table 11.

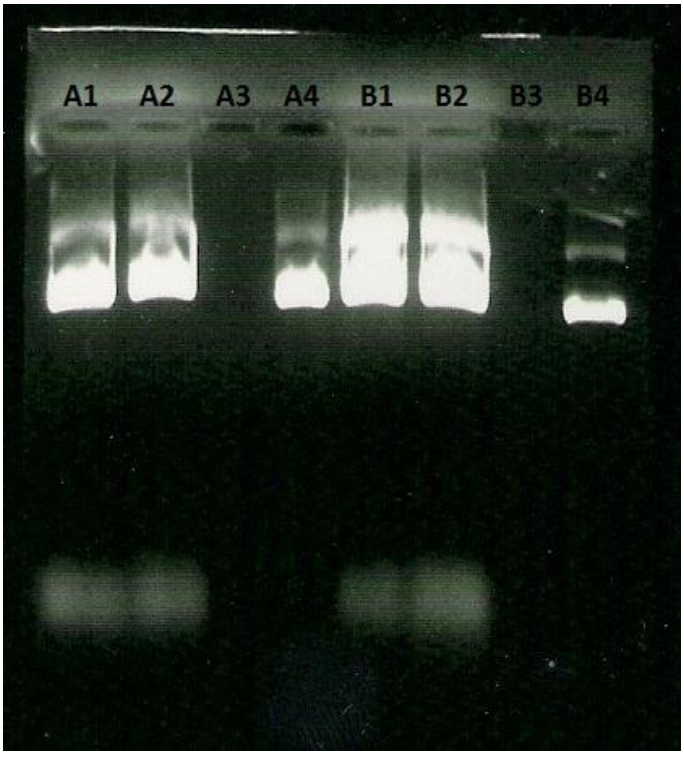


FIGURE 8 AGAROSE GEL ANALYSIS OF THE PLASMID PURIFICATION PROCEDURE

TABLE 11 LANES IDENTIFICATION AND RESULTS ANALYSIS

Lane	Identification	Analysis
1	A1	Cleared lysate containing sc and oc pDNA and degraded RNA, as expected
2	A2	Flow-through containing the same impurities that A1, but it was supposed to be only degraded RNA → after the first step, the sample still have a high impurities degree
3	A3	Buffer QC wash fractions completely clear, as expected
4	A4	Buffer QF/QN elute with pDNA and a light contamination of genomic DNA
5	B1	Same as A1
6	B2	Same as A2
7	B3	Same as A3
8	B4	Buffer QF/QN elute with pure pDNA

5.4. RECOVER OF ANION-EXCHANGE RESINS FOR PLASMID DNA PURIFICATION

After elution of plasmid DNA following the procedures suggested by the manufacturer, the tips were washed briefly with deionized water to remove any particulate materials that are trapped on top of the packed resins and salt deposits on the outer surface of the tips before storage.

Immediately before applying the used tips for plasmid DNA purification, 10 mL of "renew" buffer (3M NaCl, 0,15% Triton X-100) is added into the tips and allowed to flow through to remove any residual material from the previous use. This process was repeated three times (Chang *et al*, 1999).

TABLE 12 COMPOSITION OF RENEW BUFFER

Reagents	Information available	Concentration required
NaCl	M = 58, 443 g/mol	3 M
Triton X-100	-	0,15%

CHAPTER 6. EXPERIMENTAL MATRICES PRODUCTION

Cellulose fibers origin:

Pine cellulose pulp obtained by Kraft method was used as a source for the microfibrillated cellulose. The pulp was treated in three steps: a refining step to increase the accessibility of the cell wall to the subsequent monocomponent endoglucanase treatment, an enzymatic step and a second refining.

Cellulose pulp was torn into small pieces (a portion corresponding to 360,6g of dry cellulose) and wetted in 4L of distilled water. After 3 hours, the cellulose suspension was mechanically refined using a Valley Beater Rafiner to 27ºSR. Cellulose pulp thus obtained was seeped out by filtration and homogenized in Karl Frank Homogenizer. Next, the enzyme was added. In this enzymatic treatment, 360,6g (calculated as dry fibers) of the refined pulp was dispersed in 9L of phosphate buffer (pH 7, final pulp concentration 4%(w/w)) with 0,11 μL of monocomponent endoglucanases per gram dry fiber and then incubated for 2 hours at 50ºC. The mixture was stirred manually every 30 minutes. Then, the samples were washed with 30L of deionized water, drained and homogenized. The monocomponent endoglucanase was denaturated by heating at 80ºC for 30 minutes and then the pulp sample was washed with 20 liters of deionized water. The pre-refined and enzyme treated pulp was refined once again with the Valley Beater, this time, to 92ºSR (Szewczyk, 2009).

The cellulose fibers (not yet in a micro scale range) that Katarzyna Szewczyk obtained were stored in a solution of water with 20% ethanol, at 4ºC.

6.1. ULTRASONIC TREATMENT

First of all, a pre-wash step was performed to remove ethanol from samples. Samples were washed by a centrifuge method: washing with water, five rounds of 1min, at 4600 rpm and 25ºC. A total of eight samples were prepared (Table 13):

TABLE 13 SAMPLES VOLUMES AFTER PRE-WASH STEP

Sample	A1	A2	A3	A4	B1	B2	B3	B4
Volume (mL)	22,0	22,0	24,0	22,0	27,5	27,0	25,0	27,0

Samples were treated with ultrasounds (*Labsonic P B Braun Biotech Intern*., amplitude 80, cycle 0,5), during 30 minutes, with a break of 30 seconds in every minute, to not overheating and homogenize the sample. The initial and final temperatures of fibers were measured (Table 14).

TABLE 14 TEMPERATURES OF THE SAMPLES BEFORE AND AFTER ULTRASONIC TREATMENT

Sample	A1	A2	A3	A4	B1	B2	B3	B4
Initial temp. (ºC)	25,0	25,5	25,0	25,0	24,0	24,5	26,0	26,5
Final temp. (ºC)	76,0	73,0	74,0	72,0	78,0	76,0	74,0	73,0

The samples were stored, at 4ºC, in a solution of water with 20% ethanol, until completion of the next step.

6.2. MODIFICATION OF CELLULOSE MICROFIBERS TO HYDROPHOBIC INTERACTION ADSORPTION

The protocol used for this step was based on Sundberg's (Sundberg and Porath, 1974). The list of reagents is at table 15.

TABLE 15 SURFACE MODIFICATION PROTOCOL REAGENTS

Reagents	Information available	Concentration required
1,4-Butanediol diglycidyl ether	M = 202,20 g/mol 62% pure	-
Sodium hydroxide	M = 40 g/mol 99% pure R35, S26, S45, S37/39	0,6 M and 1 M
Sodium borihydride	M = 37,83 g/mol 99% pure	-
Sodium thiosulphate	M = 158,11 g/mol 99% pure	1,3 M
Hydrochloric Acid	M = 36,46 g/mol 37% pure d = 1,19 R34, R37, S26, S45	0,01 M

6.2.1. ACTIVATION

Per gram of cellulose microfibers gel, the sample was mixed with 1 mL of 1,4-butanediol diglycidyl ether and 1 mL of 0,6 M sodium hydroxide solution containing 2 mg of sodium borihydride per milliliter (figure 9). The suspension was mixed by rotation for 8 hours at 25ºC and the reaction stopped by washing the gel with large volumes of water with the centrifuge-wash method. The amounts used are described in table 16.

After washing, to determine the amount of bound substance (step 6.1.2.2), were retired 500 mg samples and the rest of the gel was placed in 1M NaOH overnight, to inactivate the free epoxy groups.

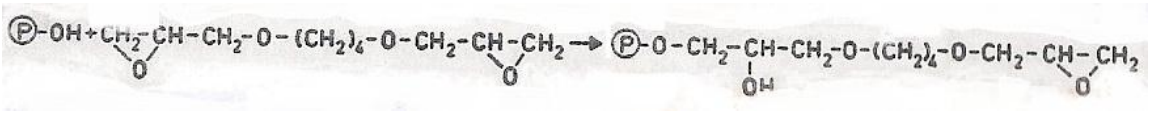


FIGURE 9 REACTION OF POLYMER ACTIVATION (ADAPTED FROM SUNDBERG AND PORATH, 1974)

TABLE 16 SAMPLES AND REAGENTS USED IN THE ACTIVATION STEP

Sample		**Identification**	(HI)I
		Primary sample	A2, A3 and A4
		Mass (g)	50,15
Volumes (mL)	**Required** / **Measured or Prepared**	**Diglycidyl ether**	50,0 / 50,0
		Sodium hydroxide 0,6 M	50,0 / 50,0
		Sodium hydroxide 1 M	500,0 / 500,0
Masses (g)	**Required** / **Measured**	**Sodium hydroxide 0,6 M**	1,22 / 1,23
		Sodium hydroxide 1 M	20,0 / 20, 408
		Sodium borihydride	0,100 / 0,101
Bath		**Temperature (ºC)**	24,9
		Rotation (U/min)	67

After 24h with 1M NaOH, the cellulose microfibers gel was washed with a great amount of water and then stored, at 4ºC, in a solution of water with 20% ethanol.

6.2.2. EPOXY GROUPS QUANTIFICATION

The epoxy groups are quantified through a typical titration method. To each 500 mg sample was added 5 mL of water and 15 mL of 1,3 M sodium thiosulphate. Until a pH = 7 was reached 0,01 M hydrochloric acid was added (figure 10). The initial and final pH were measured (table 17).

The samples were centrifuged and dry in previously weighted falcons, at 40ºC, during 48 hours[3].

With the HCl volume and the weight of dry gel samples is possible to quantify epoxy groups. The amount of ligands (in µmol per gel gram) is at table 18.

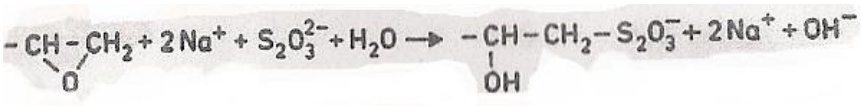


FIGURE 10 DETERMINATION OF BOUNDED GROUPS REACTION (ADAPTED FROM SUNDBERG AND PORATH, 1974)

TABLE 17 SAMPLES PH BEFORE AND AFTER TITRATION AND HCL VOLUME NEEDED

Activated sample Id.	Analyzed sample Id.	Initial pH	Final pH	0,01 M HCl vol. (mL)
I	Ia	11,79	7,07	0,60
	Ib	11,86	6,33	1,20
	Ic	11,35	7,24	0,45

[3] This period was extended to 96 hours due the observation of water in the sample.

TABLE 18 DRY GEL SAMPLES WEIGHT AND AMOUNT OF LIGANDS IN GEL SURFACE

Sample	Dry gel mass* (g)	Amount of H (µmol)	Amount of ligands (µmol/g)	
Ia	0,349	6,00	17,192	16,808
Ib	0,173	12,00	69,364	
Ic	0,274	4,50	16,423	

Note: The sample in grey was excluded to calculate the gel capacity.

*the samples weight was obtained by subtracting falcons mass from falcons with dry gel mass.

Other gel samples were prepared with the same procedure as the first one, with one only exception: gels III, IV and V were titrated with HCl 0,005 M. The amount differences were in the surface modification step and are tabulated at annex C.

6.3. MODIFICATION OF CELLULOSE MICROFIBERS TO ANION-EXCHANGE ADSORPTION

The protocol used for this step was based on Hjertén's (Hjertén *et al*, 1991). The list of reagents is at table 19.

TABLE 19 SURFACE MODIFICATION PROTOCOL REAGENTS

Reagents	Information available	Concentration required
Glycidyltrimethyl-ammonium chloride	M = 151,63 g/mol D=1,13 g/mL R45, R60, R20/21/22, R48/22, R68, R41, R43, R52/53, S53, S26, S36/37/39, S45, S61	m = 70g
Sodium hydroxide	M = 40,0 g/mol 99% pure R35, S26, S45, S37/39	0,5 M
Sodium borihydride	M = 37,83 g/mol 99% pure	-

Per 2 grams of cellulose microfibers gel, the sample was mixed with 2 mL of 0,5 M sodium hydroxide solution containing 40 mg of sodium borihydride per milliliter and 2mL of 2,3-epoxypropyl-trimethyl ammonium chloride (figure 11). The suspension was maintained under rotation (90 rpm) for 3 days at 25ºC and the reaction stopped by washing the gel with large volumes of water with the centrifuge-wash method. The amounts used are described at table 20.

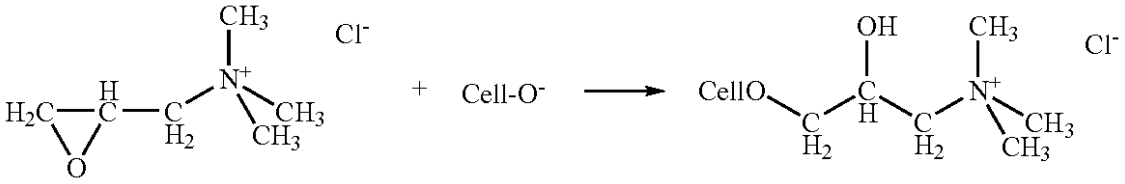


FIGURE 11 REACTION OF POLYMER ACTIVATION (ADAPTED FROM SMITHT *ET AL.*, 2007)

TABLE 20 SAMPLES AND REAGENTS USED IN THE ACTIVATION STEP

Sample	**Identification**	AE-Cel-I
	Primary sample	B4
	Mass	10,009 g
Sodium hydroxide	**Concentration required**	0,5 M
	Volume prepared	50,0 mL
	Mass	1,01 g
	Volume needed	10,0 mL
Sodium borihydride	**Mass required**	0,202 g
Glycidyltrimethyl-ammonium chloride	**Mass required**	6,0 g (d = 1,13) / 2g
	Volume measured	26,5 mL

After washing, the sample was stored, at 4ºC, in a solution of water with 20% ethanol. Other gel samples were prepared with the same procedure as the first one; the amounts used are at annex D.

6.4. MODIFICATION OF CL-6B-SEPHAROSE TO ANION-EXCHANGE ADSORPTION

This matrix was prepared from commercial CL-6B Sepharose (Sepharose™ CL-6B apbiotech, lot 287614).

A pre-wash step was performed to remove ethanol from samples (centrifuge method).

The protocol used for this step is the same used for cellulose (step 6.3.). The only thing that differs is the amounts (table 21).

TABLE 21 SAMPLES AND REAGENTS USED IN THE MODIFICATION

Sample	**Identification**	AE-Sep-I
	Primary sample	Commercial
	Mass	20,004 g
Sodium hydroxide	**Concentration required**	0,5 M
	Volume prepared	50,0 mL
	Mass	1,01 g
	Volume needed	20,0 mL
Sodium borihydride	**Mass required**	0,402 g
Glycidyltrimethyl-ammonium chloride	**Mass required**	6,0 g (d = 1,13) / 2g
	Volume measured	53,1 mL

After washing, the sample was stored, at 4ºC, in a solution of water with 20% ethanol. Other gel samples were prepared with the same procedure as the first one; the amounts used are at annex D too.

6.5. MODIFICATION OF PVA NANOFIBERS TO ANION-EXCHANGE ADSORPTION

The first step was to immerse the poly(vinyl) alcohol nanofibers in methanol, for 3 days, to dissolve and form a relatively homogeneous solution (like gel), but that did not happened. So a wash step was performed, with abundant water, to remove the methanol.

The protocol used for the modification is similar to the one used for cellulose (step 6.3.), differing the amounts (table 22).

During the 3 days suspension (under rotation), at 25ºC, nanofibers packed and took a very rigid and inflexible consistency, even after the wash step.

The sample was stored, at 4ºC, in a solution of water with 20% ethanol.

This was the only performance of PVA nanofibers modification.

TABLE 22 SAMPLES AND REAGENTS USED IN THE MODIFICATION

Sample	**Identification**	PVA
	Primary sample	PVA nanofibers electrospun
	Mass	1,52 g
Sodium hydroxide	**Concentration required**	0,5 M
	Volume prepared	50,0 mL
	Mass	1,01 g
	Volume needed	1,52 mL
Sodium borihydride	**Mass required**	0,0304 g
Glycidyltrimethyl-ammonium chloride	**Mass required**	6,0 g (d = 1,13) / 2g
	Volume measured	4,035 mL

6.6. SCANNING ELECTRON MICROSCOPY

A scanning electron microscope (SEM) operates in such a way that an electron beam having a fine spot, by being finely focused, is used to scan the surface of a specimen in its longitudinal and lateral directions and reflected electrons, or secondary electrons emitted from points of the specimen bombarded by the primary electron beam, are detected in the form of signals, which signals are then supplied to a cathode-ray tube, which is scanned in synchronized relation with the above scanning by the primary electron beam, so as to modulate brightness of the cathode-ray tube and to thereby observe the surface image of the specimen on the fluorescent screen (Koganei *et al*, 1969).

The analysis with SEM is made to realize the microfibers and nanofibers size and identified the atoms on the matrices surface, to find out if modification was succeed and the degree of substitution.

A minimum amount of samples were placed in lamellas and dried, to remove all humidity, then were coated with gold (deposited by vacuum), to protect the sample of static electric charge during electron irradiation.

6.6.1. IMAGES

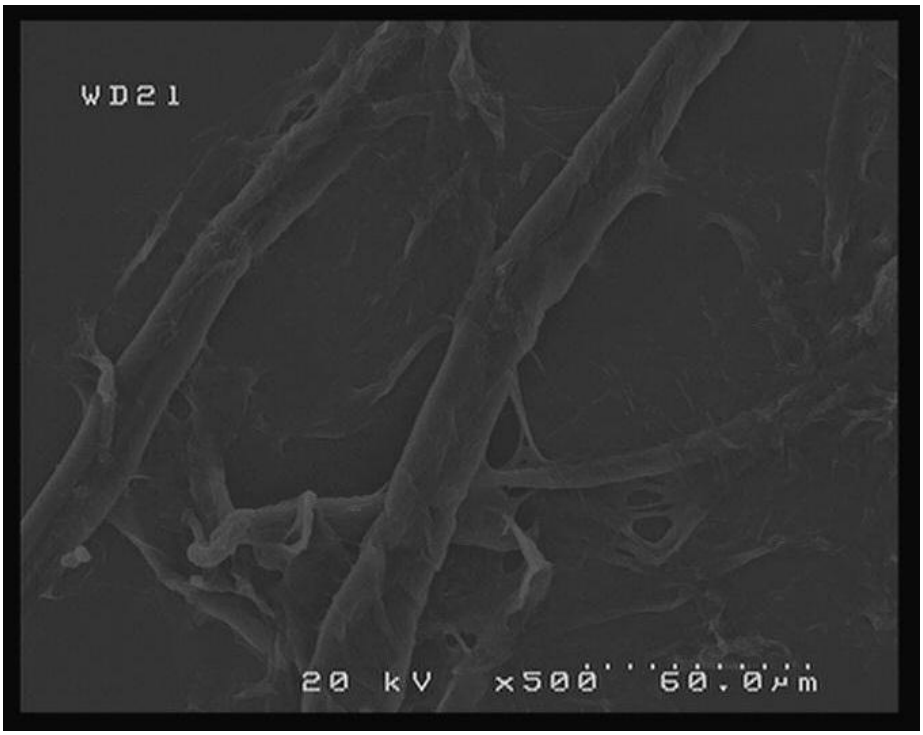


FIGURE 12 NOT MODIFIED CELLULOSE FIBERS (SAMPLE B4) MAGNIFICATION X500

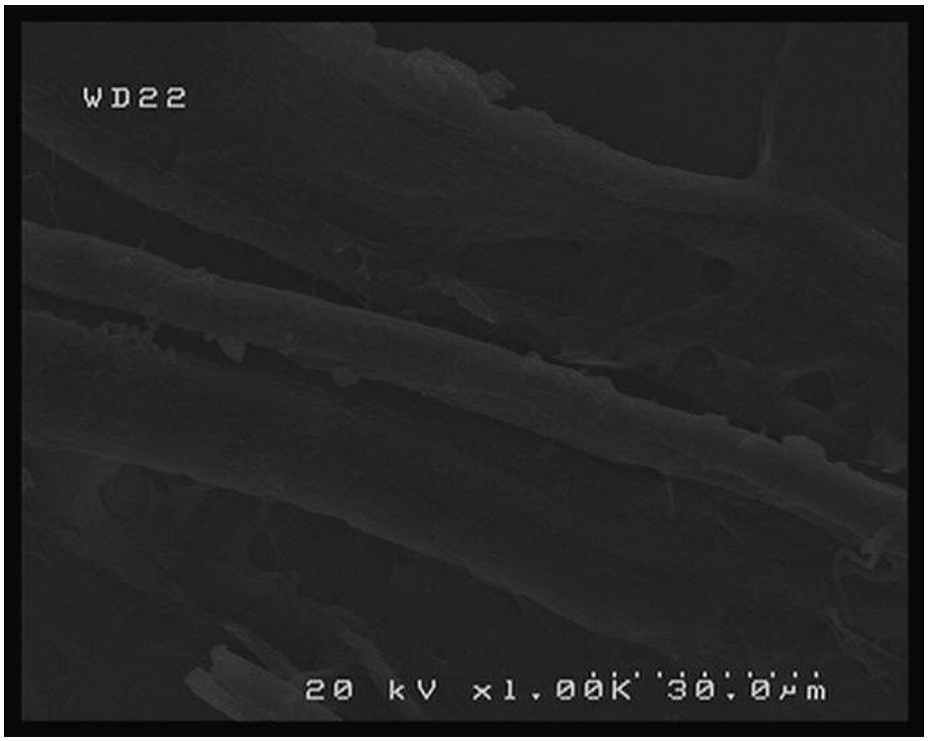


FIGURE 13 NOT MODIFIED CELLULOSE FIBERS (SAMPLE B1) MAGNIFICATION X1000

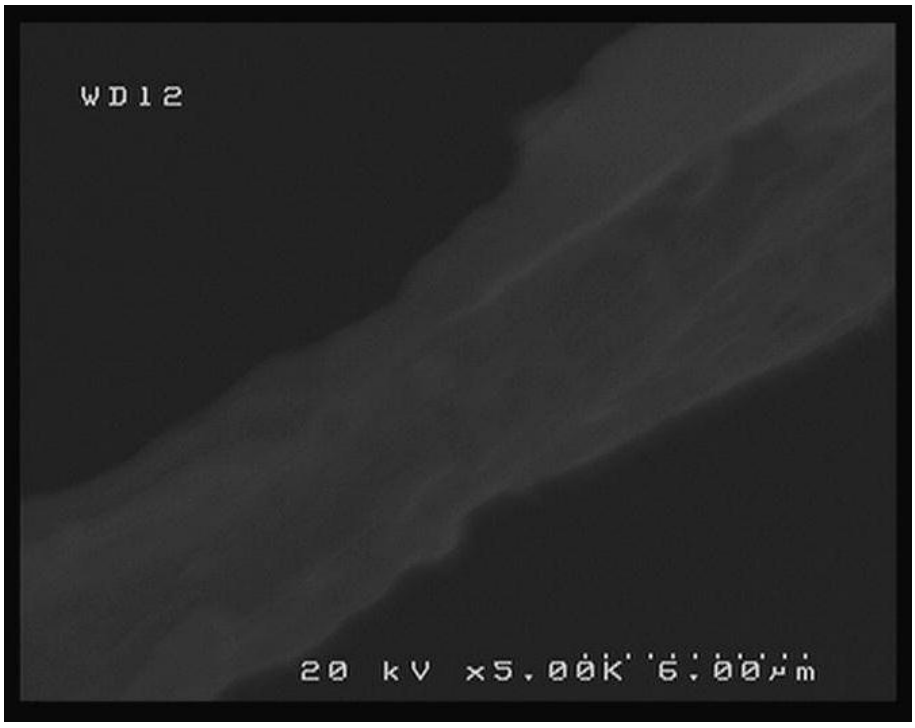


FIGURE 14 NOT MODIFIED CELLULOSE FIBERS (SAMPLE B1) MAGNIFICATION X5000

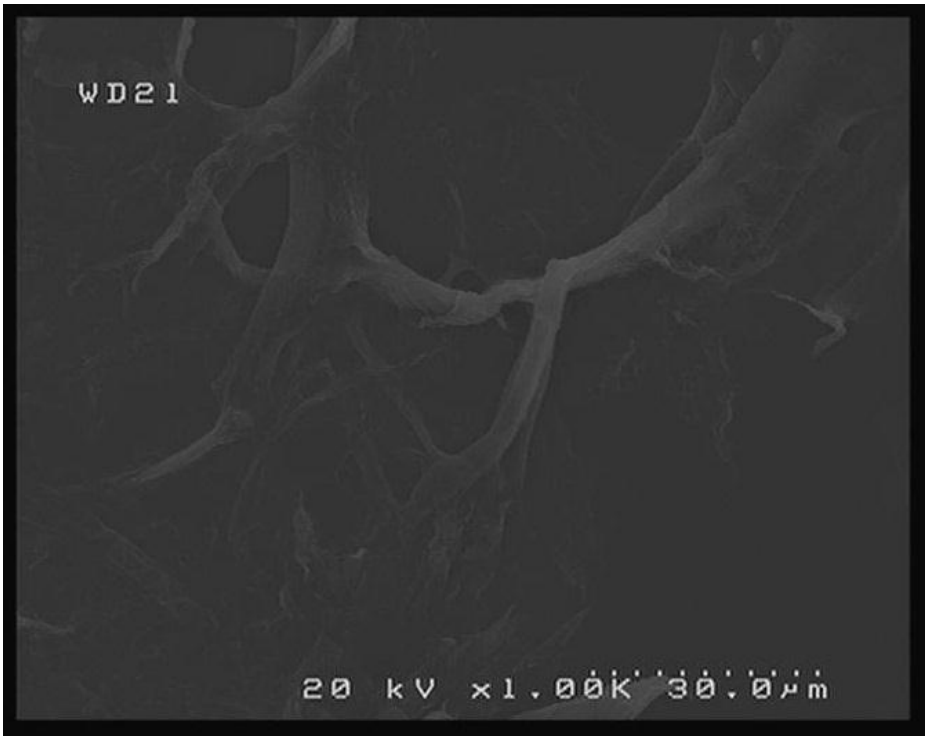


FIGURE 15 EPOXY-CELLULOSE MICROFIBERS FOR HYDROPHOBIC INTERACTION (SAMPLE (HI)II) MAGNIFICATION X1000

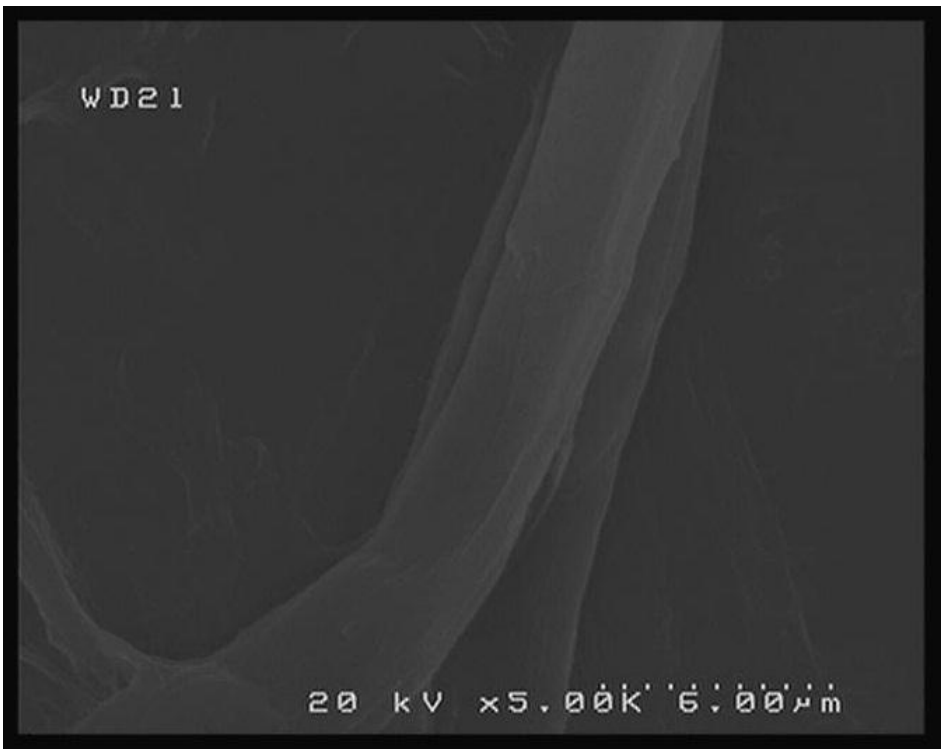


FIGURE 16 EPOXY-CELLULOSE MICROFIBERS FOR HYDROPHOBIC INTERACTION (SAMPLE (HI)II) MAGNIFICATION X5000

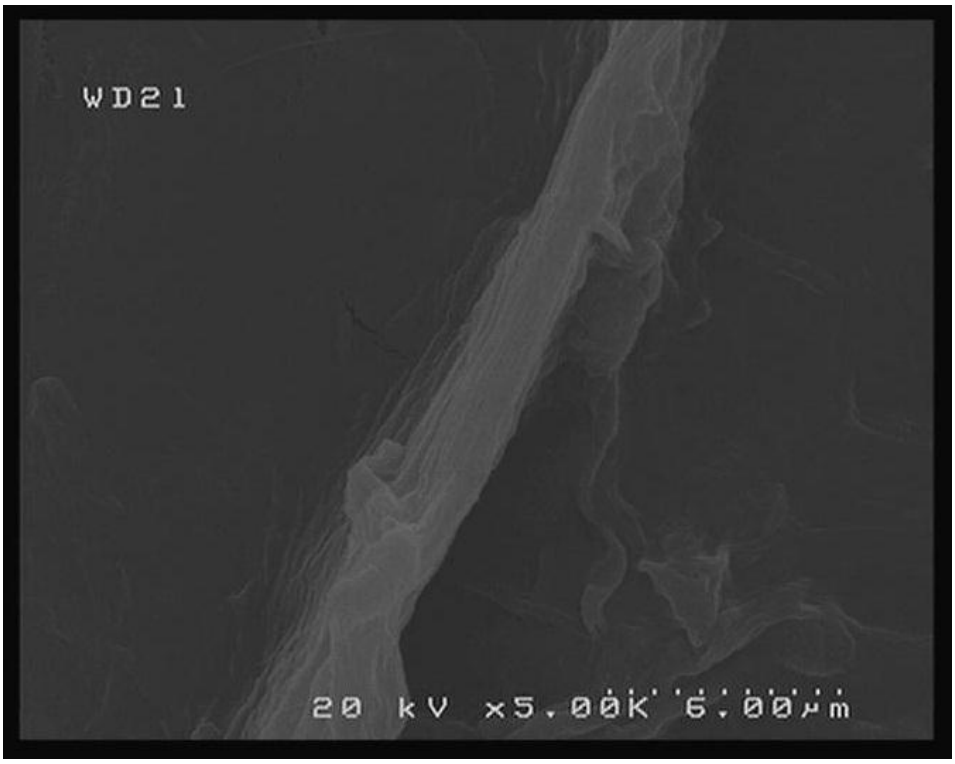


FIGURE 17 EPOXY-CELLULOSE MICROFIBERS FOR HYDROPHOBIC INTERACTION (SAMPLE (HI)I) MAGNIFICATION X5000

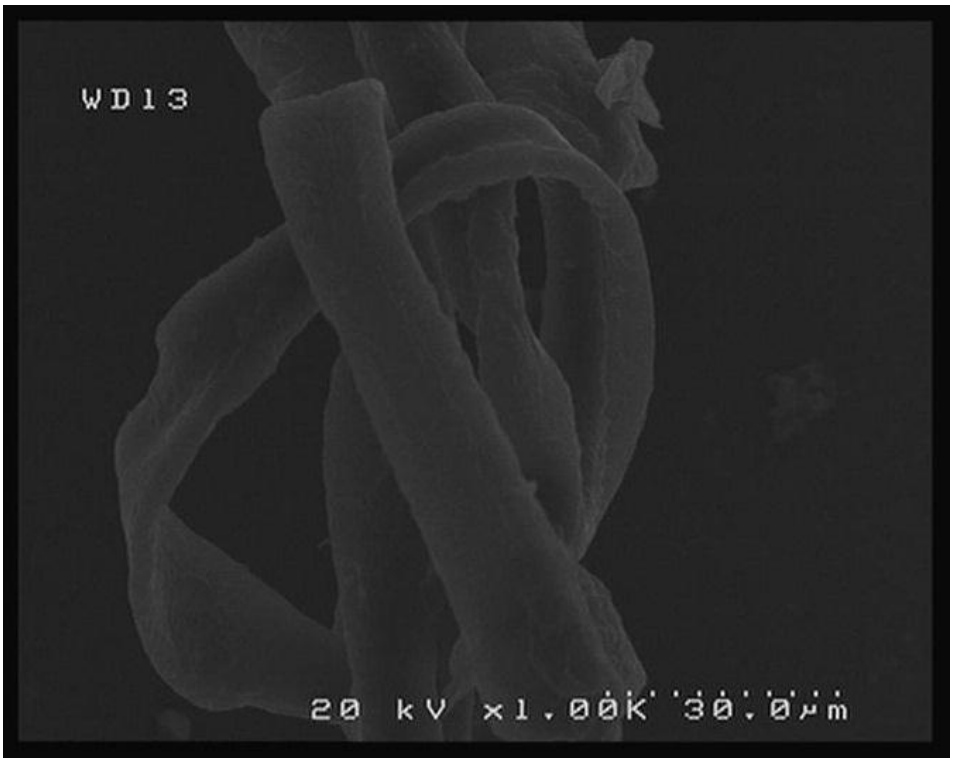


FIGURE 18 CATIONIC-CELLULOSE MICROFIBERS FOR ANION-EXCHANGE (SAMPLE AE-CEL-I) MAGNIFICATION X1000

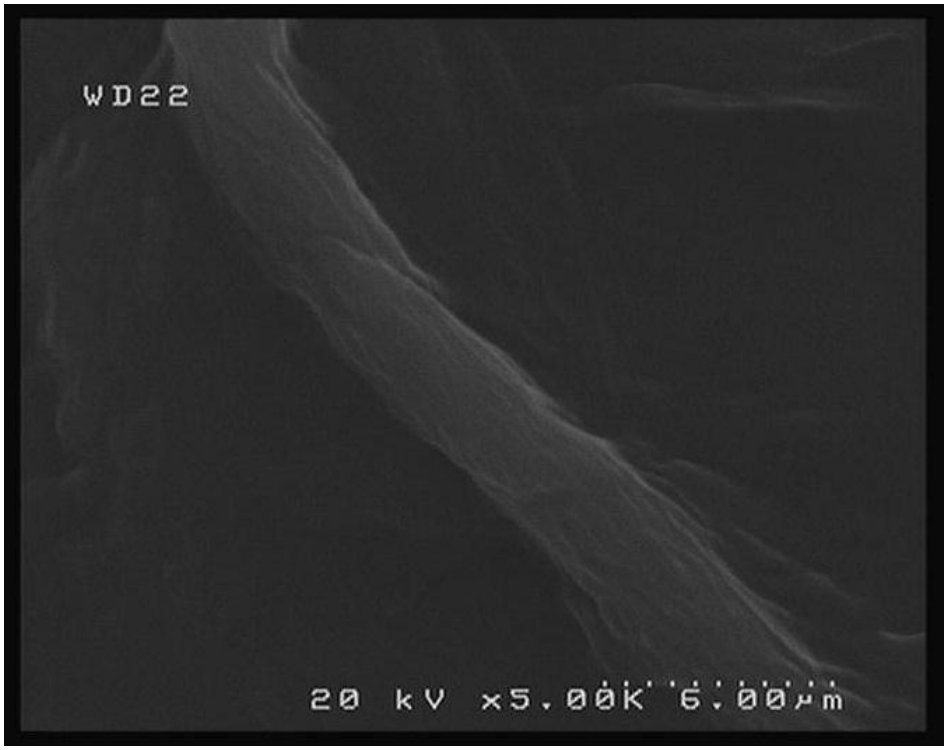


FIGURE 19 CATIONIC-CELLULOSE MICROFIBERS FOR ANION-EXCHANGE (SAMPLE AE-CEL-I) MAGNIFICATION X5000

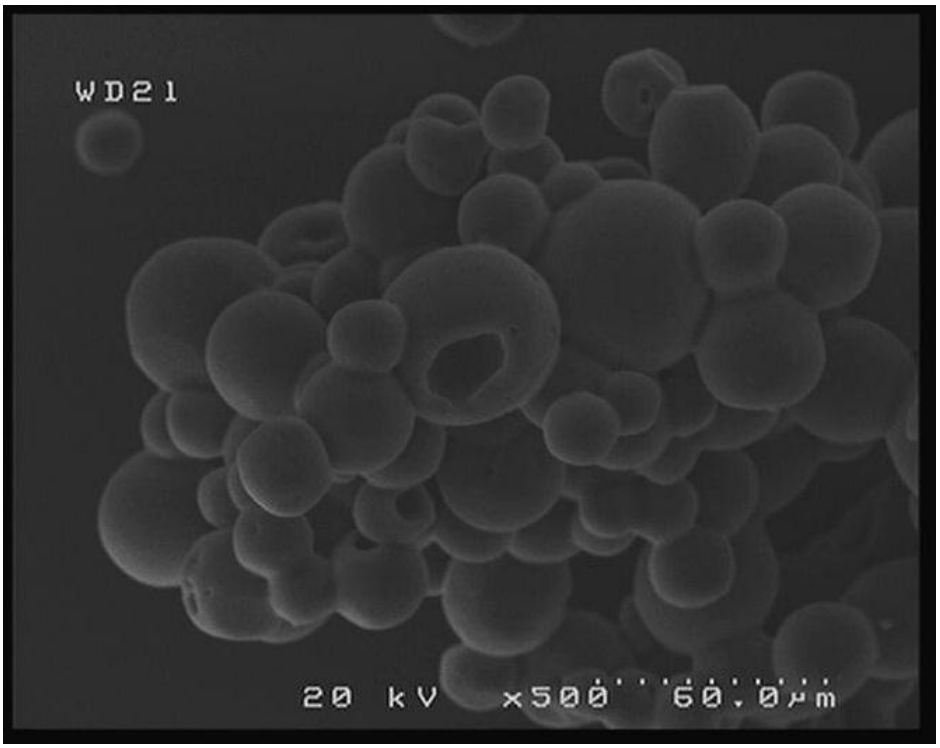


FIGURE 20 CL-6B-SEPHAROSE BEFORE MODIFICATION, MAGNIFICATION X500

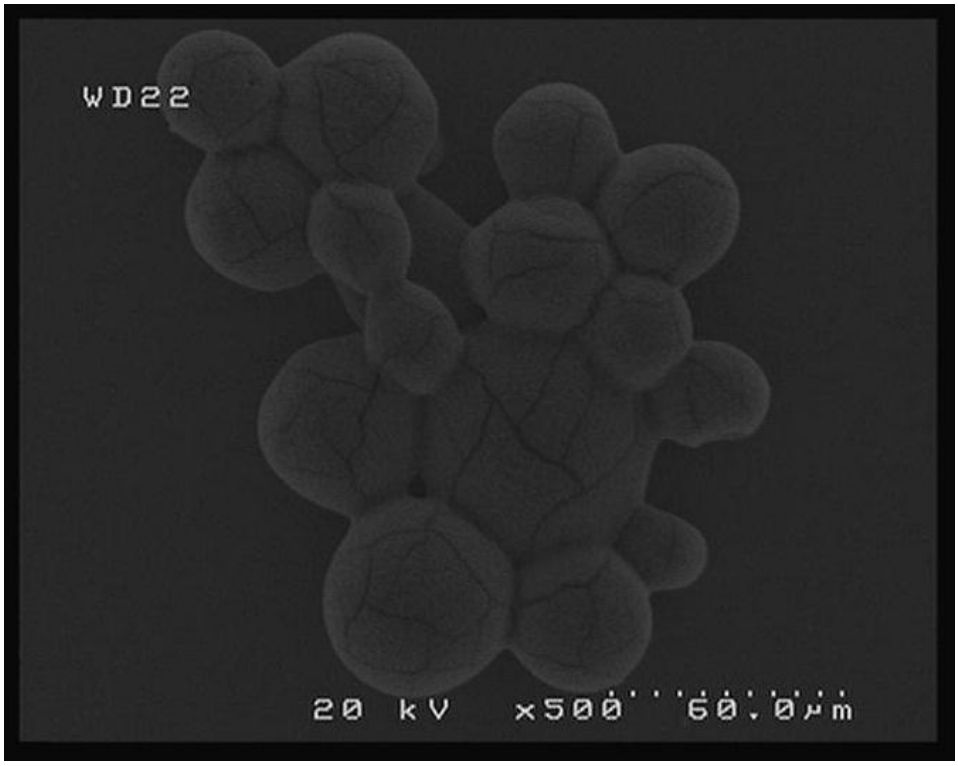


FIGURE 21 Q-SEPHAROSE MODIFIED BY US FOR ANION-EXCHANGE, MAGNIFICATION X500

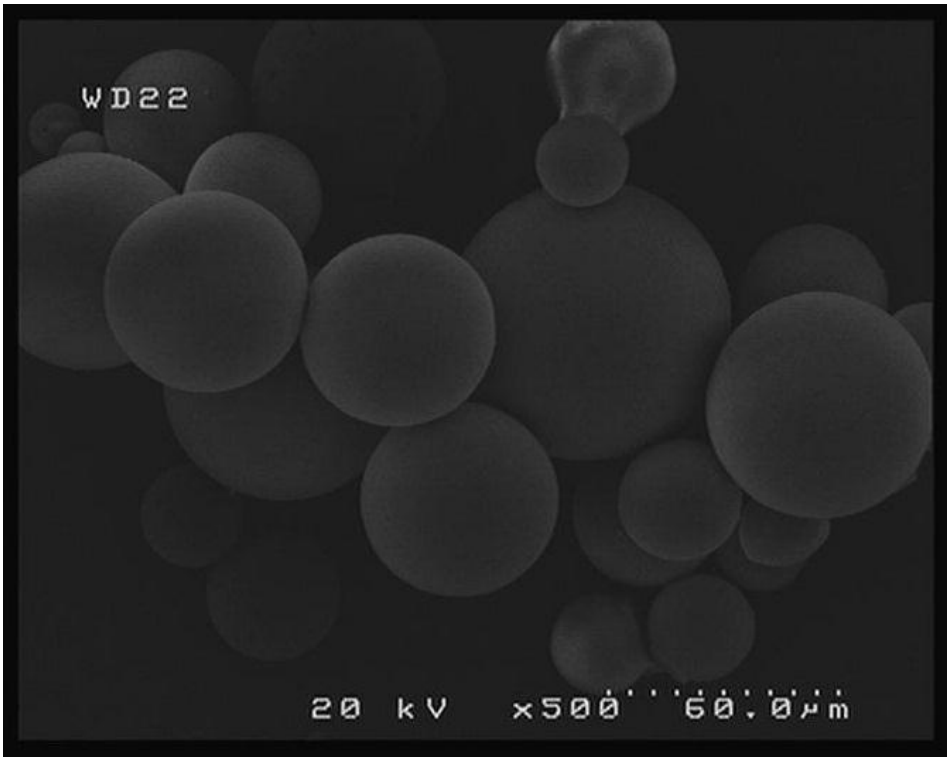


FIGURE 22 COMMERCIAL Q-SEPHAROSE FOR ANION-EXCHANGE, MAGNIFICATION X500

6.6.2. ELEMENTAL ANALYSIS

TABLE 23 ELEMENTAL ANALYSIS OF CELLULOSE MICROFIBERS MODIFIED FOR HYDROPHOBIC INTERACTION

Element	Microfibers before modification	Microfibers after modification	Another sample of microfibers modified
	Sample B1	Sample (HI)II	Sample (HI)I
C	79,3	51,68	66,91
O	16,52	30,58	22,41
Al	-	0,85	-
Si	1,38	11,59	6,66
Ag	0,32	0,34	0,40
K	0,59	1,72	1,07
Ca	-	-	-
Au	1,39	1,14	1,41
N	-	-	-
Na	-	0,85	0,30
Cl	0,36	-	-
Ti	-	0,65	0,41
Zn	-	0,61	0,43

TABLE 24 ELEMENTAL ANALYSIS OF CELLULOSE MICROFIBERS MODIFIED FOR ANION-EXCHANGE

Element	Microfibers before modification	Microfibers after modification
	Sample B4	Sample AE-Cel-I
C	74,12	61,21
O	23,39	20,56
Al	-	1,03
Si	0,45	3,38
Ag	0,40	0,40
K	-	0,75
Ca	-	0,71
Au	1,65	1,42
N*	-	9,72
Na	-	-
Cl	-	0,49
Ti	-	-
Zn	-	0,32

*with large margin of error

TABLE 25 ELEMENTAL ANALYSIS OF SEPHAROSE MODIFIED FOR ANION-EXCHANGE

Element	Sepharose before modification	Sepharose after modification	Commercial Sepharose
	Sample CL-6B-Sepharose	Sample Q-Sepharose (our)	Sample Q-Sepharose commercial
C	75,74	59,38	55,98
O	23,15	18,44	28,46
Al	-	-	3,51
Si	0,47	0,27	-
Ag	-	-	0,29
K	-	-	-
Ca	-	-	-
Au	0,65	1,10	0,86
N*	-	11,31	10,48
Na	-	0,29	-
Cl	-	8,96	-
Ti	-	-	-
Zn	-	0,24	-

*with large margin of error

TABLE 26 ELEMENTAL ANALYSIS OF PVA FIBERS MODIFIED FOR ANION-EXCHANGE

Element	Fibers after modification
	Sample PVA
C	50,16
O	43,03
Al	-
Si	0,20
Ag	0,45
K	-
Ca	1,33
Au	4,83
N	-
Na	-
Cl	-
Ti	-
Zn	-

CHAPTER 7. PLASMID DNA ADSORPTION TRIALS

7.1. HYDROPHOBIC INTERACTION WITH EPOXY-CELLULOSE MATRIX

To study hydrophobic interaction between pDNA and the epoxy-cellulose gel was used a high concentration of ammonium sulfate and the reaction lasts 24 hours (steps 7.1.2. and 7.1.3.). Just to verify that these conditions are good choices, others conditions experiments were performed (step 7.1.1.).

As said before, after activation, gel was stored in a solution of water with 20% ethanol, at 4ºC. So, before execute adsorptions assessments, a pre-wash step was performed: samples were washed with water and centrifuged (1min, 4600rpm), 5 rounds each.

7.1.1. OPTIMIZATION AND CONTROL EXPERIMENTS

First of all, it was made a study to find out the best reaction time and to verify the salt concentration influence.

The reaction takes place in eppendorfs with modified gel (about 50mg) and 200 µL of pDNA or control (only buffer with or without salt) solution. Those eppendorfs were kept in a 25ºC bath, with rotation, and an absorbency reading was made, after 8, 12 and 24 hours.

The data presented in table 27 are already a summary of the results obtained, results not significant were eliminated and those are means.

TABLE 27 CONTROL EXPERIMENT

Constitution / Time / Absorbency					Description
Gel	✓	✓	✓	✓	Cellulose microfibers
Buffer	✓	✓	✓	✓	Tris-Cl 10 mM (pH = 8.0)
Salt		✓		✓	Ammonium sulfate 3 M
pDNA			✓	✓	0, 150 mg / mL
8 hours	0,006	0,062	0,124	0,023	The presence of salt is determinant for HI. Most of the interaction happens in the first hours, but still occurs in 24hours.
12 hours	0,012	0,037	0,115	0,018	
24 hours	0,010	0,032	0,126	0,014	

7.1.2. EPOXY-CELLULOSE BATCH ADSORPTION IN AMMONIUM SULFATE 3.0 M

The reaction takes place in buffer tris-Cl 10 mM (pH = 8.0) with 3M ammonium sulfate. Reagents needed to prepare the solution are at table 28.

TABLE 28 PDNA HYDROPHOBIC INTERACTION ADSORPTION PROTOCOL REAGENTS

Reagents	**Information available**	**Concentration required**
Tris	M = 121,14 g/mol R36/37/38, S26, S37/39	10 mM
Ammonium sulfate	M = 132,14 g/mol 99% pure	3 M

Were prepared samples with different pDNA concentrations from the "stock sample" stored at -80ºC, after QIAGEN kit purification (step 5.2), and buffer tris-Cl. The samples concentrations were selected with a maximum diversity in the possible range, but the actual concentrations not equalize theoretical (table 29), because samples were prepared in a way to use maximum possible initial sample (higher concentrations), since it was so small.

The reaction takes place in eppendorfs with modified gel (about 50mg) and 200µL of pDNA solution (or control). Those eppendorfs were kept in a 25ºC bath, with rotation, and an absorbency reading was made, after 24 hours.

The first time that this experiment was performed, the theoretical concentrations were 75, 150 and 500µg/mL (two times each). Data it is describe at table 30.

TABLE 29 REAL PDNA CONCENTRATIONS

Theoretical concentration (µg/mL)	Absorbency (at 260nm)	Real concentration (µg/mL)
75	0,0785	78,5
150	0,1610	161,0
500	0,5335	533,5

TABLE 30 PDNA HYDROPHOBIC INTERACTION ADSORPTION AFTER 24 HOURS

Initial concentration (µg/mL)	Gel mass (mg)	Final concentration (µg/mL)	Adsorbed mass (µg)	Adsorption (µg pDNA/ Kg gel)
78,5	52,5	11,0	13,5	257142,86
161,0	52,0	13,5	29,5	567307,69
533,5	51,0	456,5	15,4	301960,78

Other adsorption tests valid results are summarized at table 31. Those data analysis are described later in chapter 8.

TABLE 31 PDNA HYDROPHOBIC INTERACTION ADSORPTION AFTER 24 HOURS (OTHER RESULTS)

Initial concentration (µg/mL)	Gel mass (mg)	Final concentration (µg/mL)	Adsorbed mass (µg)	Adsorption (µg pDNA/ mg gel)
33,0	48,0	12,0	4,2	0,0875
91,0	51,0	8,0	16,6	0,3255
106,0	48,0	41,0	13,0	0,2708
239,0	51,0	148,0	18,2	0,3569
239,0	52,0	155,0	16,8	0,3231
285,0	50,0	219,0	13,2	0,264
285,0	49,0	191,0	18,8	0,3837
388,0	53,0	304,0	16,8	0,3170
423,0	56,0	341,0	16,4	0,2928
423,0	51,0	353,0	14,0	0,2745
452,0	54,0	378,0	14,8	0,2741
452,0	56,0	368,0	16,8	0,3000
520,0	58,0	458,0	12,4	0,2138
520,0	54,0	475,0	9,0	0,1667

7.1.3. EPOXY-CELLULOSE BATCH ADSORPTION IN AMMONIUM SULFATE 2.0 M

This study procedure is like 7.1.2., but with a lower salt concentration. It was made to decrease the consumption of salt, but, in fact, to guarantee a good hydrophobic interaction, 2,0 M ammonium sulfate are insufficient. The maximum adsorption was 0,213 µg pDNA/ mg gel, so this test can be considered irrelevant.

7.2. ANION-EXCHANGE INTERACTION WITH CATIONIC-CELLULOSE MATRIX

The reaction takes place in buffer tris-Cl 10 mM (pH = 8.0) with 1M EDTA and 0,5 M NaCl. Reagents needed to prepare the solution are at table 32.

TABLE 32 pDNA ANION-EXCHANGE ADSORPTION PROTOCOL REAGENTS

Reagents	Information available	Concentration required
Tris	M = 121,14 g/mol R36/37/38, S26, S37/39	10 mM
EDTA	M=292,24g/mol R36, S26	1 M
Sodium chloride	M = 58, 443 g/mol	0,5 M

Were prepared samples with different pDNA concentrations from the "stock sample" stored at -80ºC, after QIAGEN kit purification (step 5.2), and buffer tris-Cl. The samples concentrations were selected with a maximum diversity in the possible range.

The reaction takes place in eppendorfs with modified gel (about 30mg) and 200µL of pDNA solution (or control). These eppendorfs were kept in a 25ºC bath, with rotation, and absorbencies readings were made after 24hours or at time (in hours) 4, 8, 20, 24, 28, 32 (two different study types).

Data of those experiments are described at tables 33 and 34 and are analyzed in chapter 8 (discussion).

TABLE 33 pDNA ANION-EXCHANGE ADSORPTION AFTER 24 HOURS (CELLULOSE)

Initial concentration (µg/mL)	Gel mass (mg)	Final concentration (µg/mL)	Adsorbed mass (µg)	Adsorption (µg pDNA/ mg gel)
33,0	42,9	23,0	2,0	0,0466
33,0	42,6	23,0	2,0	0,0469
33,0	44,2	15,0	3,6	0,0814
93,0	29,7	33,0	12,0	0,4040
93,0	27,2	22,0	14,2	0,5220
93,0	32,1	24,0	13,8	0,4299
145,0	24,5	16,0	25,8	1,0531
145,0	31,4	21,0	24,8	0,7898
145,0	36,1	17,0	25,6	0,7091
252,0	47,3	36,0	43,2	0,9133
252,0	33,5	27,0	45,0	1,3433
252,0	25,2	36,0	43,2	1,7143
479,0	33,0	104,0	75,0	2,2727
479,0	30,3	153,0	65,2	2,1518
479,0	26,6	143,0	67,2	2,5263

TABLE 34 pDNA ANION-EXCHANGE ADSORPTION UP TO 32 HOURS (CELLULOSE)

Initial concentration (µg/mL)	Gel mass (mg)	Final concentration (µg/mL)	Adsorbed mass (µg)	Adsorption (µg pDNA/ mg gel)
Time = 4 hours				
17,0	19,9	14,0	0,6	0,0302
17,0	36,7	15,0	0,4	0,0109
121,0	52,1	23,0	19,6	0,3762
121,0	29,5	28,0	18,6	0,6305
121,0	40,9	19,0	20,4	0,4988
167,0	50,5	17,0	30,0	0,5941
167,0	46,6	30,0	27,4	0,5880
Time = 8 hours				
17,0	49,1	10,0	1,36	0,0278
17,0	19,9	3,0	2,73	0,1372
121,0	52,1	34,0	16,96	0,3256
121,0	29,5	28,0	18,14	0,6147
121,0	40,9	29,0	17,94	0,4386
167,0	50,5	30,0	26,72	0,5290
167,0	46,6	26,0	27, 50	0,5900
Time = 20 hours				
17,0	49,1	13,0	0,76	0,0155
17,0	19,9	12,0	0,95	0,0477
121,0	52,1	16,0	19,95	0,3829
121,0	40,9	12,0	20,71	0,5064
167,0	50,5	18,0	28,31	0,5606
167,0	37,8	14,0	29,07	0,7690
167,0	46,6	30,0	26,03	0,5586
Time = 24 hours				
17,0	36,7	11,0	1,11	0,0302
121,0	29,5	16,0	19,42	0,6585
121,0	40,9	11,0	20,35	0,4976
167,0	50,5	17,0	27,75	0,5495
167,0	37,8	21,0	27,01	0,7146
167,0	46,6	12,0	28,68	0,6153
Time = 28 hours				
17,0	19,9	12,0	0,90	0,0452
17,0	36,7	16,0	0,18	0,0049
121,0	52,1	33,0	15,84	0,3040
121,0	29,5	23,0	17,64	0,5980
121,0	40,9	31,0	16,20	0,3961
167,0	50,5	45,0	21,96	0,4348
167,0	46,6	39,0	23,04	0,4944
Time = 32 hours				
17,0	19,9	13,0	0,70	0,0352
121,0	52,1	44,0	13,48	0,2586
121,0	29,5	38,0	14,52	0,4924
121,0	40,9	43,0	13,65	0,3337
167,0	50,5	77,0	15,75	0,3119
167,0	37,8	76,0	15,92	0,4213
167,0	46,6	49,0	20,65	0,4431

7.3. ANION-EXCHANGE INTERACTION WITH Q-SEPHAROSE MATRIX (OUR MODIFICATION)

Those studies have an identical procedure to the ones described at 7.2. (at laboratory, most of those assays were done at the same time). The results are described at tables 35 and 36 and discuss in chapter 8.

TABLE 35 PDNA ANION-EXCHANGE ADSORPTION AFTER 24 HOURS (Q-SEPHAROSE)

Initial concentration (µg/mL)	Gel mass (mg)	Final concentration (µg/mL)	Adsorbed mass (µg)	Adsorption (µg pDNA/ mg gel)
33,0	43,2	20,0	2,6	0,0602
33,0	44,2	29,0	0,8	0,0181
33,0	27,1	31,0	0,4	0,0148
93,0	36,3	25,0	13,6	0,3746
93,0	36,0	28,0	13,0	0,3611
93,0	19,8	53,0	8,0	0,4040
145,0	46,0	32,0	22,6	0,4913
145,0	51,8	47,0	19,6	0,3784
145,0	24,2	75,0	14,0	0,5785
252,0	36,5	170,0	16,4	0,4493
252,0	30,8	162,0	18,0	0,5844
252,0	48,0	152,0	20,0	0,4167
479,0	24,5	392,0	17,4	0,7102
479,0	31,7	402,0	15,4	0,4858
479,0	30,2	412,0	13,4	0,4437

TABLE 36 PDNA ANION-EXCHANGE ADSORPTION UP TO 32 HOURS (Q-SEPHAROSE)

Initial concentration (µg/mL)	Gel mass (mg)	Final concentration (µg/mL)	Adsorbed mass (µg)	Adsorption (µg pDNA/ mg gel)
Time = 4 hours				
17,0	39,4	13,0	0,8	0,0203
17,0	44,8	10,0	1,4	0,0312
121,0	49,2	61,0	12,0	0,2439
121,0	32,0	72,0	9,8	0,3062
121,0	31,0	103,0	3,6	0,1161
167,0	41,5	95,0	14,4	0,3470
167,0	40,9	105,0	12,4	0,3032
Time = 8 hours				
17,0	34,3	14,0	0,58	0,0171
17,0	39,4	13,0	0,78	0,0198
121,0	49,2	54,0	13,06	0,2655
121,0	32,0	81,0	7,80	0,2438
121,0	31,0	66,0	10,72	0,3460
167,0	41,5	64,0	20,08	0,4840
167,0	40,9	72,0	18,52	0,4529
167,0	37,4	81,0	16,77	0,4484
Time = 20 hours				
17,0	34,3	14,0	0,57	0,0166
121,0	49,2	21,0	19,00	0,3862
121,0	32,0	41,0	15,20	0,4750
121,0	31,0	36,0	16,15	0,5210
167,0	41,5	33,0	25,46	0,6135
167,0	40,9	71,0	18,24	0,4460
167,0	37,4	52,0	21,85	0,5842
Time = 24 hours				
17,0	34,3	13,0	0,74	0,0216
17,0	39,4	13,0	0,74	0,0188
121,0	49,2	23,0	18,13	0,3685
121,0	32,0	25,0	17,76	0,5550
121,0	31,0	36,0	15,72	0,5072
167,0	41,5	30,0	25,34	0,6107
167,0	40,9	40,0	23,50	0,5744
167,0	37,4	32,0	24,98	0,6678
Time = 28 hours				
17,0	34,3	11,0	1,08	0,0315
121,0	49,2	19,0	18,36	0,3732
121,0	31,0	34,0	15,66	0,5052
167,0	41,5	20,0	26,46	0,6360
167,0	40,9	33,0	24,12	0,5897
167,0	37,4	25,0	25,56	0,6834
Time = 32 hours				
17,0	34,3	11,0	1,05	0,0306
121,0	49,2	45,0	13,30	0,2703
121,0	32,0	74,0	8,22	0,2570
167,0	41,5	54,0	19,78	0,4765
167,0	37,4	62,0	18,38	0,4913

7.4. ANION-EXCHANGE INTERACTION WITH Q-SEPHAROSE MATRIX (COMMERCIAL)

The buffer used in these interaction is also tris-Cl 10 mM (pH = 8.0) with 1M EDTA and 0,5 M NaCl.

Were prepared samples with different pDNA concentrations from the "stock sample", stored at -80ºC after QIAGEN kit purification (step 5.2), and buffer tris-Cl. The reaction takes place in eppendorfs with commercial gel (about 30mg) and 200µL of pDNA solution (or control). Those eppendorfs were kept in a 25ºC bath, with rotation, and absorbencies readings were made after 24hours.

Data of those experiments are described at table 37 and are analyzed in chapter 8 (discussion).

TABLE 37 PDNA ANION-EXCHANGE ADSORPTION AFTER 24 HOURS (COMMERCIAL Q-SEPHAROSE)

Initial concentration (µg/mL)	Gel mass (mg)	Final concentration (µg/mL)	Adsorbed mass (µg)	Adsorption (µg pDNA/ mg gel)
66,0	51,0	32,0	6,8	0,1333
66,0	53,0	24,0	8,4	0,1585
114,0	53,0	66,0	9,6	0,1811
114,0	53,0	61,0	10,6	0,2000
140,0	51,0	79,0	12,2	0,2392
140,0	48,0	83,0	11,4	0,2375
172,0	50,0	103,0	13,8	0,2760
172,0	51,0	109,0	12,6	0,2471
222,0	60,0	121,0	20,2	0,3367
404,0	56,0	245,0	31,8	0,5678
404,0	57,0	254,0	30,0	0,5263

CHAPTER 8. DISCUSSION

8.1. MATRICES MODIFICATION

The first step was an ultrasonic treatment of cellulose fibers to guarantee a range of micrometers (or nanometers, if possible). Through SEM analysis were detected microfibers with a diameter from 6 to 30 micrometers (figures 12-19).

Then, was performed a modification of the cellulose microfibers surface to hydrophobic interaction. The best degree of ligand substitution detected by titration was 76,733 μmol/g (annexes table V). By elemental analysis, it is possible to calculate degree of substitution (DS) through the following equation:

$$\%C_{gel\ derivatized} = \frac{\%C_{gel\ not\ modified}}{1 + DS} + \frac{\%C_{ligand}}{1 + DS^{-1}} \quad [4]$$

Was not possible to calculate the DS for cellulose sample modified to hydrophobic interaction (comparing samples B1 with (HI)II) because of the Si contamination (11,59% detected), results in a negative value, so it is disregarded. Doing the calculation with samples B1 and (HI)I, results in a DS of 1.6, which is a good result, assuming that the primary sample of (HI)I is similar to the primary sample of (HI)II. About the Si contamination, it comes from cover glass (possibly been scraped during sample preparation) (table 23).

The same happens for the samples modified to anion-exchange adsorption, both cellulose and sepharose gels, because of Si and Cl contaminations. Cl contamination comes from a poor wash of the sample, after modification. But, besides the large margin of error, it is considered that the modification was succeeding due to detection of atom N (tables 24 and 25).

As said before, during the modification procedure, PVA nanofibers packed and took a very rigid and inflexible consistency, even after the wash step. Was not possible

[4] *DS* = degree of substitution
$\%C_{gel\ derivatized}$ = percentage of Carbon in the gel sample after modification
$\%C_{gel\ derivatized}$ = percentage of Carbon in the gel sample before modification
$\%C_{gel\ derivatized}$ = percentage of Carbon in the ligand

to analyze the structure in SEM, because is in a nanometer range, and elemental analysis revealed that the degree of ligand substitution was very low or inexistent (N atom was not detected, table 26). So, PVA nanofibers matrix was not used in pDNA adsorption tests.

8.2. pDNA ADSORPTION

Through hydrophobic interaction was possible to adsorb up to 0,4 μg of pDNA per mg of gel, with ammonium sulfate 3,0 M (figure 23). As said before, an enormous concentration of salt was needed, so the hydrophobic interaction may be due the amount of salt. When this concentration was reduced (to 2,0 M), pDNA adsorption was even lower.

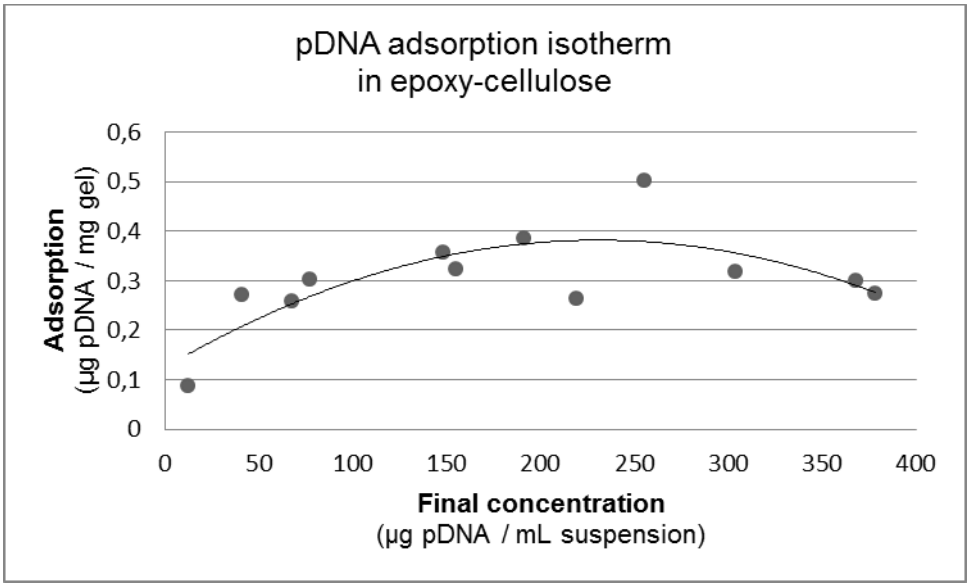


FIGURE 23 HYDROPHOBIC INTERACTION EPOXY-CELLULOSE BATCH: ADSORPTION ISOTHERM (AFTER 24HOURS)

Through anion-exchange batch, the results were much better as expected.

Adsorption over time experiments were performed with modified cellulose and sepharose gels to study how pDNA is adsorbed and to identify the best reaction time. Those experiments were made only with low pDNA initial concentration, because was not possible to purified higher pDNA quantities (at this time of master's research, was no longer possible to produce high concentrations and the factor was not identify). For the concentrations analyzed, the best reaction time recognize was 24 hours (figures 24 and 25).

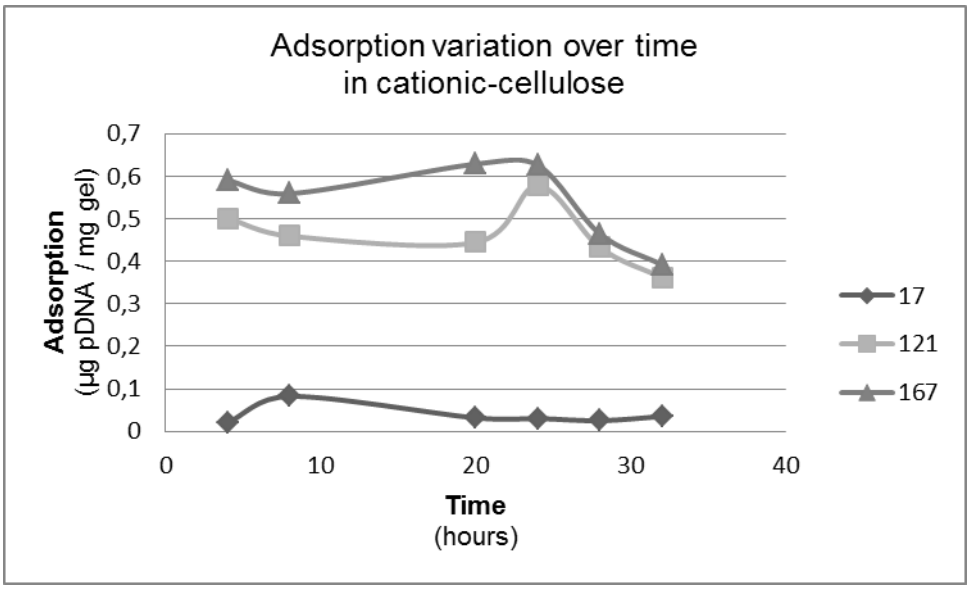


FIGURE 24 ANION-EXCHANGE CELLULOSE BATCH: ADSORPTION OVER TIME (FOR PDNA INITIAL CONCENTRATION OF 17, 121 AND 167 µg/mL)

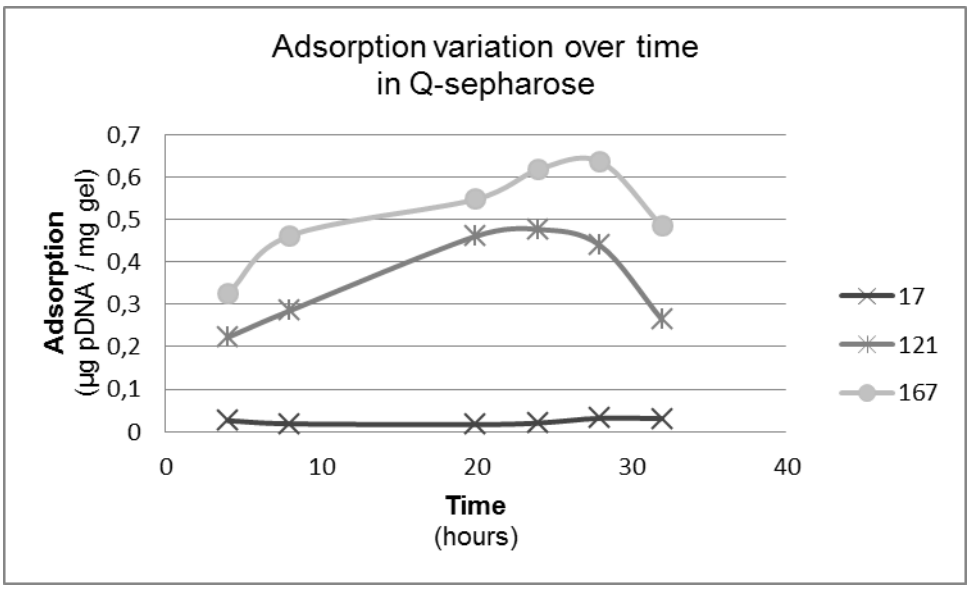


FIGURE 25 ANION-EXCHANGE Q-SEPHAROSE BATCH: ADSORPTION OVER TIME (FOR PDNA INITIAL CONCENTRATION OF 17, 121 AND 167 µg/mL)

In both cellulose and sepharose tests, is visible a decrease of adsorption after 25 hours of reaction, the possible explanations for this are: a full binding capacity of gel and starts to loose adsorption capacity, a destruction of gel because of the time exposure to buffer or a modification of pDNA structure that induces inaccuracy of the results.

In the 24 hours experiment with anion-exchange matrices, it was possible to use a bigger range of pDNA concentration, from 33,0 to 479,0 μg/mL. In cellulose based matrix, the adorption was up to 2,5 μg of pDNA per mg of gel, and there is a possibility that this adsorption can be higher with an enhanced of pDNA concentration (figure 26). Comparing with sepharose based-matrix (figure 27), cellulose microfibers are a better support, due to surface area expose to ligands.

A commercial anion-exchange support was tested too to compare with the matrices modified by us. By the same conditions, anion-exchange commercial Q-sepharose and Q-sepharose modified have a similar capacity adsorptive, although is possible that commercial Q-sepharose have a slightly higher capacity, but it was not possible do try with higher pDNA initial concentrations (figure 28).

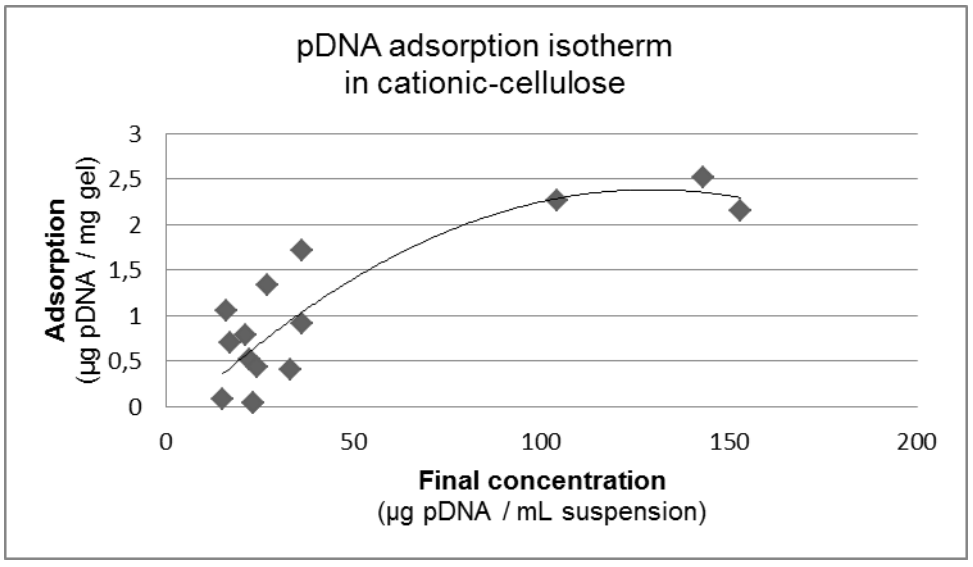


FIGURE 26 ANION-EXCHANGE CELLULOSE BATCH: ADSORPTION ISOTHERM (AFTER 24HOURS)

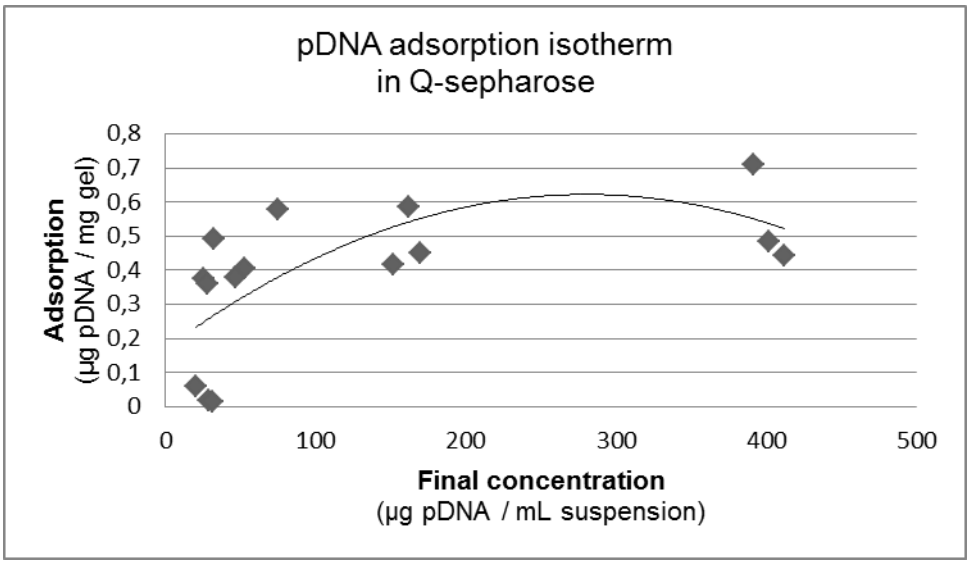


FIGURE 27 ANION-EXCHANGE Q-SEPHAROSE BATCH: ADSORPTION ISOTHERM (AFTER 24 HOURS)

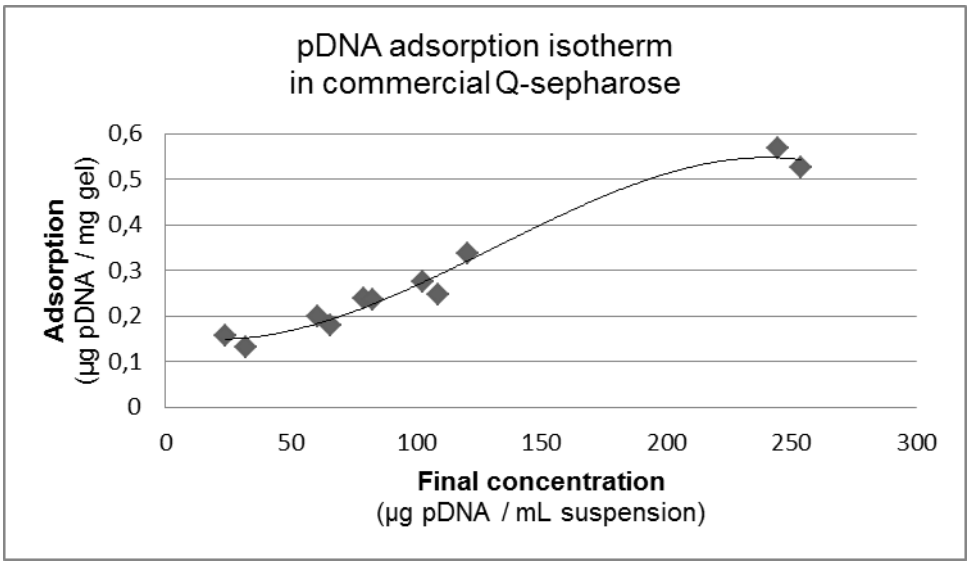


FIGURE 28 ANION-EXCHANGE COMMERCIAL Q-SEPHAROSE BATCH: ADSORPTION ISOTHERM (AFTER 24 HOURS)

CONCLUSIONS

Through hydrophobic interaction, the adsorption was up to 0,4 μg of pDNA per mg of gel, a low gel capacity and probably due the amount of salt used to perform this experiment (ammonium sulfate 3,0 M), because with a reduction of salt concentration, pDNA adsorption was even lower.

Through anion-exchange batch, the adorption was up to 2,5 μg of pDNA per mg of gel, and there is a possibility that this adsorption can be higher with an enhanced of pDNA concentration and/or a decrease of gel mass. Comparing with sepharose based-matrix (adorption was up to 0,7 μg of pDNA per mg of gel), cellulose microfibers are a better support, due to surface area expose to ligands.

The surface modification to anion-exchange support method used in the research is not the ideal to PVA nanofibers, since this modification was not succeed and PVA nanofibers structure was altered irreversibly.

Factors that can be improved in these alternative matrices to pDNA purification experiments are: an increase of pDNA initial concentrations, a decrease of gel mass and, if possible, an increase of matrix surface area, i.e. a decrease of particle/fibers size.

So, in the future could be made some improvements, specifically, changes, like size decrease, in the structure of cellulose fibers, if it demonstrate certain adsorption efficacy in micro range, sure that the results are better in nano range.

REFERENCES

Anderson W F, *Human gene-therapy*, Science 256 (**1992**) 808-813.

Anderson W F, *Human gene therapy*, Nature 392 supp (**1998**) 25-30.

Aniulyte J, Liesiene J, Niemeyer B, *Evaluation of cellulose-based biospecific adsorbents as a stationary phase for lectin affinity chromatography*, Journal of Chromatography B 831 (**2006**) 24-30.

Arakawa T and Timasheff S N, *Preferential interactions of proteins with salts in concentrated solutions*, Biochemistry 21 (**1982**) 6545–6552.

Atkinson B and Mavituna F. *In:* Biochemical Engineering and Biotechnology Handbook. MacMillan, New York (**1991**).

Bergshoef M M and Vancso G J, *Transparent nanocomposites with ultrathin, electrospun nylon-4,6 fiber reinforcement*, Advanced Materials 11 (**1999**) 1362-1365.

Boland E D, Wnek G E, Simpson D G, Palowski K J, *Bowlin G L*, *Tailoring tissue engineering scaffolds using electrostatic processing techniques: a study of poly(glycolic acid) electrospinning*. Journal of Macromolecules Science, Part A: Pure and Applied Chemistry 38 (**2001**), 1231–1243.

Bornat A, *Production of electrostatically spun products*, US Patent 4689186. **1987**.

Burger C, Hsiao B S, Chu B, *Nanofibers materials and their applications*, Annual Review of Materials Research 36 (**2006**) 333-368.

Cashion P, Sathe G, Javed A, Kuster J, *Hydrophobic affinity-chromatography of nuncleic-acids and proteins*, Nucleic Acids Research 8 (**1980**) 1167-1185.

Casper C L, Stephens J S, Tassi N G, Chase D B, Rabolt J F, *Controlling surface morphology of electrospun polystyrene fibers: Effect of humidity and molecular weight in the electrospinning process*, Macromolecules 37 (**2004**) 573-578.

Chakrabarti A, Sitaric S, Ohi S, *A procedure for large-scale plasmid isolation without using ultracentrifugation*, Biotechnology and Applied Biochemistry 16 (**1992**), 211–215.

Chandra G, Patel P, Kost T A, Gray J, *Large-scale purification of plasmid DNA by fast protein liquid-chromatography using a hi-load q-sepharose column*, Analytical Biochemistry 203 (**1992**) 169-172.

Chang V W-H, Wu R, Ho Y-S, *Recycling of Anion-Exchange Resins for Plasmid DNA Purification*, BioTechniques 26 (**1999**) 1056.

Chen L, Bromberg L, Hatton T A, Rutledge G C, *Catalytic hydrolysis of p-nitrophenyl acetate by electrospun polyacrylamidoxime nanofibers*, Polymer 48 (**2007**) 4675-4682.

Choi S W, Jo S M, Lee W S, Kim Y R, *An electrospun poly(vinylidene fluoride) nanofibrous membrane and its battery applications*, Advanced Materials 15 (**2003**) 2027-2032.

Ciccolini L A, Shamlou P A, Titchener-Hooker N J, Ward J M, Dunnill P, *Time course of SDS–alkaline lysis of recombinant bacterial cells for plasmid release*, Biotechnology and Bioengineering 60 (**1998**) 768–770.

Colote S, Ferraz C, Liautard J P, *Analysis and purification of plasmid DNA by reversed-phase high-performance liquid chromatography*, Analytical Biochemistry 154 (**1986**) 15–20.

Colpan M and Riesner D, *High-performance liquid-chromatography of high molecular-weight nucleic-acids on the macroporous ion-exchanger, nucleogen*, Journal of Chromatography 296 (**1984**) 339-353.

Crystal RG, *The gene as the drug*, Nature Medicine 1 (**1995**) 15-17.

Cuculo JA, Aminuddin N, Frey MW. *In*: Salem DR, editor. Structure formation in polymeric fiber. Munich: Hanser Gardner Publications, Inc.; **2001**, chapter 8: p. 296-328.

Cui W G, Zhou S B, Li X H, Weng J, *Drug-loaded biodegradable polymeric nanofibers prepared by electrospinning*, Tissue Engineering 12 (**2006**) 1070-1107.

Darby R A J and Hine A V, *LacI-mediated sequence-specific affinity purification of plasmid DNA for therapeutic applications*, FASEB Journal 19 (**2005**) 801.

Deitzel J M, Kleinmeyer J, Harris D, Tan N C B. *The effect of processing variables on the morphology of electrospun nanofibers and textiles*, Polymer 42 (**2001**)^{2} 261 –272.

Deitzel J M, Kleinmeyer J D, Hirvonen J K, Tan N C B, *Controlled deposition of electrospun poly(ethylene oxide) fibers*, Polymer 42 (**2001**) 8163-8170.

DeMerlis C C and Schoneker D R, *Review of the oral toxicity of polyvinyl alcohol (PVA)*, Food and Chemical Toxicology 41 (**2003**) 319-326.

Deshmukh N R and Lali A M, *Adsorptive purification of pDNA on superporous rigid cross-linked cellulose matrix*, Journal of Chromatography B 818 (**2005**) 5-10.

Diogo M M, Queiroz J A, Monteiro G A, Prazeres D M F, *Separation and analysis of plasmid denatured forms using hydrophobic interaction chromatography*, Analytical Biochemistry 275 (**1999**) 122-124.

Diogo M M, Queiroz J A, Monteiro G A, Martins S A M, Ferreira G N M, Prazeres D M F, *Purification of a cystic fibrosis plasmid vector for gene therapy using hydrophobic interaction chromatography*, Biotechnology and Bioengineering 68 (**2000**) 576–583.

Diogo M M, Queiroz J A, Prazeres D M F, *Chromatography of plasmid DNA*, Journal of Chromatography A 1069 (**2005**) 3–22.

Diogo M M, Queiroz J A, Prazeres D M F, Hydrophobic *interaction chromatography of homo-oligonucleotides on derivatized Sepharose CL-6B: application of the solvophobic theory*, Journal of Chromatography A 944 (**2002**) 119–128.

Diogo M M, Queiroz J A, Prazeres D M F, *Studies on the retention of plasmid DNA and Escherichia coli nucleic acids by hydrophobic interaction chromatography*, Bioseparation 10 (**2001**)^{2} 211-220.

Diogo M M, Ribeiro S C, Queiroz J A, Monteiro G A, Tordo N, Perrin P, Prazeres D M F, *Production, purification and analysis of an experimental DNA vaccine against rabies*, Journal of Gene Medicine 3 (**2001**) 577–584.

Eon-Duval A and Burke G, *Purification of pharmaceutical-grade plasmid DNA by anion-exchange chromatography in an RNase-free process*, Journal of Chromatography B 804 (**2004**) 327-335.

FDA (**2003**) Talk paper: FDA places temporary halt on gene therapy trials using retroviral vectors in blood stem cells. Jan 14, T03-04.

Ferreira G N M, Cabral J M S, Prazeres D M F, *Purification of supercoiled plasmid DNA using chromatographic processes*, Journal of Molecular Recognition 11 (**1998**) 250-251.

Ferreira G N M, Monteiro G A, Prazeres D M F, Cabral J M S, *Downstream prossecing of plasmid DNA for gene therapy and DNA vaccine applications*, Trends in Biotechnology 18 (**2000**) 380-388.

Ferreira G N M, Cabral J M S, Prazeres D M F, *Development of process flow sheets for the purification of plasmid vectors for gene therapy applications*, Biotechnology Progress 15 (**1999**) 725–731.

Fong H, Chun I, Reneker D H, *Beaded nanofibers formed during electrospinning*, Polymer 40 (**1999**) 4585;4592.

Fong H and Reneker D H. *In*: Salem DR, editor. Structure formation in polymeric fiber. Munich: Hanser Gardner Publications, Inc.; **2001**, chapter 6: p. 225-246.

Formhals A, *Process and apparatus for preparing artificial threads*, **1934**, Vol. 1, 975, 504, US Patent.

Fox J L, *Gene therapy: safety issues come to fore*. Nature Biotechnology 17 (**1999**) 1153.

Frenot A and Chronakis I S, *Polymer nanofibers assembled by electrospinning*, Current Opinion in Colloid and Interface Science 8 (**2003**) 64-75.

Gemeiner P, Polakovic M, Mislovicova D, Stefuca V, *Cellulose as a (bio)affinity carrier: properties, design and applications*, Journal of Chromatography B 715 (**1998**) 245-271.

Greiner A and Wendorff J H, *Electrospinning: a fascinating method for the preparation of ultrathin fibres*, Angewandte Chemie-International Edition 46 (**2007**) 5670-5703.

Groman E V and Wilchek M, *Recent developments in affinity-chromatography supports*, Trends in Biotechnology 5 (**1987**) 220-224.

Handbook of Pharmaceutical Excipients, **1994**, second ed. A. Wade, P.J. Weller (Eds), pp. 383–384. American Pharmaceutical Association, Washington, DC.

Hjertén S, Mohammad J, Eriksson K O, Liao J L, *General Methods to Render Macroporous Stationary Phases Nonporous and Deformable, Exemplified with Agarose and Silica Beads and Their Use in High-Performance Ion-Exchange and Hydrophobic-Interaction Chromatography of Proteins*, Chromatographia 31 (**1991**) 1/2:85-94.

Huang Z M, Zhang Y Z, Kotaki M, Ramakrishna S, *A review on polymer nanofibers by electrospinning and their applications in nanocomposites*, Composites Science and Technology 63 (**2003**), 2223–2253.

Huber C G, *Micropellicular stationary phases for high-performance liquid chromatography of double-stranded DNA*, Journal of Chromatography A 806 (**1998**), 3–30.

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

Janson J and Rydén L, *In*: Protein purification, 2nd ed. Wiley-VCH Inc., **1998**, New York, pp 283-296.

(The) **Japanese Pharmaceutical Excipients Directory** (**1996**), Monograph on Polyvinyl Alcohol, p.355.

Jayaraman K, Kotaki M, Zhang Y Z, Mo X M, Ramakrishna S, *Recent advances in polymer nanofibers*, Journal of Nanoscience and Nanotechnology 4 (**2004**) 52-65.

Kelly B D and Hatton TA, *The fermentation downstream processing interface*. Bioseparation 1 (**1991**) 333–349.

Kim C and Yang K S, *Electrochemical properties of carbon nanofiber web as an electrode for supercapacitor prepared by electrospinning*, Applied Physics Letters 83 (**2003**) 1216-1218.

Kim C W and Rha C, *Selective adsorption/desorption of nucleic acids on submicron-sized polymeric particles*, Biotechnology and Bioengineering 33 (**1989**) 1205-1209.

Kim J S and Reneker D H, *Mechanical properties of composites using ultrafine electrospun fibers*, Polymer Composites 20 (**1999**) 124-131.

Koganei H K, Hachioji H T, Kodairi M M, Kokubunji H H, *Scanninh Electron Microscope Patent*, United States Patent Office, October 21, **1969**.

Krisky D M, Wolfe D, Goins W F, Marconi P C, Ramakrisknan R, Mata M, Rouse R J D, Fink D J, Glorioso J C, *Deletion of multiple immediate-early genes from herpes simplex virus reduces cytotoxicity and permits long-term gene expression in neurons*, Gene Therapy 5 (**1998**) 1593-1603.

Legendre J Y, Haensler J, Remy J S, *Non-viral gene delivery systems*, Medicine Sciences 12 (**1996**) 1334-1341.

Levy M S, Collins I J, Yim SS, Ward J M, Titchener-Hooker N, Shamlou P A, Dunnill P, *Effect of shear on plasmid DNA solution*, Bioprocess Engineering 20 (**1999**) 7–13.

Li D, Wang Y L, Xia Y N, *Electrospinning of polymeric and ceramic nanofibers as uniaxially aligned arrays*, Nano Letters 3 (**2003**) 1167-1171.

Li D, Xia Y N, *Electrospinning of nanofibers: Reinventing the wheel?*, Advanced Materials 16 (**2004**) 1151--1170.

Li Y, Dong X Y, Sun Y, *High-speed chromatographic purification of plasmid DNA with a customized biporous hydrophobic adsorbent*, Biochemical Engineering Journal 27 (**2005**) 33–39.

Li D, McCann J T, Xia Y, *Electrospinning: A simple and versatile technique for producing ceramic nanofibers and nanotubes*, Journal of the American Ceramic Society 89 (**2006**) 1861-1869.

Lin T, Wang H X, Wang X G, *Self-crimping bicomponent nanoribers efectrospun from polyacrylonitrile and elastomeric polyurethane*, Advanced Materials 17 (**2005**) 2699.

Liu H C, Fried J R, *Breakthrough of lysozyme through an affinity membrane of cellulose-cibacron blue*, Aiche Journal 40 (**1994**) 40-49.

Liu T Q, *Preparation of a novel micro/nano tubes via electrospun fiber as a template*, Journal of Materials Science and Technology 20 (**2004**) 613-616.

Luo D and Saltzman W M, *Synthetic DNA delivery systems*, Nature Biotechnology 18 (**2000**) 33-37.

Luu Y K, Kim K, Hsiao B S, Chu B, Hadjiargyrou M, *Development of a nanostructured DNA delivery scaffold via electrospinning of PLGA and PLA-PEG block copolymers*, Journal of Controlled Release 89 (**2003**) 341-353.

Ma P X and Zhang R, *Synthetic nano-scale fibrous extracellular matrix*, Journal of Biomedical Materials Research 46 (**1999**) 60-72.

Ma Z W, Kotaki M, Ramakrishna S, *Electrospun cellulose nanofiber as affinity membrane*, Journal of Membrane Science 265 (**2005**) 115-123.

Marquet M, Horn N A, Meek J A, *Process development for the manufacture of plasmid DNA vectors for use in gene therapy*, Biopharm- The Technology and Business of Biopharmaceuticals 8 (**1995**), 26–37.

Matthews J A, Wnek G E, Simpson D G, Bowlin G L, *Electrospinning of Collagen Nanofibers*, Biomacromolecules 3 (**2002**), 232–238.

Melander W R, Corradini D, Horváth C, *Salt-mediated retention of proteins in hydrophobic interactions chromatography: application of solvophobic theory*, Journal of Chromatography 317 (**1984**) 67–85.

Min B M, Lee G, Kim S H, Nam Y S, Lee T S, Park W H, *Electrospinning of silk fibroin nanofibers and its effects on the adhesion and spreading of normal human keratinocytes and fibroblasts in vitro*, Biomaterials 25 (**2004**) 1289-1297.

Mountain A, *Gene therapy: the first decade*, Trends in Biotechnology 18 (**2000**) 119-128.

Narayanan S R and Crane L J, *Affinity-chromatography supports - a look at performance requirements*, Trends in Biotechnolology 8 (**1990**) 12-16.

Ondarçuhu T and Joachim C, *Drawing a single nanofiber over hundreds of microns*, Europhysics Letters 42 (**1998**) 215-220.

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

Prazeres D M F, Ferreira G N M, Monteiro G A, Cooney C L, Cabral J M S, *Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy: problems and bottlenecks*, Trends in Biotechnology 17 (**1999**) 169-174.

Prazeres D M F, Monteiro G A, Ferreira G N M, Diogo M M, Ribeiro S C, Cabral J M S, *In*: M.R. El-Gewely (Ed.), Biotechnology Annual Review, Elsevier, Amsterdam (**2001**) p. 1.

Prazeres, D M F, Schluep T, Cooney C, *Preparative purification of supercoiled plasmid DNA using anion-exchange chromatography*, Journal of Chromatography A 806 (**1998**) 31–45.

QIAGEN Plasmid Purification Handbook

Queiroz J A, Tomaz C T, Cabral J M S, *Hydrophobic interaction chromatography of proteins*, Journal of Biotechnology 87 (**2001**) 143-159.

Reneker D H and Chun I, *Nanometer diameter fibers of polymer, produced by electrospinning*, Nanotechnology 7 (**1996**) 216-223.

Robbins P D, Tahara H, Ghivizzani S C, *Viral vectors for gene therapy*, Trends Biotechnology 16 (**1998**) 35-40.

Robinson H L, Montefiori D C, Johnson R P, Manson K H, Kalish M L, Lifson J D, Rizvi T A, Lu S, Hu S L, Mazzara G P, Panicali D L, Herndon J G, Glickman R,

Candido M A, Lydy S L, Wyand M S, McClure H M, *Neutralization antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations*, Nature Medicine 5 (**1999**) 526-534.

Sambrook J and Russell D, *Molecular cloning: a laboratory manual*, 3rd ed., vol. 1. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; **2001**.

Sanda M G, Smith D C, Charles L G, Hwang C, Pienta K J, Schlom J, Milenic D, Panicali D, Montie J E, *Recombinant vaccinia-PSA (prostvac) can induce a prostate-specific immune response in androgen-modulated human prostate cancer*, Urology 53 (**1999**) 260-266.

Scherman D, *Towards nonviral gene therapy*, Bulletin de l Academie Nationale de Medecine 185 (**2001**) 1683-1697.

Schleef M, Issues of large-scale plasmid DNA manufacturing. *In* Recombinant Proteins, Monoclonal Antibodies and Therapeutic Genes (Biotechnology) (Vol. 5a) (Mountain, A. et al., eds), pp. 443–469, **1999**, Wiley–VCH.

Scopes R K, Protein Purification: Principles and Practice, Springer Verlag, New York (**1982**).

Sewell P A, *Theory of Liquid Chromatography*, UK, Akademic Press, **2000**.

Shin Y M, Hohman M M, Brenner M P, Rutledge G C. *Experimental characterization of electrospinning: the electrically forced jet and instabilities*, Polymer 42 (**2001**) 9955-9967.

Sinden R R, *DNA Structure and Function*, Academic Press, San Diego, CA, **1994**.

Smith C B, Mohamed M H, Hauser P J, *Product and method for treating cotton*, United States Patent, Jan. 23, **2007**.

Sousa F, Prazeres D M F, Queiroz J A, *Affinity chromatography approaches to overcome the challenges of purifying plasmid DNA*, Trends in Biotechnology 26 (**2008**) 447-449.

Stasiak M, Studer A, Greiner A, Wendorff J H, *Polymer fibers as carriers for homogeneous catalysts*, Chemistry – A European Journal 13 (**2007**) 6150-6156.

Summers D K, *The Biology of Plasmids, Blackwell*, Oxford, UK, **1996**.

Sun Z C, Zussman E, Yarin A L, Wendorff J H, Greiner A, *Compound core-shell polymer nanofibers by co-electrospinning*, Advanced Materials 15 (**2003**) 1929-1932.

Sundberg L and Porath J, *Preparation of adsorbents for biospecific affinity chromatography 1. Attachment of group-containing ligands to insoluble polymers by means of bifunctional oxiranes*, Journal of Chromatography 90 (**1974**) 87-98.

Szewczyk K, *Master's Thesis: The use of cellulose nanofibers in chromatography for the purification of bimolecules* (**2009**).

Theodossiou I, Collins I J, Ward J M, Thomas O R T, Dunnill P, *The processing of plasmid based gene from Escherichia coli: primary recovery by filtration*, Bioprocess Engineering 16 (**1997**) 175–183.

Theron A, Zussman E, Yarin A L, *Electrostatic field-assisted alignment of electrospun nanofibers*, Nanotechnology 12 (**2001**), 384–390.

Tighe H, Corr M, Roman M, Raz E, *Gene vaccination: plasmid DNA is more than just a blueprint*, Immunology Today 19 (**1998**) 89–97.

Van Oss C J, Good R J, Chaudhury M K, *The role of Van der Waals forces and hydrogen bonds in hydrophobic interaction between biopolymers and low energy surfaces*, Journal of Colloid and Interface Science 111 (**1986**) 378-390.

Wang X Y, Lee S H, Ku B C, Samuelson L A, Kumar J, *Synthesis and electrospinning of a novel fluorescent polymer PMMA-PM for quenching-based optical sensing*. Journal of Macromolecular Science, Part A: Pure and Applied Chemistry A39 (**2002**) 1241-1249.

Welle A, Kroger M, Doring M, Niederer K, Pindel E, Chronakis I S, *Electrospun aliphatic polycarbonates as tailored tissue scaffold materials*, Biomaterials 28 (**2007**) 2211-2219.

Widera G, Austin M, Rabussay D, Goldbeck C, Barnett S W, Chen M, Leung L, Otten R G, Thudium K, Selby M J, Ulmer J B, *Increased DNA Vaccine Delivery and*

Immunogenicity by Electroporation In Vivo, The Journal of Immunology 164 (**2000**) 4635-4640.

Xiong Y, Fang J, Zeng Q H, Liu Q L, *Preparation and characterization of cross-linked quaternized poly(vinyl alcohol) membranes for anion exchange membrane fuel cells*, Journal of Membrane Science 311 (**2008**) 319–325.

Yamakawa H, Higashino K I, Ohara O, *Sequence-dependent DNA separation by anion-exchange high-performance liquid chromatography*, Analytical Biochemistry 240 (**1996**) 242-250.

Yao L, Hass T W, Guiseppi-Elie A, Bowlin G L, Simpson D G, Wnek G E, *Electrospinning and stabilization of fully hydrolyzed poly(vinyl alcohol) fibers*, Chemistry of Materials 15(**2003**) 1860-1864.

Yarin A L, Koombhongse S, Reneker D H, *Bending instability of nanofibers*, Journal of Applied Physics 89 (**2001**), 3018-3026.

Yarin A L, Koombhongse S, Reneker D H, *Taylor cone and jetting from liquid droplets in electrospinning of nanofibers*, Journal of Applied Physics 90 (**2001**)^{2}, 4836-4846.

Yoon K, Kim K, Wang X F, Fang D F, Hsiao B S, Chu B, *High flux ultrafiltration membranes based on electrospun nanofibrous PAN scaffolds and chitosan coating*, Polymer 47 (**2006**) 2434-2441.

Zeleny J, *The electrical discharge from liquids points, and a hydrostatic method of measuring the electrical intensity at their surfaces*, Physical Reviews 3(2) (**1914**) 69-91.

Zhang L, Menkhaus T J, Fong H, *Fabrication and bioseparation studies of adsorptive membranes/felts made from electrospun cellulose acetate nanofibers*, Journal of Membrane Science 319 (**2008**) 176-184.

ANNEXES

ANNEX A - OTHER PERFORMANCES OF PLASMID DNA PRODUCTION

ANNEXES TABLE I DATA FROM OTHER FERMENTATIONS

Sample C	
Pre-fermentation time	3h30min
Absorbency after pre-fermentation (at 600nm)	3,10 (0,620 with 1:5 dilution)
Pre-fermentation volume to fermentation	8,621 mL
Fermentation time	24h
Absorbency fermentation (at 600nm)	12,90 (0,645 with 1:20 dilution)
Sample D	
Pre-fermentation time	3h30min
Absorbency after pre-fermentation (at 600nm)	3,10 (0,620 with 1:5 dilution)
Pre-fermentation volume to fermentation	8,621 mL
Fermentation time	24h
Absorbency fermentation (at 600nm)	14,18 (0,709 with 1:20 dilution)
Sample E	
Pre-fermentation time	4h
Absorbency after pre-fermentation (at 600nm)	2,925 (0,585 with 1:5 dilution)
Pre-fermentation volume to fermentation	9,174 mL
Fermentation time	24h
Absorbency fermentation (at 600nm)	7,99 (0,799 with 1:10 dilution)
Sample F	
Pre-fermentation time	4h
Absorbency after pre-fermentation (at 600nm)	2,925 (0,585 with 1:5 dilution)
Pre-fermentation volume to fermentation	9,174 mL
Fermentation time	24h
Absorbency fermentation (at 600nm)	6,98 (0,698 with 1:10 dilution)
Sample G	
Pre-fermentation time	4h30
Absorbency after pre-fermentation (at 600nm)	5,18 (0,518 with 1:10 dilution)
Pre-fermentation volume to fermentation	5,02 mL
Fermentation time	25h
Absorbency fermentation (at 600nm)	6,37
Sample H	
Pre-fermentation time	4h30
Absorbency after pre-fermentation (at 600nm)	5,18 (0,518 with 1:10 dilution)
Pre-fermentation volume to fermentation	5,02 mL
Fermentation time	25h
Absorbency fermentation (at 600nm)	8,15
Sample I	
Pre-fermentation time	4h30

Absorbency after pre-fermentation (at 600nm)	5,18 (0,518 with 1:10 dilution)
Pre-fermentation volume to fermentation	5,02 mL
Fermentation time	25h
Absorbency fermentation (at 600nm)	9,70
Sample J	
Pre-fermentation time	4h30
Absorbency after pre-fermentation (at 600nm)	5,18 (0,518 with 1:10 dilution)
Pre-fermentation volume to fermentation	5,02 mL
Fermentation time	25h
Absorbency fermentation (at 600nm)	9,04
Sample K	
Pre-fermentation time	3h00
Absorbency after pre-fermentation (at 600nm)	2,83 (0,518 with 1:10 dilution)
Pre-fermentation volume to fermentation	9,00 mL
Fermentation time	25h30min
Absorbency fermentation (at 600nm)	5,83
Sample L	
Pre-fermentation time	3h00
Absorbency after pre-fermentation (at 600nm)	2,83 (0,518 with 1:10 dilution)
Pre-fermentation volume to fermentation	9,00 mL
Fermentation time	25h30min
Absorbency fermentation (at 600nm)	6,95
Sample M	
Pre-fermentation time	3h00
Absorbency after pre-fermentation (at 600nm)	2,83 (0,518 with 1:10 dilution)
Pre-fermentation volume to fermentation	9,00 mL
Fermentation time	25h30min
Absorbency fermentation (at 600nm)	5,57
Sample N	
Pre-fermentation time	3h00
Absorbency after pre-fermentation (at 600nm)	2,83 (0,518 with 1:10 dilution)
Pre-fermentation volume to fermentation	9,00 mL
Fermentation time	25h30min
Absorbency fermentation (at 600nm)	5,62

ANNEXES TABLE II COMPOSITION OF BUFFERS AND REAGENTS

Buffer	Function	Composition
P1	Resuspension	50mM Tris-Cl, pH 8,0 10mM EDTA 100µg/mL RNase A
P2	Lysis	200mM NaOH, 1%SDS
P3	Neutralization	3,0M potassium acetate, pH 5,5
QBT	Equilibration	750mM NaCl 50 mM MOPS, pH 7,0 15%isopropanol 0,15% Triton X-100
QC	Wash	1,0M NaCl 50 mM MOPS, pH 7,0 15%isopropanol
QF	Elution	1,25M NaCl 50 mM Tris-Cl, pH 8,5 15%isopropanol
QN	Elution	1,6M NaCl 50 mM MOPS, pH 7,0 15%isopropanol

QIAGEN kit General Procedure

with some alterations, signalized in grey

- Add RNase A solution to Buffer P1;
- Check Buffer P2 for SDS precipitation;
- Pre-chill Buffer P3 to 4ºC

Bacterial culture, harvest and lysis

- Homogeneously resuspend 125mL of bacterial pellet in 10mL Buffer P1;
- Add 10mL Buffer P2, mix carefully by inverting 4-6 times, and incubate at room temperature for 5 min.;
- Add 10mL Buffer P3, mix carefully by inverting 4-6 times, and incubate in ice for 20 min.;
-

Bacterial lysate clearing

- Centrifuge at ≥20.000g for 30min at 4ºC. Re-centrifuge the supernatant at ≥20.000g for 15min at 4ºC.

Bind, wash and elute plasmid DNA

- Equilibrate a QIAGEN-tip by applying 10mL Buffer QBT and allow column to empty by gravity flow.
- Apply the supernatant to the QIAGEN-tip and allow it to enter the resin by gravity flow.
- Wash QIAGEN-tip with 2×30mL Buffer QC. Allow Buffer QC to through thr QIAGEN-tip by gravity flow.
- Elute DNA with 15mL Buffer QF into clean 50mL vessel.

Precipitate, wash and redissolve plasmid DNA

- Precipitate DNA by adding 10,5mL room-temperature isopropanol to the eluted DNA and mix. Centrifuge at ≥15.000g for 30min at 4ºC. Carefully decant supernatant.
- Dissolve DNA 1,5mL tris-CL 10mM (pH=8) and storage at -80ºC.

ANNEX C - OTHER PERFORMANCES OF HYDROPHOBIC INTERACTION CELLULOSE MATRIX PREPARATION

ANNEXES TABLE III SAMPLES AND REAGENTS USED IN THE ACTIVATION STEP

Sample		**Identification**	II	III	IV
		Primary sample	B1	B4	B2
		Mass (g)	15,03	10,0	7,0
Volumes (mL)	**Required / Measured or Prepared**	**Diglycidyl ether**	15,0 / 15,0	10,0 / 10,0	7,0 / 7,0
		Sodium hydroxide 0,6 M	15,0 / 25,0	10,0 / 25,0	7,0 / 50,0
		Sodium hydroxide 1 M	150,0 / 200,0	100,0 / 100,0	70,0 / 100,0
Masses (g)	**Required / Measured**	**Sodium hydroxide 0,6 M**	0,60 / 0,59	0,60 / 0,60	1,20 / 1,202
		Sodium hydroxide 1 M	8,0 / 8,063	4,0 / 4,0	4,0 / 4,02
		Sodium borihydride	0,030 / 0,030	0,020 / 0,020	0,014 / 0,014
Bath		**Temperature (ºC)**	24,6	25,0	25,8
		Rotation (U/min)	76	70	70

ANNEXES TABLE IV EPOXY GROUPS QUANTIFICATION - SAMPLES PH BEFORE AND AFTER TITRATION AND HCL VOLUME NEEDED

Activated sample Id.	Analyzed sample Id.	Initial pH	Final pH	HCl vol. (mL)
II	IIa	10,83	5,87	0,30
	IIb	11,47	7,07	0,80
	IIc	11,55	7,74	0,85
III	IIIa	10,7	7,0	3,2
	IIIb	11,0	7,0	3,1
	IIIc	11,0	7,0	3,6
IV	IVa	11,15	7,0	0,75
	IVb	11,19	6,9	0,70

ANNEXES TABLE V EPOXY GROUPS QUANTIFICATION – DRY GEL SAMPLES WEIGHT AND AMOUNT OF LIGANDS

Sample	Dry gel mass (g)	Amount of H (µmol)	Ligands Amount (µmol/g)	
II				
IIa	0,147	3,00	20,408	**48,239**
IIb	0,170	8,00	47,059	
IIc	0,172	8,50	49,419	
III				
IIIa	0,344	16,00	46,512	**76,733**
IIIb	0,208	15,50	74,519	
IIIc	0,228	18,00	78,947	
IV				
Iva	0,250	3,75	15,000	**15,716***
IVb	0,213	3,50	16,432	

Note: The samples in grey were excluded to calculate the gel capacity

*this sample titration was not made right after modification, so it is possible that the real capacity is higher than that.

ANNEX D - OTHER PERFORMANCES OF ANION-EXCHANGE MATRICES PREPARATIONS

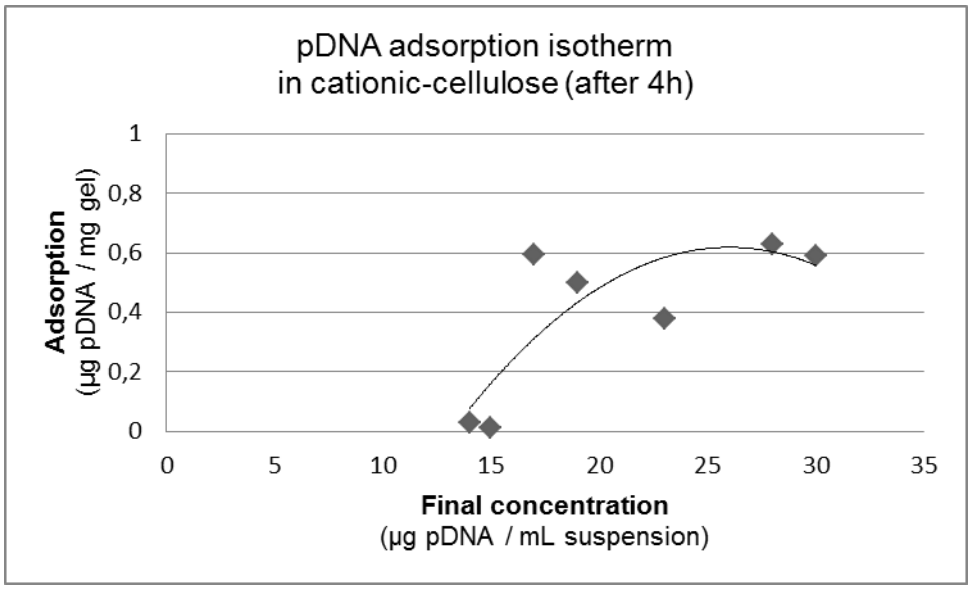
ANNEXES TABLE VI SAMPLES AND REAGENTS USED IN THE MODIFICATION (CELLULOSE)

Sample	**Identification**	AE-Cel-II	AE-Cel-III
	Primary sample	B1	A3 e A4
	Mass	0,512 g	0,500 g
Sodium hydroxide	**Concentration required**	0,5 M	0,5 M
	Volume prepared	50,0 mL	50,0 mL
	Mass	1,01 g	1,01 g
	Volume needed	0,50 mL	0,50 mL
Sodium borihydride	**Mass required**	0,005 g	0,005 g
Glycidyltrimethyl-ammonium chloride	**Mass required**	6,0 g (d = 1,13) / 2g	6,0 g (d = 1,13) / 2g
	Volume measured	1,327 mL	1,327 mL

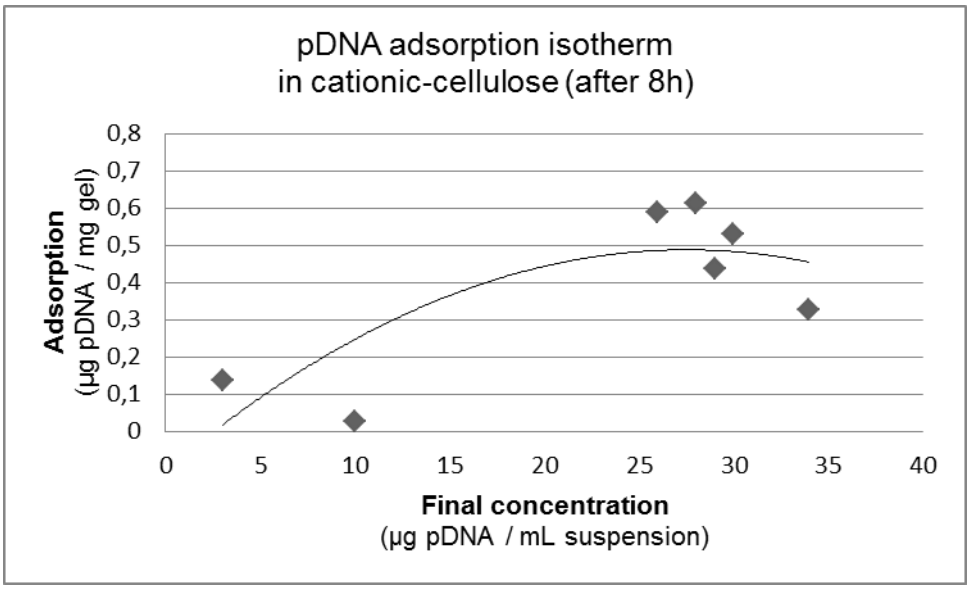
ANNEXES TABLE VII SAMPLES AND REAGENTS USED IN THE MODIFICATION (SEPHAROSE)

Sample	**Identification**	AE-Sep-II	AE-Sep-III
	Primary sample	Commercial	Commercial
	Mass	0,505 g	0,506 g
Sodium hydroxide	**Concentration required**	0,5 M	0,5 M
	Volume prepared	50,0 mL	50,0 mL
	Mass	1,01 g	1,01 g
	Volume needed	0,50 mL	0,50 mL
Sodium borihydride	**Mass required**	0,005 g	0,005 g
Glycidyltrimethyl-ammonium chloride	**Mass required**	6,0 g (d = 1,13) / 2g	6,0 g (d = 1,13) / 2g
	Volume measured	1,327 mL	1,327 mL

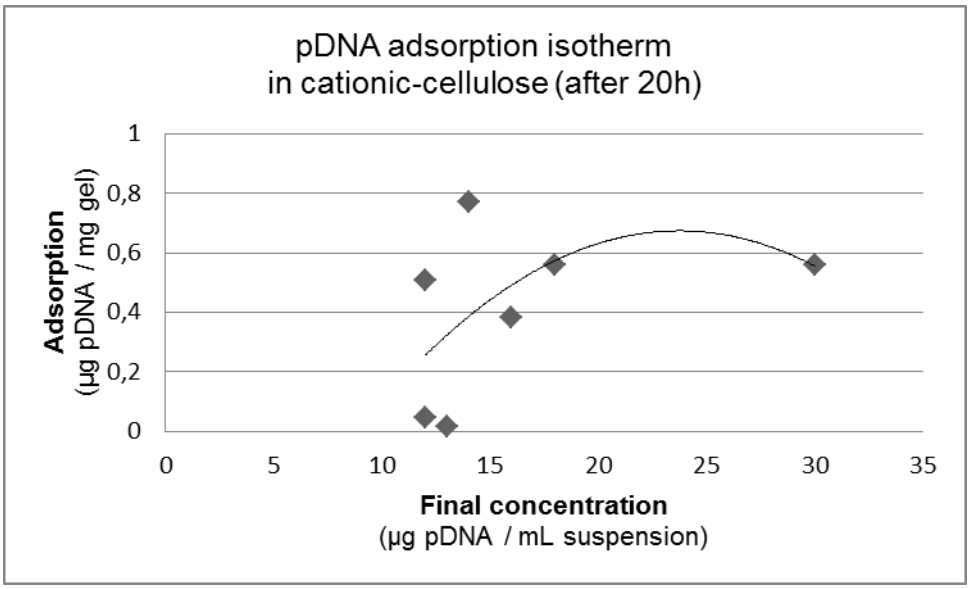
ANNEX E – PDNA ADSORPTION ISOTHERMS UP TO 32 HOURS



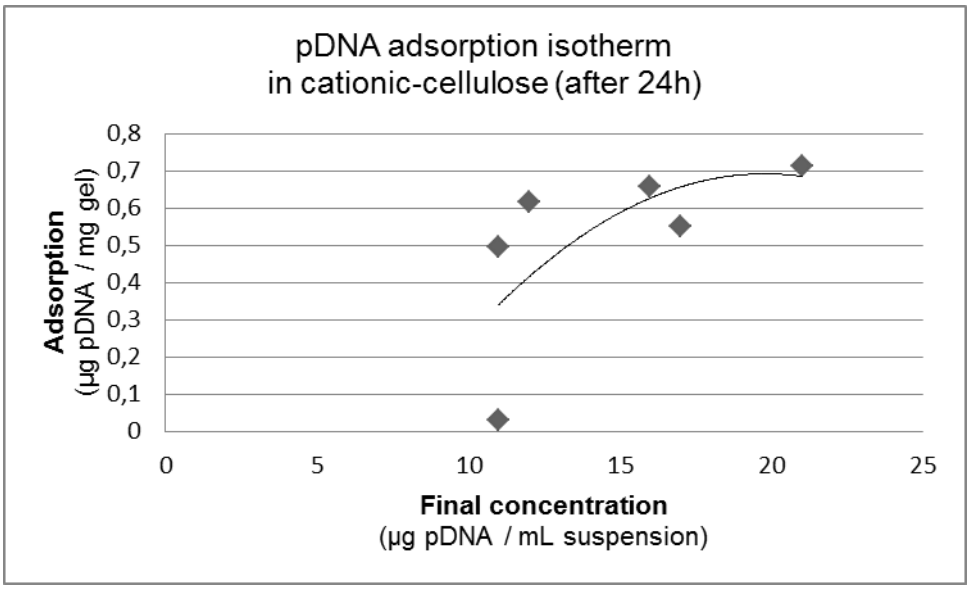
ANNEXES FIGURE I ANION-EXCHANGE CELLULOSE BATCH: ADSORPTION ISOTHERM (AFTER 4 HOURS)



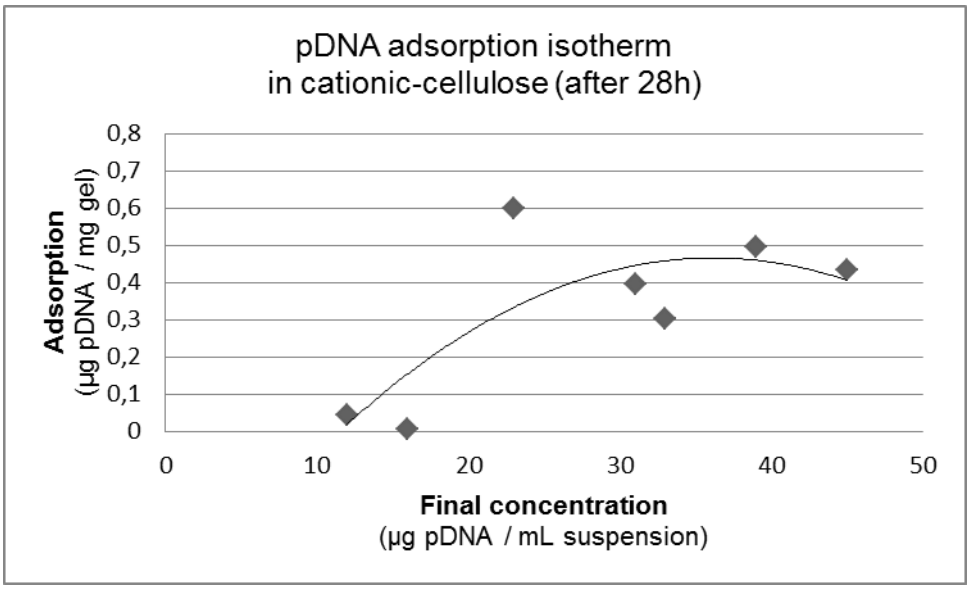
ANNEXES FIGURE II ANION-EXCHANGE CELLULOSE BATCH: ADSORPTION ISOTHERM (AFTER 8 HOURS)



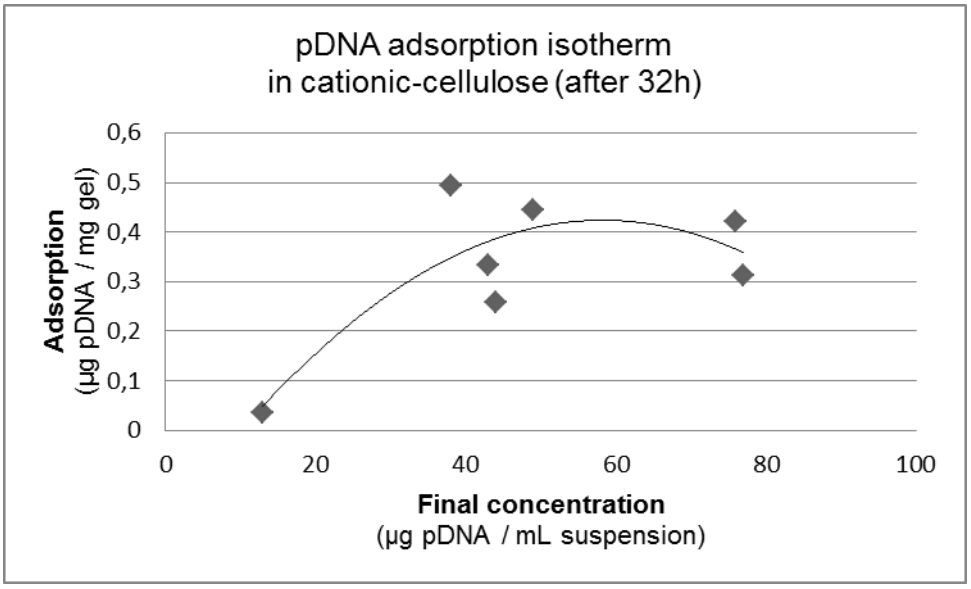
ANNEXES FIGURE III ANION-EXCHANGE CELLULOSE BATCH: ADSORPTION ISOTHERM (AFTER 20 HOURS)



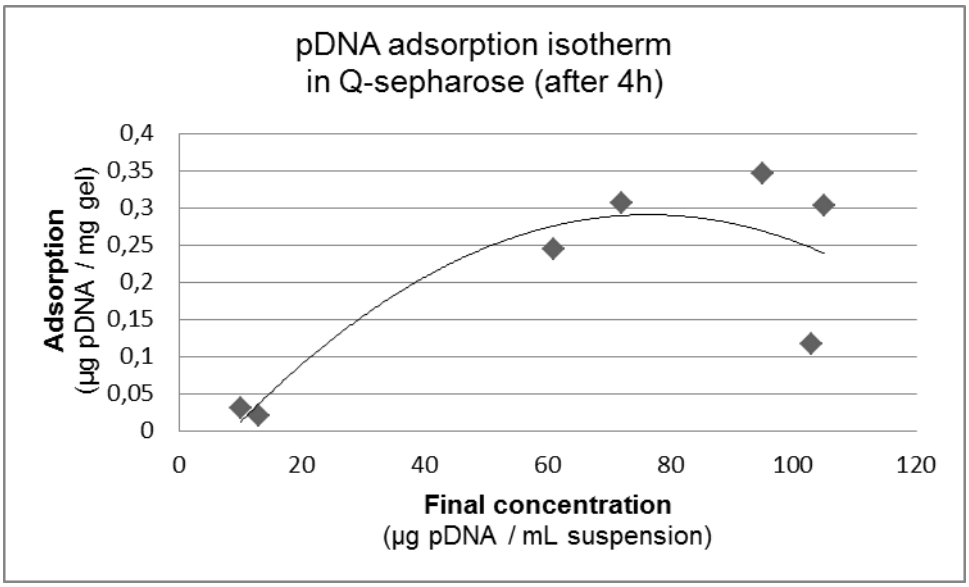
ANNEXES FIGURE IV ANION-EXCHANGE CELLULOSE BATCH: ADSORPTION ISOTHERM (AFTER 24 HOURS)



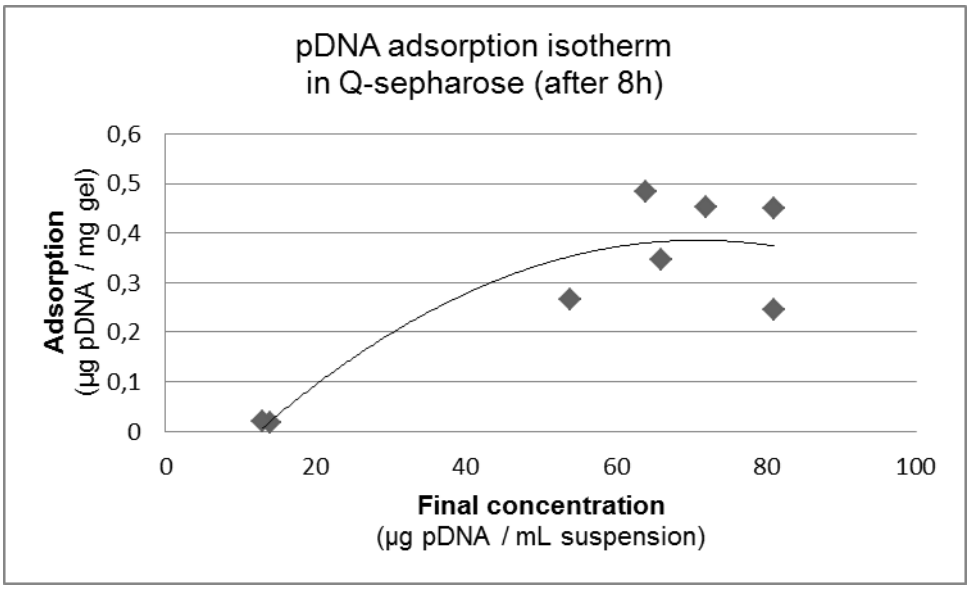
ANNEXES FIGURE V ANION-EXCHANGE CELLULOSE BATCH: ADSORPTION ISOTHERM (AFTER 28 HOURS)



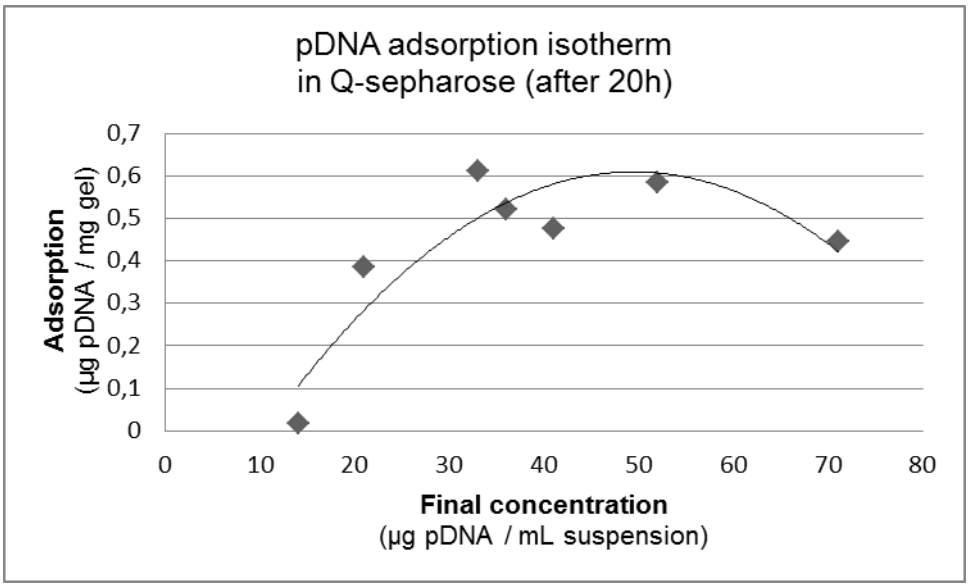
ANNEXES FIGURE VI ANION-EXCHANGE CELLULOSE BATCH: ADSORPTION ISOTHERM (AFTER 32 HOURS)



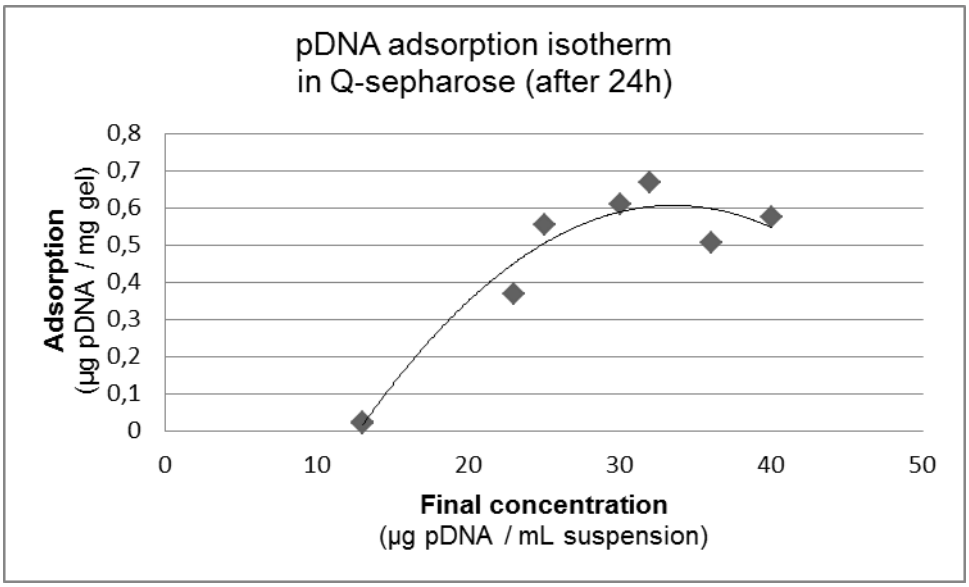
ANNEXES FIGURE VII ANION-EXCHANGE Q-SEPHAROSE BATCH: ADSORPTION ISOTHERM (AFTER 4 HOURS)



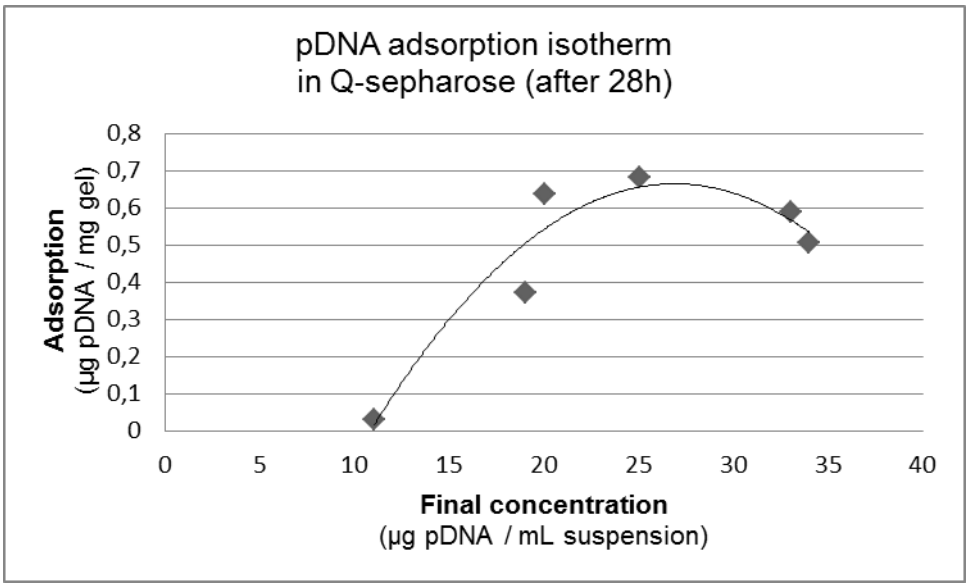
ANNEXES FIGURE VIII ANION-EXCHANGE Q-SEPHAROSE BATCH: ADSORPTION ISOTHERM (AFTER 8 HOURS)



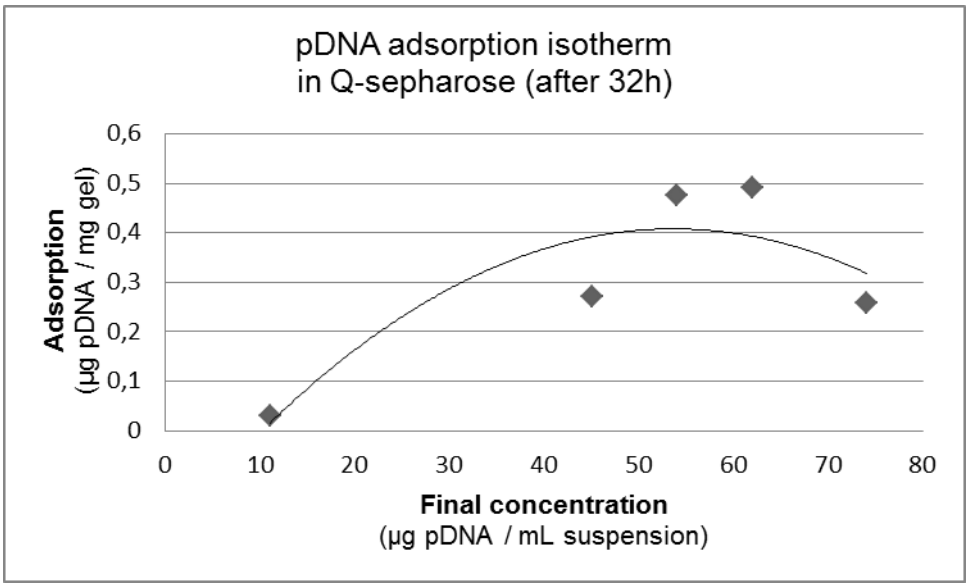
ANNEXES FIGURE IX ANION-EXCHANGE Q-SEPHAROSE BATCH: ADSORPTION ISOTHERM (AFTER 20 HOURS)



ANNEXES FIGURE X ANION-EXCHANGE Q-SEPHAROSE BATCH: ADSORPTION ISOTHERM (AFTER 24 HOURS)



ANNEXES FIGURE XI ANION-EXCHANGE Q-SEPHAROSE BATCH: ADSORPTION ISOTHERM (AFTER 28 HOURS)



ANNEXES FIGURE XII ANION-EXCHANGE Q-SEPHAROSE BATCH: ADSORPTION ISOTHERM (AFTER 32 HOURS)