TÉCNICO LISBOA

Plasmid DNA purification with synthetic protein-mimic affinity ligands

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Resumo

A purificação de plasmídeos é crucial no progresso da terapia génica e na produção de vacinas de DNA. A cromatografia de afinidade tem um papel importante neste empreendimento ao possibilitar a purificação de biomoléculas por interações seletivas com ligandos específicos. Apesar da elevada seletividade, os ligandos biológicos apresentam baixa capacidade de ligação, elevados custos de produção e fraco potencial de *scale-up.* Ligandos sintéticos podem ultrapassar estas desvantagens, sendo resistentes à degradação química e biológica e uma alternativa económica, mas a sua aplicação não foi descrita para purificação de plasmídeos. Num trabalho anterior, um ligando baseado na triazina, selecionado de uma biblioteca combinatorial com 22 elementos e imobilizado num suporte monolítico CIM® (ligando 6/5) foi considerado promissor na purificação de sc pDNA usando cromatografia de afinidade. Neste trabalho, para confirmar estes resultados, este suporte foi avaliado no sistema de purificação ÄKTA, em condições hidrófobas (usando $(NH_4)_2SO_4$ no tampão de lavagem) e hidrófilas (com NaCl no tampão de eluição). No entanto, o ligando não permitiu purificar a isoforma superenrolada do plasmídeo pVAX1/lacZ. Alternativamente, outros ligandos de afinidade da biblioteca combinatorial sintetizada em Sepharose CL-6B, com composição distinta (hidrófoba e mista), foram selecionados e testados na purificação da isoforma superenrolada do mesmo plasmídeo. Após avaliação por cromatografia em bancada, os ligandos 2/1 e 8/1 foram considerados ligandos-líder, sendo posteriormente testados no sistema ÄKTA. Os melhores resultados foram obtidos para o ligando 2/1, em condições hidrófobas, em que se purificou a isoforma superenrolada com rendimento de recuperação de 75,1%.

Palavras-chave: purificação de plasmídeos, pVAX1/lacZ cromatografia de afinidade, ligandos sintéticos de afinidade, Sepharose CL-6B, discos monolíticos CIM®.

Abstract

Plasmid DNA purification is a crucial process to be considered nowadays for the progress of gene therapy and DNA vaccine production processes. Affinity chromatography plays a powerful role in this endeavor by enabling the purification of biomolecules by selective interaction with a specific ligand. Despite their high selectivity, biological ligands are associated to low biding capacity, high costs of production and low scale-up potential. Synthetic affinity ligands can overcome these disadvantages, being resistant to chemical and biological degradation and a cost-effective alternative, though their application has not been described for plasmid DNA purification. In a previous preliminary work, a triazine-scaffolded ligand selected from a 22-membered combinatorial library and immobilized in a CIM® monolithic support (ligand 6/5) was considered as promising in supercoiled plasmid DNA purification using affinity chromatography. In this work, to confirm these results, this support was evaluated in an ÄKTA purifying system, under hydrophobic (using $(NH_4)_2SO_4$ in washing buffer) and hydrophilic conditions (using NaCl as elution buffer). However, purification of supercoiled isoform of pVAX1/lacZ plasmid was not achieved. Alternatively, several affinity ligands from the same library of triazine-based protein-mimic ligands, synthesized in Sepharose CL-6B, with distinct composition (hydrophobic and mixed-behavior), were selected and screened for pVAX1/lacZ purification and supercoiled isoform isolation. From the screening in bench-scale chromatography assays, ligands 2/1 and 8/1 emerged as leads and were further tested in the ÄKTA system. The best results were obtained for ligand 2/1, under hydrophobic environment, which enabled plasmid DNA supercoiled isoform purification with a recovery yield of 75.1%.

Keywords: plasmid purification, pVAX1/lacZ, affinity chromatography, synthetic affinity ligands, Sepharose CL-6B, CIM ® monolithic disks.

List of abbreviations

AC: Affinity Chromatography
ACS: American Chemical Society
Ala: Alanine
Asp: Aspartic acid
ATPS: Aqueous-two phase system
BCA: bicinchoninic acid
BGH: Bovine Growth Hormone
bp: base pairs
cDNA: complementary DNA
CIM: Convective Interaction Media
CMV: cytomegalovirus
CV: Column Volumes
DNA: Deoxyribonucleic Acid
***E. coli*:** *Escherichia Coli*
EDA: Ethylenediamine
E.U.: Endotoxin Units
FDA: Food and Drug Administration
gDNA: genomic DNA
Gln: Glutamine
Glu: Glutamic acid
Gly: Glycine
GRAS: Generally Recognized As Safe
HCl: Chloridric Acid
HIC: Hydrophobic Interaction Chromatography
HPLC: High Pressure Liquid Chromatography
Ile: Isoleucine
K_2HPO_4: Dipotassium Phosphate
LAL: Limulus Amoebocyte Lysate
LB: Luria-Bertani
Leu: Leucine
LPS: Lipopolysaccharide
Lys: Lysine
mRNA: messenger RNA
NaCl: Sodium Chloride
$NaHCO_3$: Sodium Bicarbonate
$(NH_4)_2SO_4$: Ammonium Sulphate
oc: open circular
O.D.: Optical Density
ORF: Open Reading Frame
pDNA: plasmid DNA
PEG: Polyethylene glycol
Phe: Phenylalanine
RNA: Ribonucleic Acid
RPC: Reverse Phase Chromatography
Rpm: rotations-per-minute
sc: supercoiled
SEC: Size Exclusion Chromatography
Thr: Threonine
Tyr: Tyrosine
USP: United Stated Pharmacopeia
WHO: World Health Organization

Table of contents

List of Tables

List of Figures

1. Introduction

1.1. DNA and plasmid DNA

From the past years, numerous studies have been performed to find and develop efficient therapeutics based on gene delivery and manipulation, also referred as gene therapy, to treat a wide range of diseases from infections, genetic disorders to cancer and other gene-related illnesses [1].

As such, several potential DNA therapeutics have been developed and studied in which it is possible to manipulate the genes from host organisms due to gene delivery, introducing useful and benefic properties to the host cells [1].

Plasmid DNA molecules (or pDNA) are small circular double stranded DNA molecules (Figure 1), ranging between 5000 to 40000 bp (in natural occurring bacterial plasmids) that are able to replicate in bacteria separately from the host bacterial chromosome [2,3]. Those molecules can be artificially introduced in bacterial cells in an operation called transformation [2]. Transformation of bacterial cells can be performed by two different methods. The first one, places the cells and plasmid DNA at 0ºC in a solution of calcium chloride followed by a change of temperature to 37-45ºC, which promotes heat shock, allowing DNA uptake [2]. On the other hand, a process called electroporation can be applied in which the cells and the desired plasmid are incubated together and subjected to a high-voltage pulse, resulting in a transient permeability of the bacterial membrane to large molecules like pDNA [2].

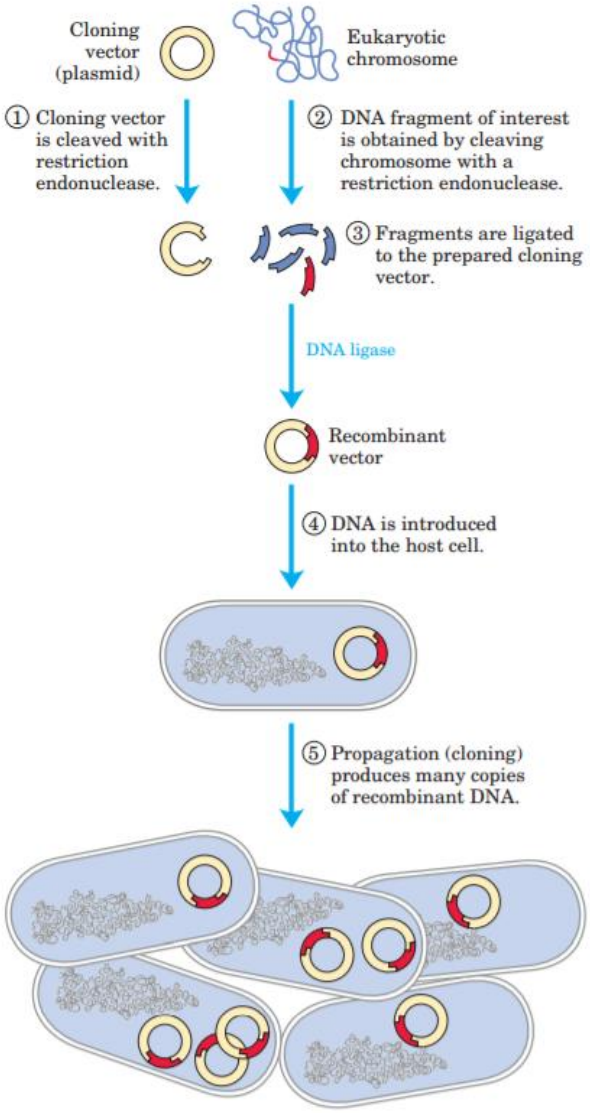


Figure 1: Schematic diagram for DNA cloning. Adapted from [2].

Those molecules and their introduction into host cells are the basis of recombinant technology or molecular cloning. As such, a DNA fragment of interest can be inserted in a plasmid, originating a recombinant molecule or vector [2,3]. This DNA material is introduced in the host cell, where it replicates, proliferates and can be isolated yielding a high amount of pDNA copies containing the DNA fragment of interest (Figure 1) [2,3].

The previous method can be applied in DNA-based vaccines in which bacterial plasmids are developed to express protein immunogens in vaccinated hosts to promote immune response [4].

1.1.1. DNA delivery methods

There are two major methods for DNA delivery in host organisms, viral and non-viral methods. The first group of methods is performed using viruses containing recombinant viral vectors. Those vectors are introduced in the cell by the viruses and have the advantage of mimicking the real infection. However, this therapeutic has several limitations like the inherent immunogenicity, that limits its repeated use, the delivery capacity of only small DNA fragments and the potential risk of integration into the host genome, which can promote oncogene activation and tumor development [1].

Regarding those limitations, this alternative has been changed by non-viral vector systems. An example of a non-viral system is the use of recombinant plasmid DNA, such as DNA-vaccines, which represents a safer alternative to viral vectors [5].

Unlike conventional protein vaccines, pDNA-based vaccines are able to induce not only humoral or antibody-mediated response but also cellular-immune response without the use of live attenuated pathogens or complex biochemical techniques, producing stronger immune responses and resulting in longer lasting production of antigenic proteins compared to conventional vaccines [6-8].

These vaccines are highly specific, producing antigens that are subjected to the same post-translational modifications and glycosylation processes observed in natural viral infections, avoiding the risk of infection reported in viral-delivery methods. [6,8] Moreover, pDNA vaccines are stable at high temperatures as well as for storage and shipping, representing a cost-effective alternative to conventional vaccines (in terms of production, transport and storage) [4,6,9]. Nevertheless, DNA vaccines are associated to reduced levels of immunogenicity in humans, requiring optimization [6,10].

Currently, several human trials with DNA vaccines developed against various diseases as malaria, dengue or tuberculosis to AIDS or cancer are in progress. The development of this field is associated to an increased demand for plasmid DNA, which requires high-yield and cost-effective production and purification processes [11].

Moreover, in order to be accepted and applied as therapeutic drugs, high-quality phamarceutical-grade pDNA is mandatory [11]. As such, plasmid DNA production and purification must follow specific guidelines from regulatory agencies, like FDA and WHO [12]. Several specifications, related to bacterial cell lysate (resultant from cell lysis), bulk plasmid product (purified plasmid before final formulation) and final product content that are established by those agencies, are presented in Table 1. Regarding the development and application of DNA vaccines and gene therapy in the past decades and in the future, these specifications must be considered flexible and may be susceptible to revision and change in the future.

Table 1: Specifications of bacterial cell lysates, bulk plasmid product and “accepted” level of impurities in the final product obtained from FDA and WHO guidelines. Adapted from[12,13,14]. E.U. stands for Endotoxin Units; sc: supercoiled isoform; oc: open circular isoform of pDNA.

<table>
<tr><th colspan="2" rowspan="2">Content of bacterial cell lysates</th><th colspan="2">Content of Bulk Plasmid Product</th><th colspan="2">Final Product</th></tr>
<tr><th>Component</th><th>Range of acceptance</th><th>Component</th><th>Range of acceptance</th></tr>
<tr><td>55 % Proteins</td><td rowspan="6">21 % RNA</td><td>Endotoxins</td><td><40E.U./mg pDNA</td><td>Endotoxins</td><td><10 E.U./mg pDNA</td></tr>
<tr><td>3 % Host cDNA</td><td>Host DNA, RNA and protein</td><td><1 %</td><td>cDNA</td><td><2 µg/mg pDNA</td></tr>
<tr><td>3 % LPS</td><td rowspan="3">sc form</td><td rowspan="3">>= 80 %</td><td>RNA</td><td><0.2 µg/mg pDNA</td></tr>
<tr><td>>3 % Plasmid</td><td>Protein</td><td><3 µg/mg pDNA</td></tr>
<tr><td>15 % Others</td><td>sc over oc form</td><td>>97 %</td></tr>
</table>

1.1.2. Plasmid DNA isoforms

In plasmid DNA production and purification, different topological morphologies of this molecule can be present. During the recircularization of DNA, several enzymes can add or remove twists in the double helix of DNA, being responsible for the introduction of helical stresses in the molecule that promotes it to twist in the opposite direction of double-helix which generates negative coils, resulting in a compact super-coiled structure, termed as supercoiled pDNA or sc pDNA (Figure 2) [15,16].

Although sc pDNA is the most predominant isoform, other topological isoforms can occur from sc pDNA, which depends on the properties of DNA sequence, supercoiling stress or unfavorable external conditions like extreme pH and temperature [16,17]. Thus, when one of the strands of the double strand suffers a break, the helical stresses are released as well as the super-coiling effect, originating an open-circular isoform, or oc pDNA. On the other hand, when both strands from double helix are broken it results in a linear isoform of the plasmid [15].

From the above mentioned isoforms, the supercoiled isoform has the highest transfection efficiency and biological activity in eukaryotic cells being the preferred isoform in gene therapy [16,18]. It was previously reported that DNA vaccination is more effective in terms of inducing an immune response in the presence of plasmids containing mainly the supercoiled isoform compared to open circular isoform [15,18,19]. This may be related to the fact that the negative supercoiling of sc pDNA helps in the opening of the double-helix, and the excess of energy released is used in cellular mechanisms like replication, transcription or protein binding [1,16,20]. Also, its structure is less prone to the activity of enzymes like exonucleases compared to linear DNA [21]. On the other hand, open-circular and linear pDNA structures may be damaged at random gene locations, possibly affecting transfection efficiency, especially if important gene coding regions or promoters are compromised[1].

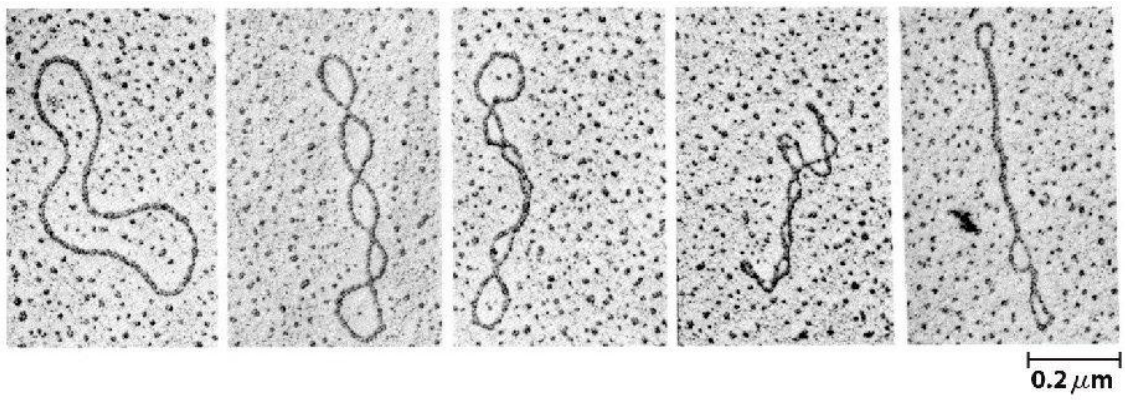


Figure 2: Electron micrograph of relaxed or open circular (oc) plasmid DNA (on the leftmost picture) and increasing levels of supercoiling of plasmid DNA (further four pictures). Adapted from [22].

To be applied in pharmaceutical industry, pDNA final product must have high content in homogeneous supercoiled isoform. Namely, FDA recommends that the bulk plasmid product should contain at least 80% of this specific isoform, as previously indicated in Table 1 [14]. As such, separation methods must be applied to separate supercoiled pDNA from the other isoforms (open circular, linear isoforms) and host impurities present in the bacterial lysate [23].

However, the process of purification of sc pDNA has some challenges and limitations associated. First of all, pDNA is present in low amount in *E. coli* cells, corresponding to less than 3% dry weight (depending on copy number and plasmid size) [24]. Moreover, this isoform is associated to mechanical lability (which is discussed in topic 1.1.3) and to the presence of similar characteristics with other macromolecules that represent impurities in pDNA samples (Table 2).

Table 2: Properties in supercoiled pDNA that are similar to other macromolecules that can be present in plasmid products[24-26].

Properties in supercoiled pDNA	Macromolecules with similar characteristics
Negative Charge	RNA, oc pDNA, gDNA, endotoxins
High Molecular Mass	oc pDNA, gDNA, endotoxins
Bases exposition	RNA
Hydrophobicity	Endotoxins

To overcome those limitations, it is needed to maximize the amount of final plasmid product, which is accomplished by the optimization of specific key-steps in pDNA manufacturing processes (Figure 3)[24]. Plasmid DNA production can be included into two categories: upstream and downstream processing [1]. Upstream processing involves the construction of the plasmid with the gene of interest and its introduction into host bacterial cells, as discussed in topic 1.1. The suspension of bacterial cells containing the plasmid can be stored and aliquoted into cell banks, to be used in further plasmid propagation (plasmid production) [27].

Plasmid production is followed by downstream processing, which includes the recovery or isolation of plasmid molecules from the cells and further steps of purification (specially focused in this work) that increases plasmid purity, ideally reaching the specifications of regulatory agencies, in order to be applied at pharmaceutical level [1]. These steps are discussed in detail in the further topics.

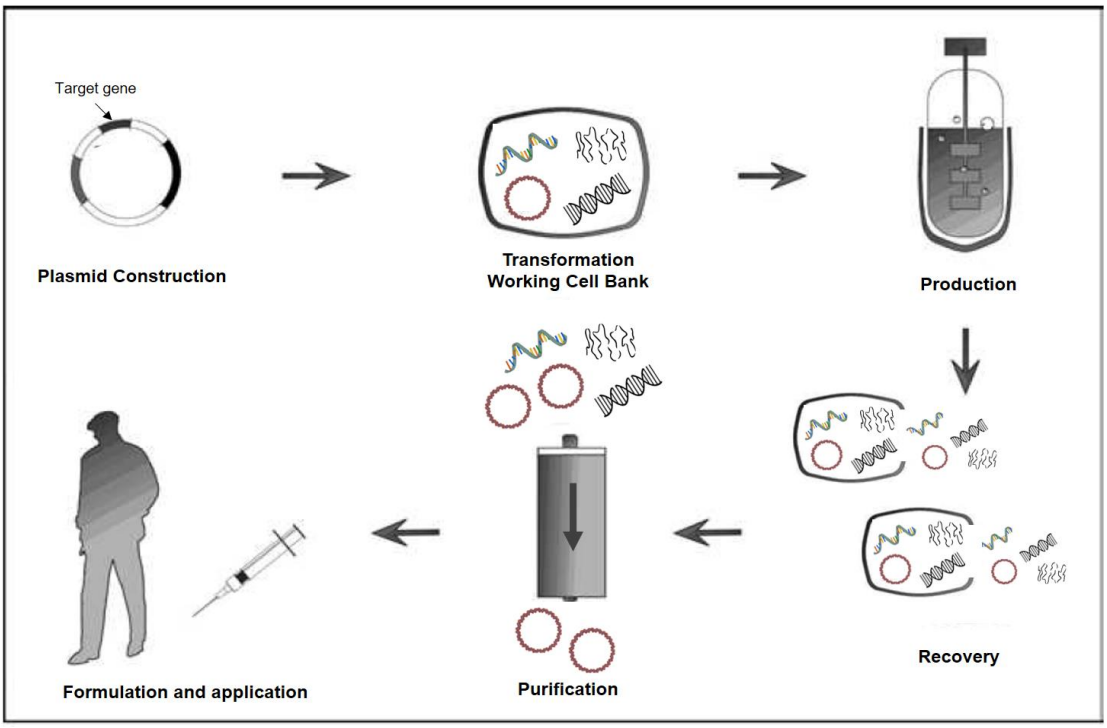


Figure 3: Schematic diagram of the manufacturing process of plasmid DNA, at both laboratorial and industrial scales. Plasmid DNA production involves the construction of the plasmid with the gene of interest and its introduction into host bacterial cells. The bacterial cells containing the plasmid can be used in further plasmid propagation (plasmid production). After plasmid production, plasmid molecules must be recovered and isolated from the cells and purified, ideally reaching the specifications of regulatory agencies, to be applied at pharmaceutical level. Adapted from [1,27].

1.1.3. Plasmid DNA production

1.1.3.1. Choice of bacterial strain and plasmid DNA

As already mentioned, plasmid DNA is introduced in bacterial cells to be replicated and proliferated.

The bacterial strains used for this operation must be carefully chosen since it will influence the plasmid production yield, downstream processing yield, isoform content and presence of endotoxins [7, 8].

Usually, *Escherichia Coli* cells are used in this process. The choice of this bacteria for plasmid production is associated to its rapid growth rate, the ability to grow on chemically defined media and facilitated transformation and genetic manipulation [28]. Regarding *E. coli* strains available, *E. coli* K12 strains, including DH5α, DH5, DH1 or BL21 strains, are considered safe to human health and are commonly used for this process [1,29,30]. In this study, *E. coli* DH5α cells are used. This strain is well-known and versatile, is commonly used in laboratory cloning, having high transformation efficiency and a significant insert stability and plasmid yield, due to mutations in specific genes of the genome [31,32].

Plasmid DNA typically applied in DNA-based therapeutics must have several features that allows them not only to propagate into *E. coli* cells but also to contain therapeutic efficiency and, most importantly, to ensure that are safe models in human health [14,30].

First, to propagate in *E. coli* cells, these plasmids must have an origin of replication that promote their proliferation in those cells [1,4,6]. The presence of a mammalian origin of replication would increase the risk of integration into the human genome [1].

Moreover, the plasmid may be engineered with a marker for selective growth in the host cell, usually a gene that confers resistance to antibiotics, like kanamycin [1,4,6,33]. These markers will exert selective pressure upon the host cells, keeping the plasmids inside the cells[1]. It is recommended that this type of gene does not confer resistance to beta lactam antibiotics, like penicillin, since it can induce severe allergic responses in humans [11].

The therapeutic effectiveness of DNA vectors in mammalian cells requires an efficient expression of these plasmids *in vivo*, which is accomplished by the presence of a strong viral promoter, such as cytomegalovirus or simian virus 40, as well as by an efficient transcription termination and polyadenylation of mRNA, applying, for example, a bovine growth hormone (or BGH) polyadenylation signal [4,6,34].

From the wide range of plasmids available for DNA-based therapeutics, pVAX1/lacZ is a 6050 bp vector that meets the conditions above mentioned and is the one used during this work (Figure A1 – Appendix I). Thus, it is a commercial plasmid containing elements like a cytomegalovirus early-promoter, BGH polyadenylation signal and a kanamycin resistance gene[35]. Also, it is a pUC origin-containing plasmid, which can yield between 500 to 700 copies per bacterial cell [30, 33].

1.1.3.2. Cell Fermentation

Plasmid DNA is introduced in host cells and proliferated due to cell fermentation. During this process, it is desired to obtain a high cell density and plasmid copy number to improve plasmid yield after downstream processing. These parameters are influenced by the host strain, fermentation mode, medium composition and harvesting point. [1,36]

Usually, the production of plasmid DNA is based on cell banks, which are homogeneous suspensions of bacterial cells, containing the plasmid of interest, that are divided into aliquots, required to initiate cell growth [1,27].

At analytical or laboratorial scale, cell fermentation is performed in shake-flasks at a standard speed between 200 to 250 rpm and at a temperature of 37 ºC [32,37]. During this process, an oxygen-limited environment should be avoided since it can trigger the production of toxic metabolites by the cells, like acetic acid, which limits the growth an may lead to cell death [38]. These phenomena can be avoided by let enough empty volume inside the shake flasks to allow an efficient aeration.

Plasmid applied for pharmaceutical purposes contain an antibiotic resistance gene for selection in *E. coli* culture, and thus antibiotic is usually added to the culture media for selective growth. The application of antibiotic is not problematic in terms of human health, since its clearance is achieved during downstream processing of plasmid product. [38].

Regarding culture media suitable for *E. coli* cultivation, there is a wide range of culture media available in the market or formulations that can be followed in the literature, being their selection dependent on the purpose of the bacterial culture [38]. Once again, culture media can influence considerably physiological properties of cells and, consequently, will affect biomass and plasmid productivity [38-40].

From the range of culture media available for bacterial cultivation, two main classes are distinguished: defined and complex media.

Defined or synthetic media is prepared with precise amounts of inorganic and organic compounds that are adapted for a specific bacterium [41]. This type of media is advantageous in terms of improved control of the physiology of the host cells, turning out possible to perform a better control of a specific growth rate or to reach optimal carbon to nitrogen ratio, increasing plasmid and biomass yields [42-44].

On the other hand, complex media contains digest products from microbes, plants or animals, like casein, beef extract, soy beans (tryptic soy broth) or yeast extract, corresponding to carbon and nitrogen sources highly nutritious but with undefined composition. Their unprecise composition corresponds to a disadvantage, since it may contain carbon to nitrogen ratio different from the optimal value, which possibly will decrease the plasmid yield and affect cell wall polysaccharide synthesis [41], [43-45]. Nevertheless, this type of media is commercially available, low-priced, providing biosynthetic intermediates to the cells and being associated to fast growth [41,44,45].

A known example of complex media is Luria-Bertani or LB medium, a commercially available media that is usually used for laboratory research. It is composed of tryptone peptone, yeast extract and sodium chloride and may include other compounds like amino acids, vitamins, metal ions. Also, yeast extract can also contain considerable amounts of adenosine, trehalose and lactate, important in bacterial growth [44,45]. Although this formulation yields low cell mass compared to defined media, it is adequate at a laboratorial-scale [38].

E. coli is a microorganism that is able to grow in both complex organic media and in salt-based chemically defined media containing organic carbon sources [38]. Namely in this work, the plasmid used is produced at a laboratorial-scale, which validates the use of LB medium in this process.

Considering the ideal harvesting-point for *E. coli* DH5α cells, it is advisable to harvest the cells from incubation at an optical density (O.D. at 600 nm) around 3.0 , corresponding to mid-exponential phase, since it was reported that cells harvested at this point have higher efficiency of transformation [46].

To harvest the cells, a step of centrifugation or microfiltration is used not only to concentrate the cell slurry but also to remove the excess of fermentation broth which may affect further steps of cell lysis and purification [38].

1.1.4. Plasmid Purification Strategies

1.1.4.1. Primary isolation of plasmid DNA

After fermentation and cell harvesting, it is needed to disrupt the cells to remove their inner content. Mechanical methods, like high pressure homogenization, in which cell suspensions are forced to pass through narrow gaps at high pressures to promote their lysis can be applied [47]. However they should be avoided since they induce shear stress in genomic and plasmid DNA, producing fragments of gDNA that difficult further purification steps [36,48]. At analytical or laboratorial scales, enzymatic reagents are usually applied to perform enzymatic degradation, such as RNAse or lysozyme. Nevertheless such procedures are not recommended by regulatory agencies, rising concerns about human health safety, and are not a cost-effective alternative [9,36]. Also, several purification kits are commercially available to isolate plasmid DNA, consisting in disposable chromatographic columns or resins, that despite their efficiency, are expensive alternatives and are not suitable for high demanding processing [49].

For preparative and large-scale processing, regarding the purpose of the final product, it is mandatory to use only reagents that are considered to be safe for human health by regulatory agencies like FDA (known as GRAS Reagents), which includes reagents without enzymatic and animal origin [9],[50].

Alkaline lysis method, originally described by Birboim et al., is a promising alternative not only to release the inner content but also to perform a primary isolation of plasmid DNA, which can be applied to scales up to 5 liters of cell culture [51,52]. In this method the cells are subjected to an isotonic solution containing glucose, followed by the addition of an alkaline solution with SDS and sodium hydroxide that disrupts the cell membrane and denature proteins and genomic DNA (which becomes single-stranded due to complementary strand separation or cleavage) [17, 51, 53-55]. This suspension is neutralized with a solution containing acetic acid and potassium acetate, which precipitates SDS-protein complexes and RNA, that are removed by centrifugation, while plasmid DNA remains soluble in the supernatant [51].

The use of alkaline lysis can denature pDNA, reducing the amount of supercoiled covalently packed DNA and increasing the amount of open circular isoform, since the structure of supercoiled isoform is unfolded during this process [12],[49],[56]. The level of pDNA denaturation depends on alkali concentration, time of exposure and pH neutralization after potassium acetate addition (renaturation was previously reported at a pH value bellow 12.5), thus these parameters must be optimized in order to avoid significant plasmid DNA losses [55,56].

In order to reduce the presence of RNA and other impurities, a primary isolation can be performed after alkaline lysis, Figure 4, in which isopropanol is used to concentrate nucleic acid molecules, that are recovered from the pellet after centrifugation, being followed by the addition of ammonium sulphate, used to precipitate proteins and RNA, allowing partial isolation of pDNA, present in the supernatant [17]. This strategy was the one adopted to reduce the level of impurities in the cell lysates used in the present work.

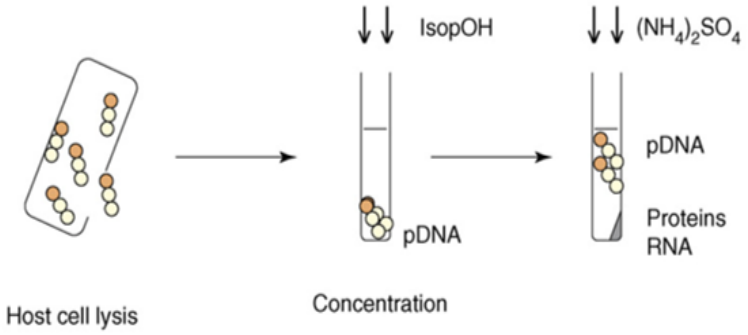


Figure 4: Schematic representation of the method of primary isolation adopted in the present work. To perform primary plasmid isolation, alkaline lysis procedure is applied to break host cells and release the inner content and isopropanol (IsopOH) is applied to concentrate pDNA, which is recovered in the pellet by centrifugation. Further addition of ammonium sulphate ($(NH_4)_2SO_4$), precipitates impurities such as proteins and RNA, isolating plasmid DNA in the supernatant. Adapted from [17].

It was reported that an additional precipitation of the previous suspension with a polymer - PEG-8000 - in a NaCl solution can enrich pDNA concentration, reduce the amount of RNA and decrease the presence of salt from the ammonium sulphate step, yielding a more selective isolation of plasmid DNA [57,58].

Alternatively, specific reagents, like organic solvents, toxic chemicals or animal-derived solutions, can be used to remove individual impurities such as RNA, endotoxins or nucleases [9,58]. However, those techniques are associated to costs of production due to its high price per activity unit and cannot be used for pharmaceutical purposes since these enzymes are derived from animal sources [8,10]. On the other hand, at preparative and large-scale of production of plasmid DNA, membrane processes, such as tangential flow filtration, can be applied for selective isolation of macromolecules from the lysate [9,59]. In this method, the clarified lysate passes through a membrane that, due to different molecular size, allows the removal of RNA through the membrane pores (to the permeate) while plasmid DNA remains in suspension (in the retentate), allowing direct purification of pDNA from the lysate. Nevertheless, this technique was reported to be insufficient for endotoxins and RNA removal, requiring optimization of the process [59].

It is important to mention that temperature and ionic strength are parameters that may affect the overall structure of supercoiled isoform. Thermal fluctuations may promote changes in the tridimensional structure of DNA, due to partial removal of negative coils, resulting in different degrees of supercoiling due to molecular overwound or underwound [60]. On the other hand, the presence of salt in plasmid solution will interact with the negative charges of DNA, promoting the bending of the molecule which results in a highly compact structure [61].

To avoid plasmid losses and to obtain a homogeneous and pharmaceutical-grade plasmid product, several steps should be adopted to keep supercoiled isoform integrity. As such, the *E. coli* pellets from fermentation and the lysates resultant from primary isolation should be stored at -20ºC till further processing, which keeps sc pDNA stable up to 8 weeks or 12 weeks for the cells resultant from growth [55]. Regarding salt-containing samples, it is advisable to remove salt before storing using

desalting methods, such as micro-dialysis, in which the samples are placed in contact with a dialysis membrane removing the salt present in solution [57].

1.1.4.2. Final plasmid purification

After removal of impurities excesses from the cell lysate, it is aimed to perform a final polishing of the clarified lysate to increase the level of purification of the plasmid DNA isoform of interest, ideally reaching a final product that is suitable for pharmaceutical-grade application, fulfilling the requirements of regulatory agencies. This process usually includes one or more purification steps that can be divided into two major classes: non-chromatographic and chromatographic methods, as discussed in the next items[36].

1.1.4.2.1. Non-chromatographic methods

In this class, several methods can be applied to isolate or partially isolate the molecule of interest, including affinity precipitation and aqueous two-phase extraction.

Affinity precipitation results from the interaction of DNA or RNA molecules with specific ligands that are attached to polymers. A lysate containing the desired molecule is incubated with those polymers followed by the induction of precipitation of the target molecule while other remain soluble. This technique has been applied for plasmid DNA purification due to the presence of specific affinity ligands, such as oligonucleotides or metal chelating ligands, that binds selectively to plasmid DNA or RNA, producing a complex that precipitates and can be recovered after precipitation (Figure 5) [62,63]. This technique was reported to obtain pDNA recovery yields between 70 to 90 % [62].

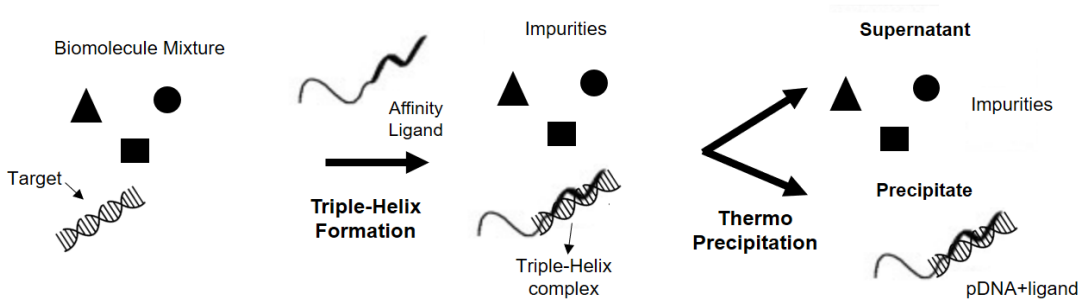


Figure 5: Principle of affinity precipitation using an affinity ligand containing an oligonucleotide for plasmid DNA precipitation and recovery. The target molecule present in an initial mixture, plasmid DNA, binds to the affinity ligand forming a triple helix complex that precipitates due to temperature increase (thermoprecipitation), while the remaining molecules stay solubilized in the supernatant. After, the precipitate can be recovered and the DNA molecules dissociated from the ligands. Adapted from [62].

Aqueous two-phase systems (ATPS) are composed of two immiscible phases, generally one composed of a polymer (usually PEG), corresponding to the top phase and another corresponding to the bottom phase that contains and a salt (like phosphate, sulphate or citrate) or a second- polymer [64,65]. It is possible to separate different molecules according to the properties of the molecule (molecular size, chemical properties) and the properties of two-phase system, which determine the partitioning of the molecules in a sample between both phases [64,65]. Systems composed of PEG,

namely PEG 600, and a K_2HPO_4 salt were reported to be able to separate plasmid DNA which was partitioned to the bottom-phase, obtaining recovery yields up to 67 %, from proteins and genomic DNA while RNA was mainly found between phases.[64]

1.1.4.2.2. Chromatographic methods

Chromatography is a simple technique in which the sample under study can interact with two phases physically different – a mobile phase and a stationary or solid phase.

Mobile phase can be a gas (gas chromatography) or a liquid (liquid chromatography) that allows the interaction of the sample through the stationary phase. The molecules contact with both phases. If the molecules have higher affinity to the stationary phase it is expected that they are retained closed to this phase for a longer time. On the other hand, when a given molecule has a weak affinity to the stationary phase, it will be rapidly eluted with the mobile phase [66]. Regarding the aim of pDNA purification, chromatography, mainly liquid chromatography, has been widely applied and is considered the method with highest resolution, which explains its use for pDNA therapeutic-grade [36].

In general, liquid chromatography is composed of six key steps, presented in Table 3. In this technique, the plasmid molecules of interest are distributed between mobile and stationary phase, thus the nature of mobile phase should be adapted to the nature of the stationary phase, not only to ensure the stability of sc pDNA but also to avoid significant losses of this isoform [26]. The range of techniques already tested for this purpose includes size-exclusion chromatography (or SEC), anion-exchange chromatography, reverse-phase chromatography (RPC), hydrophobic-interaction chromatography (HIC) or affinity chromatography.

Table 3: General workflow in liquid chromatography. Adapted from [67,68]

Chromatographic Steps in Liquid Chromatography	
Column Equilibration	Buffer compatible with the resin and target molecule passes through the column (equilibration buffer).
Sample Loading	The sample to be purified is added to the column generally conditioned in the equilibration buffer.
Washing Step	The molecules weakly or nonspecifically attached to the resin are removed from the column, using a buffer (washing buffer) that is usually the same as equilibration buffer.
Elution Step	The molecules that are strongly attached to the resin are removed from the column due to change of buffer composition (elution buffer), which weakens the molecule-matrix interactions.
Column Regeneration	The molecules still attached to the resin after the elution step are eluted from the column using a regeneration buffer (e.g. containing NaOH or NaCl)
Column storage	The column is filled with storage buffer, generally ethanol 20%(v/v) to avoid microbial contamination.

Size-Exclusion Chromatography, or SEC, is based on the different molecular size of molecules in a sample where the smaller molecules stay retained inside column pores for longer time while the larger ones are directly eluted since they cannot penetrate inside the pores [66]. It was

previously reported that, using Sepharose as solid support, it was possible to separate pDNA from a bulk sample with RNA and other impurities, mainly by increasing the concentration of the salt present in the buffer (ammonium sulphate) [12]. However, this method has a low resolution and there is a risk of co-elution of pDNA isoforms and gDNA, due to similar size (Table 2) [17].

Anion-exchange chromatography is commonly used for preparative and analytical scale pDNA purification [12]. In this approach, the solid support has positively charged functional groups and negative counter-ions being called an anion-exchanger [66]. In previous works, anion-exchange chromatography has been reported to offer a good selectivity for pDNA allowing its separation from RNA and other impurities. However, it is associated to low resolution and co-elution of endotoxins with nucleic acids [12,17].

Another technique used in plasmid purification is reverse- phase chromatography, or RPC. This term results from the fact that the eluent used is more polar than the stationary phase [69]. In fact, this chromatography is based on hydrophobic interactions between the solute and a non-polar stationary phase, resulting in the elution of solution in decreasing order of polarity. This technique had demonstrated to be effective in endotoxin removal from *E. coli* lysates being mainly applied at laboratorial-scale [12,53]. Nevertheless, this method requires the use of organic solvents that are not recommended by regulatory agencies [17].

Regarding hydrophobic Interaction chromatography, or HIC, macromolecule separation is based on the different surface exposition to hydrophobic bases. Namely, HIC is able separate single-stranded from double-stranded nucleic acids – pDNA - due to different interaction with the stationary phase[56]. Single stranded nucleic acids, such as RNA, oligonucleotides or denatured gDNA and pDNA (that may contain single-stranded regions), show higher exposure of hydrophobic aromatic bases compared to double-stranded nucleic acids, such as plasmids, in which the hydrophobic bases are shielded and packed inside the double helix [53]. As such, the species with less hydrophobic regions exposed will be eluted first while the denatured species and RNA are retained for longer time, due to non-covalent interactions, like van der Waals forces, with the hydrophobic ligands derivatized in the stationary phase [53,56].

To promote hydrophobic interaction in HIC, a salt-containing buffer must be used. The salt-type chosen is determinant for a successful separation in HIC. The interactions between ions and water and ions and molecules can be inferred by the Hofmeister series (Figure 6), in which the ions are ordered from strongly hydrated, or kosmotropic ions, and weakly hydrated, or chaotropic ions. Kosmotropic ions present a stabilizing effect or salting-out effect on proteins and macromolecules, while chaotropic ions have a destabilizing effect, or salting-in, on proteins and macromolecules. Thus, a kosmotropic salt is desired in this method. Ammonium sulphate (a concentration between 1.0 M and 2.4 M) is usually used for this chromatographic method, but sodium citrate was also reported as a possible alternative to the former [70]. The use of this type of salt at high concentration represents a drawback, regarding the difficulty in waste treatment rising concerns about the effects of environmental disposal [70].

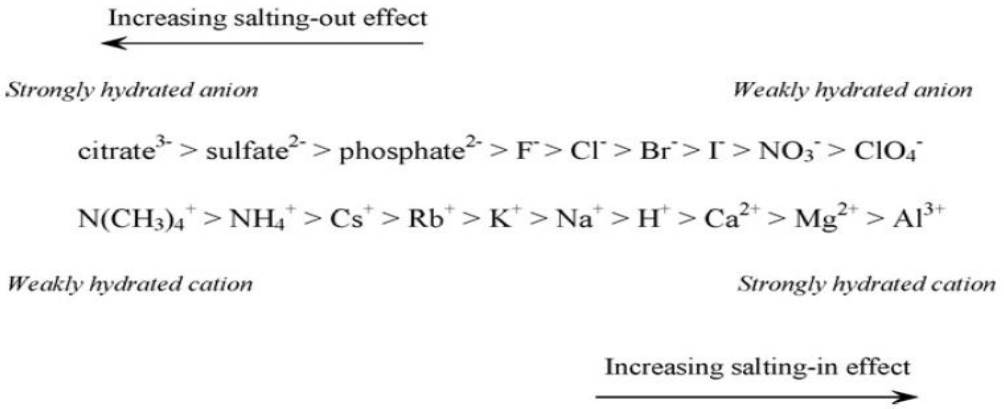


Figure 6: Hofmeister series containing the ions ordered by their affinity to water and respective effect on macromolecule solubility. Adapted from [70].

Affinity chromatography is another approach that has shown significant advances and potential results for pDNA purification. This chromatographic class will be discussed in the next topic.

At industrial-scale, it is usual to apply sequential steps with different chromatographic methods resulting in an efficient way of recovering plasmid from cell lysate, yielding considerable content in supercoiled pDNA and reducing the amount of impurities in the final product, compared to other single-step chromatographic protocols [36,52,70].

1.1.5. Affinity chromatography applied for plasmid purification

Affinity chromatography, or AC, was developed from the need of higher specificity in chromatographic approaches [66]. The basis of this type of chromatography is molecular recognition. In this approach, if a given molecule prone to purification has specific sites that can be recognized by a specific molecule, the latter can be immobilized in a insoluble stationary phase support that is packed in a chromatographic column [66,72]. Then, a cell lysate containing the desired molecule can be passed through the column, where the molecules that have affinity with the ligand are retained inside the column, due to strong but reversible interaction with affinity ligand, while other components that do not interact with the ligands are directly washed out from the column [66,72,73]. To elute the desired molecule from the column, external conditions like pH, ionic strength or solvents can be modulate in order to weaken the interactions between this biomolecule and the ligand and to remove it to the liquid phase [72,74]. The interactions between the ligand and the molecule under purification emerge from non-covalent interactions, such as electrostatic and hydrophobic interactions, van der Waals or hydrogen bonding. Those interactions are responsible for the high selectivity and resolution of this chromatographic approach [17].

This technique allows the purification of a broad range of molecules: antibodies, antigens, enzymes, hormone-binding proteins, glycoproteins, RNA and DNA, bacteria, viruses and phages and other molecules [72].

Affinity chromatography allows the elimination of additional steps in downstream processing, however, the type and origin of the ligands can be a limitation to its use, as discussed in the next item [17].

Despite important advances in ligand design have been reached for protein purification, this area has not been extensively described under the light of plasmid purification [17].

1.1.5.1. Type of affinity ligands

There are two main classes of ligands used in affinity chromatography: biospecific/natural ligands and pseubiospecific ligands.

Biospecific ligands are derived from natural sources and represent the most traditional adsorbents used in affinity chromatography. These ligands include antigens, antibodies, bacterial immunoglobulin-binding proteins (like protein A or G), amino acids and others [74]. These molecules have high selectivity and affinity for their complementary targets. Despite the previous characteristics, high costs of production and purification are associated with natural ligands as well as low binding capacities for the target molecules, limited scale-up potential, difficulties in sterilization and reduced life cycles. [75,76] Moreover, the use of these ligands can result in final product contamination due to ligand degradation since stronger dissociation methods may be needed to elute the bound molecules [74].

From the biospecific ligands used in affinity chromatography, amino acids are a promising alternative that may overcome the above-mentioned limitations. Affinity chromatography using amino-acids is based on the natural interactions found between amino acid residues in proteins or isolated amino acid molecules and different regions of nucleic acids [77]. In this case, instead of using complex proteins, whose production and purification is expensive, amino acids are used as affinity-ligands since they are highly stable (physically and chemically) and are possible to obtain in large amounts at low-costs [73,77].

Several types of interactions have been detected between amino acid and nucleic acids, like electrostatic interactions, hydrogen bonds, van der Waals, water-mediated, hydrophobic, stacking and cation-π interactions, which will be further discussed in detail in this section [78].

While hydrophobic interactions involve non-polar groups, such as aromatic rings, electrostatic interactions occur between positively charged amino acids, such as lysine and arginine, and negatively charges in the nucleic acids [22]. Regarding hydrogen bonding, it usually occurs between amino acids and an acceptor group, such as the oxygen atom of phosphate group, or an hydrogen donor in the nucleic acid [77]. The potential positions for this type of interaction are mainly located in DNA grooves (Figure 7), which result from the spaces between adjacent turns of the double helix, that can have different dimension, namely a minor groove and a major groove. Most of interactions occur in positions W2, in major DNA groove, and S1, in minor DNA groove, due to the presence of potential hydrogen-bonding atoms (Figure 7). Polar (Asparagine, Glutamine, Histidine, Serine and Threonine) and positively charged amino acids (Arginine and Lysine) are the ones more likely to interact with

nucleic acids by hydrogen-bonding [77]. Water-mediated bonds are non-specific and indirect hydrogen bonds, acting as space-fillers in the interface protein-DNA, which may contribute to the recognition process [77,79]. These interactions occur between amino-acids and the oxygen atom from phosphate group, generally involving polar or positively charged amino acids, but also Glutamic Acid and Aspartic Acid, since they are able to interact at distance [77].

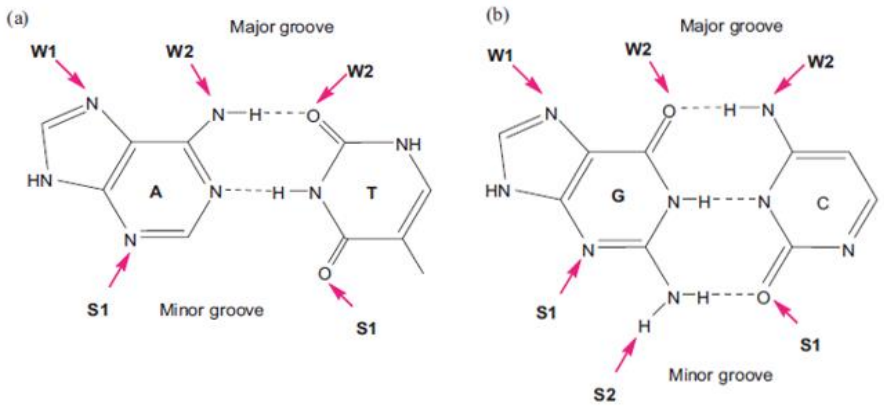


Figure 7: Schematic diagrams containing the potential positions (marked by the arrows) for hydrogen bond in DNA grooves. W stands for the recognition sites in major groove, while S stands for the recognition sites in minor groove. (a) Base pairing between Adenine (A) and Thymine (T), containing two hydrogen-bonds among them (shaded-line) and (b) Base pairing between Guanine (G) and Cytosine (C) with the respective three hydrogen-bonds between them (shaded-line). Adapted from [77].

Van der Waals interactions occur mainly between amino acids and the phosphate group in nucleic acid bases, due to its high exposure on DNA surface, but also between amino acids and the sugar group of nucleic acids [79]. Amino acids most involved in this type of interaction include Arginine, Threonine, Phenylalanine, Isoleucine, Glutamine, Lysine, Glycine, Serine or Histidine.

Previous studies have also reported the presence of cation-π interactions between amino acids and DNA. This type of interaction occurs between positively charged groups of amino acids, mainly from Arginine, and the aromatic rings of DNA (Figure 8) [77,80]. Moreover, amino acids such as Phenylalanine, Proline and Histidine have high affinity for many base types and for this reason may also perform π-π stacking interactions (aromatic rings of amino acid and nucleic acids are overlapped parallel to each other) with the aromatic rings of deformed DNA structures (Figure 8) [79,81].

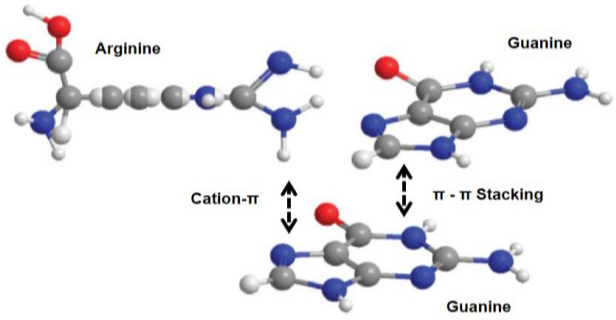


Figure 8: Schematic diagram of cation-π and π- π stacking interactions between arginine and guanine. The carbon atoms are colored in grey, hydrogen in white and nitrogen in blue. Adapted from [77].

In amino acid affinity chromatography, the elution of a target molecule is possible by changing the ionic strength, pH or polarity of the elution buffer. For example, hydrophobic interactions are strengthened by the presence of high salt concentration, from kosmotropic salts like ammonium sulphate or sodium citrate (Figure 6). Moreover, a competing agent, such as a solution containing amino acids can be used to bind to the target molecule or the solid-support, which depends on their amino acids properties, weakening the interaction between ligand and target molecules [78].

Affinity chromatography based on amino acid-nucleic interaction have been explored in the past years for the purification of supercoiled plasmid isoform, yielding promising results in this field. Amino acids already explored for plasmid purification include Histidine, L-methionine, Lysine, Arginine or L-tryptophan.

With histidine-based chromatography, sc pDNA isolation from other isoforms and host impurities from a clarified *E. coli* lysate was possible by using a stepwise elution with decreasing concentration of ammonium sulphate, retaining sc isoform and RNA, through hydrophobic interactions between histidine-support and this isoform, being sc pDNA firstly eluted followed by RNA elution, while oc isoform and gDNA were eluted in flowthrough [82].

L-methionine derivatized supports were able to purify sc pDNA from clarified *E. coli* lysates, reducing significantly the amount of proteins, gDNA and RNA, by applying a stepwise elution with initial high concentration of ammonium sulphate at low temperature. The occurrence of hydrophobic interaction with the nucleic acids allow the selective separation, similar to the one observed in histidine chromatography [83].

Chromatography approaches using lysine as affinity ligands yielded purified sc pDNA isolated from other isoforms and containing host impurities below the levels recommended by regulatory agencies. Selective separation was possible when crescent ionic strength, retaining sc pDNA and eluting oc pDNA in flow through. The interactions between lysine and nucleic acids include electrostatic interaction between positively charged amino acid and phosphate groups from nucleic acids, as well as hydrogen-bonding [85,86]. Previous studies suggested that this amino acid has specific recognition of the supercoiled isoform, explaining the selective retention of this isoform [85].

Arginine-affinity chromatography was also used for plasmid purification, since arginine interacts with guanine bases via hydrogen-bonding. Isolation of sc pDNA was obtained with high purity and stability, using increasing concentrations of NaCl, in which oc pDNA is eluted al lower ionic strength , followed by sc pDNA elution at higher ionic strength, due to stronger interaction with arginine [86].

Recently, L-tryptophan was used as a potential ligand in affinity chromatography, using a stepwise elution with ammonium sulphate, where sc isoform was retained due to hydrophobic and stacking interactions and isolated from other isoforms [73].

Regarding the second class of affinity ligands, pseudobiospecific ligands are small and chemically defined molecules that can have biological or synthetic origin [74,88]. This group can overcome the limitations of biospecific ligands, being easily produced at lower costs [74,89].

Moreover, these ligands have lower toxicity and immunogenicity as well as increased resistance to chemical and biochemical degradation, which allows *in situ* sterilization without loss of quality [74,89]. Pseudobiospecifc ligands can be divided in non-mimic and biomimetic affinity ligands [74].

Considering non-mimic affinity ligands, there are several examples, such as hydroxyapatite crystals, chelating compounds or multi-modal.

Hydroxyapatite chromatography uses hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$] which have a structured arrangement of interactions points. Immobilizing these crystals to a solid support it is possible to retain positively charged molecules due to interactions with negative charges of phosphate groups, while negatively charged macromolecules coordinate with calcium sites of the crystal. It is possible to elute the retained molecules by changing the ionic strength or adding agents with high affinity to calcium [74]. These ligands have shown to be selective for RNA, promoting the removal of RNA in excess in cell lysates. However it may difficult the further removal of endotoxins and genomic DNA [12].

Another non-mimic- ligand based technique is immobilized metal affinity chromatography, or IMAC, which is based on the use of chelating compounds covalently attached to a solid support. Those compounds are responsible for metal ion entrapment ($Ni^{2+,}$ Zn^{2+}, Cu^{2+} or Co^{2+}) which act as affinity ligands for target molecules [74]. Despite being widely used for protein purification, IMAC approach can also be used for the retention of single-stranded nucleic-acids like RNA and endotoxins for which it was demonstrated to have high binding affinity, while showing low binding affinity to plasmid or genomic DNA.[33,34].

Mixed-Mode or multimodal ligands are composed of a hydrophobic core, like a hydrocarbon or hetero-aromatic ring molecule, and a hydrophilic or ionic group attached to it, which can be an amino or carboxyl group. The binding strength to the target molecule depends on the pH value rather than ionic strength, being the elution performed through pH variation. [74] A Multimodal ligand, namely Capto™Adhere Ligand (Figure 9), was recently reported to efficiently isolate sc pDNA from other impurities from a clarified *E. coli* lysate, like oc pDNA and RNA, containing levels of gDNA and protein according to specifications. This behavior was obtained applying a stepwise elution with a NaCl-buffer, used to modulate hydrophobic and charge interactions between the several elements of the multimodal ligand and the nucleic acids [57].

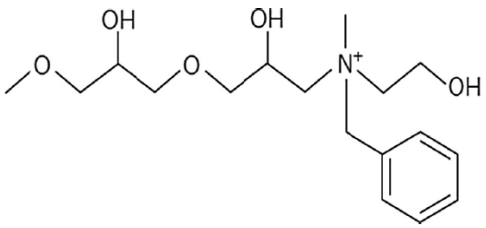


Figure 9: Example of a multimodal ligand, Capto™Adhere, namely N-benzyl-N-methyl ethanolamine, which is able to establish anion-exchange (due to charged nitrogen), hydrogen bonding (with the hydroxyls) and hydrophobic interactions (due to the presence of phenyl ring). Adapted from [57].

Regarding the other class of pseudobiospecific ligands, biomimetic ligands**,** it includes fully synthetic molecules [74]. A biomimetic ligand mimics a natural ligand being an improved version of the latter, due to its chemical resistance to degradation, increased scale-up potential, ability for sterilization and high ligand specificity [74,75].

To improve the chemical and biological properties of these ligands, the rational design of small and stable compounds must be adopted, as discussed in topic 1.1.6. [74].

Synthetic ligands based on a triazine structure have been developed for target molecule purification using affinity chromatography. These synthetic ligands contain non-fissile bonds and use a triazine derivative, respectively cyanuric chloride or 1,3,5-trichloro-sym-triazine (Figure 10 a), as the scaffold molecule for the design of ligand libraries [88]. The structure of cyanuric chloride allows the introduction of natural and non-natural amino acid side chain residues, due to the presence of three chlorines that are prone to be replaced by reactive amine substituents (Figure 10 a) [91]. This triazine scaffold can be immobilized into a solid support, which is possible by the displacement of one of the chlorines by the amine groups present in the resin used, such as aminated agarose, which yields cyanuric chloride activated agarose, presented in Figure 10 b.

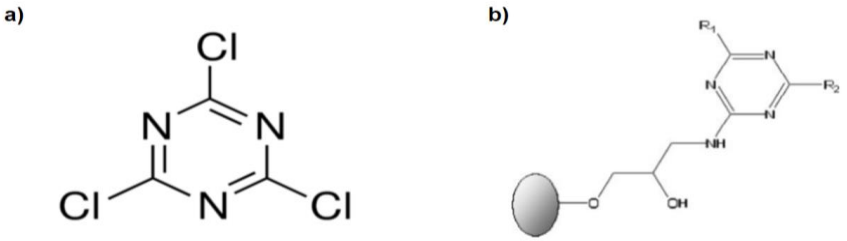


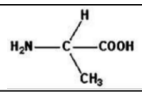
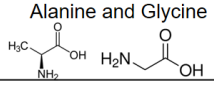
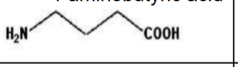
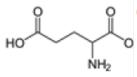
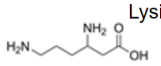
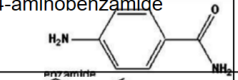
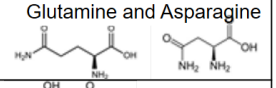
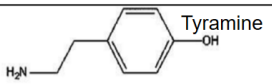
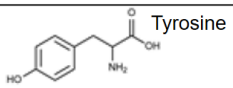
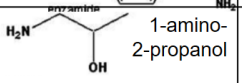
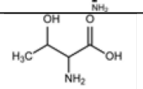
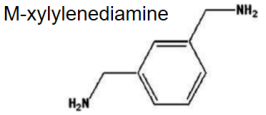
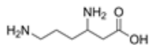
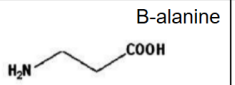
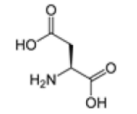
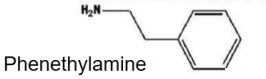
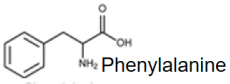
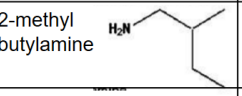
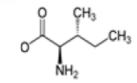
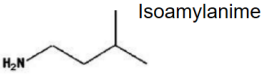
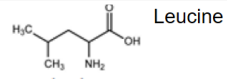
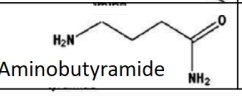
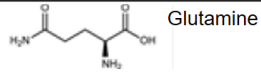
Figure 10: General structure of a) cyanuric chloride (1,3,5-trichloro-sym-triazine) and b) the solid-phase triazine-based ligands used in this study. R1 and R2 positions are replaced by the amines presented in table. Adapted from [113].

Different amine substituents, respectively at R1 and R2 positions can be used to replace the chlorines of the cyanuric chloride molecules (Figure 10). The substituents can be aliphatic or aromatic amines that mimic the side chains of specific amino acids, being able to simulate the natural interactions between amino acids residues from proteins and nucleic acids. Thus, the resultant ligands will have similar characteristics of the amino acids used amino acid-based chromatography and similar elution conditions can be applied, according to the target molecules and properties of the ligand.

Affinity-chromatography using triazine-based ligands has been extensively explored for the purification of antibodies, Fab fragments and cutinase, in which it was reported that those ligands are able to mimic natural protein adsorbents, such as protein A (SpA) or protein L (PpL), maintaining the affinity and specificity for the target molecules [75]. However, there are still no published studies describing and applying this type of ligands for plasmid purification (only preliminary studies in master thesis), representing an innovative technique in this field.

In this study, affinity chromatography using triazine-based ligands was used to study their ability to purify supercoiled pDNA from other impurities presents in an *E. coli* lysate, such as open-circular, linear pDNA or RNA. The ligands chosen were based on a synthetic chemical library whose substituents R1 and R2 are listed and presented in Table 4 [75].

Table 4: Structure of the substituents used in the construction of a large combinatorial library that was used to select ligands, and the respective analogue amino acids. Adapted from [75]. In this list, NH_3 is considered to be amine 0.

Number	Chemical Structure	Analogue amino acid	Number	Chemical Structure	Analogue amino acid
1	 L-alanine	 Alanine and Glycine	7	 4-aminobutyric acid	 Glutamic Acid
2	 1,5-diaminopentane	 Lysine	8	 4-aminobenzamide	 Glutamine and Asparagine
3	 Tyramine	 Tyrosine	9	 1-amino-2-propanol	 Threonine
4	 M-xylylenediamine	 Lysine	10	 B-alanine	 Aspartic Acid
5	 Phenethylamine	 Phenylalanine	11	 2-methyl butylamine	 Isoleucine
6	 Isoamylanime	 Leucine	12	 Aminobutyramide	 Glutamine

Substituent 1, or L-alanine, is analogue to alanine, a non-polar amino acid that is able to perform hydrophobic and van der Waals interactions as well as hydrogen bonding, and glycine , a simple and small amino acid also involved in van der Waals interactions [22,77]. On the other hand, 1,5 diaminopentane (2) and M-xylylenediamine (4) mimic Lysine, a positively charged amino acid that is able to perform electrostatic interactions with negatively charged groups, acting as hydrogen-donor [22,77]. Tyramine (substituent 3) is analogue to tyrosine, containing an aromatic ring that confers hydrophobic character to the molecule, participating in hydrophobic interactions, and a hydroxyl group that can form hydrogen bonds with others groups [22].Substituent 5, phenethylamine, mimics phenylalanine, containing an aromatic ring prone to hydrophobic interactions, including stacking interactions [77]. Isoamylamine is an analogue of Leucine, another amino acid with non-polar side chain that is involved in hydrophobic interactions. Amine 7, 4-aminobutyric acid, is analogue to glutamic acid, while amine 10 (β-alanine) is analogue to aspartic acid, two amino acids that have negative charged group at pH 7, which may involve unfavorable electrostatic interactions with negatively charged phosphate groups of nucleic acids [22]. Nevertheless, these amino acids can be involved in water-mediated bonds [77]. 4-aminobenzamide is similar to glutamine and asparagine, which contain polar amide groups, but includes an extra aromatic ring that increases its hydrophobic profile [22]. Substituent 9, 1-amino-2-propanol , mimics a polar amino acid (Threonine) that is involved in van der Waals interactions and hydrogen bonds and that was reported to stabilize DNA backbone [22,77,79]. 2-methyl-butylamine is analogue of Isoleucine, a non-polar molecule that can interact by

van der Waals and hydrophobic interactions [22,77]. To finish, substituent 12 is analogue to glutamine, as substituent 8.

These substituents are inexpensive and commercially available, except for compound 12, that needs to be previously synthesized [75]. The use of these substituents instead of amino acids may increase target molecule specificity. Amine substituents were rationally designed to contain the key features in interaction with target molecules and not entire amino acid structure, while amino acids may present groups (e.g. NH_2 or CO groups) that can interfere in target molecule biorecognition [76]. Moreover, triazine ligands allow the introduction of two different amine substitutions, thus these ligands act as dipeptides, unlike natural amino acids (Table 4).

Regarding the synthesis of the ligands used for plasmid purification , they are a result of a solid-phase assembly process in a agarose support, in which the chlorines from the cyanuric chloride molecule are sequentially substituted by the amine substituents (Table 4) [75]. The first chlorine on cyanuric chloride is replaced by aminated agarose at 0 ºC for 2 hours, yielding dichlorotriazinyl agarose or cyanuric chloride activated agarose (Figure 11). After that, the second chlorine is substituted by the first or R1 amine substituent (R1 substitution) by incubating cyanuric chloride activated agarose with this substituent in excess at 30ºC for 24 hours. The third chlorine is then replaced by the second or R2 amine substituent (R2 substitution) by subjecting the R1 substituted molecule to R2 substituent for 72 hours at 83 ºC, yielding a final ligand R1/R2 (Figure 11) [93,94].

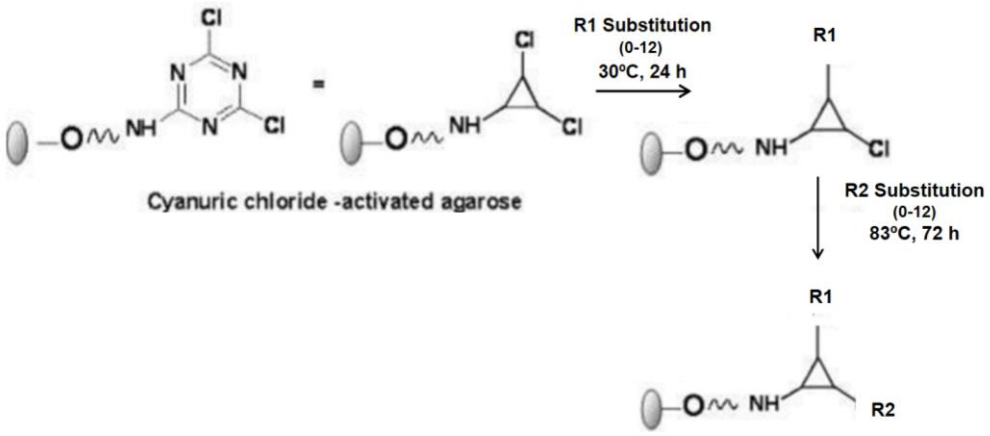


Figure 11: General solid-phase synthesis of the synthetic triazine-scaffolded mimetic ligands. Substituents presented in table 4 are used R1 and R2 substitution. Adapted from [93].

1.1.5.2. Solid supports used in affinity chromatography

There are several chemical supports available on the market that can be classified according to their chemical properties as natural polymers, synthetic polymers, inorganic material and composite materials, as presented in Table 5 [94].

In general, for a solid support to be used in affinity chromatography, the functional groups present in the matrix must be firstly activated followed by the immobilization/coupling of a specific ligand to the functional groups [66].

Table 5: Example of chemical properties of solid supports applied to affinity chromatography. Adapted from [26].

Category	Basic Material	Physical Shape	Trade name
Natural Polymer	Cellulose	Fibrous crosslinked	CELBEADS
	Nitrocellulose	Fibrous	-
	Dextran	Particles	Sephacryl S
		Particles cross linked	Sephadex G
	Agarose	Particles	Sepharose 4B
Synthetic Polymer	Polyacrylamide derivative	Cryogel	Trisacyl or Hyper
	Polymethacrylate	Pous continuous bed	CIM
	Polystyrene	Microparticles	-
Inorganic Material	Hydroxyapatite	Porous crystal	-
	Silica	Particles	-
	Glass	Coated with polystyrene	-
Composite Material	Methacrylate+Cryogel	Supermacroporous	PHEMAH

In this work, two different matrixes were used: Sepharose CL-6B and a EDA CIM® monolithic disk.

Sepharose CL-6B™, is a cross-linked agarose matrix. This medium has a long hydrophilic spacer arm, useful for the immobilization of small molecules [95]. This matrix has been used as a solid support for affinity chromatography since the material accomplishes the exigencies of solid-phase synthesis and the qualities needed for affinity chromatography [88]. However, this matrix has non-uniform pore distribution which may affect the flow rate of liquid phase [96]. This matrix was used in this work as solid-support for immobilization of the tested ligands, following a procedure described in section 3.6., and packed in chromatographic columns.

CIM® monolithic disks were also used in this work. CIM® or Convective Interaction Media is based on convective transport, due to the presence of open and highly interconnected pores in the monoliths [97]. These properties force the mobile phase to flow through these pores directing the sample molecules to the binding sites without flow rate limitations, overcoming the limitations observed for Sepharose matrix. Moreover, these monoliths exhibit high porosity and high binding capacity for larger molecules like plasmid DNA [97]. Ethylene diamino (EDA) CIM disk was used in this work as an alternative to Sepharose CL-6B This disk is a weak anion exchanger that contains free amine groups used for ligand immobilization [68]. This monolithic disk is stable at a range of temperatures between 4 to 40ºC as indicated by the supplier [98]. In a previous work, this type monolith was subjected, for the first time, to ligand immobilization as indicated in Figure 11, which includes a step of 72 hours at 83ºC. Despite the extreme thermal conditions, the stability and quality of the disk was apparently maintained after this process and a different behavior of the monolith after immobilization suggests that ligand derivatization was efficiently performed [92].

1.1.6. Construction of combinatorial libraries of synthetic ligands

In this work, the ligands tested were based on a ligand library developed by a method pioneered by Lowe and co-workers, for *de novo* design and synthesis of biomimetic ligands using cyanuric-chloride molecule as scaffold [88]. This method is based on several key steps. First of all, it includes searching of structural information, based on NMR or X-Ray crystallography data, about the target biomolecules or possible complexes with natural ligands. From the information obtained and using computer-assisted modelling tools it is possible to design *in silico* several chemical compounds that mimic key residues involved in a specific molecular recognition, in a "template-guided" approach [99]. On the other hand, design of compounds that can interact by affinity-complementarity with specific residues can be achieved, due to identification of key amino acid residues in proteins that are feasible to perform multiple affinity interactions with specific compounds, in a method termed as "templateless" or "structure-guided" approach [99,100]. Design of potential chemical compounds is followed by the synthesis of a solid-phase combinatorial library comprising disubstituted triazine-based ligands in agarose support, in which the first and second chlorine of this scaffold are substituted by all potential designed groups (known as "split synthesis" method), generating a library with high molecular diversity [99,100]. To evaluate the potential of the ligands of this library, those compounds must be screened in terms of binding to the target molecule using high throughput assays, which allows the selection of lead ligands. Lead ligands are characterized and can be subjected to solution-phase synthesis, performed in solution or using soluble polymers as supports, followed by optimization of chromatographic conditions. In some cases, a second-generation library of ligands can be designed to increase the selectivity of potential lead ligands [99].

Lowe and co-workers studied and identified the key structural features responsible for interactions between proteins, such as protein L and human antibody fragment Fab or antibodies and immunotoxin ricin, using computer modelling tools or X-ray crystallography [76,89,93] Those key features, generally specific amino acid residues (in this case Ala, Asp, Gln, Glu, Gly, Ile, Leu, Lys, Phe, Thr, and Tyr) inspired the rational design of 12 amino substituents analogs to the side chains of those amino acids, presented in Table 4 [75,99]. The combination of those compounds as substituents of second and third chlorine of triazine-based scaffold originated an original library composed of 169 possible ligands [75].

This library was screened in terms of oligonucleotide binding in a preliminary work performed by João Belchior, using a high throughput assay based on fluorescein isothiocyanate-labeling of homo-oligonucleotides (or FITC-labeling) which enabled the identification of ligands with strong-binding behavior, originating a smaller library containing 22 ligands [99,101].

In this work, ligands based on the previous 22-membered ligand library were re-selected. Several criteria were considered for selection. First, substituents mimicking predominantly hydrophobic amino acids or predominantly charged amino acids from table 4 were identified. The latter were combined according to their properties, resulting a combinatorial library that was divided in hydrophobic (containing two hydrophobic substituents), positively charged ligands (with two charged ligands) and

mixed-behavioral ligands (containing one hydrophobic and one charged ligand). This list was compared with the 22-ligand library, discarding the ligands that were not available in the lab and ligands that were not included in this library, selecting only the ones that demonstrated significant binding to nucleic acids [101]. This selection originated a list containing 9 ligands that were tested in affinity chromatography for plasmid purification, namely 11/8, 8/11,3/5, 8/1 (predominantly hydrophobic ligands) 4/11, 3/4 and 2/1 (mixed-behaviour ligands) and 4/2 and 2/4 (charged ligands). Ligands with hydrophilic character were discarded regarding previous indications that those ligands should not be selective for any of plasmid isoforms, due to high affinity to nucleic acids of substituents [86,101]

2. Background and objectives for this work

In the past decades, several DNA therapeutics, such as gene therapy vehicles or DNA-vaccines have been developed and studied. In this field, supercoiled plasmid DNA is used to introduce benefic genes in host organisms. Regarding the nature of these therapeutic drugs, high quality and pharmaceutical grade pDNA is mandatory. An increased demand for plasmid DNA is therefore expected. As such, efficient purification and isolation methods must be adopted to obtain a final plasmid product isolated from other impurities present in host bacterial cells. From the methods available, chromatography has been widely applied in plasmid purification. Among the chromatographic techniques, affinity chromatography is the one with higher specificity for the target molecule. In this technique, biomimetic ligands mimicking the natural interactions between amino acids and a target molecule were designed using a triazine scaffold. Although those ligands have been extensively explored for the purification of proteins or antibodies, only preliminary results mention these ligands for plasmid purification [94,101].

In a preliminary work, an initial library containing 169 ligands was screened under hydrophilic and hydrophobic binding conditions. From this screening, 22 strong binding ligands were selected and tested in terms of binding to double stranded DNA sequences, purified plasmid, followed by plasmid purification from *E. coli* extracts using affinity chromatography. From these studies, two ligands (designated as 1/2 and 6/7) were able to purify plasmid DNA using low concentrations of ammonium sulphate as elution buffer [101].

The previous work was followed by the study of ligands 6/5 and 5/6, which yields promising results in the previous work [101]. Those ligands were tested under hydrophilic and hydrophobic conditions using Sepharose CL-6B as solid-support. In this work, ligand 6/5 was immobilized in EDA CIM® monolithic disks for the first time using a well-established solid-phase protocol and tested for plasmid purification, using the same buffer conditions used for Sepharose resin. From Sepharose derivatized with ligand 6/5, plasmid separation from RNA was achieved using hydrophobic conditions (0.4 to 1.5M ammonium sulphate and 0.8M sodium citrate). In CIM®-derivatized disk containing 6/5, similar behavior was observed for pDNA and RNA using the same conditions. Nevertheless, higher plasmid

yield was obtained in CIM-monolith [92]. Despite this results, the reproducibility in some cases was not fully proven, which required additional studies to confirm this data [92].

The aim of the present study was to evaluate the potential of a synthetic mimic compound (ligand 6/5), previously synthesized either in aminated Sepharose CL-6B and on a EDA CIM® monolithic disk, in isolation and purification of plasmid DNA, ideally the super-coiled isoform, from other undesired isoforms and RNA, using affinity chromatography under hydrophobic and hydrophilic binding environments, applying higher concentrations ranges of salt in both conditions and optimizing the conditions previously used. Additionally, several ligands were selected from the 22-ligand library based on the identification of ligands with promising results in binding nucleic acids, namely 11/8, 8/11, 3/5, 8/1, 4/11, 3/4 and 2/1 and their potential for plasmid and, ideally, supercoiled purification will be evaluated using bench-scale chromatographic columns, under the same conditions of ligand 6/5. Potential efficient ligands were further tested at a later stage in ÄKTA purifying system to confirm the bench scale chromatographic results.

3. Materials and methods

3.1. Reagents

The methods described in this section were performed using the reagents presented in Table 6

Table 6: Description of the manufacturer, grade and minimum purity of the reagents used in this work.

Reagent	Manufacturer	Grade	Minimum Purity (%)
Luria-Bertani (LB) Broth	Nzytech	Molecular Biology	-
Glycerol	VWR Chemicals	Molecular biology	99
Kanamycin	AMRESCO	USP	ultrapure
D-Glucose anhydrous	Fisher Chemical	ACS	-
Tris	Eurobio	Molecular Biology	-
HCl	Fisher Chemical	For analysis	37
EDTA	Fisher Bioreagents	For Electrophoresis	99.5
Sodium Hydroxide	Fisher Chemical	ACS	98.3
SDS	Merck	For synthesis	95
Potassium acetate	Acros Organic	ACS	99.5
Acetic Acid	Fisher Chemical	For analysis	99.8
Isopropanol	Fisher Chemical	ACS	99.9
Ammonium Sulphate	Applichem Panreac	ACS	99
Epichlorohydrin	Sigma-Aldrich	For synthesis	99
Ammonium hydroxide	Honeywell	-	30
Cyanuric Chloride	Merck	For synthesis	99
Acetone	Labchem	-	-
L-alanine	Alfa-Aesar	For synthesis	99
1,5-diaminopentane	Aldrich	For synthesis	95
Sodium Bicarbonate	Merck	ACS	99.7
Ethanol	Fisher Chemical	ACS	99.9
Bromophenol Blue	Sigma	For electrophoresis	-
Ethidium Bromide	Sigma	-	-

3.2. Cell Banks

For the preparation of cell banks, 10 µL of a Cell Bank of *E. coli* DH5α containing a 6.1 kb plasmid pVAX1/lacZ , previously prepared by Cátia Jorge, was inoculated in 5 mL of sterile LB medium (autoclaved at 121 °C, 20 minutes) supplemented with 5 µL of kanamycin (30 µg/mL) in a 15 mL *Falcon* Tube[92]. This suspension was incubated overnight at 37°C and 250 rpm. After overnight incubation, an appropriate volume of suspension for an initial optical density (O.D.) of 0.1 (measured at 600 nm – $O.D._{600nm}$) was determined and centrifuged using an Eppendorf 5810R centrifuge (3 minutes at 6000xg). The resultant pellet was resuspended in 5 ml sterile LB medium supplemented with 5 µL of kanamycin (30 µg/mL) in a 15 mL *Falcon* Tube and incubated at 37ºC and 250 rpm till an $O.D._{600nm}$ between 1 and 1.5 was reached. After this step, 10 cell banks were prepared adding 65 µL of cell suspension to 35 µL of 50 % glycerol (v/v) and were stored at -80 °C till further processing.

3.3. Cell Fermentation

At an initial phase, 20 µL of one of the cell banks, previously prepared and stored (topic 3.2), was pre-inoculated in 100 mL shake flasks containing 30 mL of LB medium, previously autoclaved (at 121ºC for 20 minutes), supplemented with 30 µL of kanamycin (30 µg/mL) and incubated at 37 °C and 250 rpm. After overnight growth, the volume of pre-inoculum needed to start the growth with an initial $O.D._{600nm}$ of 0.1, measured at 600 nm, was determined (Equation 1) and centrifuged at 6000xg for 3 minutes. The resultant pellet was resuspended and inoculated in 2 L shake-flasks with 500 mL of sterilized LB medium (at 121 ºC for 20 minutes) and 500 µL of kanamycin (30 µg/ml). In the following equation, $O.D._P$ and $O.D._I$ represent the optical densities (at 600 nm) on the pre-inoculum and on the inoculum and Vp and Vi stand for the volumes of pre-inoculum and inoculum, respectively.

$$O.D._P * V_P = O.D._I * V_I \quad \text{(Equation1)}$$

The inoculated cells were grown in the shake flasks at 37°C and 250 rpm, until they reached an $O.D._{600nm}$ value around 3. The resultant broth was equally divided into *Beckman* 500 mL flasks and subjected to a centrifugation step at 6000 xg at 15 minutes and 4 °C, using SLC-3000 rotor in an *Sorvall RC6* Ultracentrifuge. The supernatant was discarded and the pellet stored at -20 °C for further work.

3.4. Cell lysis and Plasmid Primary Isolation

The perform cell lysis, alkaline lysis was performed, a method based in a protocol originally described by Birnboim et al. [51]. The pellets obtained from fermentation were resuspended in P1 buffer (50 mM Glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0) using a vortex, until total resuspension. To perform alkaline lysis, this suspension was divided into 50 mL centrifuge tubes, containing a volume of buffer P2 (0.2 M NaOH, 1 % SDS (w/v)) in a final ratio of 1:1 (v/v), being gently homogenized and the tubes were placed to rest for 10 minutes at room temperature. To stop lysis and neutralize the lysate, a determined volume of buffer P3 (5 M potassium acetate, acetic acid) was added to each centrifuge tube, followed by gentle homogenization, until the precipitate acquired a

white color (indicative of pH decrease) and the tubes were left to rest on ice for 10 minutes. The volume of buffers P1, P2 and P3 required for cell lysis were determined according to equation 2, where $O.D._{growth}$ and $O.D._{F}$ represent the final optical densities (at 600 nm) of the pellets resultant from cell growth and the desired final optical density ($O.D._{F}$(600 nm) = 60), respectively. On the other hand, V_{growth} stands for the volumes of growth medium and $V_{P1,}$ V_{P2} and V_{P3} represent the volumes of buffers P1, P2 and P3, respectively.

$$V_{P1} = V_{P2} = V_{P3} = \frac{O.D._{growth} * V_{growth}}{O.D._{F}} \quad \text{(Equation 2)}$$

The resultant neutralized lysate was directly centrifuged in a *Sorvall RC6* Ultracentrifuge (rotor SA-300) at 13000 rpm for 30 minutes at 4 °C, for the removal of cell debris, proteins and chromosomal DNA. The supernatants were placed in new tubes and centrifuged again in the same conditions, discarding the pellets at the end. To perform the primary plasmid DNA purification, a volume of isopropanol corresponding to 70 % (v/v) of the total supernatant volume, was added to the supernatant and gently mixed. The mixture was divided into 50 mL centrifuge tubes and placed at -18 °C for 2 hours, to promote the precipitation of all nucleic acid molecules, namely pDNA, RNA and traces of gDNA.

After this precipitation step, the mixture was centrifuged at 13000 rpm for 30 minutes at 4°C using a *SLC-3000* Sorvall RC6 Ultracentrifuge, being the supernatant discarded and the tubes inverted on adsorbent paper and placed overnight at 4 °C, to remove traces of isopropanol. After this period, Tris-HCl (20 mM; pH 8.0) buffer was added to the tubes in fractions of 100 µL until total resuspension of the pellets, being collected in 1.5 mL *Eppendorf* tubes. An amount of ammonium sulphate required to obtain a concentration of 2.5 M of this salt was dissolved in the previous suspension and the latter was left on ice for 15 min. This step was followed by the centrifugation of the mixture at 11000 rpm and 4°C for 35 minutes in an Eppendorf 5810R centrifuge, being the resultant supernatant transferred to new tubes. The resultant lysate was dialyzed as described in section 3.4. and stored at -20 ºC till further use.

3.5. Micro-dialysis of *E. coli* clarified lysates and fractions collected during chromatographic approaches

The samples for desalting were placed in 1.5mL *Eppendorf* tubes with their caps removed. The top of each tube was covered with squares of OrbDial D14 dialysis membranes, containing a molecular weight cut-off (MWCO) between 12 to 14 kDa and a thickness of 23 µm (Orange Scientific), previously washed 3 times in distilled water, that were tightened with a rubber band. The tubes were inverted and shaked twice firmly to ensure that all the inner solution was in contact with membrane, being placed overnight in a 2 L Erlenmeyer flask with 1 L of buffer Tris-HCl (20 mM, pH 8.0) with gentle agitation. After this period, the rubber bands and membranes were removed from the tubes and the samples could be directly used (in chromatographic assays or analyzed by gel electrophoresis) or stored at -20ºC till further processing.

3.6. Concentration of *E. coli* clarified lysates and fractions collected during chromatographic approaches

If a given sample, from a lysate produced or from fractions collected during chromatographic assays, needed to be concentrated for better analysis, the sample would be subjected to evaporation using a Vacuum Concentrator (Thermo Scientific™ Savant™ DNA 120 SpeedVac ™Concentrator), until total liquid evaporation, being directly analysed by gel electrophoresis, by previous resuspension of the dried pellet with 30 µL Tris-HCl (20 mM, pH 8.0), or stored at 4 ºC, till further use.

3.7. Solid-Phase Synthesis of triazine-based ligands

The synthesis of triazine based-ligands 1/2 and 0/0 was performed by a well-established protocol used to obtain ligands for affinity purification of biomolecules, using Sepharose CL-6B as solid-support [102,103].

3.7.1. Epoxy activation of Sepharose CL-6B

Epoxy activation of Sepharose CL-6B (Sigma) was performed according to method developed by Fillipusson et al. [104]. Sepharose was washed with abundant distilled water on a sinter funnel for the removal of ethanol (storage solution), and suspended in 0.8 mL of 1 M NaOH per gram of gel. Epichlorohydrin was added to the previous suspension, in a *hotte* system, at a proportion of 0.1 mL per gram of gel, followed by overnight incubation at 30 °C in a rotary shaker (*Heidolph – Titramax 1000*) at 100 rpm.

3.7.2. Amination of epoxy-activated Sepharose CL-6B

Epoxy-activated agarose was aminated as described in a protocol developed by Roque et. al [75]. Epoxy-activated gel was washed with water after overnight incubation, and resuspended with 1.5 mL of ammonia (33 % v/v) per gram of moist gel and incubated overnight in a rotary shaker at 30 ºC with gentle agitation (200 rpm). The suspension was thoroughly washed with distilled water in a sinter funnel for ammonia removal, till a pH close to pH of distilled water, controlled with a pH-indicator paper. Part of aminated supports was used immediately for cyanuric chloric activation and the remaining was stored in 20 % ethanol at 4ºC for further column packing or evaluation in terms of nucleic acid binding (topic 4.2.1.2).

3.7.3. Activation of aminated Sepharose with cyanuric chloride

The preparation of dichlorotriazinyl gel was performed according to a method that was adapted from literature [74,102,103]. Aminated agarose was suspended in acetone/water 50 %(v/v) (1 mL/g moist gel). The slurry was maintained in an ice bath at 0 ºC with an agitation between 150 and 300 rpm. An amount corresponding to 5 molar equivalent of cyanuric chloride (relative to the extent of amination) was dissolved in acetone (8.6 mL/gram of cyanuric chloride) and divided into four aliquots. In this work, the extent of amination was assumed to be 20 µmol amine groups/g moist gel as previously reported [92,104]. Each aliquot was added to the aminated slurry in 30 minutes intervals, maintaining

a mixture at constant shaking and at 0 ºC. The pH was monitored with a pH-indicator paper and maintained around 7 with addition of NaOH 1M. After cyanuric chloride addition, the gel was washed twice with each acetone/water mixtures (v/v), in the following order: 1:1, 1:3, 0:1, 1:1, 3:1, 1:0; and then washed with abundant water for cyanuric chloride removal. From this gel, part (about 5 g) was packed in bench chromatographic column and evaluated in terms of nucleic acid binding (topic 4.2.1.3) and the remaining gel was immediately used for R1 nucleophilic substitution.

3.7.4. Nucleophilic substitution of the second chlorine atom of dichlorotriazinyl Sepharose (R1 substitution)

Dichlorotriazinyl gel was divided into 2 aliquots, each of them for the substitution of the second available chlorine in triazine structure with different aminated compound, in this case, L-alanine (1) (for ligand 1/2) and ammonia (0) (for ligand 0/0). An amount of 2 molar equivalent of each amine (relative to the extent of amination) was dissolved in distilled water. For amine 1, one equivalent of $NaHCO_3$ was also added to the previous solution. The total volume of solvent was 1mL per gram of gel. Each aliquot with dichlorotriazinyl gel containing L-alanine or ammonia solutions, was incubated for 24 h, at 30ºC in a rotary shaker (150-300 rpm). After this period, each aliquot was abundantly washed with distilled water in a sinter funnel and used for nucleophilic R2 substitution.

3.7.5. Nucleophilic substitution of the third chlorine atom of dichlorotriazinyl Sepharose (R2 substitution)

Subsequent R2 substitution was performed using amine 2 (for ligand 1/2) and amine 0 (for ligand 0/0). In this step, 5-molar equivalent (related to the extent of amination) was dissolved in distilled water (3 mL solvent/g moist gel) and the substitution was carried out at 83 ºC for 72 h in an hybridization oven/shaker (Amersham Pharmacia Biotech). After incubation, both aliquots were washed thoroughly with distilled water and stored in ethanol 20 % (v/v) at 4ºC.

3.8. Chromatography assays with selected ligands

3.8.1. Bench-scale chromatography assays

For bench-scale chromatographic assays, 1 mL of Sepharose-CL 6B derivatized with selected ligands or controls were packed in a 4 mL (0.8 x 6 cm) PD-10 column from Amersham-Pharmacia Biosciences. To regenerate the column, 8 mL of regeneration buffer (NaOH (0.1 M) in 30% (v/v) isopropanol in distilled water) were passed through the column, followed by 8mL of distilled water.

Under hydrophobic binding environment, column equilibration was performed with 10 mL of the same buffer used in the washing step, namely 2.5 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl, pH 8.0 for multiple step washing assays; and 1.0 M or 0.5 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl, pH 8.0 for single step-assays. After column equilibration, 250 µL of clarified *E. coli* lysate, prepared as described in topic 3.3. and 3.4. was conditioned with ammonium sulphate to achieve the same concentration of equilibration and washing buffers and was loaded to the column. For multiple-step washing assays, washing step was performed by applying 1.5 mL of each buffer containing decreasing concentrations of ammonium sulphate in Tris-HCl 20 mM, pH 8.0 in the following order: 2.5 M, 2.0 M, 1.5 M, 1.0 M and 0.5 M $(NH_4)_2SO_4$. During this step, fractions of 200 µL were collected. For elution, 5 mL of Tris-HCl 20mM, pH 8.0 were passed

through the column while fractions of 500 µL were collected. On the other hand, in single-step assays, washing step was performed by applying 3 mL of 0.5 M or 1.0 M $(NH_4)_2SO_4$, while fractions of 200 µL were collected. For elution, 5 mL of Tris-HCl 20 mM, pH 8.0 were applied to column and fractions of 500 µL were collected during this process.

For hydrophilic chromatographic assays, column equilibration was performed with 10mL of 20Mm Tris-HCL, pH 8.0. After column equilibration, 250 µL of clarified *E. coli* lysate, prepared as described in topic 3.3. and 3.4., was conditioned with similar conditions of equilibration buffer and loaded to the column. After lysate loading, washing step was performed by applying 3mL of Tris-HCl 20 mM, pH 8.0, while fractions of 200 µL were collected. For elution, 1.5 mL of each buffer with increasing concentrations of NaCl was applied in the following order: 0.5 M, 1.0 M, 1.5 M, 2.0 M and 2.5 M NaCl. During elution, fractions of 500 µL were collected.

To store the column, 5 mL of distilled water was applied followed by 2 mL of ethanol 20% (v/v), being stored at 4°C.

3.8.2. Chromatography assays using CIM® monolithic disk in ÄKTA purifying system (ÄKTA 10)

In these assays, EDA CIM® monolithic disks with a bed volume of 0.34 mL (Bia Separations), were connected to ÄKTA purifying system (ÄKTA 10). One of the monolithic disks, was previously derivatized with ligand 6/5, as described by Cátia Jorge (derivatized monolith) while the other was not subjected to derivatization (non-derivatized monolith) [92]. As performed for the sepharose columns, these monoliths were tested under hydrophobic and hydrophilic conditions, performed at a flow rate of 1mL/min. For both assays, 100 µL clarified E. coli lysate, prepared as described in topic 3.3. and 3.4., was loaded into the system. For hydrophobic chromatographic assays, lysate was previously conditioned with ammonium sulphate to achieve the concentration of equilibration and washing buffers. During these assays, fractions of 200 µL and 500µL were collected for the washing and elution steps, respectively.

To perform the assays under hydrophobic conditions, the column was filled and equilibrated with 15 CV (1CV=0.34mL) of 2.5M (NH4)2SO4 in 20 mM Tris-HCl (pH 8.0). The lysate was then injected, and washing was performed with decreasing gradient of 100 CV starting with 2.5 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl , pH 8.0 followed by 20 CV of Tris-HCl 20 mM (elution buffer). To wash the system, distilled water was passed through till the stabilization of UV signal, followed by 20 CV of regeneration buffer (2 M NaCl), washing with water to achieve stabilization of UV signal and pH and 30 CV of ethanol 20 % (v/v).

For the assays under hydrophilic conditions, the channels were filled and equilibrated with 15CV Tris-HCl 20 mM pH 8.0. The lysate was loaded, followed by washing of unbound molecules with 30 CV Tris-HCl 20 mM pH 8.0. Elution was carried out with gradient between 0 to 2.5 M NaCl in 20 mM Tris-HCl (pH 8.0), during 100 CV. Channels and monolith were passed through distilled water till stabilization of UV signal, followed by 20 CV of regeneration buffer (2 M NaCl), washing with distilled water to stabilize UV signal and pH and 30 CV of ethanol 20% (v/v).

The fractions collected were examined by gel electrophoresis and the resultant chromatograms were analyzed using UNICORN™ software, version 5.11 (GE Healthcare).

3.8.3. Chromatography assays using Sepharose CL-6B resins with selected ligands in ÄKTA purifying system (ÄKTA 10)

The chromatographic assays were performed using a Tricorn™10/50 column containing 2 mL Sepharose CL-6B (=1 CV) derivatized with ligand 2/1 or 8/1. Assays were performed under hydrophobic conditions, applying a decreasing gradient with $(NH_4)_2SO_4$ in 20 mM Tris-HCl, pH 8.0 followed by Tris-HCl 20mM as elution buffer; and under hydrophilic conditions, using Tris-HCl 20mM, pH 8.0 as equilibration and washing buffers and increasing gradient with NaCl in the same buffer for elution. For both assays, 1000 µL clarified *E. coli* lysate, prepared as described in topic 3.3. and 3.4., was loaded into the system and the assays were performed at flow rate of 1 mL/min. This feed was diluted 1:2, 1:3 and 1:6 with Tris-HCl 20mM, pH 8.0, as discussed in topic 4.3. For hydrophobic chromatographic assays, lysate was previously conditioned with ammonium sulphate to achieve the concentration of equilibration and washing buffers. During these assays, fractions of 250 µL were collected for washing and elution steps.

To perform the assays under hydrophobic conditions, the column was regenerated with 8CV of regeneration buffer (NaOH (0.1 M) in 30% (v/v) isopropanol in distilled water), washed with abundant distilled water till stabilization of UV signal and pH value. The column and channels were then filled and equilibrated with 1.5 M or 1.05 M$(NH_4)_2SO_4$ in 20 mM Tris-HCl , pH 8.0 (for ligand 2/1 and 8/1 respectively) , till stabilization of UV signal. The lysate was then injected, the column was equilibrated with 10 CV equilibration buffer and washing was performed with decreasing salt gradient in 20 CV starting with 1.5 M (for ligand 2/1) or 1.05 M (for 8/1) $(NH_4)_2SO_4$ in 20 mM Tris-HCl (pH 8.0) followed by 5 CV of Tris-HCl 20 mM (elution buffer). To wash the channels distilled water to achieve UV signal and pH stabilization and 3 CV of ethanol 20% (v/v).

To perform the assays under hydrophilic conditions, the column was regenerated with 8CV of regeneration buffer used in hydrophobic conditions, washed with abundant distilled water till stabilization of UV signal and pH value. The column and system were then filled and equilibrated with 20 mM Tris-HCl (pH 8.0), till stabilization of UV signal. The lysate was then injected, the column was equilibrated with 10 CV equilibration buffer and washing was performed with 5 CV 20 mM Tris-HCl (pH 8.0), followed by an increasing in 20 CV gradient with final concentration of 1.5 M (for ligand 8/1) or 1.05 M NaCl (for ligand 2/1) for elution step. To wash the column and channels distilled water was passed through till UV signal and pH stabilization, followed by 3 CV of ethanol 20 % (v/v).

The fractions collected were examined by gel electrophoresis and the resultant chromatograms were analyzed using UNICORN™ software, version 5.11 (GE Healthcare). To obtain pDNA and supercoiled pDNA step yield, densitometry analysis and (HIC)-HPLC analysis were performed in parallel, and the results were compared.

3.9. Agarose Gel Electrophoresis

Samples collected were previously dialyzed and were analyzed by electrophoresis in 1% (v/v) of agarose horizontal gel (20 cm gels) in 1x TAE buffer (40 mM Tris base, 1 mM EDTA and 20 mM acetic acid). This approach was performed in an *Amersham Pharmacia Electrophoresis EPS-301 Power Supply* using an American Biosciences HE99X submarine electrophoresis unit. NZYDNA DNA ladder III (NZYTECH) was used as DNA weight marker for the applied samples. Amount of fractions collected, lysate, DNA ladder and loading buffer (40 % (w/v) sucrose, 0.25 % (w/v) bromophenol blue), added to gel wells are presented in table 7. To run the samples in the gel, a voltage of 120 V was used during 1h30. After gel running, the gel was stained with 0.4 mg/mL ethidium bromide and analyzed using Stratagene EagleEye II Video Imaging System.

Table 7: Amount of fractions collected, feed and DNA ladder (M), as well as, loading buffer loaded to gel electrophoresis

	Amount added/well (µL)	**Amount loading buffer/well (µL)**
DNA ladder III (M)	4	-
Feed (F)	5	1
Fractions collected from chromatography	30	5
Fractions concentrated	20	5

3.10. Densitometry analysis

Plasmid and supercoiled pDNA yield were determined by densitometry analysis of the band intensities in feed and fractions of interest, using the ImageJ software, which gives the respective band areas, and equation 3, where $A_{Pfractions}$ and A_{Pfeed} are, respectively, the area of band intensities related to plasmid DNA or one of the isoforms in the fractions under analysis and the area bands of plasmid of isoforms in feed. FVC stands for the factor of volume correction regarding the lower amount of feed charged compared to amount loaded for fractions. The amount of supercoiled isoform over open-circular isoform was also determined using ImageJ software and equation 4, where A_{sc} correspond to the area of bands of supercoiled isoform and $A_{oc,}$is the area of bands related to open-circular isoforms. For equations 3 and 4, FC stands for the factor of concentration and was only applied for samples that were previously concentrated.

$$Plasmid\ or\ Isoform\ yield\ (\%) = \frac{\sum(A_{pfractions)/FC}}{A_{pfeed}*FVC} * 100 \quad \text{(Equation 3)}$$

$$Sc\ over\ oc\ isoform\ (\%) = \frac{\sum(A_{sc})/FC}{\sum(A_{sc}+A_{oc})/FC} * 100 \text{ (Equation 4)}$$

3.11. Quantification of fractions collected during chromatography assays using 2/1 ligand by (HIC)-HPLC analysis

The plasmid DNA collected during chromatographic elution experiments was quantified by analytical hydrophobic interaction chromatography (HIC)-HPLC. In this work, plasmid DNA was quantified in several samples recovered during downstream processing, namely after alkaline lysis, after

isopropanol overnight precipitation and ammonium sulphate precipitation, which allowed the determination of each step yield (equation 5). Adittionally, samples from the loading feed in the chromatographic assays performed with ligand 2/1 in AKTA purifier system and from the peaks obtained during those assays were also collected, allowing the determination of plasmid (equation 5) and supercoiled plasmid yield (equation 6). The column used was a 15 PHE-PE column (4.6 mmx10 cm) (GE Healthcare) connected to an ÄKTA purifier system (ÄKTA 10) The column was firstly equilibrated at 1 mL/min with 1.5 M ammonium sulphate in 10 mM Tris-HCl pH 8.0 Then 50 µl of each sample was loaded to be analyzed. Elution of pDNA isoforms was performed at 1ml/min in a step mode with 10 mM Tris-HCl, pH 8.0. The absorbance during the process was recorded at 260 nm. A calibration curve was constructed with standard plasmid (pVAX1-GFP) concentrations, prepared in a concentration range between 0 and 100 µg , to quantify plasmid DNA (presented in Appendix VII).

$$Step\ yield\ (\%) = \frac{m_{pDNA-after\ step}}{m_{pDNA-before\ step}} * 100 \quad \text{(Equation 5)}$$

$m_{pDNA\ –\ after\ step}$ and $m_{pDNA\text{-}before\ step,}$ correspond to the mass of pDNA (in µg) present after the step and before being subjected to this step, respectively.

$$sc\ pDNA\ yield\ (\%) = \frac{m_{pDNA-peak2} * \%scpDNA_{peak2}}{m_{pDNA-feed} * \%sc\ pDNA_{feed}} * 100 \quad \text{(Equation 6)}$$

$m_{pDNA\ –\ peak2}$ and $m_{pDNA\text{-}feed,}$ correspond to the massa of pDNA (in µg) present in the peak containing sc pDNA isolated and in loading feed, respectively. %sc $pDNA_{peaks2}$ and %$scpDNA_{feed}$ correspond to the amount of supercoiled isoform in the peak containing sc pDNA isolated and in loading feed, determined by densitometry analysis.

4. Results and Discussion

4.1. Cell Growth

E. coli DH5α cells containing pVAX1/lacZ plasmids were grown in conditions previously described in section 3.1 and 3.2. After inoculation, the absorbance at 600 nm was registered at specific time intervals to follow the cell growth. After fermentation and cell lysis and primary isolation, the cell lysates were subjected to membrane dialysis and their nucleic acid content was evaluated by gel electrophoresis. Regarding the high demand for plasmid DNA during this work, seven cell fermentations were performed during this period. The growth curve corresponding to cell fermentation (4*500 mL flasks) performing during this work is presented in Figure 12. The remaining growth curves of the other growths performed in this work are presented in Appendix III – Cell growths performed during this study.

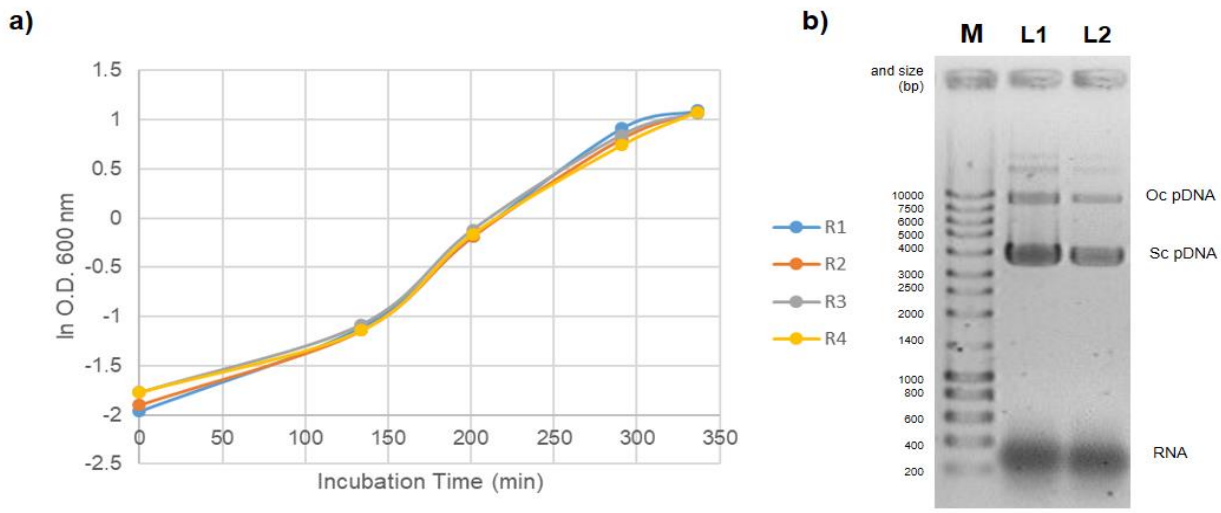


Figure 12: a) Growth of *E. coli* DH5α cells containing the plasmid pVAX1/lacZ, in 500 mL LB medium, over incubation time (in minutes) performed in 26th May 2017. R1 to R4 – replicates containing 500 mL of cell broth each. b) Gel electrophoresis obtained after alkaline lysis and primary isolation to evaluate nucleic acid content; M- DNA Ladder III (NZYtech); L1 and L2- lysates resultant from cell lysis and plasmid primary isolation.

Regarding that the growth of DH5α is well-known, it was established that the cells are in exponential phase at an optical density ($O.D._{600nm}$) around 3 (corresponding to ln ($O.D._{600nm}$) around 1.1). When this optical density was reached, the growth was stopped and the cells were recovered to proceed to cell lysis and plasmid DNA purification. In general, cell fermentations followed similar growth behavior (Figure 12a; Figures A1 to A8 - appendix III) and yielded cell lysates with significant amounts of open-circular and supercoiled isoforms and RNA, needed for the chromatographic steps (Figure 12b); Figure A1 to Figure A8 - appendix III). Cell lysates containing low amounts of one of the above-mentioned nucleic acids (Figure A6 – appendix II), were rejected and not used for further chromatographic evaluation.

4.2. Screening of selected ligands for pDNA isolation

4.2.1. Control chromatographic assays

First, to test the influence of the solid support and cyanuric-chloride backbone in the interactions between the matrix and nucleic acids, several assays were performed with different resins, namely Sepharose CL-6B, aminated sepharose, aminated sepharose with cyanuric chloride, ligand 0/0, and CIM® monolithic disk to evaluate their potential in pVAX1/lacz purification. In all assays, the resins were tested under hydrophobic and hydrophilic binding environments.

4.2.1.1. Sepharose CL-6B

As mentioned, Sepharose CL-6B was the resin selected for the immobilization of the ligands chosen for screening. About 1 mL of this resin was packed in a chromatographic column (bench-scale) and tested under hydrophilic binding conditions, performing a stepwise elution with increasing

concentrations of sodium chloride (from 0.5 M to 2.5 M) and under hydrophobic binding environmental, applying a stepwise elution with decreasing concentrations of ammonium sulphate (2.5 M to 0.5 M), as described in topic 3.8.1. The results obtained are presented in Figures 13 to 16.

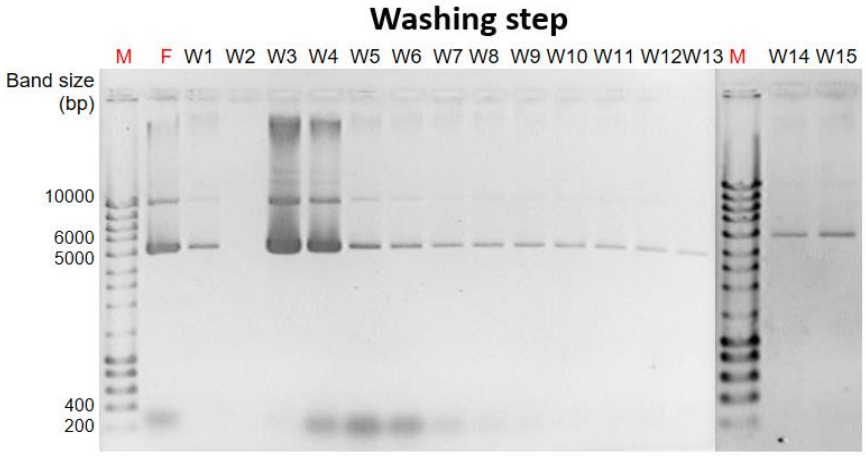


Figure 13: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL Sepharose Cl-6B packed in a 4 mL column in hydrophilic conditions. W1 to W15- Fractions collected, in increasing order, during the washing step using Tris-HCl 20mM as washing buffer; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); oc – open-circular; sc- supercoiled. DNA ladder applied in this gel electrophoresis is the same for all gel electrophoresis performed during this work and the size of the bands from this ladder is equal in all gel electrophoresis. Complete band size description is presented in appendix II.

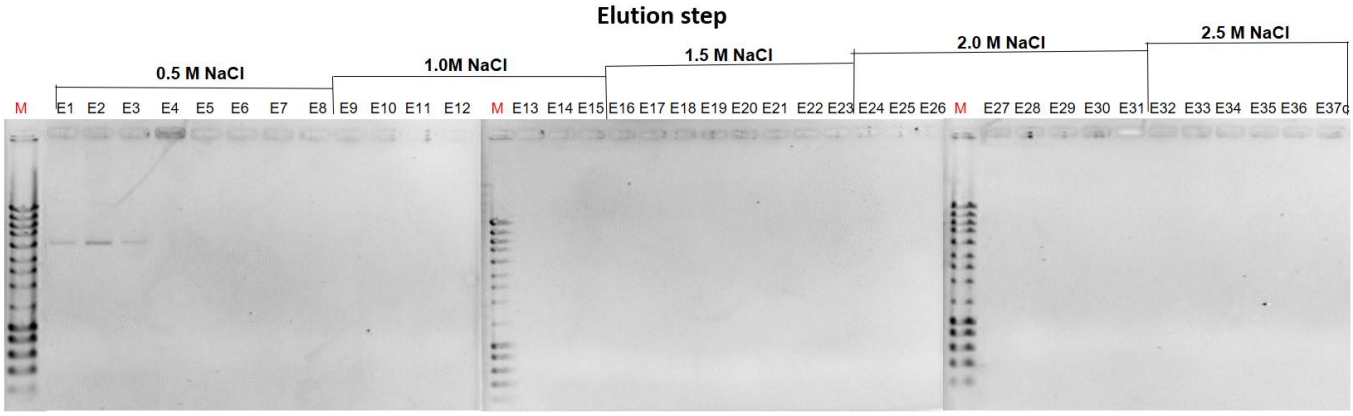


Figure 14: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL Sepharose Cl-6B packed in a 4 mL column in hydrophilic conditions (stepwise elution from 0.5 M to 2.5 M NaCl). E1 to E37- Fractions collected, in increasing order, during the elution step, where the concentration of elution buffer was increased from 0.5 M to 2.5 M NaCl; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophilic conditions, a significant amount of plasmid DNA and RNA is co-eluted in the flow through in the first fractions of the washing step (Figure 13) Traces of supercoiled and open circular pDNA are still detected after the initial co-elution in washing step and in the first fractions of elution step with 0.5 M NaCl (Figures 13 and 14).

The co-elution of a significant part of pDNA isoforms and RNA mainly in the first fractions of washing step, indicates that most of nucleic acids were not retained in the column and no separate elution is observed, suggesting that this resin is not able to perform a separate elution of nucleic acids present and is not selective for any of these species under hydrophilic environment.

When Sepharose CL-6B is evaluated under hydrophobic conditions, oc pDNA rich-fractions are detected in the first fractions, followed by the elution of pDNA in high concentration, isolated from RNA, at a concentration of 2.0 M $(NH_4)_2SO_4$ and some sc pDNA rich-fractions. RNA is retained in the column till a concentration of 1.5 M $(NH_4)_2SO_4$, where fractions containing RNA contaminated with sc pDNA are detected (Figure 15a). To confirm this fraction pattern, the initial fractions containing pDNA isoforms (W1 to W22) were fully concentrated as described in topic 3.6. and re-analyzed by gel electrophoresis (Figure 15b), where similar results were obtained. All the nucleic acids are eluted during washing step, not being detected in fractions when salt concentration is decreased to zero.

RNA retention and separation from plasmid DNA, as well as, isoform selective separation, suggest that this resin may be selective for the nucleic acids under hydrophobic conditions. This behavior may be related to molecular sieving effects. Thus, single stranded molecules, such as RNA, are retarded when compared to supercoiled plasmid since its size fits in the fractionation range of sepharose matrix, whereas higher molecular weight molecules, like double stranded material, are directly eluted in the void volume without entering the matrix pores[53]. Supercoiling reduces hydronamic radius of sc pDNA, maybe close to size exclusion limit, explaining higher retention time for sc pDNA compared to oc pDNA [106].

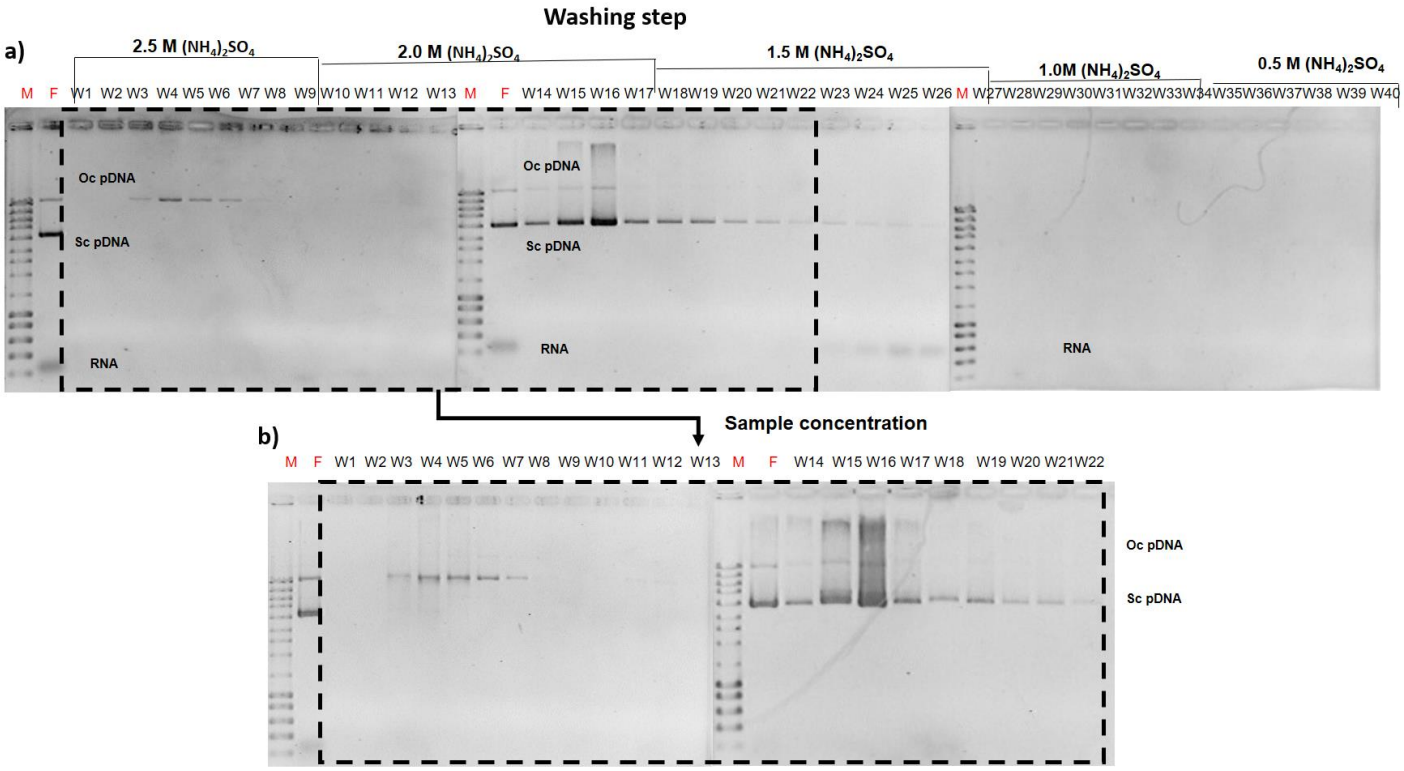


Figure 15: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL Sepharose Cl-6B packed in a column in hydrophobic conditions (stepwise washing/elution from 2.5 M to 0.5 M $(NH_4)_2SO_4$). a)

Fractions collected during washing step; W1 to W40- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5M to 0.5M $(NH_4)_2SO_4$; Fractions included in dashed area were the ones selected for further sample concentration to confirm the band pattern observed. b) Fractions selected for concentration in a) that were totally concentrated and suspended with 30 µL Tris-HCl 20mM, pH 8.0 after concentration. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled. Complete band size description of DNA ladder is presented in appendix II.

Figure 16: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL Sepharose Cl-6B packed in a 4 mL column in hydrophobic conditions. E1 to E9 - Fractions collected, in increasing order, during the elution step, where the 20mM Tris-HCl was used as elution buffer; M- DNA Ladder III (NZYtech).

4.2.1.2. Aminated Sepharose Cl-6B

To immobilize the selected ligands, sepharose CL-6B is subjected to well-stablished protocol described in 3.6. This protocols includes, at first steps, the epoxidation of Sepharose CL-6B, to activate functional epoxy groups, and amination of epoxy-activated agarose with ammonia, to include amino groups that mediate ligand immobilization, yielding aminated Sepharose [107]. About 1 mL of this resin was packed in a chromatographic column (bench-scale) and tested under hydrophilic binding conditions, performing a stepwise elution with increasing concentrations of sodium chloride (from 0.5 M to 2.5 M) and under hydrophobic binding environmental, applying a stepwise washing/elution with decreasing concentrations of ammonium sulphate (2.5 M to 0.5 M), as described in topic 3.8.1. The results obtained are presented in Figures 17 to 20.

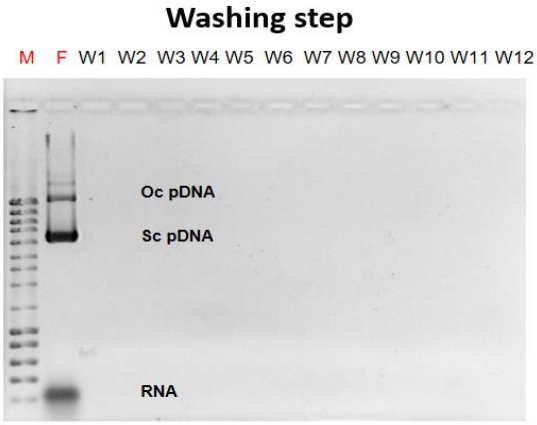


Figure 17: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL Aminated Sepharose Cl-6B packed in a 4 mL column in hydrophilic conditions. W1 to W12- Fractions collected, in increasing order, during the washing step using Tris-HCl 20mM as washing buffer; M- DNA Ladder III (NZYtech); F- Feed (clarified

lysate added to the column); oc – open-circular; sc- supercoiled. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophilic conditions, pDNA and RNA are retained inside the column during washing step, using Tris-HCl 20 mM, pH 8.0 as washing buffer (Figure 17). Apparently, co-elution of a significant part pDNA and RNA is only detected in the beginning of elution step, when 0.5 M NaCl is applied, being detected in seven fractions (Figure 18a) followed by elution of supercoiled and open-circular isoforms in lower concentrations (Figure 18b).

Nucleic acid retention in the absence of salt suggests that aminated sepharose has affinity for all the nucleic acid molecules under these conditions. Further increasing of ionic strength promotes co-elution of pDNA and RNA, indicating that electrostatic interactions between elution buffer and nucleic acids overcome possible interactions between support and nucleic acid molecules but do not allow a separate elution of the different molecules. Retention of pDNA and RNA in the absence of salt, may be related to the fact that amine groups present in the solid-support are positively charged at pH 8.0, interacting with negative charges from phosphate group in nucleic acids. With the introduction of salt, electrostatic interactions between solid-support and nucleic acids are disrupted and the latter are eluted from the column. These interactions may also be involved in ligands derivatized in solid supports if the derivatizion process is not efficiently performed, and there exist free amino groups available to react.

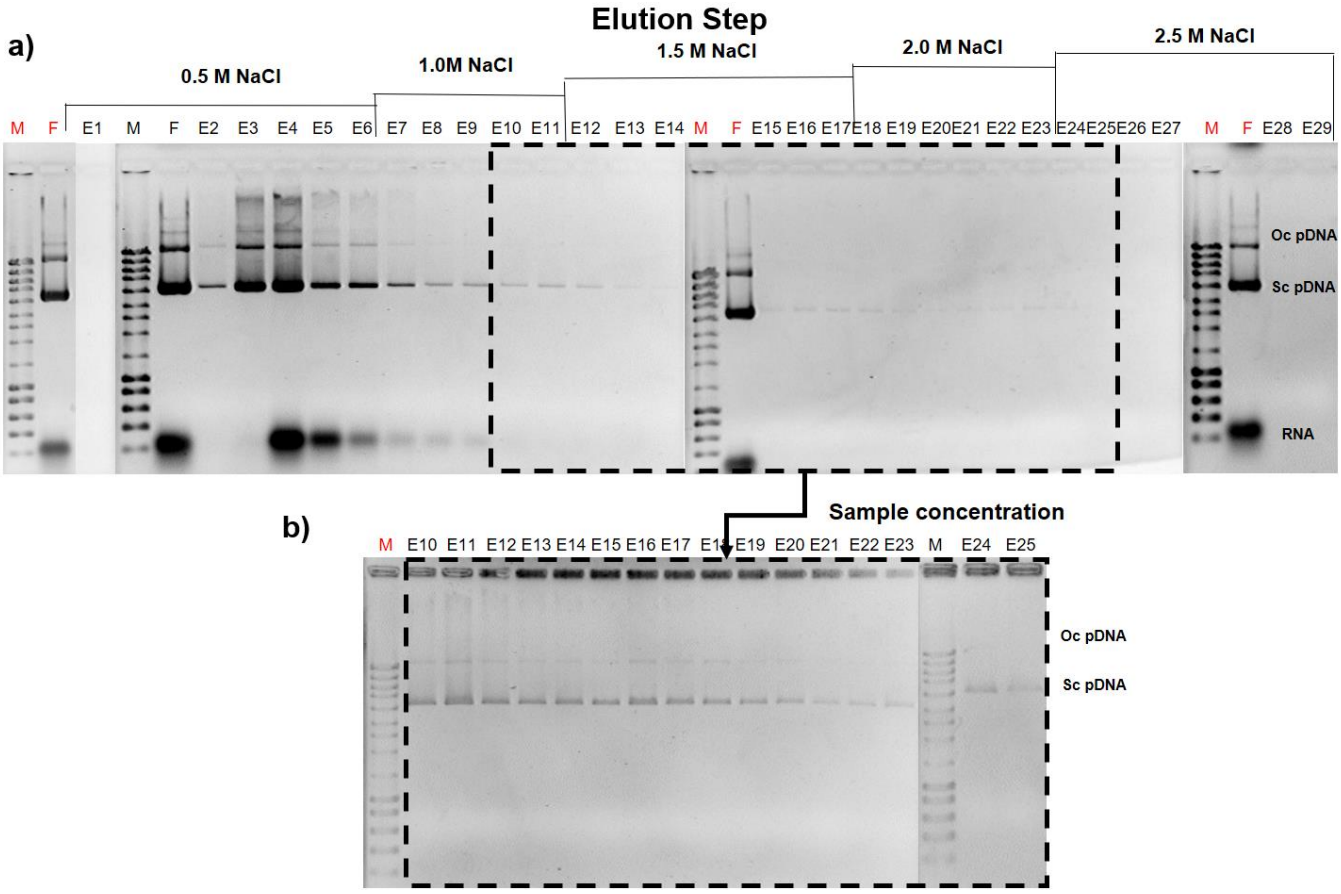


Figure 18: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Aminated Sepharose CL-6B packed in a 4 mL column in hydrophilic conditions (stepwise elution from 0.5 to 2.5M NaCl). a) Fractions collected during elution; E1 to E29- Fractions collected, in increasing order, during elution, where the concentration of elution buffer was increased from 0.5 M to 2.5 M NaCl; Fractions included in dashed area were the ones selected for further sample concentration to confirm the band pattern observed. b) Fractions selected for concentration in a) that were totally concentrated and suspended with 30 µL Tris-HCl 20 mM, pH 8.0 after concentration. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; open-circular. Complete band size description of DNA ladder is presented in appendix II.

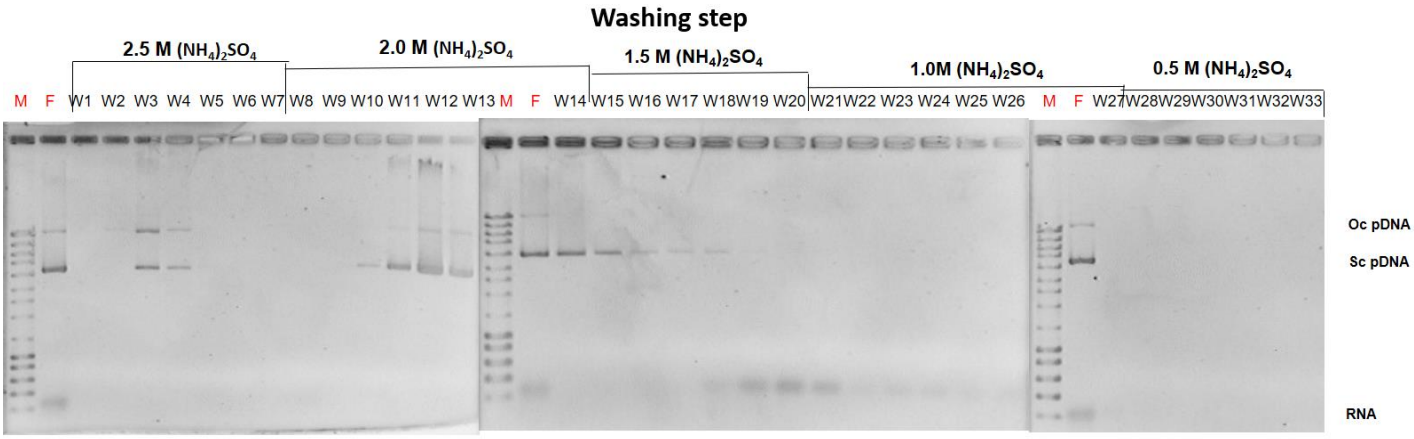


Figure 19: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Aminated Sepharose Cl-6B packed in a 4 mL column in hydrophobic conditions (stepwise washing/ elution from 2.5 M to 0.5 M $(NH_4)_2SO_4$). a) Fractions collected during washing step; W1 to W33- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$;.M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc-open-circular. Complete band size description of DNA ladder is presented in appendix II.

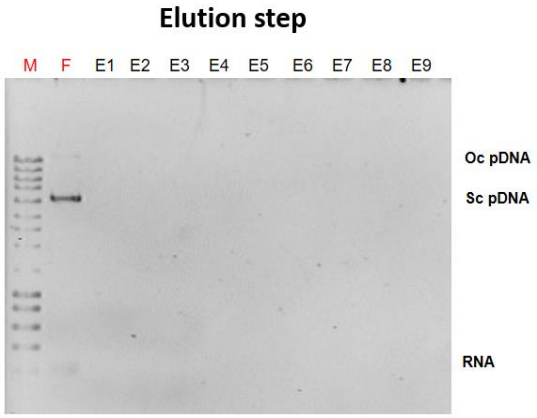


Figure 20: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL aminated Sepharose Cl-6B packed in a 4 mL column in hydrophobic conditions. E1 to E9- Fractions collected, in increasing order, during the “elution step”, where the 20 mM Tris-HCl was used as elution buffer; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc-open-circular. Complete band size description of DNA ladder is presented in appendix II.

When aminated sepharose is tested under hydrophobic binding conditions (Figure 19 and 20), the elution pattern observed is different from the one described under hydrophilic environment. In this case, all nucleic acid molecules are apparently eluted in the washing, where part of supercoiled and open-circular isoforms are eluted in third and fourth fraction collected from washing at a salt-concentration of 2.5 M ammonium sulphate, followed by the elution of remaining plasmid isoforms

apparently without RNA, when 2.0 M ammonium sulphate is applied, and fractions with RNA containing supercoiled contamination in the 17th and 18th fractions.

Selective elution between plasmid DNA and RNA in the washing, even though initially contaminated with supercoiled isoform, may suggest that this resin may be selective for those species under hydrophobic conditions. Regarding the fact that aminated sepharose is composed mainly by free amino groups, able to perform electrostatic interactions, those interactions are not expected to occur in this binding conditions compared to hydrophilic environment and thus plasmid and RNA retention may be related to molecular sieving effects as discussed previously [107].

4.2.1.3. Aminated Sepharose CL-6B activated with cyanuric chloride

After amination of epoxy-activated Sepharose CL-6B, the support can be modified by immobilization of cyanuric chloride, which is the basis of triazine-based ligands (see Figure 10a). The activation of aminated agarose with cyanuric chloride includes the substitution of one of the chlorines from cyanuric chloride by the amino groups from the resin, yielding dichlorotriazinyl agarose (vide Figure 11). During ligand synthesis and support derivatization, part of dichlorotriazinyl agarose (about 1 mL) was directly packed in a column to be evaluated under hydrophilic conditions, performing a stepwise elution with increasing concentrations of sodium chloride (from 0.5M to 2.5M) and under hydrophobic binding environmental, applying a stepwise elution with decreasing concentrations of ammonium sulphate (2.5 M to 0.5 M), as described in topic 3.8.1..Regarding the instability of resin, namely the reactivity of chlorines in cyanuric chloride structure, column equilibration and feed loading were performed at 4ºC, to slow down the rate of possible non-specific interactions with the buffers added[108]. The results obtained are presented in Figures 21 to 24.

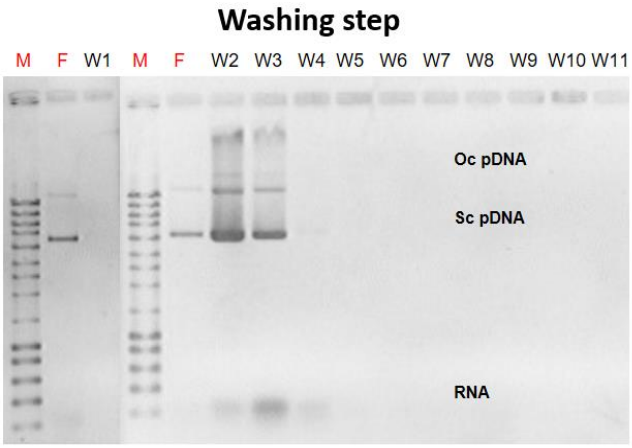


Figure 21: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Aminated Sepharose CL-6B actiated with cyanuric chloride packed in a 4 mL column in hydrophilic conditions. W1 to W11- Fractions collected, in increasing order, during washing, using Tris-HCl 20 mM, pH 8.0; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

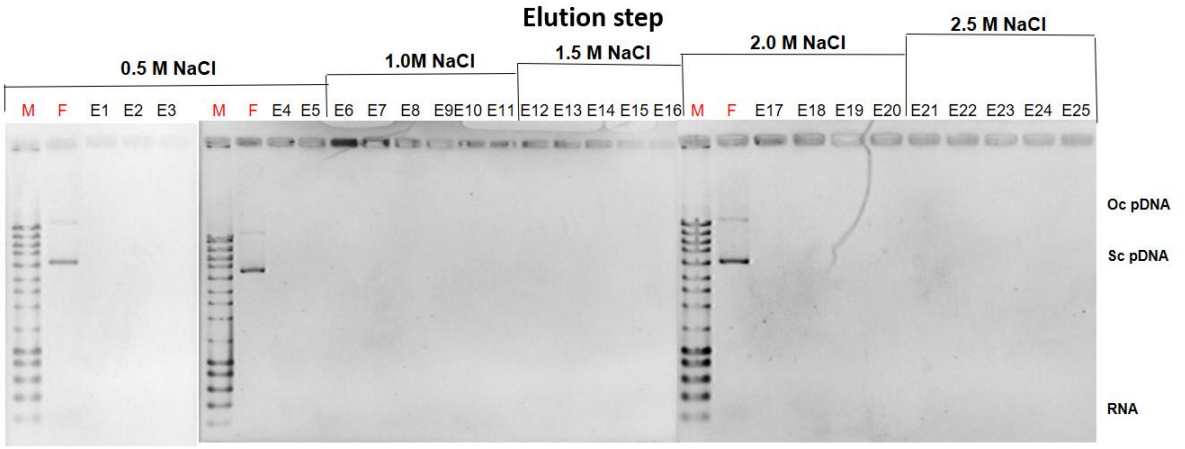


Figure 22: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Aminated Sepharose CL-6B activated with cyanuric chloride packed in a 4 mL column in hydrophilic conditions (stepwise elution from 0.5 to 2.5 M NaCl). a) Fractions collected during elution; E1 to E29- Fractions collected, in increasing order, during elution, where the concentration of elution buffer was increased from 0.5 M to 2.5 M NaCl; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc-open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophilic conditions, apparently all the pDNA and RNA are co-eluted in the beginning of washing (Figure 21) since no bands are detected in fractions collected during elution (Figure 22). The co-elution of a pDNA isoforms and RNA mainly in the first fractions of washing step, indicates that nucleic acids are not retained in the column and no separate elution is observed, suggesting that this resin is not able to perform a separate elution of nucleic acids present and is not selective for any of these species under hydrophilic environment.

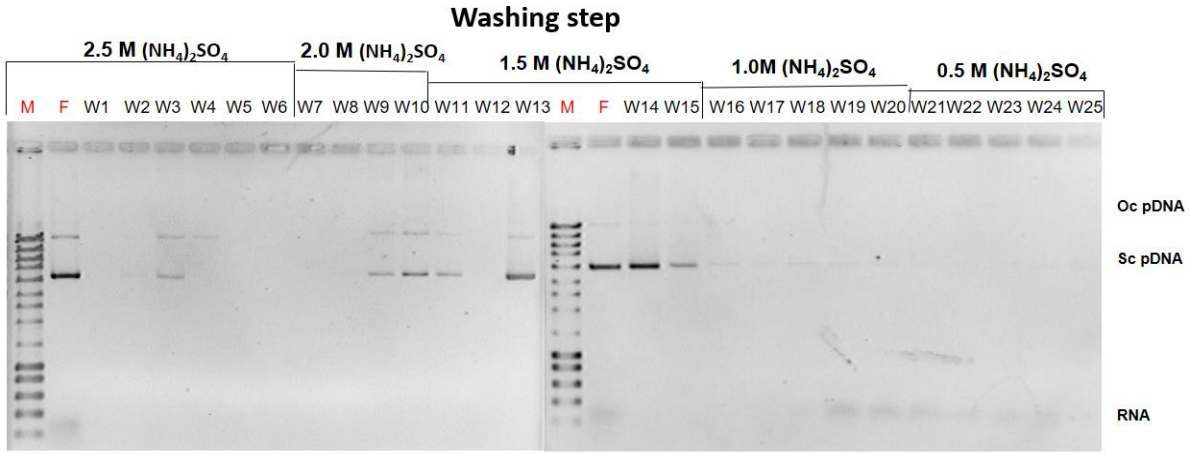


Figure 23: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Aminated Sepharose Cl-6B activated with cyanuric chloride packed in a 4 mL column in hydrophobic conditions (stepwise washing/ elution from 2.5 M to 0.5 M $(NH_4)_2SO_4$). W1 to W25- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$;.M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc-open circular. Complete band size description of DNA ladder is presented in appendix II.

Figure 24: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL aminated Sepharose Cl-6B activated with cyanuric chloride packed in a 4 mL column in hydrophobic conditions. E1 to E9- Fractions collected, in increasing order, during the "elution step", where the 20mM Tris-HCl was used as elution buffer; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

When aminated Sepharose CL-6B activated with cyanuric chloride is evaluated in terms of selective elution, under hydrophobic binding environment, all the nucleic acids from feed loading are apparently eluted in the washing (Figure 23). As such, part of plasmid isoforms, isolated from RNA, are eluted in 3rd and 4th fractions, at a concentration of 2.5M ammonium sulphate and from 9th and 15th fractions, with concentrations of ammonium sulphate ranging from 2.0 to 1.5 M, followed by the elution of RNA.

Separation between pDNA and RNA indicates that this resin is selective for one of the species under hydrophobic conditions, where RNA is retained to the column for longer time than pDNA isoforms. This selective elution can be related to the fact that triazine sccafold contains an aromatic ring that can be involved in hydrophobic or stacking interaction with nucleic acids, corresponding to an additional contribution for hydrophobic interactions in triazine scaffolds with hydrophobic substituents [109]. Plasmid DNA is a double-stranded molecule that contains its hydrophobic bases shielded inside the double helix while RNA has its bases more exposed, thus interacting strongly with triazine structure and being retained for longer time compared to pDNA [56,109].

Also, the fact that the column was equilibrated and feed loaded under 4ºC before both assays may also influence the binding of supercoiled isoform. It was previously reported that when temperature of assay is increased, retention time for supercoiled isoform is decreased. This isoform contains their bases more exposed than open-circular due to negative supercoiling that induces deformations in the torsional strain of DNA, favoring their interaction with support. An increase in temperature remove negative supercoils, which promotes structure relaxation and decrease of the number of exposed bases and the interaction with the support is decreased [16].

4.2.1.4. Ligand 0/0

Ligand 0/0, corresponds to aminated triazine scaffold, where R1 and R2 chlorines were substituted by NH_3 groups. This ligand is considered a "negative" control since it was reported in a

previous preliminary work that this ligand is not able to bind oligonucleotides under hydrophilic conditions, those being washed away in the washing steps [101]. This ligand was synthesized and derivatized in Sepharose CL-6B in this work, as described in topic 3.7., and packed in a column to be evaluated under hydrophilic conditions, performing a stepwise elution with increasing concentrations of sodium chloride (from 0.5 M to 2.5 M) and under hydrophobic binding environment, applying a stepwise washing with decreasing concentrations of ammonium sulphate (2.5 to 0.5M), as described in topic 3.8.1. To confirm that this ligand was efficiently derivatized in the solid-support, these tests were repeated in resin containing ligand 0/0, previously synthesized by João Belchior, and compared with the results from newly synthesized resin. The results obtained are presented in Figures 25 to 28.

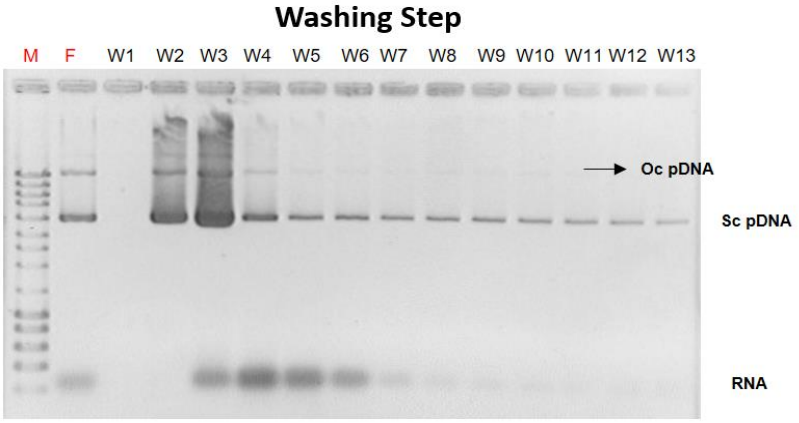


Figure 25: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 0/0 packed in a 4 mL column and evaluated in hydrophilic conditions. W1 to W13- Fractions collected, in increasing order, during washing, using Tris-HCl 20 mM, pH 8.0; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc-open-circular. Complete band size description of DNA ladder is presented in appendix II.

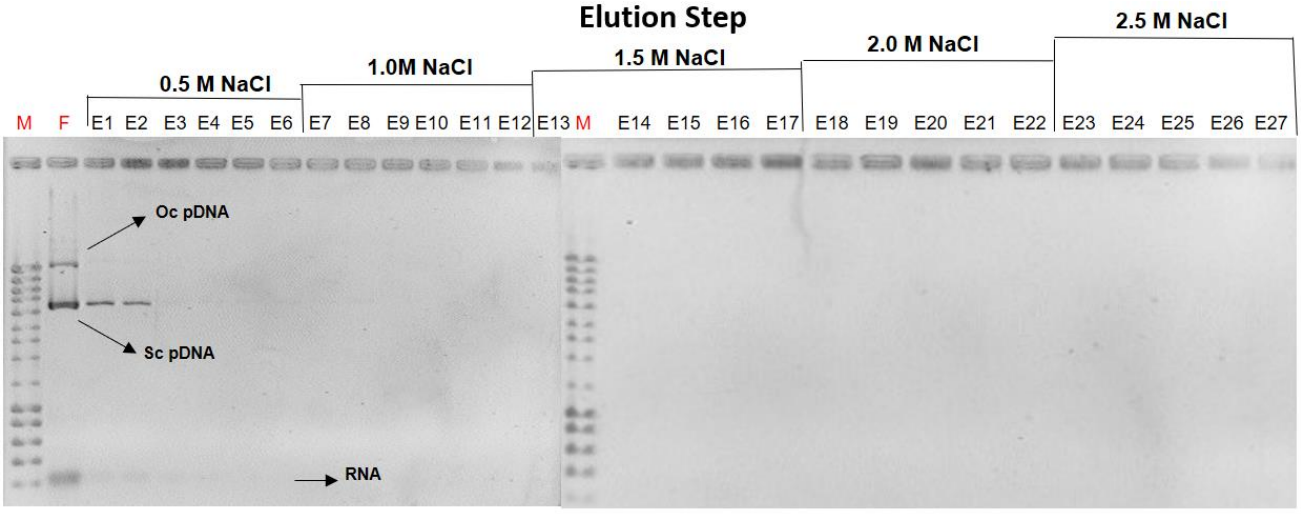


Figure 26: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 0/0 packed in a 4 mL column in hydrophilic conditions (stepwise elution from 0.5 to 2.5 M NaCl). a) Fractions collected during elution; E1 to E27- Fractions collected, in increasing order, during elution, where the concentration of elution buffer was increased from 0.5 M to 2.5 M NaCl; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

When ligand 0/0 is tested under hydrophilic binding environment, co-elution of a significant part of pDNA and RNA is observed mainly in the beginning of the washing. This pattern is still

detected in the further fractions of washing and in the first 2 fractions collected during elution but in lower amount. Co-elution of a significant part of pDNA isoforms and RNA in the washing indicates that these molecules are not retained by the ligand, suggesting that this ligand is not selective for any of these nucleic acid molecules and a selective separation does not occur. These results were similar to those observed in the resin previously derivatized with ligand 0/0, under hydrophilic environment and is in accordance with the binding studies using oligonucleotides in ligand 0/0, in which it was considered to be a non-binder [101]. Moreover, these results are different from those observed in aminated Sepharose, a resin also comprising free amine groups, in which nucleic acids are retained in the absence of salt under hydrophilic conditions (Figurres 17 and 18). Despite the fact that both resins include free amine groups to react (respectively NH_3 groups in cyanuric chloride scaffold in ligand 0/0 and free amine groups in aminated Sepharose), probably the amine groups are not charged in ligand 0/0 under these conditions (unlike the amine groups in aminated Sepharose), not interacting with the phosphate groups from nucleic acid molecules and explaining direct flow trough of these molecules in the washing step.

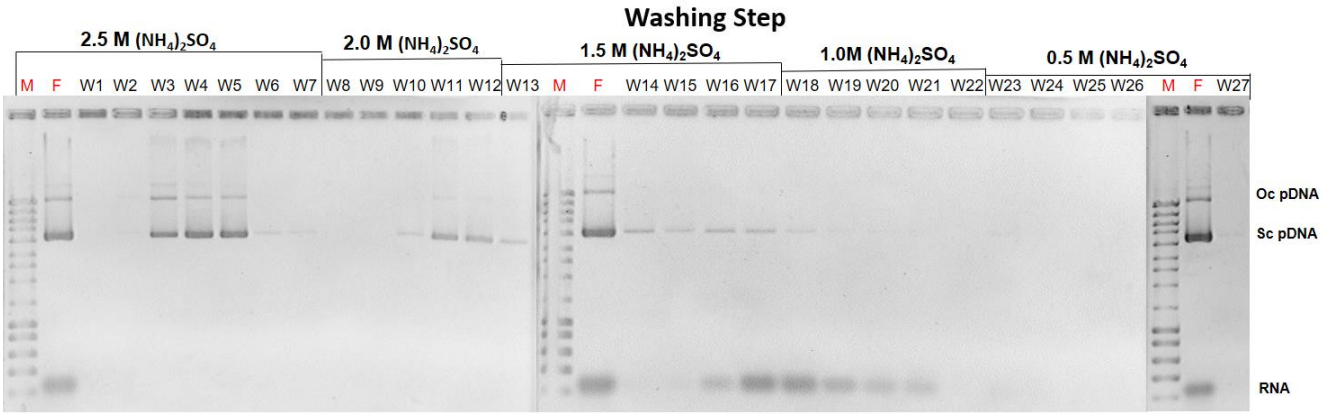


Figure 27: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 0/0 packed in a 4 mL column in hydrophobic conditions (stepwise washing/elution from 2.5 M to 0.5 M $(NH_4)_2SO_4$). W1 to W27- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$;.M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc-open circular. Complete band size description of DNA ladder is presented in appendix II.

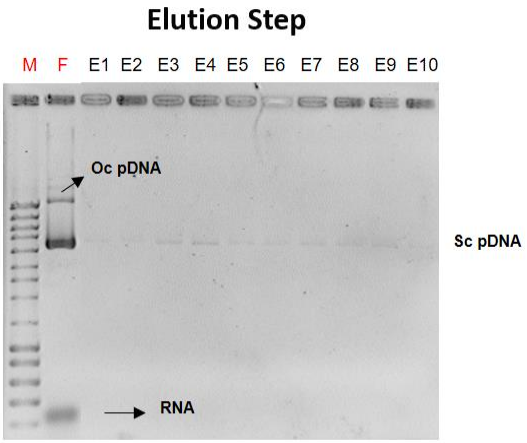


Figure 28: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 0/0 in a 4 mL column in hydrophobic conditions. E1 to E10- Fractions collected, in increasing order, during the "elution step", where the 20 mM Tris-HCl was used as elution buffer; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophobic binding conditions, oc and sc plasmid isoforms are detected from the 3rd fraction, being eluted in the further 5 fractions and, apparently the remaining plasmid molecules are eluted from fraction number 10, at a concentration of ammonium sulphate between 2.0 M and 1.5 M. RNA elution is observed with sc pDNA contamination between 1.5 M and 1.0 M ammonium sulphate in the washing step. During elution, where Tris-HCl is used as elution buffer in the absence of salt, traces of sc pDNA, apparently isolated from other isoforms and RNA, are detected in the fractions collected. The presence of contamination of other nucleic acid molecules in these fractions could have been confirmed by concentration of the final fractions of elution containing the traces of sc pDNA. The retention of plasmid, mainly sc isoform, and RNA as well as different retention times between species indicates that this ligand may be selective for the supercoiled pDNA and RNA under hydrophobic binding environment, allowing selective separation. In this case, influence of aromatic ring of triazine structure can explain separate elution between pDNA and RNA, due to hydrophobic or stacking interactions, suggesting that ligands tested may already have binding capacity *a priori* due to the presence of this aromatic ring [109]. Results from a previous work have shown that ligand 0/0 did not bind oligonucleotides under hydrophilic conditions but binding under hydrophobic environment was not evaluated[101].

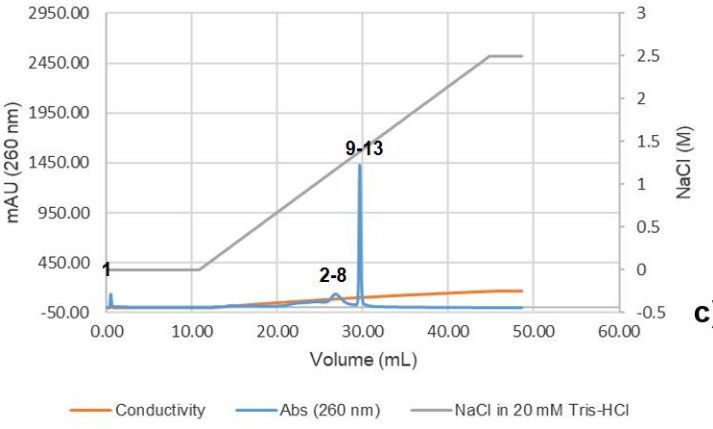
In general, the order of elution of the nucleic acid molecules is similar to the one observed with resin with 0/0 that was already available in the lab (from this previous work), under hydrophobic conditions (Figure A9 to Figure A12 – Appendix IV) [101]. Nevertheless, those molecules are retained in the column for shorter time than the oldest resin 0/0. This fact may be related to several factors, such as the atmospheric temperature of each assay, since it was reported to influence retention time and resolution in chromatographic assays but also due to possible different packaging of the resins inside the chromatographic columns [16].

4.2.1.5. EDA CIM® Monolithic disk

As mentioned before (see topic 3.8.2), ligand 6/5 was previously synthesized and derivatized in an EDA CIM® monolithic disk with a bed volume of 0.34 mL and evaluated in terms of pVAX1/lacz purification, in an ÄKTA purifying system, namely ÄKTA 10. In parallel to this derivatized monolithic disk, a non-derivatized EDA CIM® monolithic disk control was also evaluated in the same chromatographic system. Comparison of these two supports should allow to infer whether derivatization of the EDA CIM® monolith with ligand 6/5 had been successfully achieved with a protocol that was well-established and efficient for agarose [92].

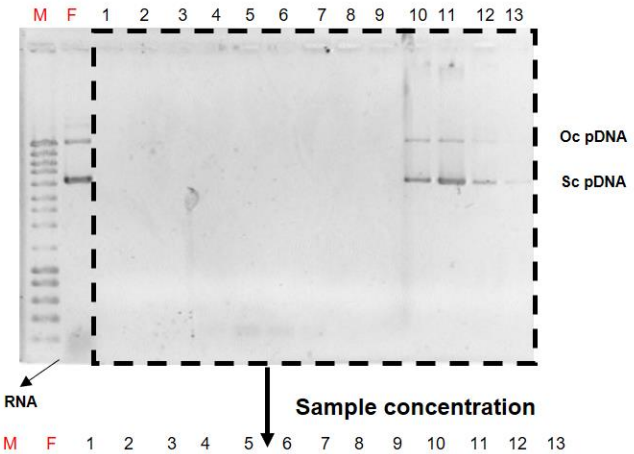
The chromatographic assays were performed under hydrophobic conditions, applying a decreasing gradient from 2.5 M to 0 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl, pH 8.0 followed by Tris-HCl 20mM as elution buffer; and under hydrophilic conditions, using Tris-HCl 20 mM, pH 8.0 as equilibration and washing buffers and a gradient between 0 and 2.5 M of NaCl in the same buffer for elution, as described in topic 3.8.2. The results obtained in non-derivatized CIM monolithic disk are presented in Figures 29 and 30.

a)



Fractions	Chromatographic Step
1	Washing
2-13	Elution

b)



c)

Sample concentration

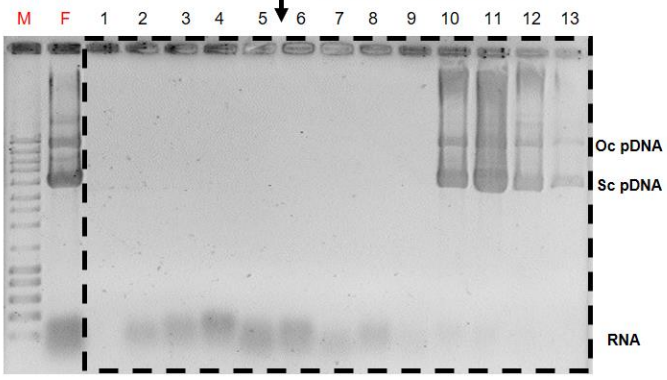


Figure 29: a) Chromatography in a EDA CIM® monolith disk performed under hydrophilic conditions (using a gradient from 0 M to 2.5 M NaCl in 20 mM Tris-HCl, pH 8.0 and Tris-HCl 20 mM, pH 8.0 as washing buffer. 100 µL of clarified *E. coli* lysate was injected after column equilibration with Tris-HCl 20mM, pH 8.0. In the chromatogram, the conductivity measured during the assay is indicated in mS/cm and the scale for this data is presented in the left vertical axis.b) Gel electrophoresis of 30µL of each fraction collected in a), the fractions included in the dashed area were selected for fully concentration and further suspension with 30 µL Tris-HCl 20mM pH 8.0. c) Gel electrophoresis of 20 µL of each fraction that was concentrated; M- DNA ladder III (NZYtech); F-Feed; oc pDNA – open-circular; sc- supercoiled. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophilic conditions, three peaks are detected in the chromatogram (Figure 29a), two small peaks and a third peak more intense. When the fractions collected from those peaks are analyzed by gel electrophoresis (Figure 29b), no bands are detected in the fraction collected from first peak, which can be due to conductivity peaks within the system. On the other hand, RNA is present in the fractions collected on the second peak, which was confirmed by concentration of those fractions and re-analysis by gel electrophoresis (Figure 29c), while plasmid-rich fractions are detected in the third peak. These results indicate that EDA CIM® monolithic disk is selective for the nucleic acid molecules, being able to separate RNA from plasmid isoforms (oc and sc). A similar elution pattern was reported in a previous work using this type of monolithic disk, in which it was possible to separate RNA from oc and sc plasmid isoforms, applying increasing concentrations of NaCl [111].

As mentioned above, EDA CIM monolith is a weak anion-exchanger, meaning that is positively charged and can interact with negatively charged molecules, such as RNA or pDNA, which explains nucleic acid retention, under a hydrophilic binding environment, till a concentration between 0.5 and 1 M NaCl (Figure 29a).Plasmid DNA retention may be associated to high charge density of supercoiled pDNA [56,109,110]. The presence of salt interferes in the electrostatic interactions between the matrix and the nucleic acids, in which the salt compete with bound molecules for the interaction with the matrix, allowing the elution of bound molecules [110,111].

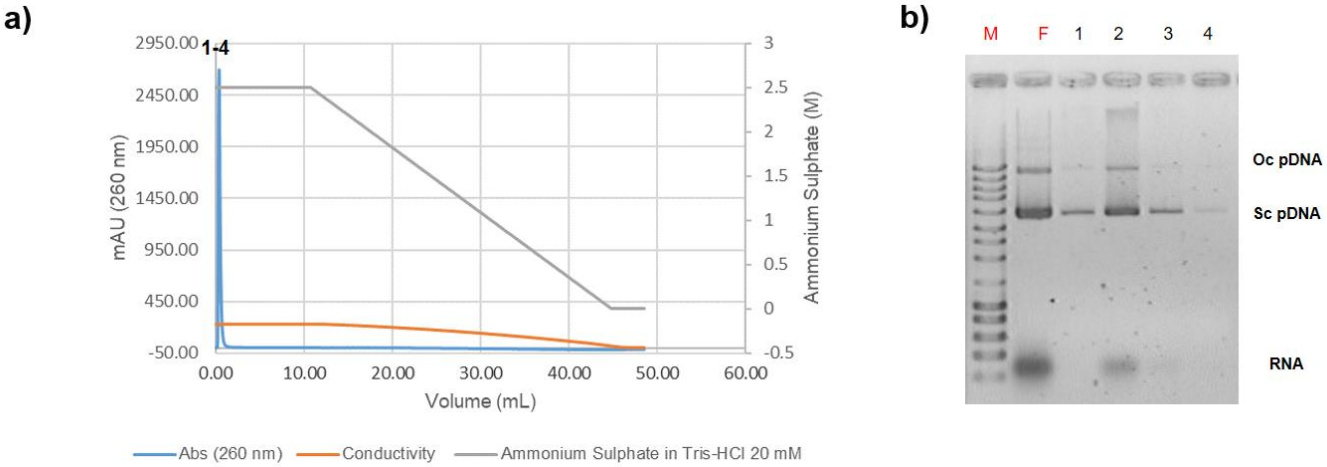


Figure 30: a) Chromatography in a EDA CIM® monolith disk performed under hydrophobic conditions (using a gradient from 2.5M to 0M ammonium sulphate in 20 mM Tris-HCl, pH 8.0 followed by Tris-HCl 20 mM, pH 8.0 as elution buffer. 100 µL of clarified *E. coli* lysate was injected after column equilibration with 2.5M ammonium sulphate in Tris-HCl 20 mM, pH 8.0. In the chromatogram, the conductivity measured during the assay is indicated in mS/cm and the scale for this data is presented in the left vertical axis. b) Gel electrophoresis of 30µL of each fraction collected in a); M- DNA ladder III (NZYtech); F-Feed; oc pDNA – open-circular; sc- supercoiled. Complete band size description of DNA ladder is presented in appendix II.

Regarding chromatography with EDA CIM® monolithic disk performed under hydrophobic binding environment, one single peak is detected in the beginning of the assay (Figure 30a), in which open-circular and supercoiled pDNA as well as RNA are co-eluted (Figure 30b).

Direct elution of all nucleic acids present in the feed indicates that those molecules are not retained by the disk, which suggests that this support is not selective for any of these species when

hydrophobic binding conditions are applied, explaining the absence of nucleic acid separation. In this case, electrostatic interactions between the positive groups monolith and nucleic acids are not favored by the application of a hydrophobic binding environment, which may explain the elution of all species in the flow through.

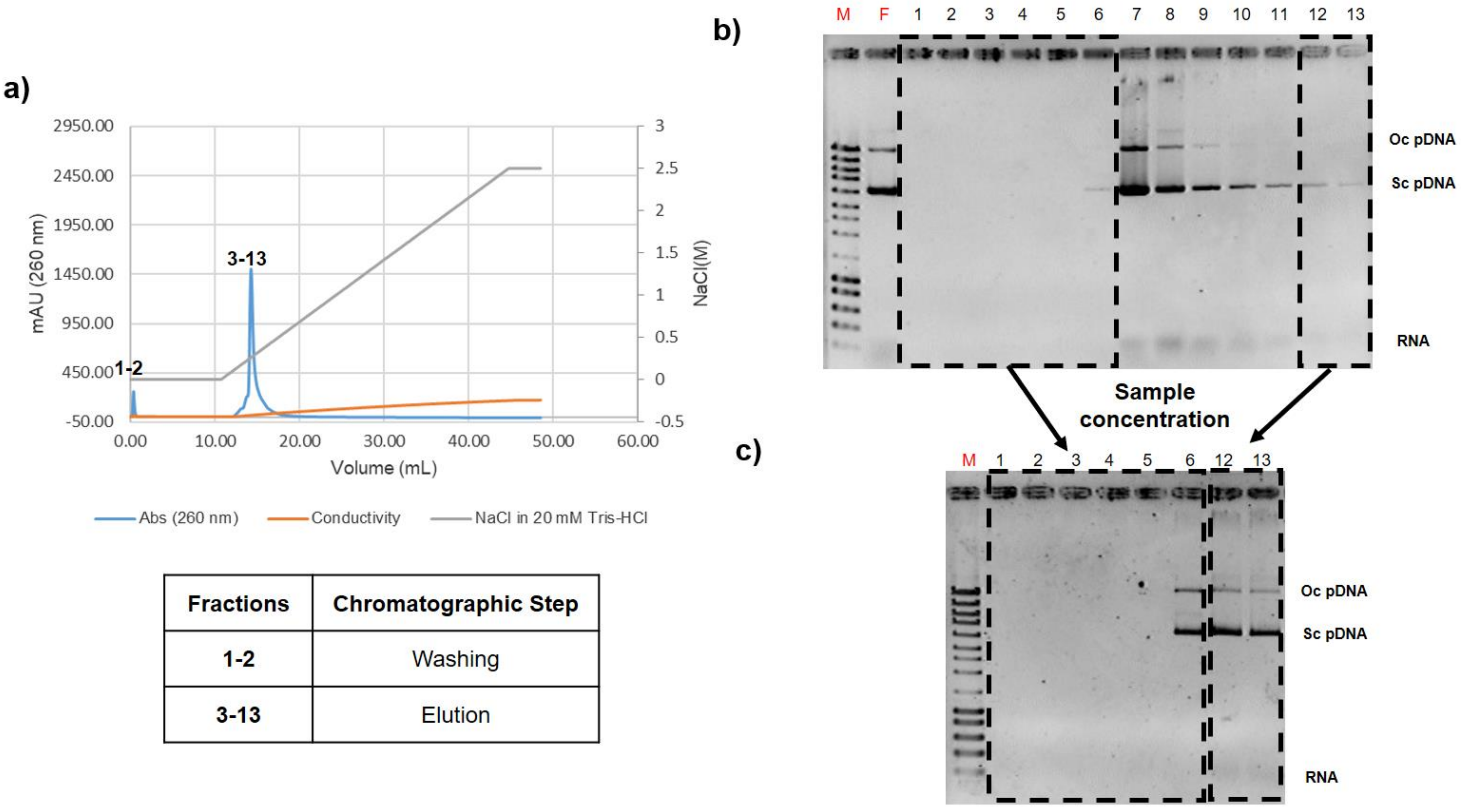
4.2.2. Predominantly hydrophobic ligands

In the present work, ligands from a previously screened 22-membered library to bind to double stranded oligonucleotides were selected to be tested for pDNA purification from *E. coli* cellular extracts[101]. From the selection of ligands available for testing, several predominantly hydrophobic ligands, namely 6/5, 11/8, 8/11, 3/5 and 8/1, were evaluated in terms binding to nucleic acids and pVAX1/lacz purification. The selection of this set of ligands was based on results previously obtained with those ligands as potential strong binders to nucleic acids [101]. Ligands screening was performed by affinity chromatography in bench chromatographic columns, at an initial phase (except for ligand 6/5), under a hydrophobic binding environment, applying a stepwise washing with decreasing concentrations of ammonium sulphate (between 2.5 and 0.5M) in Tris-HCl 20 mM, pH 8.0 followed by Tris-HCl 20 mM, pH 8.0 as elution buffer, as described in topic 3.8.1. These conditions were used to exploit possible interactions between hydrophobic ligands and hydrophobic bases of nucleic acids. The results obtained are presented in the following topics.

4.2.2.1. Ligand 6/5

As mentioned before, ligand 6/5 was previously synthesized and immobilized for the first time in a EDA CIM® monolithic disk with a bed volume of 0.34 mL using a well-established solid-phase protocol and tested for plasmid purification in a previous preliminary work [92]. In this preliminary study, sc pDNA isolation in some fractions and plasmid separation from RNA was achieved under hydrophobic conditions, using 0.4 and 1.5 M ammonium sulphate. Nevertheless, the reproducibility in some chromatographic assays was not fully proven, which required additional studies to confirm this data [92].

As such, in this work, several chromatographic assays were performed to evaluate the potential of a CIM monolithic disk derivatized with ligand 6/5 in terms of plasmid purification (namely pVAX1/lacz). Parallel assays were performed in similar conditions of non-derivatized CIM monolithic disk. Both monolithic supports were tested under hydrophilic conditions, using Tris-HCl 20 mM, pH 8.0 as equilibration and washing buffer and a gradient between 0 and 2.5 M of NaCl in the same buffer for elution, and under a hydrophobic environment, applying a decreasing gradient from 2.5 M to 0 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl, pH 8.0 followed by Tris-HCl 20 mM pH 8.0 as elution buffer, as described in topic 3.8.2. The results of affinity chromatographic assays with ligand 6/5 are presented in Figures 31 to 33.



Fractions	Chromatographic Step
1-2	Washing
3-13	Elution

Figure 31: a) Chromatography in a EDA CIM® monolith disk derivatized with ligand 6/5, performed under hydrophilic conditions (using a gradient from 0 M to 2.5 M NaCl in 20 mM Tris-HCl and Tris-HCl 20mM as washing buffer. 100 µL of clarified *E. coli* lysate was injected after column equilibration with Tris-HCl 20mM, pH 8.0. In the chromatogram, the conductivity measured during the assay is indicated in mS/cm and the scale for this data is presented in the left vertical axis. b) Gel electrophoresis of 30µL of each fraction collected in a), the fractions included in the dashed area were selected for concentration and further suspension with 30 µL Tris-HCl 20mM, pH 8.0. c) Gel electrophoresis of 20 µL of each fraction that was concentrated; M- DNA ladder III (NZYtech); F-Feed; oc pDNA – open-circular; sc- supercoiled. Complete band size description of DNA ladder is presented in appendix II.

In the derivatized monolith tested under hydrophilic conditions (Figure 31a), a small peak is detected in the beginning of washing, whereas a more intense peak is detected during elution. From the first peak, having about 265 mAU, fractions 1 and 2 were collected and analyzed by gel electrophoresis and, apparently, no nucleic acids were present Figure 31), which was confirmed by the concentration of those fractions (figure 31c). This result is probably due to high conductivity in the system that affects UV absorbance.

The second peak is more intense, about 1505 mAU, and is detected during the elution step, at a NaCl concentration of 0.26 M. Analysis by gel electrophoresis of the fractions collected form this peak (fractions 3 to 13) showed that open-circular, supercoiled pDNA and RNA are co-eluted.

Retention of nucleic acids till a concentration of 0.26 M NaCl, indicates that this derivatized monolith has affinity for these molecules, the affinity decreasing with increasing ionic strength, promoting nucleic acids elution. On the other hand, co-elution of all nucleic acids present in the feed, suggests that this ligand is not able to perform a separate elution, not being selective for any of the species under an hydrophilic environment.

In non-derivatized monolith, nucleic acids retention during washing step was also observed (Figure 29), but, unlike in the presence of ligand 6/5, separation between RNA and pDNA isoforms was achieved. The comparison of chromatographic results with non-derivatized (Figure 29 and 30) and derivatized EDA CIM® support seems to indicate that ligand 6/5 was bound, at least to some extent, to the derivatized support, altering the differential interactions between the EDA support and nucleic acids. The presence of ligand, which is bound to amine groups, alters the behavior of the polymer as a weak-anion exchanger by decreasing the number of charged amine groups available to interact with negatively charged phosphate groups in nucleic acids [111,112].

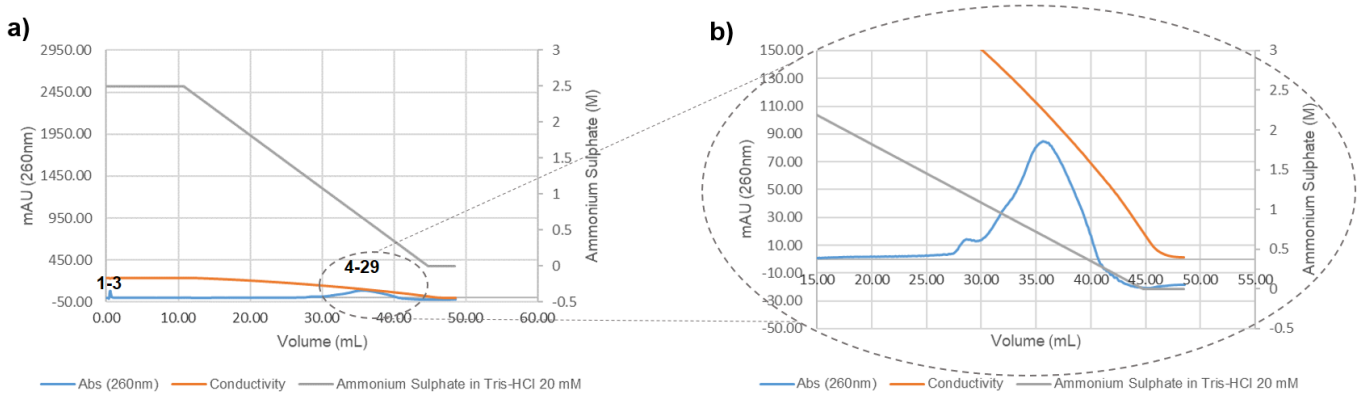


Figure 32: a) Chromatography in a EDA CIM® monolith disk derivatized with ligand 6/5 performed under hydrophobic conditions (using a gradient from 2.5 M to 0 M ammonium sulphate in 20 mM Tris-HCl and Tris-HCl 20 mM as elution buffer. 100 µL of clarified *E. coli* lysate was injected after column equilibration with 2.5 M ammonium sulphate in Tris-HCl 20 mM, pH 8.0..b) Zoomed chromatogram from a) between a volume of 15 and 55 mL. In the chromatogram, the conductivity measured during the assay is indicated in mS/cm and the scale for this data is presented in the left vertical axis. Complete band size description of DNA ladder is presented in appendix II.

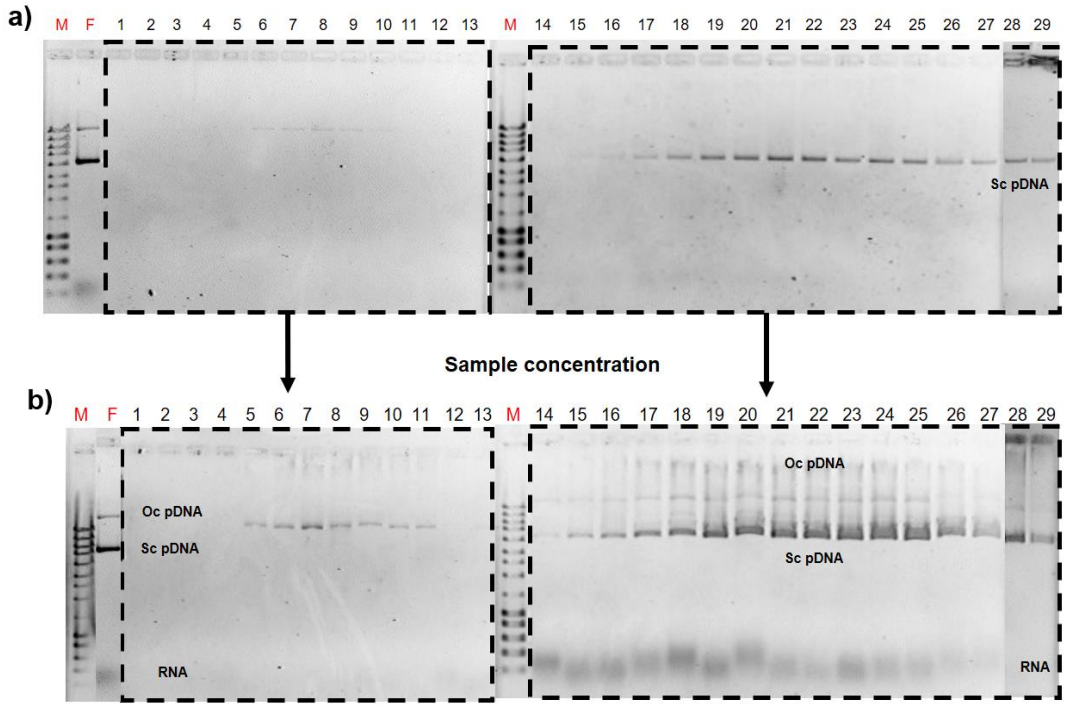


Figure 33: Gel electrophoresis of 30 µL of each fraction collected from chromatography (in figure 32); the fractions included in the dashed area were selected for concentration and further suspension with 30 µL Tris-HCl 20 mM, pH 8.0. c) Gel electrophoresis of 20 µL of each fraction that was concentrated; M- DNA ladder III (NZYtech); F-

Feed; oc pDNA – open-circular; sc- supercoiled. Complete band size description of DNA ladder is presented in appendix II.

The results of chromatographic assays with EDA CIM® monolith derivatized with ligand 6/5 under hydrophobic conditions are presented in Figure 32. In this assay, a low-intensity peak is detected in the beginning of washing at high concentration of $(NH_4)_2SO_4$ (2.5 M), while a second broad base peak is detected during elution, when the concentration of $(NH_4)_2SO_4$ is close to zero.

Regarding the first peak, fractions 1 to 3 (Figure 33) were collected from the latter and analyzed by gel electrophoresis and, apparently, no nucleic acids were detected. This peak may be due to high conductivity in the system that affects UV signal, generating a peak.

The second peak detected also has low-intensity and starts to be detected at a concentration of ammonium sulphate of 1.23 M. As it can be seen in Figure 32c, this peak seems to be composed of two overlapping peaks, one smaller followed by other with larger base and higher intensity (about 85 mAU). When the fractions collected from this broad peak are analyzed by gel electrophoresis, apparently oc pDNA are eluted in low amounts, followed by the elution of supercoiled isoform (Figure 33a). To confirm this elution pattern, these fractions were concentrated and re-analyzed by gel electrophoresis. The initial elution pattern is not observed after fraction concentration (Figure 33b), in which fractions containing isolated oc pDNA are, in fact, eluted firstly but this is followed by co-elution of a significant amount of pDNA isoforms (supercoiled and open-circular) and RNA. The difference in fraction content after concentration, highlights the importance of fraction concentration as key-step to confirm the nucleic acid content in diluted samples in gel electrophoresis, optimizing bands visualization and avoiding incorrect interpretation of the gels.

Retention of nucleic acids till a concentration of ammonium sulphate around 1.23 M, indicates that ligand 6/5 immobilized in EDA CIM® disk can retain those molecules under hydrophobic binding environment. Nucleic acid retention is also observed in ligand 6/5 immobilized in Sepharose CL-6B, under similar conditions (Figure A14). Moreover, this molecular retention is not observed in the non-derivatized EDA CIM® disk, in which nucleic acid molecules are directly eluted in the flow-through (Figure 30), suggesting that this phenomenon may be related to ligand interaction with nucleic-acid molecules. In fact, ligand 6/5 is composed of isoamylamine and phenethylamine as substituents of the two chlorines in the cyanuric chloride structure (Table 4, Figure 10), which are analogues of amino acids leucine and phenylamine, respectively, both with hydrophobic character, resulting in a ligand with predominant hydrophobic profile, prone to hydrophobic and stacking interactions (due to the aromatic ring in phenethylamine structure) [4,76,92]. As such, nucleic acid retention may be related to hydrophobic interactions with the hydrophobic ligand 6/5, that are weakened due to decrease in ammonium sulphate concentration, promoting their elution.

Partial isolation of open-circular isoform from other isoforms and RNA in the second peak, may suggest that this molecule is less retained in the column compared to the remaining molecules, and that this ligand may be selective for the nucleic acids, being able to separate this isoform from others, which can be explained the presence of lower hydrophobic bases exposed compared to sc pDNA and RNA [56,109]. Nevertheless, only a slight part of open-circular was isolated, being a

significant part co-eluted later with the remaining isoforms, which may indicate that this ligand has not a significant selectivity for nucleic acid molecules, having similar affinity for all nucleic acids. Thus, these results seem to point out to a low selectivity of ligand 6/5 for the different pDNA isoforms, once the ligand was not able to separate supercoiled form from other isoforms or to achieve pDNA separation from RNA under hydrophobic and hydrophilic binding environment, unlike was reported in previous preliminary results [92]. However, the different behavior observed for non-derivatized (Figure 30) and derivatized EDA CIM® disk also under hydrophobic conditions confirms that derivatization of the monolithic disk with ligand 6/5 was successfully achieved. This is an important result that was not proven in preliminary work with these supports.

4.2.2.2. Ligand 11/8

Ligand 11/8 comprises 2-methyl butylamine (amine 11), mimicking isoleucine, and 4-aminobenzamide (amine 8), analogue to glutamine and asparagine, as R1 and R2 substituents in the triazine scaffold (Table 4). The results of affinity chromatography assays with ligand 11/8 are presented in Figures 34 to 36.

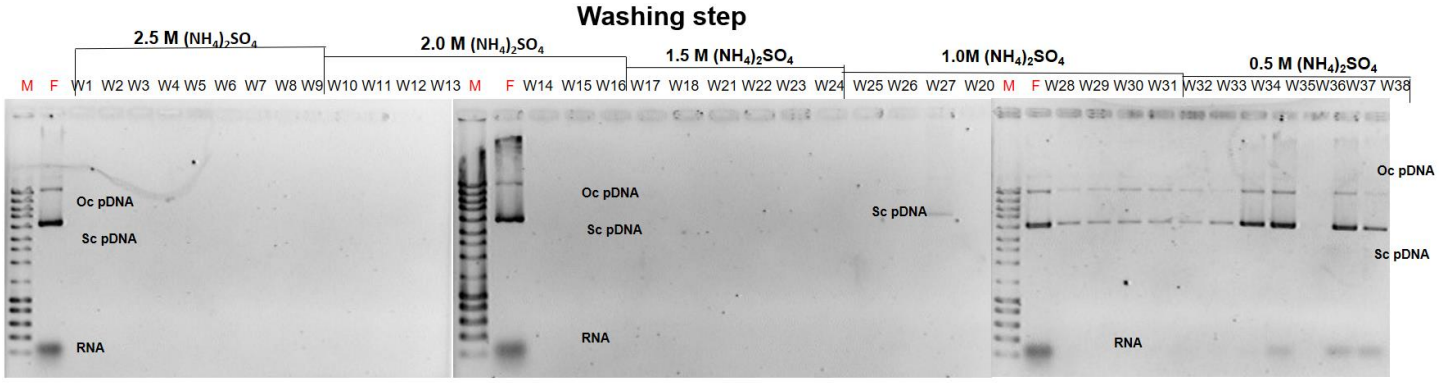


Figure 34: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 11/8 packed in a 4 mL column in hydrophobic conditions (stepwise washing/elution from 2.5M to 0.5M $(NH_4)_2SO_4$). W1 to W38- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$;.M- DNA Ladder III (NZYtech); F-Feed (clarified lysate added to the column); sc- Supercoiled; Oc-open circular. Complete band size description of DNA ladder is presented in appendix II.

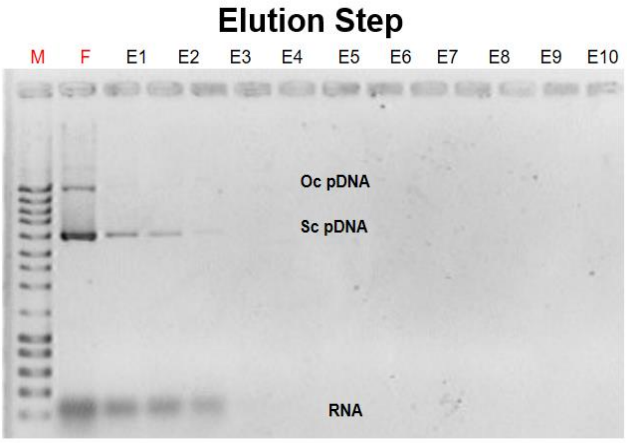


Figure 35: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 11/8 in a 4 mL column in hydrophobic environment. E1 to E10- Fractions collected, in increasing order, during the elution step, where the 20 mM Tris-HCl pH 8.0 was used as elution buffer; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophobic environment, pDNA is retained inside the column during washing step till a concentration around 1.0 M ammonium sulphate, in which seven fractions containing part of open-circular and supercoiled pDNA are eluted, without isoforms separation (Figure 34). This is followed by co-elution of pDNA and RNA, at concentration of $(NH_4)_2SO_4$ around 0.5 M, which is maintained till the end of washing step and beginning of elution (Figure 35).

After this initial study, additional chromatographic studies were performed applying a single step elution with 1.0 M ammonium sulphate, as described in 3.8.1., to evaluate possible plasmid isoforms separation (Figure 36). In this assay, RNA is retained under hydrophobic conditions, in the presence of ammonium sulphate, being eluted when ammonium sulphate is removed, whereas part of plasmid molecules (open-circular and supercoiled) are co-eluted in low-amount since the beginning of washing, which is confirmed by fraction concentration (Figure 36b).

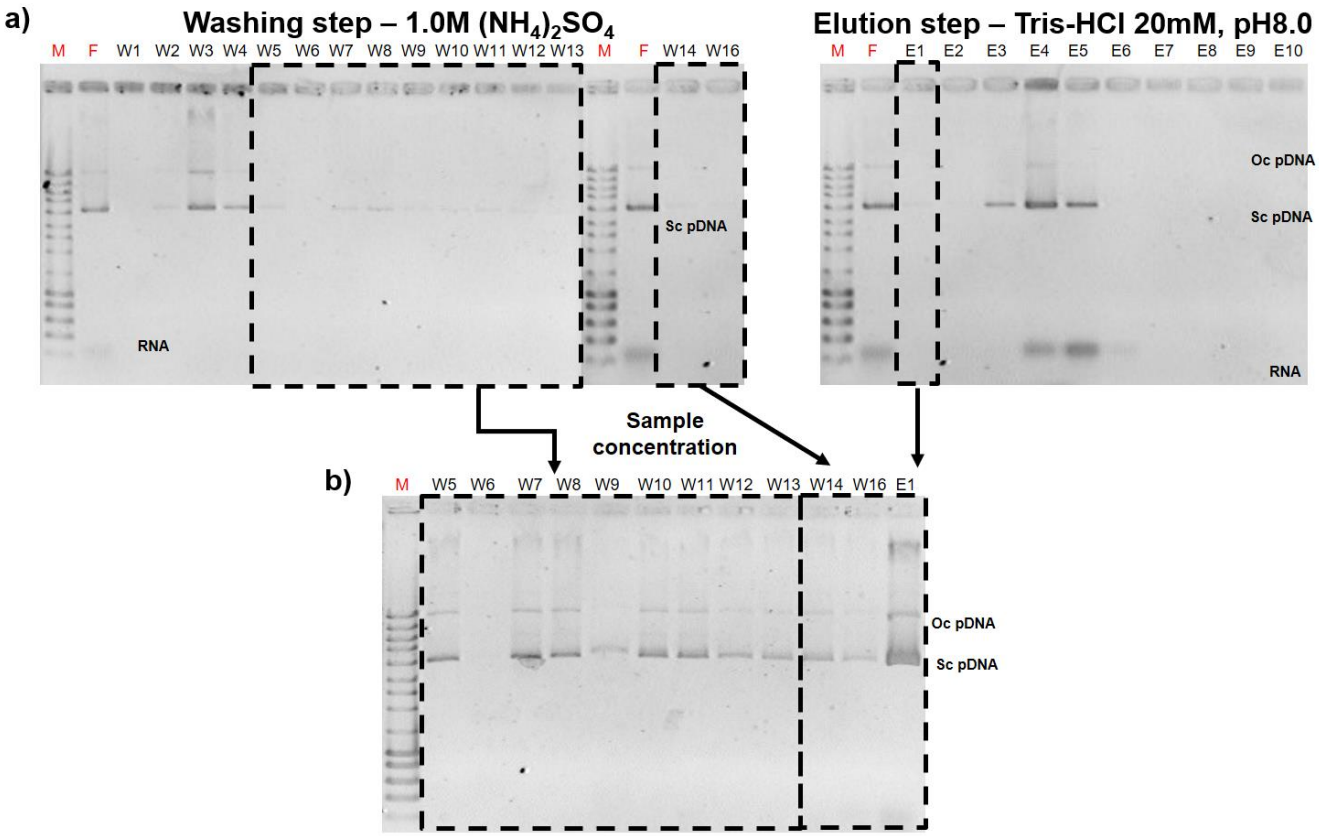


Figure 36: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 11/8 packed in a 4 mL column in hydrophobic conditions (single step washing with 1.0 M $(NH_4)_2SO_4$). a) fractions collected during washing, using 1.0M $(NH_4)_2SO_4$) in 20 mM Tris-HCl, pH 8.0 as washing buffers and elution step, applying Tris-HCl 20 mM, pH 8.0 as elution buffer. Fractions included in dashed area were the one selected for further concentration and analysis by gel electrophoresis. W1 to W16- Fractions collected, in increasing order, during washing; E1 to E10- Fractions collected, in increasing order, during the elution step. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Nucleic acid retention in both assays under strong hydrophobic environment suggest that this ligand retains those molecules, indicating that 11/8 may have affinity for all nucleic acid molecules under these conditions, namely above 1.0 M ammonium sulphate. Ligand 11/8 contain as substituents, 2-methyl butylamine, a mimetic of Isoleucine, which is a non-polar molecule able to perform van der Waals and hydrophobic interactions; and 4-aminobenzamide, a molecule with an aromatic group that confers hydrophobicity to the molecule. The combination of those substituents results in molecule predominantly hydrophobic, prone to hydrophobic interactions with nucleic acid molecules [22,77].

The retention of part of plasmid molecules (open-circular and supercoiled) for shorter time than RNA, suggests the presence of different hydrophobic interactions between this ligand and different nucleic acids present. Under hydrophobic environment, double stranded molecules, such as plasmid DNA, have their hydrophobic bases shielded and packed inside the helix, thus little or no retention is expected in these conditions. On the other hand, single stranded molecules, such as RNA, have higher exposure of hydrophobic bases, being retained for longer time under hydrophobic environment, due to stronger interactions with hydrophobic ligand 11/8 [56,109]. This hypothesis is

supported by the fact that RNA is retained inside the column only in the presence of ammonium sulphate in the single step assay using 1.0 M ammonium sulphate, being eluted only when ammonium sulphate is removed, while plasmid DNA is directly eluted from the column in the flow through (Figure 36). Nevertheless, a significant part of plasmid molecules is still co-eluted with RNA in both assays, which means that plasmid DNA is not completely isolated from RNA. These results seem to indicate that the hydrophobic contribution of ligand 11/8 is not as significant in separate elution as expected, once in the presence of the ligand, an efficient separation between plasmid isoforms or even between plasmid and RNA is not achieved.

4.2.2.3. Ligand 8/11

Ligand 8/11 contains 4-aminobenzamide (amine 8), mimetic of glutamine and asparagine, and 2-methyl butylamine (amine 11), analogue to isoleucine, as R1 and R2 substituents in the triazine scaffold (Table 4), corresponding to a symmetrical structure of ligand 11/8. Due to the uncertainty of nucleophilic substitution in R1 and R2 positions in solid-phase synthesis, symmetrical ligands may have a distinct behavior [99,104]. As so, it seemed pertinent to test ligand 8/11 and compare these results with those obtained for ligand 8/11. The results of affinity chromatography assays with ligand 8/11 are presented in Figures 37 to 39.

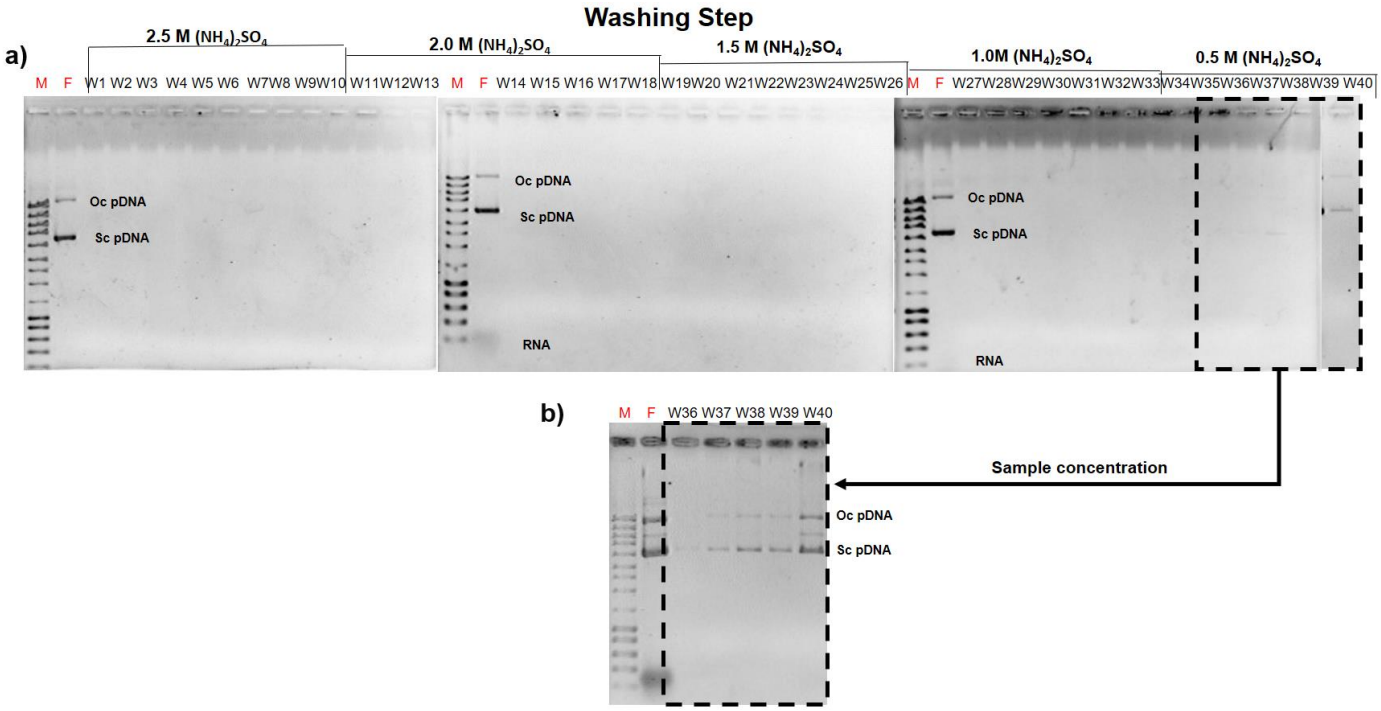


Figure 37: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 8/11 packed in a 4 mL column in hydrophobic conditions (stepwise washing/elution from 2.5 M to 0.5 M $(NH_4)_2SO_4$). W1 to W40- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$; The fractions included in the dashed area were selected for fully concentration and further suspension with 30 µL Tris-HCl 20mM, pH 8.0 to be re-analysed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction that was concentrated.M-DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc-open circular. Complete band size description of DNA ladder is presented in appendix II.

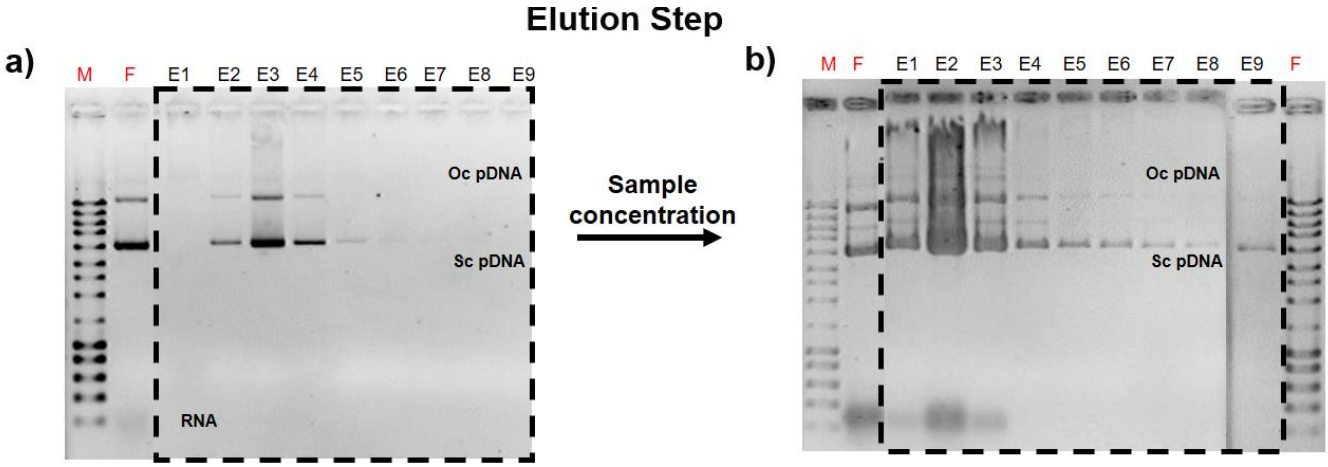


Figure 38: *a)* Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 8/11 in a column in hydrophobic conditions. E1 to E9- Fractions collected, in increasing order, during the elution step, where the 20 mM Tris-HCl, pH 8.0 was used as elution buffer. The fractions included in the dashed area were selected for fully concentration and further suspension with 30 µL Tris-HCl 20 mM, pH 8.0 to be re-analysed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction that was concentrated; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

When the ligand is tested under hydrophobic conditions, traces of open-circular and supercoiled pDNA are co-eluted at concentration of ammonium sulphate around 0.5 M in the washing, without isoform separation. After removal of salt in elution step, where Tris-HCl 20mM is applied, RNA starts to be eluted with a significant amount of plasmid isoforms, followed by the elution of five fractions containing mainly supercoiled isoform but in low amount, which is only visualized by fraction concentration (Figure 38b). A similar elution pattern is obtained in a chromatographic assay where a single-step elution with 0.5 M ammonium sulphate (Figure 39).

Nucleic acid retention under strong hydrophobic conditions, above 0.5 M ammonium sulphate and retention of plasmid isoforms for shorter timewere also observed in the assays performed with ligand 11/8 and these results were discussed previously in topic 4.2.2.2.

When compared to ligand 11/8, ligand 8/11 also retains nucleic acids under stronger hydrophobic binding environment and is not able to separate completely plasmid from RNA molecules. However, for ligand 8/11, hydrophobic binding environment, and nucleic acid retention, is maintained for a larger range of ammonium sulphate concentrations (from 2.5 M to 0.5 M) compared to ligand 11/8, in which nucleic acid elution is detected from a concentration around 1.0 M ammonium sulphate. Those slight differences may suggest that, despite both ligands being composed of equal substituents, their composition in terms of both substituents is not likely symmetrical as it can be expected in solid-phase synthesized ligands [105].

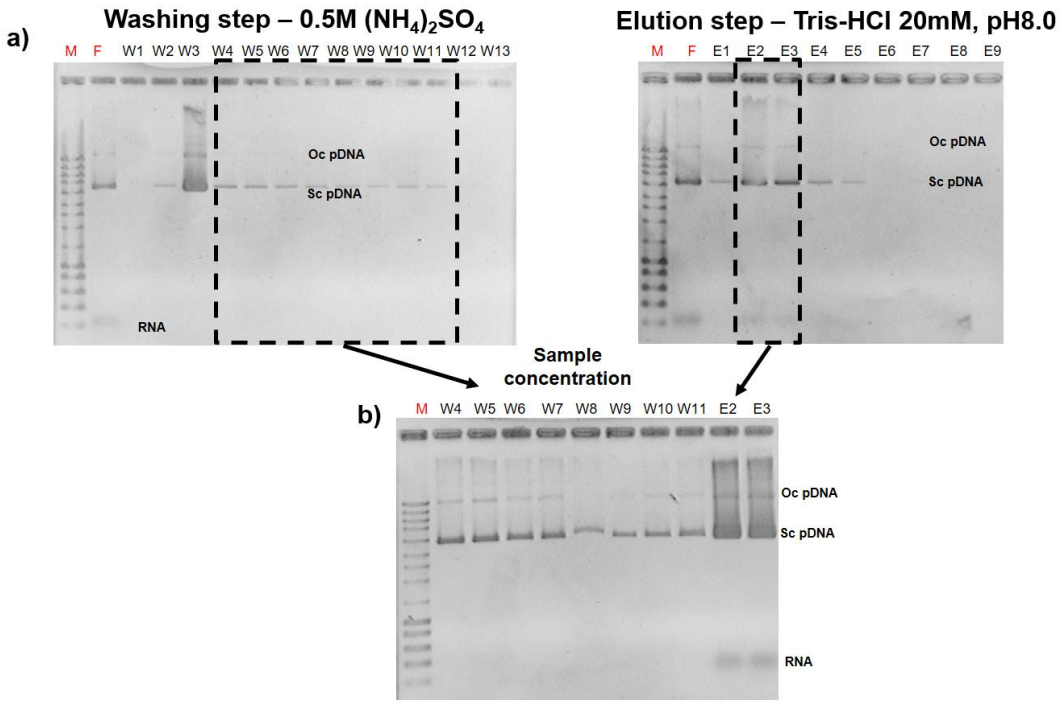


Figure 39: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 8/11 packed in a 4 mL column in hydrophobic conditions (single step washing with 0.5 M $(NH_4)_2SO_4$). a) Fractions collected during washing, using 0.5 M $(NH_4)_2SO_4$) in 20 mM Tris-HCl, pH 8.0 as washing buffers and elution step, applying Tris-HCl 20 mM, pH 8.0 as elution buffer. Fractions included in dashed area were the one selected for further concentration and analysis by gel electrophoresis. W1 to W13- Fractions collected, in increasing order, during washing; E1 to E9- Fractions collected, in increasing order, during the elution step. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

4.2.2.4. Ligand 3/5

Ligand 3/5 contains tyramine (amine 3), analogue of tyrosine, and phenethylamine (amine 5), mimetic phenylalanine, as R1 and R2 substituents in the triazine scaffold (Table 4). The results obtained in the affinity chromatography assays with ligand 3/5 are presented in Figures 40 to 42.

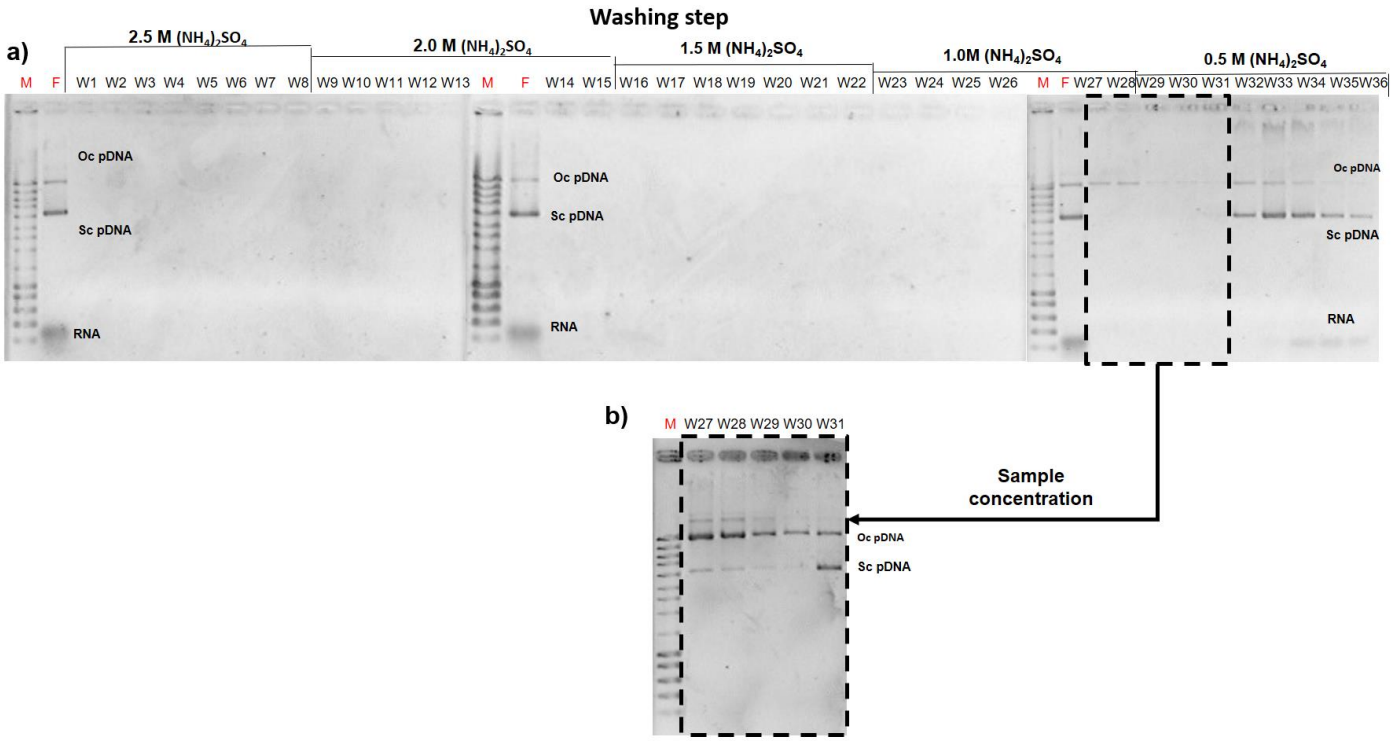


Figure 40: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 3/5 packed in a 4mL column in hydrophobic conditions (stepwise washing/elution from 2.5 M to 0.5 M $(NH_4)_2SO_4$). W1 to W36- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$; The fractions included in the dashed area were selected for fully concentration and further suspension with 30 µL Tris-HCl 20 mM, pH 8.0 to be re-analysed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction that was concentrated.M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc-open circular. Complete band size description of DNA ladder is presented in appendix II.

Figure 41: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 3/5 in a 4 mL column in hydrophobic conditions. E1 to E9- Fractions collected, in increasing order, during the elution step, where the 20 mM Tris-HCl was used as elution buffer. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophobic conditions, plasmid DNA is retained inside the columns during washing step till a concentration of ammonium sulphate around 1.0 M. At this concentration, five fractions containing mainly open-circular in low amount, followed by three fractions containing mainly supercoiled but also open-circular isoforms isolated from RNA are eluted. Bellow 0.5 M $(NH_4)_2SO_4$, co-

elution of pDNA isoforms with RNA is observed till the end of washing (Figure 40). The initial fractions collected in elution with 20 mM Tris-HCl pH 8.0 are composed of a significant amount of supercoiled DNA and RNA which are eluted in smaller amounts along this elution step (Figure 41).

After this assay, a chromatographic approach was perform applying a single step method with 1.0 M ammonium sulphate to evaluate plasmid separation from RNA under abrupt decrease in hydrophobic environment. As can be seen in Figure 42, part of plasmid molecules is eluted without RNA and isoform separation in the beginning of washing, followed by co-elution of a significant amount of pDNA and RNA when hydrophobic environment decreases with removal of ammonium sulphate.

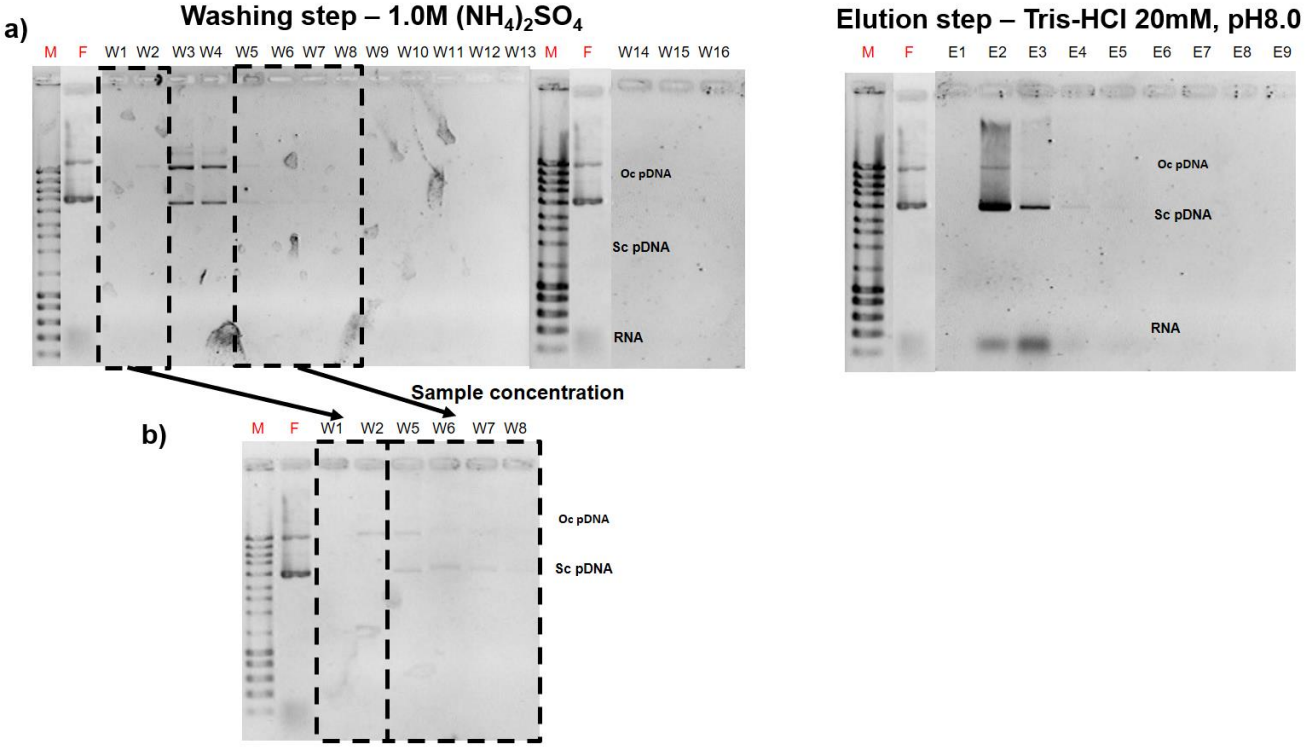


Figure 42: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 3/5 packed in a column in hydrophobic conditions (single step washing with 1.0 M $(NH_4)_2SO_4$). a) fractions collected during washing, using 1.0 M $(NH_4)_2SO_4$) in 20 mM Tris-HCl, pH 8.0 as washing buffers and elution step, applying Tris-HCl 20 mM, pH 8.0 as elution buffer. Fractions included in dashed area were the one selected for further concentration and analysis by gel electrophoresis. W1 to W16- Fractions collected, in increasing order, during washing; E1 to E9- Fractions collected, in increasing order, during the elution step. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Nucleic acid retention in the first chromatographic assay (Figure 41) under strong hydrophobic conditions, indicates that those molecules are retained by ligand 3/5, suggesting that this ligand may have affinity for all nucleic acids under these conditions. Moreover, the fact that, at least, a part of plasmid molecules is eluted at concentration around 1.0 M ammonium sulphate for both assays, while RNA is retained for longer time inside the column, suggesting that this ligand may interact differently with different nucleic acids present. These results may be related with the properties of ligand 3/5. As mentioned, ligand 3/5 substituents comprises tyramine (amine 3), analogue of tyrosine, and phenethylamine (amine 5), mimetic of phenylalanine, both containing aromatic rings that are prone to

hydrophobic and stacking interactions [4,76]. It is expected that the combination of both substituents yields a highly hydrophobic ligand, able to perform hydrophobic interactions with the hydrophobic bases of nucleic acids, retaining them under hydrophobic conditions and eluting them with decreasing of ammonium sulphate concentration. Plasmid DNA lower retention compared to RNA is also observed in other ligands and these results were already discussed (topic 4.2.2.2.). On the other hand, the elution of oc pDNA rich-fractions preceded the sc pDNA rich fractions during plasmid co-elution in the first assay (Figure 41), can be related to supercoiled isoform properties. This isoform contains deformations due to its negative supercoiling that make its bases more exposed than the bases of oc pDNA, favouring their interaction with the support and higher retention time, which explains the elution of fractions richer in sc pDNA after the fractions rich in oc pDNA [16].

Nevertheless, this ligand was not able to completely separate pDNA from RNA, since a significant amount of pDNA is still eluted with RNA or either to perform a separate elution between plasmid isoforms under tested conditions.

4.2.2.5. Ligand 8/1

Ligand 8/1 contains 4-aminobenzamide (amine 8), mimetic of glutamine and asparagine, and L-alanine, analogue to alanine and glycine, as R1 and R2 substituents in the triazine scaffold (Table 4). The results of affinity chromatographic assays with ligand 8/1 are presented in Figures 43 to 47.

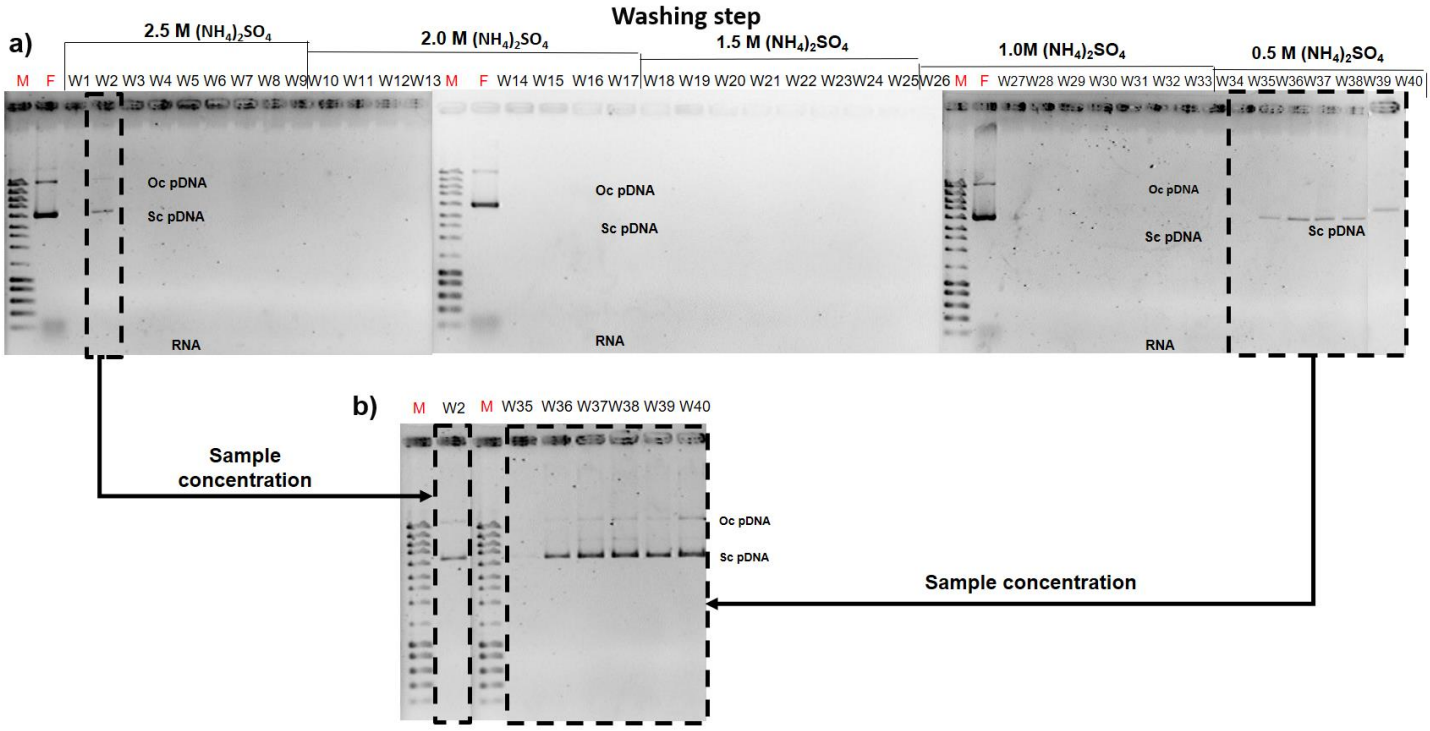


Figure 43: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 8/1 packed in a 4 mL column in hydrophobic conditions (stepwise washing/elution from 2.5 M to 0.5 M $(NH_4)_2SO_4$). W1 to W40- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$; The fractions included in the dashed area were selected for concentration and further suspension with 30 µL Tris-HCl 20 mM, pH 8.0 to be re-analysed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction that was concentrated.M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc-open circular. Complete band size description of DNA ladder is presented in appendix II.

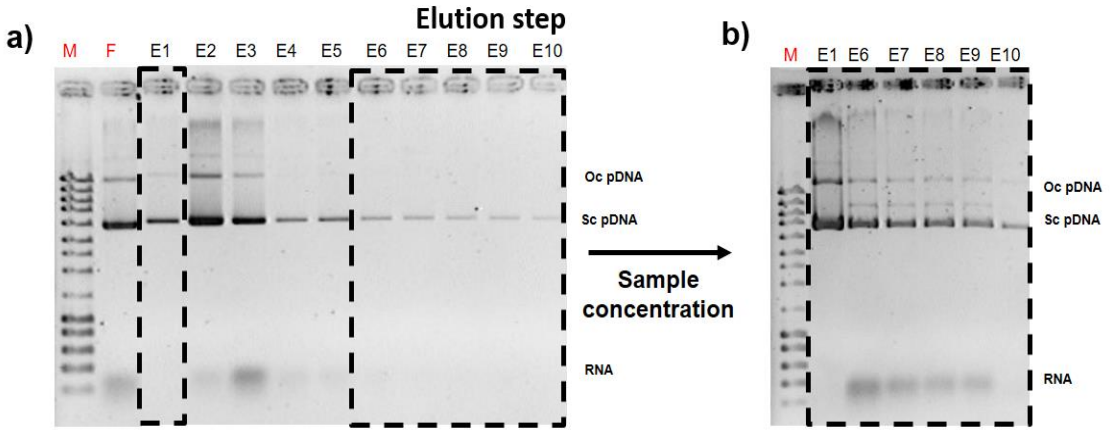


Figure 44: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 8/1 in a column in hydrophobic conditions. E1 to E10- Fractions collected, in increasing order, during the elution step, where the 20 mM Tris-HCl, pH 8.0 was used as elution buffer. The fractions included in the dashed area were selected for fully concentration and further suspension with 30 µL Tris-HCl 20 mM, pH 8.0 to be re-analyzed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction that was concentrated; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophobic conditions, performing a stepwise washing from 2.5 M to 0.5 M ammonium sulphate, traces of open-circular and supercoiled isoforms are detected in the second fraction collected from washing (Figure 43). Since part of plasmid isoforms are eluted in the flow through, it is possible that the column capacity has been surpassed in this assay [114]. After this fraction, nucleic acids are only eluted at a concentration around 0.5 M $(NH_4)_2SO_4$, and, apparently supercoiled isoform is eluted in low-amounts isolated from other isoforms and RNA (Figure 43a). After sample concentration, performed as described in section 3.5., fractions containing supercoiled isoform have also some traces of open-circular isoform (Figure 43b). During elution step, where 20 mM Tris-HCl pH 8.0 is applied, a significant amount of pDNA is co-eluted with RNA (Figure 44), which is confirmed by sample concentration (Figure 44b).

By densitometry analysis of the band intensities corresponding supercoiled and open-circular in feed and fractions rich in supercoiled isoform – w35 to w40 - in gel electrophoresis (Figure 43b), as described in section 3.10, it was possible to obtain only 11.2 % of the supercoiled isoform present in the loading feed, against 65.3 % that is apparently eluted in the remaining fractions, yielding about 76.5 % of total sc pDNA recovery. A total recovery of sc pDNA apparently is not achieved in this assay probably due to incorrect quantification of band intensities of gel electrophoresis by densitometry analysis. Nevertheless, it was possible to determine that sc pDNA rich-fractions are composed of 95.4 % supercoiled isoform over oc isoform.

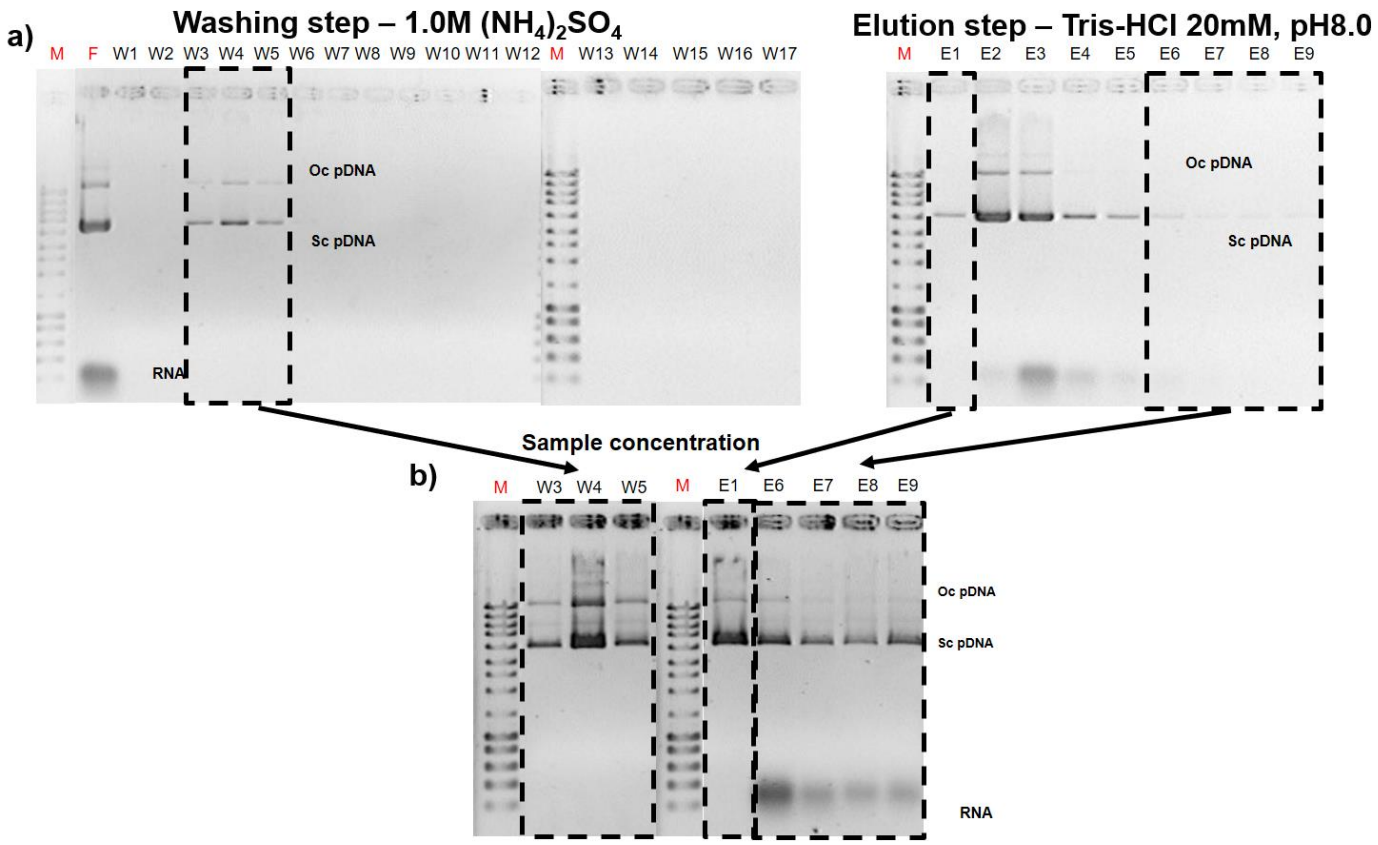


Figure 45: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 8/1 packed in a 4 mL column in hydrophobic conditions (single step washing with 1.0M $(NH_4)_2SO_4$). a) fractions collected during washing, using 1.0 M $(NH_4)_2SO_4$) in 20 mM Tris-HCl, pH 8.0 as washing buffers and elution step, applying Tris-HCl 20 mM, pH 8.0 as elution buffer. Fractions included in dashed area were the one selected for further concentration and analysis by gel electrophoresis. W1 to W17- Fractions collected, in increasing order, during washing; E1 to E9- Fractions collected, in increasing order, during the elution step. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

To evaluate the potential to selective separation between plasmid isoforms and between pDNA and RNA, a single-step method using 1.0 M or 0.5 M ammonium sulphate in 20 mM Tris-HCl, pH 8.0 was performed. Using 1.0 M ammonium sulphate, part of open-circular and supercoiled isoforms are co-eluted isolated from RNA between the third and fifth fractions collected during washing (Figure 45a*Figure 45*), which was confirmed by sample concentration (Figure 45b). When ammonium sulphate is decreased to zero in elution step, RNA starts to be eluted with plasmid DNA contamination (Figure 45a). To understand if plasmid elution in fractions W3 to W5 in figure 45 was related to excess of capacity of the column rather than support hydrophobic properties, this assay was repeated loading half of the feed (clarified *E. coli* lysate) (Figure 46). In this assay, initial elution of traces of plasmid DNA is also detected, suggesting that this may be related to support properties rather than excess of matrix capacity. After initial plasmid elution, the remaining nucleic acids are retained under hydrophobic conditions promoted by the presence of ammonium sulphate being eluted only during elution, namely RNA with a significant amount of sc and oc pDNA (Figure 46b).

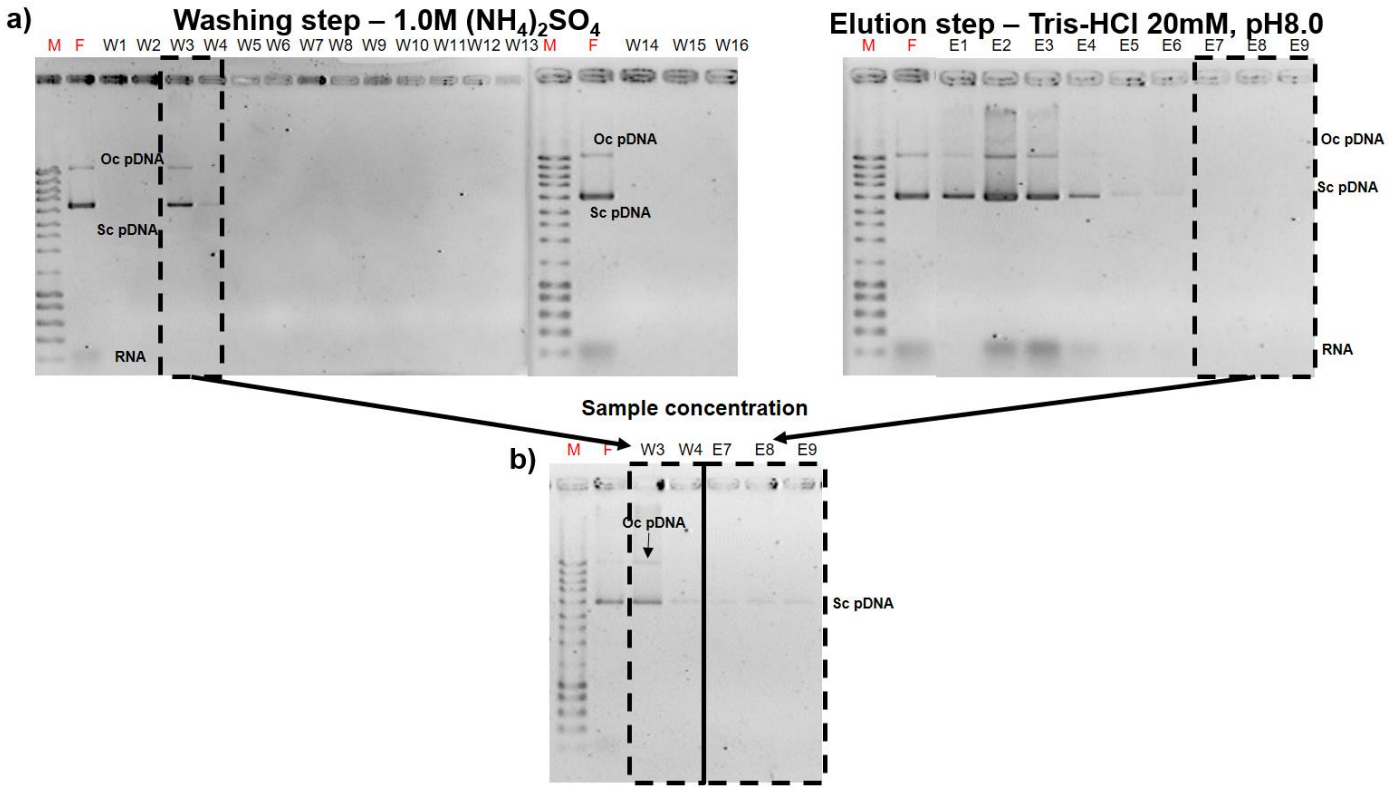


Figure 46: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL Sepharose CL-6B derivatized with ligand 8/1 packed in a 4 mL column in hydrophobic conditions (single step washing with 1.0 M $(NH_4)_2SO_4$). In this assay, instead of loading the column with 250 µL of feed, 125 µL was loaded to evaluate the capacity of the column. a) fractions collected during washing, using 1.0 M $(NH_4)_2SO_4$) in 20 mM Tris-HCl, pH 8.0 as washing buffers and elution step, applying Tris-HCl 20 mM, pH 8.0 as elution buffer. Fractions included in dashed area were the one selected for further concentration and analysis by gel electrophoresis. W1 to W16- Fractions collected, in increasing order, during washing; E1 to E9- Fractions collected, in increasing order, during the elution step. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

When ligand 8/1 was tested with 0.5 M ammonium sulphate to promote the hydrophobic environment, an initial part of plasmid isoforms (oc and sc) are eluted in first fractions collected in washing , as observed in the previous assays, followed by detection in some fractions of traces of supercoiled isoform isolated from other nucleic acid molecules (namely fractions w7, w9, w11,w12 and w13), corresponding to 0.8 % of supercoiled pDNA loaded in the column (obtained by densitometry analysis of the band intensities of these fractions) (Figure 47). The remaining fractions apparently recover 75.8 % of supercoiled isoform, according to densitometry analysis of band intensities, being lower than expected (99.2 %), to complete the mass balance in this process, which can be related to uncertain quantification of band intensities using this technique.

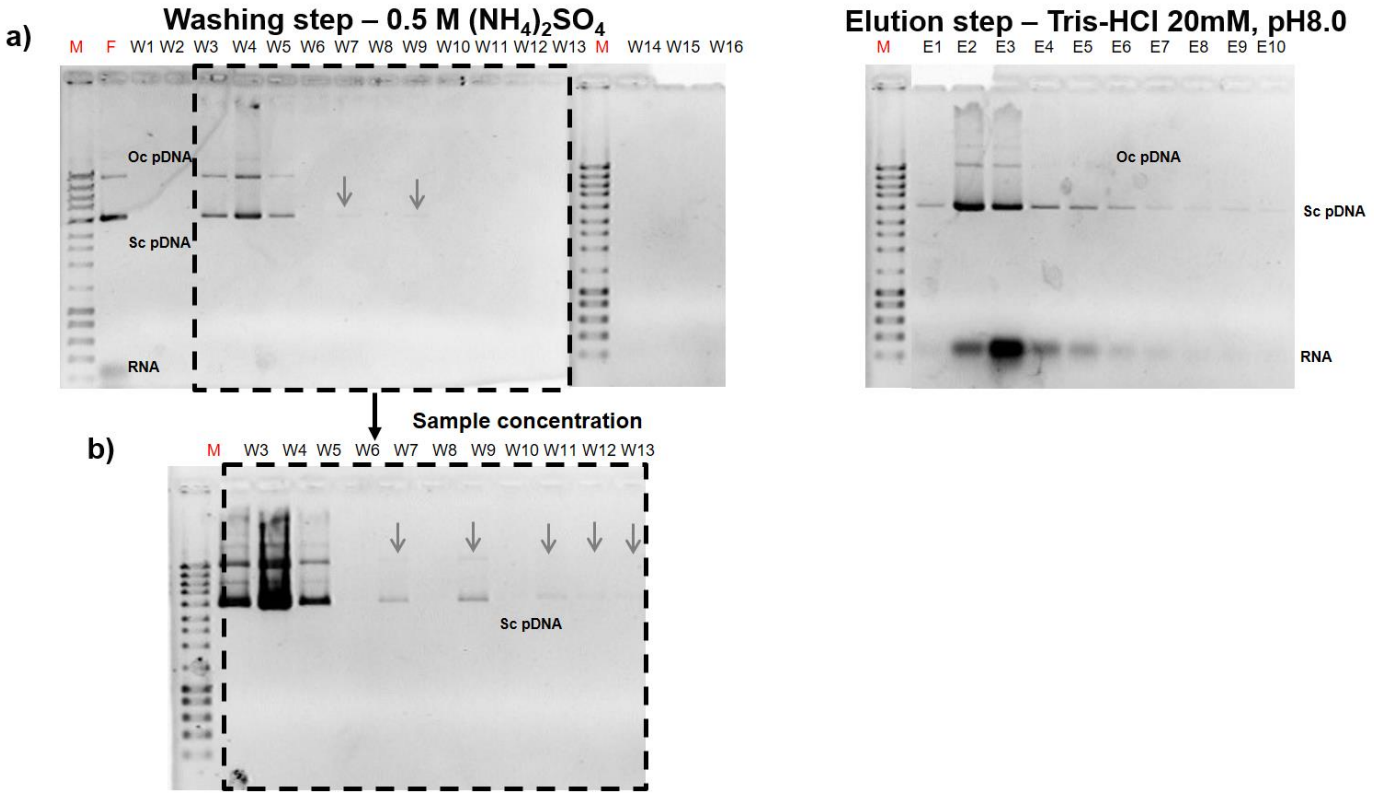


Figure 47: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 8/1 packed in a 4 mL column in hydrophobic conditions (single step washing with 0.5M $(NH_4)_2SO_4$). a) fractions collected during washing, using 0.5 M $(NH_4)_2SO_4$) in 20 mM Tris-HCl, pH 8.0 as washing buffers and elution step, applying Tris-HCl 20 mM, pH 8.0 as elution buffer. Fractions included in dashed area were the one selected for further concentration and analysis by gel electrophoresis. W1 to W16- Fractions collected, in increasing order, during washing; E1 to E10- Fractions collected, in increasing order, during the elution step. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis.Grey arrows indicated the presence of traces of supercoiled isoform in the respective fraction. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Retention of part of plasmid DNA for a shorter time than RNA in all the assays performed can be also associated to the properties of ligand 8/1. As mentioned, ligand 8/1, comprises in one hand, 4-aminobenzamide (amine 8), which contains an aromatic ring that confers hydrophobicity to the structure. On the other hand, amine 1, L-alanine, is analogue to alanine and glycine, two amino acids with small side chains that are able to perform hydrophobic, van der Waals and hydrogen bonding [4,76,78]. It is expected that the combination of both substituents will yield a ligand with hydrophobic profile, mainly by the properties of amine 8, since amino acids with larger side chain may hinder the access of shorter side chains (from alanine and glycine) to DNA [79]. As such, regarding that RNA contains higher number of hydrophobic exposed than plasmid DNA, it will interact strongly with hydrophobic ligand, perhaps with aromatic ring, and will be retained for longer time [56,109]

Despite the low yield in supercoiled isoform in fractions rich in this isoform for the assays performed with ligand 8/1, it was still possible to isolate supercoiled pDNA in some fractions, that were composed of 95.4 % of this isoform over open-circular, almost reaching the range of acceptance by

regulatory agencies for a final plasmid product (Table 1). Thus, this ligand was selected as one of the lead ligands for further studies in AKTA purifier system, using a Tricorn™10/50 column.

4.2.3. Mixed-behavior ligands (hydrophobic and hydrophilic substituents)

The other ligands selected from the 22-membered library [101] to be evaluated for plasmid DNA purification contained simultaneously a hydrophobic substituent and substituent with hydrophilic character, in R1 and R2 positions of triazine structure, respectively, namely ligands 4/11, 3/4 and 2/1. The combination of hydrophobic and charged substituents, yield ligands that can act as hydrophobic or hydrophilic ligands, depending on the type of buffers used, acting in a similar way as multimodal ligands, previously described.

Ligands screening was performed by affinity chromatography in bench chromatographic columns, under both hydrophobic, using similar conditions applied in topic 4.2.2., and hydrophilic binding conditions, applying Tris-HCl 20 mM, pH 8.0 as washing buffer and a stepwise elution with increasing concentrations of NaCl (between 0.5 M and 2.5 M) in Tris-HCl 20 mM, pH 8.0, as described in topic 3.8.1., to promote electrostatic interactions between positively charged-groups of ligands and negative charges from nucleic acids.

4.2.3.1. Ligand 4/11

Ligand 4/11 contains M-xylylenediamine (amine 4), mimetic of lysine and 2-methyl butylamine (amine 11), analogue of isoleucine, as R1 and R2 substituents in the triazine scaffold (Table 4). The results of chromatographic screening assays with this ligand under both hydrophilic and hydrophobic conditions are presented in Figures 48 to 51.

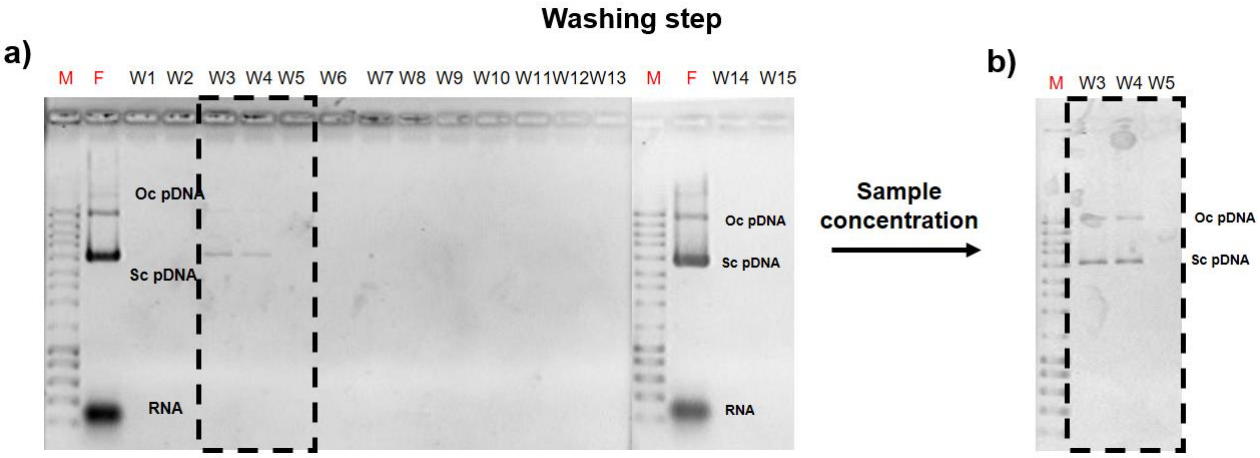


Figure 48: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 4/11 packed in a 4 mL column and evaluated in hydrophilic conditions. W1 to W15-Fractions collected, in increasing order, during washing, using Tris-HCl 20 mM, pH 8.0. The fractions included in the dashed area are the ones selected for concentration, resuspension in 30µL Tris-HCl, pH 8.0 and re-analysis

by gel electrophoresis. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

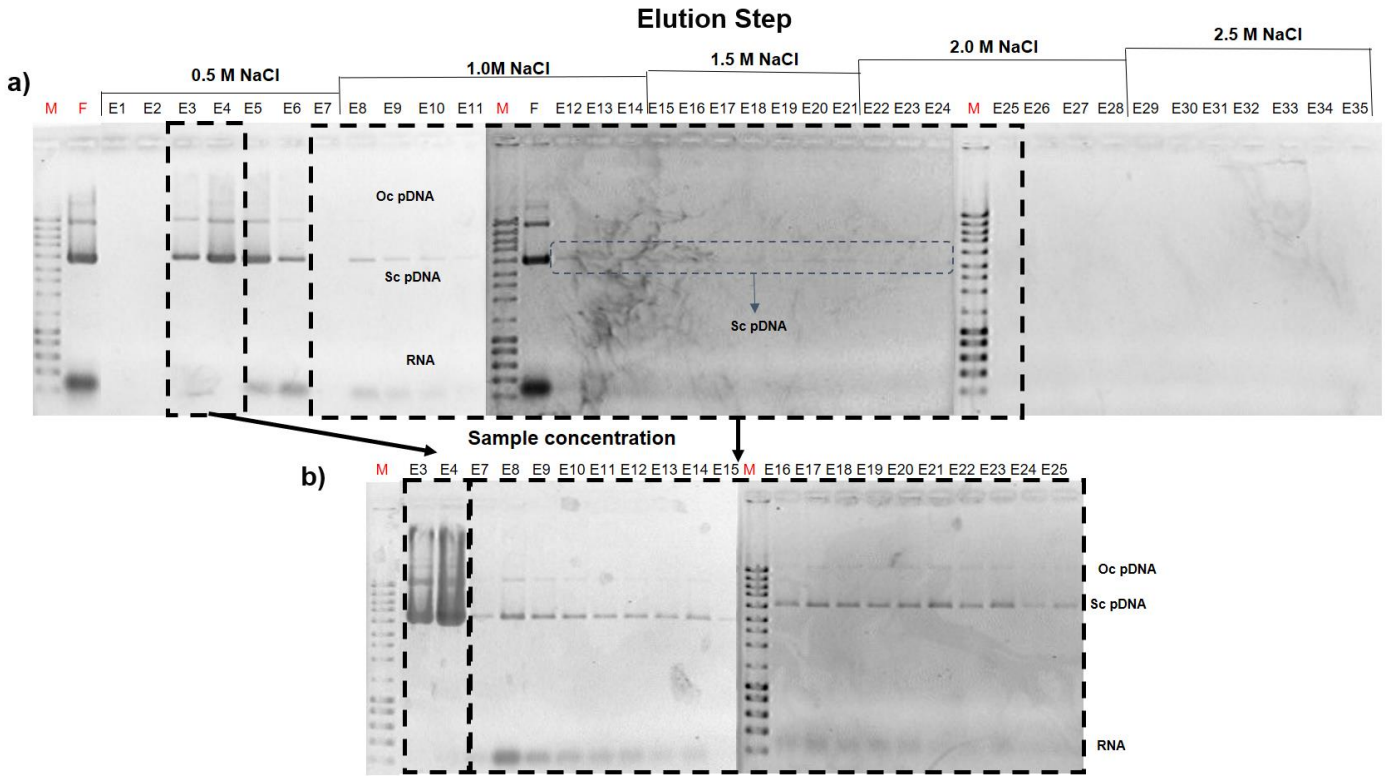


Figure 49: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 4/11 packed in a 4 mL column in hydrophilic conditions (stepwise elution from 0.5 to 2.5 M NaCl). a) Fractions collected during elution; E1 to E35- Fractions collected, in increasing order, during elution, where the concentration of elution buffer was increased from 0.5 M to 2.5 M NaCl; Fractions included in dashed area were the ones selected for further sample concentration to confirm the band pattern observed. b) Fractions selected for concentration in a) that were totally concentrated and suspended with 30 µL Tris-HCl 20mM, pH 8.0 after concentration. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophilic conditions, traces of plasmid isoforms (oc and sc) are eluted in the first fractions of washing, namely third and fourth fraction, isolated from RNA (Figure 48a), which is confirmed by sample concentration (Figure 48b). During elution, a significant amount of supercoiled and open-circular isoforms is detected in two fractions, at a concentration of 0.5 M NaCl, followed by the co-elution of pDNA and RNA along elution step till a concentration around 2.0 M NaCl (Figure 49).

Elution of RNA and a significant part of supercoiled isoform at higher concentrations of NaCl, indicates that ligand 4/11 has affinity for the nucleic acids. This ligand comprises M-xylylenediamine as R1 substituent of triazine structure (Table 4), which is mimetic of positive charged amino acid (lysine) that promotes electrostatic interactions with negative charges of nucleic acids, under hydrophilic conditions[4,76,84]. However, the ligand has shown inefficient to separate plasmid DNA from RNA and is not selective for pDNA isoforms under hydrophilic binding environment.

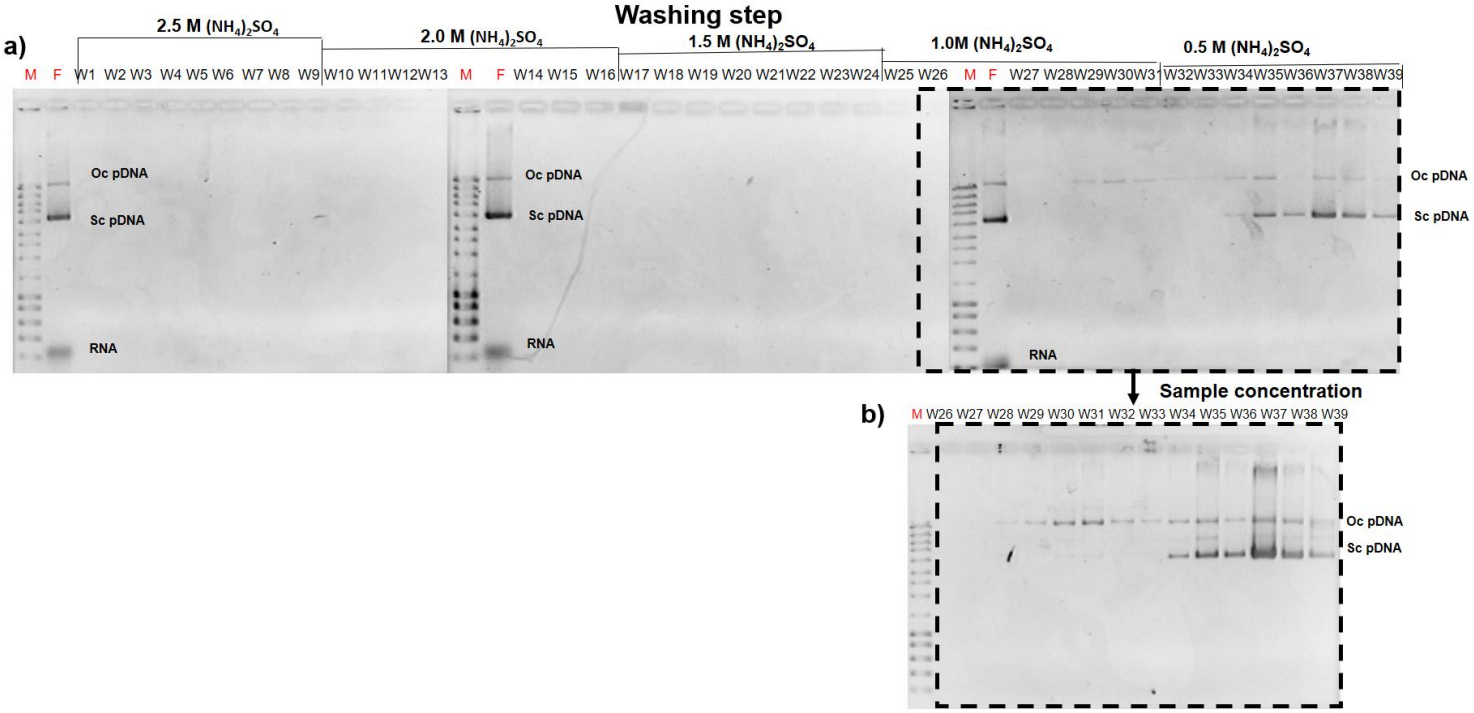


Figure 50: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 4/11 packed in a 4 mL column in hydrophobic conditions (stepwise washing/elution from 2.5 M to 0.5 M $(NH_4)_2SO_4$). W1 to W39- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$; The fractions included in the dashed area were selected for fully concentration and further suspension with 30 µL Tris-HCl 20 mM, pH 8.0 to be re-analysed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction collected in a) that was concentrated.M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc- open circular. Complete band size description of DNA ladder is presented in appendix II.

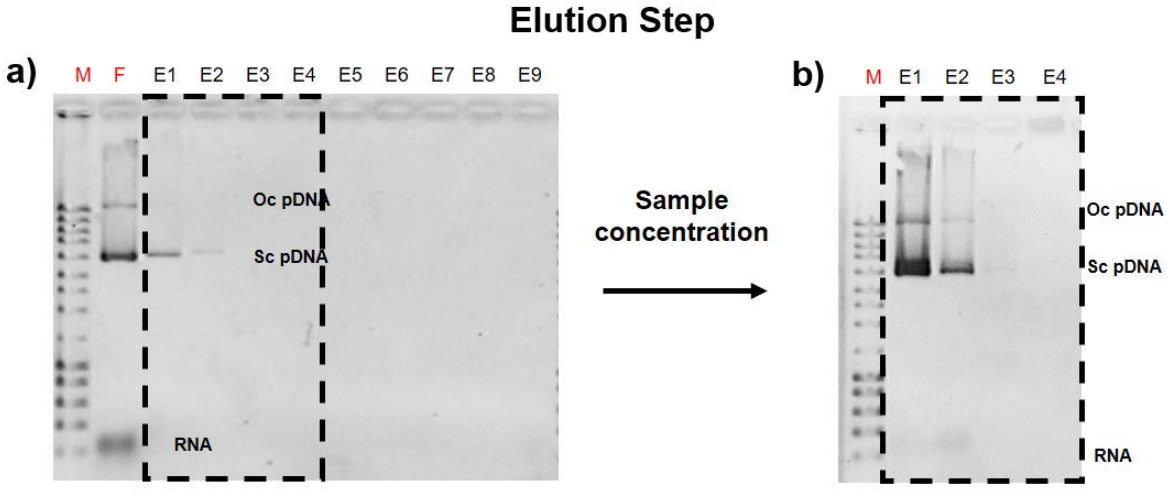


Figure 51: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 4/11 in a 4 mL column in hydrophobic conditions. E1 to E9- Fractions collected, in increasing order, during the elution step, where the 20 mM Tris-HCl was used as elution buffer. The fractions included in the dashed area were selected for concentration and further suspension with 30 µL Tris-HCl 20 mM, pH 8.0 to be re-analyzed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction collected in a) that was concentrated; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

At hydrophobic conditions, fractions rich in oc pDNA are eluted at a salt concentration around 1.0 M , followed by the co-elution of both isoforms without RNA till the end of washing step (Figure 50). When Tris-HCl 20mM, pH 8.0 is applied without salt as elution buffer, 2 fractions with both pDNA isoforms and a low amount of RNA are detected (Figure 51). The amount of RNA eluted compared to the feed suggests that part of RNA may be retained after the assay. To evaluate this hypothesis, densitometry analysis based on band intensities of RNA in fraction E2 and feed was performed. It was obtained that only 1 % of RNA was recovered in this fraction which means that ligand 4/11 retains the major part of RNA, even without hydrophobic binding environment promoted by ammonium sulphate. Further treatment of the column with a regeneration buffer, such as NaOH 0.1 M in 20 % ethanol, was mandatory to assure RNA removal, which should be followed by fraction collection and analysis to confirm RNA removal.

Nucleic acid retention under hydrophobic retention can be related to the hydrophobic properties of ligand 4/11. As mentioned, ligand 4/11 comprises substituent M-xylylenediamine and 2-methyl butylamine in R1 and R2 position of triazine scaffold, respectively. 2-methyl butylamine is analogue of isoleucine, which is able to perform hydrophobic and van der Waals interactions [4,76]. On the other hand, M-xylylenediamine, mimetic of lysine, that despite being a charged amino acid, its aliphatic chain and the extra aromatic ring (in mimetic structure) can perform hydrophobic interaction if promoted by hydrophobic environment [115]. Hydrophobic character of ligand 4/11 is enhanced under strong hydrophobic conditions, interacting with hydrophobic bases of nucleic acids and retaining them under these conditions. Moreover, elution of part of open-circular isoform before supercoiled isoform and RNA under hydrophobic environment can be related to different recognition of the different pDNA isoforms and RNA (with different exposure of bases) by the ligand, as already discussed for other ligands (see topic 4.2.2.2).

Regarding RNA retention inside the column, since it was manly retained during the assay, even in the absence of hydrophobic environment, may indicate that other type of interactions may be involved in this ligand interaction with RNA. Amine 11, 2-methyl butylamine substituent can also perform van der Waals interactions and M-xylylenediamine (amine 4) mimics an amino acid that is able to perform multiple types of interactions, due to the ability of behave as acceptor and donor of hydrogen atoms[4,76,84]. Thus, those interactions may also contribute to RNA-ligand 4/11 interaction and RNA retention.

Initial elution of traces of plasmid in only observed under hydrophilic conditions which support the idea that this behavior is related to matrix interactions with nucleic acids rather than excess of capacity of the column.

Regarding the results obtained, ligand 4/11 is a candidate ligand to plasmid isolation from RNA, specially under hydrophobic conditions, in which the main part of RNA is retained inside the column, not being eluted during the assay. Nevertheless, ligand 4/11 was shown unselective for plasmid isoforms, not being able to separate both isoforms under the hydrophilic and hydrophobic conditions tested.

4.2.3.2. Ligand 3/4

Ligand 3/4 contains tyramine (amine 3), mimetic of tyrosine and m-xylylenediamine (amine 4), analogue of lysine, as R1 and R2 substituents in the triazine scaffold (Table 4). The results of chromatographic screening assays with ligand 3/4 under both hydrophilic and hydrophobic conditions are presented in Figures 52 to 55.

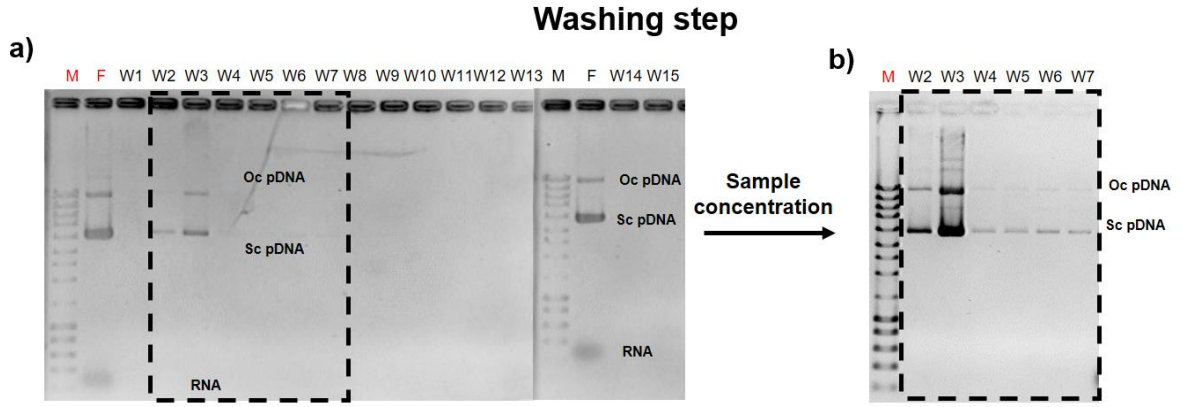


Figure 52: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 3/4 packed in a 4 mL column and evaluated in hydrophilic conditions. W1 to W15- Fractions collected, in increasing order, during washing, using Tris-HCl 20 mM, pH 8.0. The fractions included in the dashed area are the ones selected for concentration, resuspension in 30µL Tris-HCl, pH 8.0 and re-analysis by gel electrophoresis. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophilic environment, using Tris-HCl 20 mM pH 8.0, fractions containing pDNA isoforms oc and sc without RNA are detected between fraction 2 and fraction 7 (Figure 52a). Between fraction 4 and 7, only traces of plasmid isoforms are detected, being their presence confirmed by fraction concentration (Figure 52b). Despite being composed mainly by supercoiled isoform, densitometry analysis of band intensities revealed that only 3 % of this isoform was recovered in these fractions, that contained about 20 % of open-circular contamination. In the elution step, plasmid isoforms (oc an sc) starts to be detected in the beginning of this step, namely in third and fourth fraction, which is followed by co-elution of plasmid DNA and RNA (Figure 53). Between 1 M and 1.5 M NaCl apparently supercoiled isoform is eluted, isolated from other isoforms and RNA (Figure 53a).To confirm this elution pattern, fraction E10 to E24 were concentrated and analyzed by gel electrophoresis. The initial elution pattern observed in Figure 53a is not observed and those fractions contain oc and sc pDNA co-eluting with RNA (Figure 53b). Elution of RNA only in the elution step indicates a higher retention compared to plasmid DNA. Ligand 3/4 comprises M-xylylenediamine as R2 substituent of triazine structure mimicking lysine (Table 4) which is a positively charged group prone to perform hydrogen bonding with phosphate groups and N atoms of nucleic acids [22,77,84]. In double stranded molecules such as plasmid DNA, part of these groups are shielded in DNA grooves (Figure 7) and those molecules interact less with amino acids compared to single-stranded molecules (e.g. RNA) and are less retained by the ligand [78]. Nevertheless, the ligand has shown to be inefficient to separate RNA from sc plasmid DNA and not selective for pDNA isoforms.

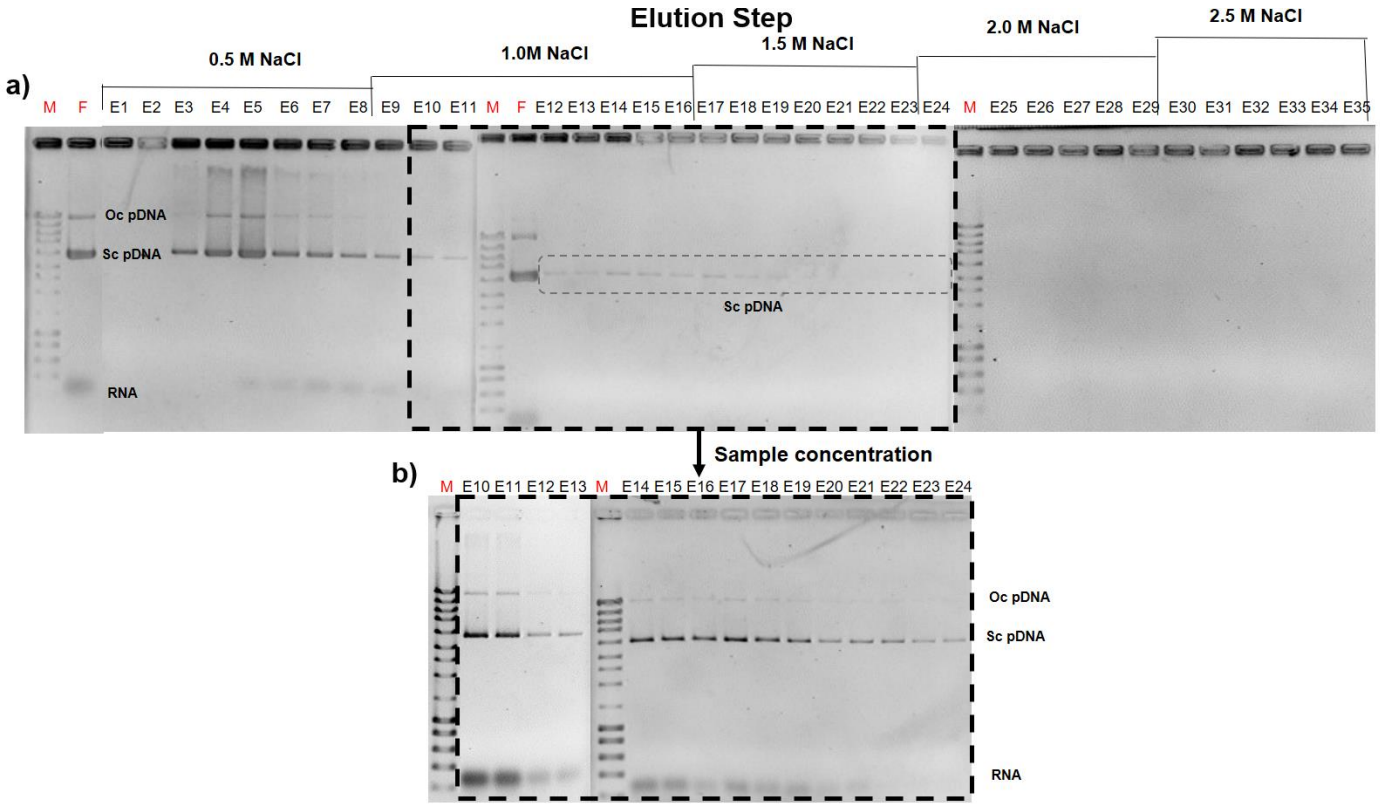


Figure 53: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 3/4 packed in a column in hydrophilic conditions (stepwise elution from 0.5 to 2.5 M NaCl). a) Fractions collected during elution; E1 to E35- Fractions collected, in increasing order, during elution, where the concentration of elution buffer was increased from 0.5 M to 2.5 M NaCl; Fractions included in dashed area were the ones selected for further sample concentration to confirm the band pattern observed. b) Fractions selected for concentration in a) that were totally concentrated and suspended with 30 µL Tris-HCl 20 mM, pH 8.0 after concentration. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophobic conditions (Figures 54 and 55), nucleic acid molecules are retained inside the column till a concentration around 0.5 M ammonium sulphate. At this concentration, fractions rich in open-circular isoform are firstly detected, followed by co-elution of supercoiled and open-circular isoforms (Figure 54). When ammonium sulphate is removed in elution step, sc and oc pDNA are both eluted. Under hydrophobic conditions, apparently RNA is not detected in the fractions collected during this assay, suggesting that this ligand has a high affinity to RNA and retains it even without hydrophobic binding conditions (Figure 55). This is compatible with the higher retention of RNA under hydrophilic binding conditions and is likely related to the mixed behavior of this ligand. Separate elution of RNA could probably be achieved by high concentration of NaCl (not tested). Elution of RNA can be performed in parallel with initial washing under hydrophobic conditions, for plasmid recovery, exploiting the potential of ligand 3/4.

Nucleic acid retention under hydrophobic retention can be related to the hydrophobic properties of ligand 3/4. This ligand contains Tyramine and M-xylylenediamine as substituents in R1 and R2 position of triazine scaffold, respectively. Tyramine mimics tyrosine that contains aromatic rings that are prone to hydrophobic and stacking interactions [4,76]. On the other hand, M-xylylenediamine, mimics lysine, that despite being a charged amino acid, has an aliphatic chain and the extra aromatic

ring (in mimetic structure) that can also perform hydrophobic interaction if promoted by an hydrophobic environment [115]. It is then expected that the combination of both substituents yield a ligand with hydrophobic character, able to perform hydrophobic interactions with the hydrophobic bases of nucleic acids, retaining them under hydrophobic conditions and eluting them with decreasing of ammonium sulphate concentration.

Ligand 3/4 is a candidate ligand to plasmid isolation from RNA, namely under hydrophobic conditions, in which RNA is retained inside the column, not being eluted during the assay. Nevertheless, ligand 3/4 is not fully selective for plasmid isoforms, not being able to completely separate both isoforms under the conditions tested.

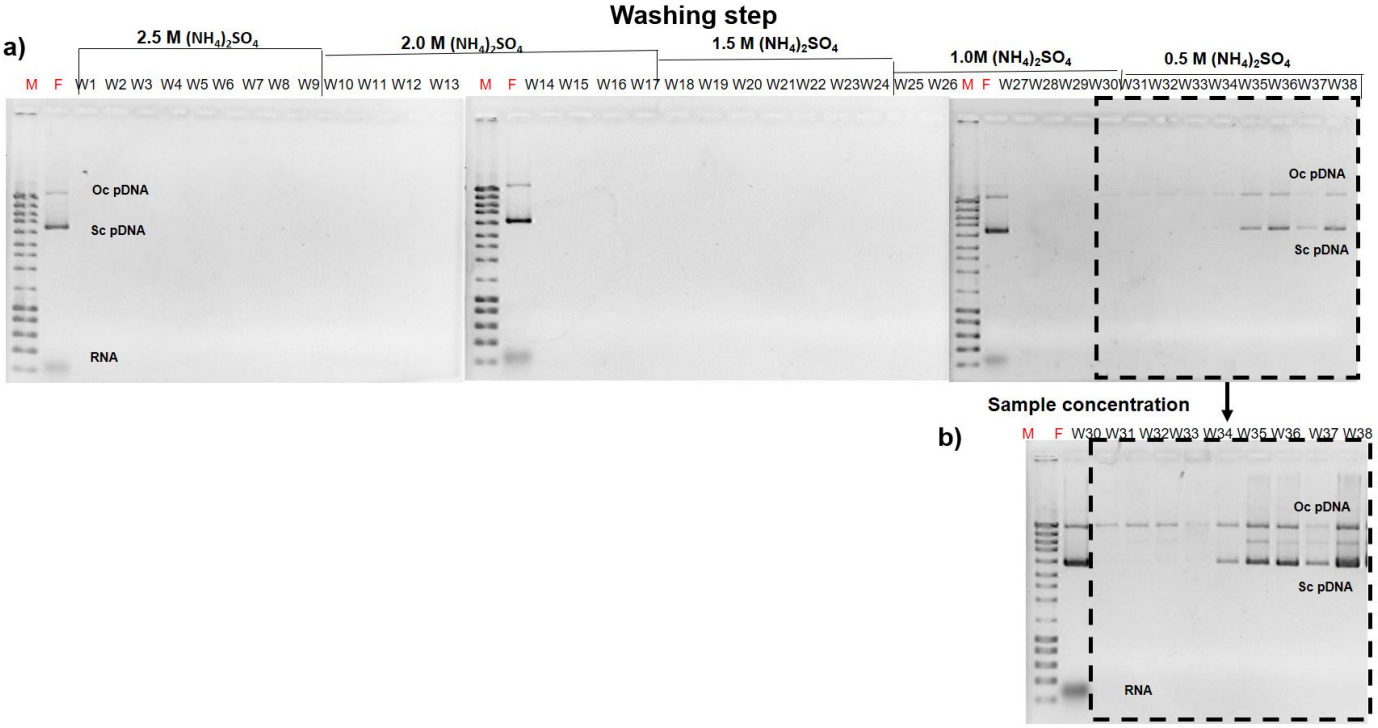


Figure 54: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 3/4 packed in a 4 mL column in hydrophobic conditions (stepwise washing/elution from 2.5 M to 0.5 M $(NH_4)_2SO_4$). W1 to W38- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$; The fractions included in the dashed area were selected for fully concentration and further suspension with 30 µL Tris-HCl 20 mM, pH 8.0 to be re-analysed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction collected in a) that was concentrated.M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc- open circular. Complete band size description of DNA ladder is presented in appendix II.

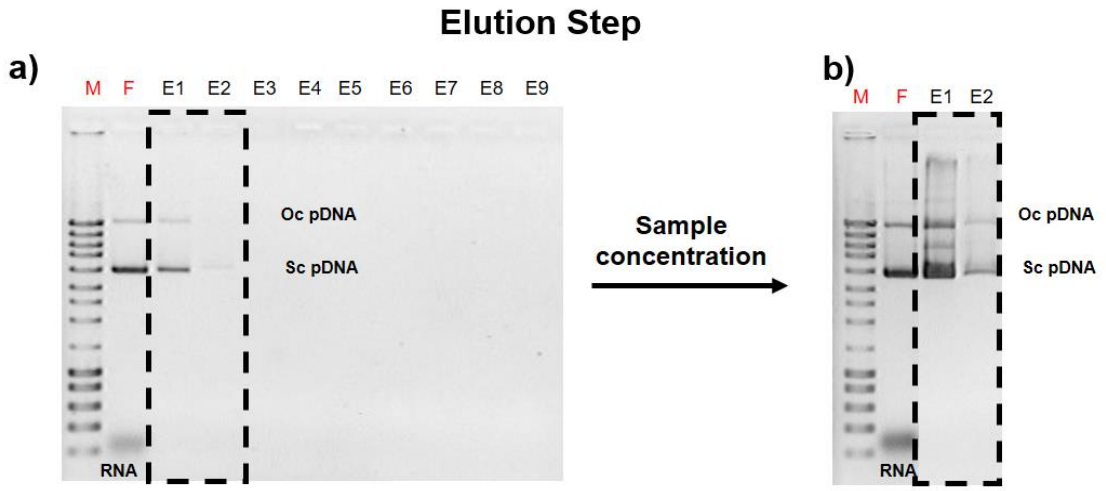


Figure 55: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 3/4 in a 4 mL column in hydrophobic conditions. E1 to E9- Fractions collected, in increasing order, during the elution step, where the 20 mM Tris-HCl was used as elution buffer. The fractions included in the dashed area were selected for fully concentration and further suspension with 30 µL Tris-HCl 20 mM, pH 8.0 to be re-analyzed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction collected in a) that was concentrated; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

4.2.3.3. Ligand 2/1

Ligand 2/1 comprises 1,5-diaminopentane (amine 2), mimetic of lysine and L-alanine (amine 1), analogue of alanine and glycine, as R1 and R2 substituents in triazine scaffold (Table 4). The results of screening by affinity chromatography with ligand 2/1 are presented in Figures 56 to 59.

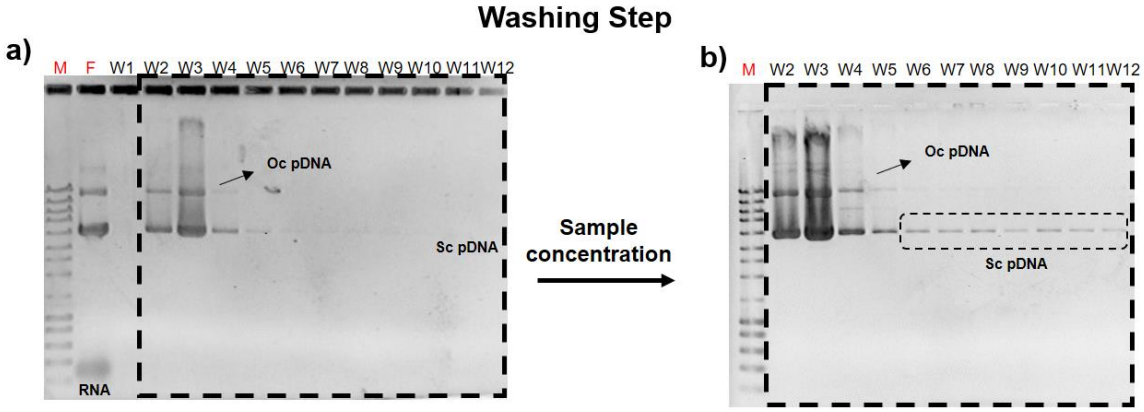


Figure 56: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 2/1 packed in a 4 mL column and evaluated in hydrophilic conditions. W1 to W12- Fractions collected, in increasing order, during washing, using Tris-HCl 20mM, pH 8.0. The fractions included in the dashed area are the ones selected for concentration, resuspension in 30 µL Tris-HCl, pH 8.0 and re-analysis by gel electrophoresis. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

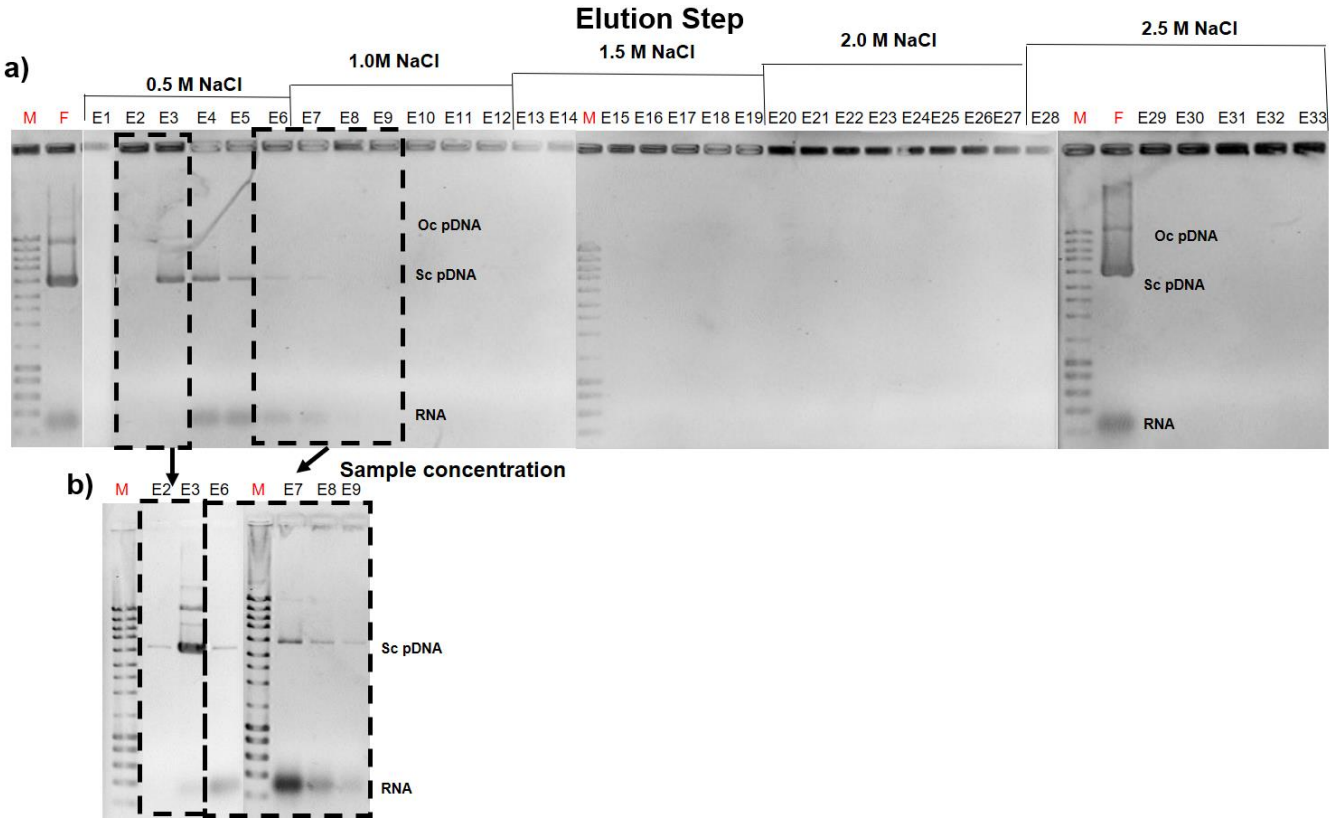


Figure 57: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 2/1 packed in a 4 mL column in hydrophilic conditions (stepwise elution from 0.5 to 2.5 M NaCl). E1 to E33- Fractions collected, in increasing order, during elution, where the concentration of elution buffer was increased from 0.5 M to 2.5 M NaCl; Fractions included in dashed area were the ones selected for further sample concentration to confirm the band pattern observed. b) Fractions selected for concentration in a) that were totally concentrated and suspended with 30 µL Tris-HCl 20 mM, pH 8.0 after concentration. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophilic binding environment, sc and oc pDNA are washed out from the column, at a significant amount, in the beginning of washing, which is followed by the collection of eight fractions rich in supercoiled isoform (Figure 56a), confirmed by sample concentration (Figure 56b). By densitometry analysis of the band intensities from feed and fractions rich in supercoiled isoform – w5 to w12 - in gel electrophoresis (Figure 56b), about 3.3 % of supercoiled isoform is recovered in theses fractions, being composed of 81.4 % of this isoform over open-circular pDNA. Densitometry analysis of intensity of the bands corresponding to supercoiled isoform in the remaining fractions, determine a isoform recovery around 52.6 %, which is lower than expected (96.7 %), as observed for ligand 8/1, which is probably due to uncertain band intensity measurements using this quantification method (which is increased in gel electrophoresis images with low band definition).

During elution, the remaining nucleic acids loaded are apparently eluted in the presence of 0.5 M NaCl, with initial fractions containing both isoforms and RNA and the remaining mainly composed of supercoiled and RNA (Figure 57).

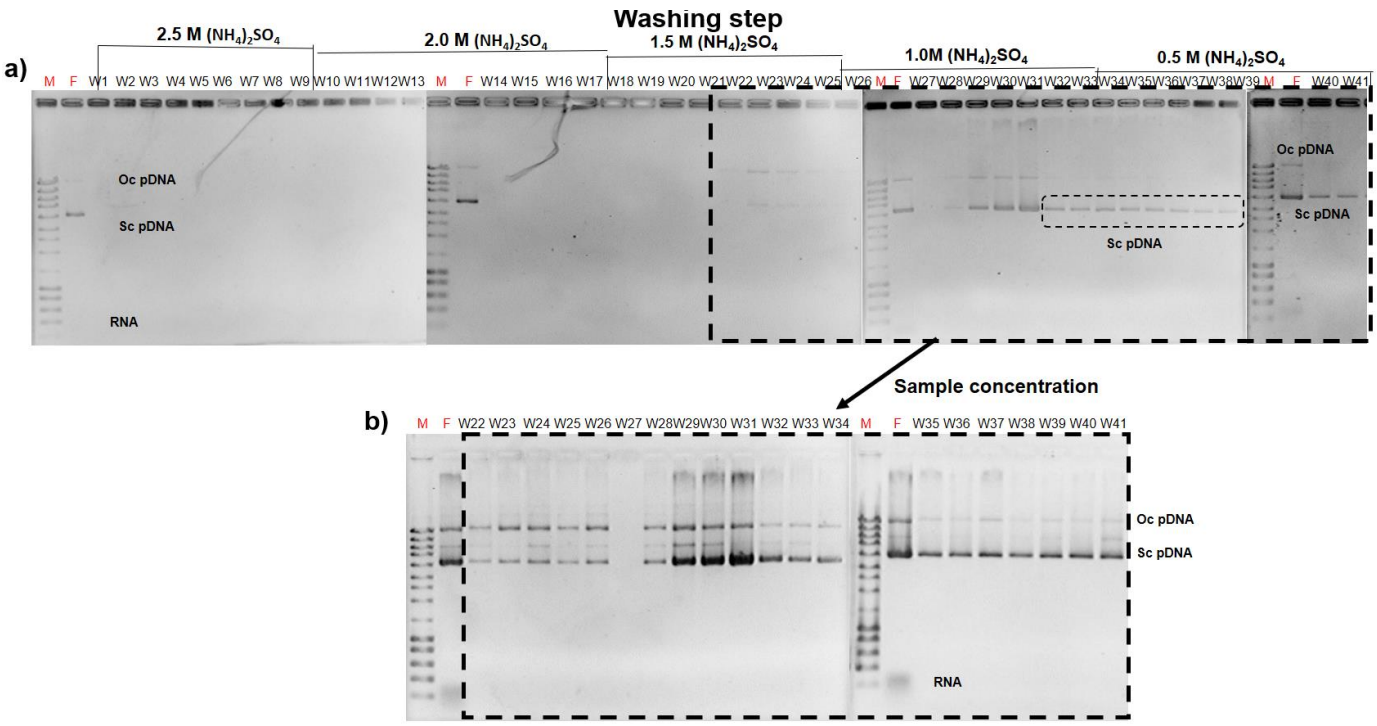


Figure 58: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 2/1 packed in a 4 mL column in hydrophobic conditions (stepwise washing/ elution from 2.5M to 0.5M $(NH_4)_2SO_4$). W1 to W41- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$; The fractions included in the dashed area were selected for fully concentration and further suspension with 30 µL Tris-HCl 20 mM, pH 8.0 to be re-analysed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction collected in a) that was concentrated.M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc- open circular. Complete band size description of DNA ladder is presented in appendix II.

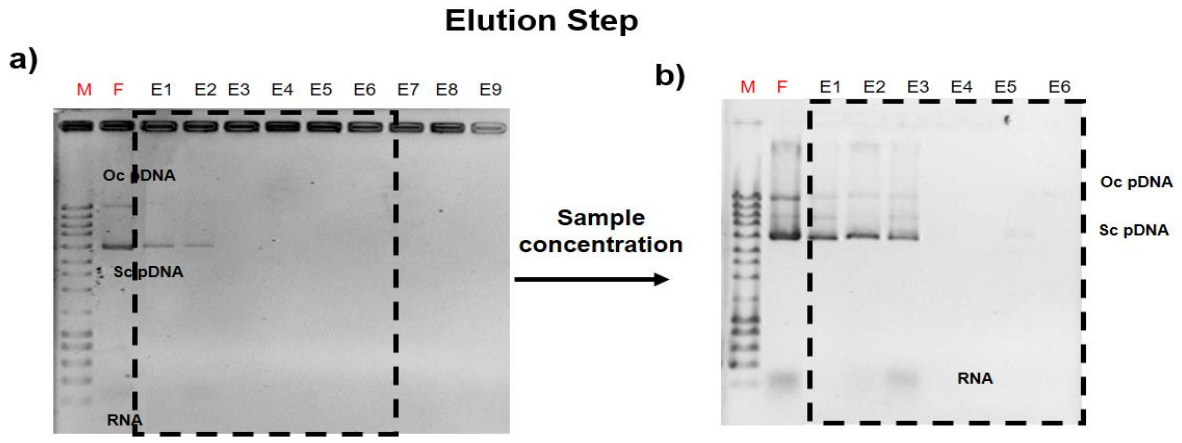


Figure 59: a) Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 2/1 in a 4 mL column in hydrophobic conditions. E1 to E9- Fractions collected, in increasing order, during the elution step, where the 20mM Tris-HCl was used as elution buffer. The fractions included in the dashed area were selected for fully concentration and further suspension with 30 µL Tris-HCl 20mM, pH 8.0 to be re-analyzed by gel electrophoresis. b) Gel electrophoresis of 20 µL of each fraction collected in a) that was concentrated; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophobic binding environment, nucleic acid molecules loaded to the column are retained till a concentration of 1.0 M ammonium sulphate , in which a sc and oc pDNA are co-eluted from fraction

w22 to w31(Figure 58a). From fraction W32, fractions rich in supercoiled isoform are collected till the end of washing (Figure 58b). Densitometry analysis of band intensities indicates that about 79.9 % of supercoiled isoform is recovered in these fractions, being composed of 93.3 % supercoiled isoforms over remaining contaminants (open-circular isoform, in this case) (Figure 56b).

Elution pattern is similar in hydrophobic and hydrophilic conditions, namely initial washing out of part of pDNA, elution of fractions rich in supercoiled isoform followed by the elution of remaining nucleic acids, which indicates that this ligand is able to separate supercoiled isoform in both conditions.

In hydrophobic binding environment, RNA is retained for longer time than plasmid DNA, which can be related different base exposure of nucleic acids, as discussed previously (topic 4.2.2.2.), that result in stronger interactions with ligand 2/1 and higher retention time [56,109]. The same is applied for supercoiled isoform, that is eluted at higher concentrations compared to open-circular pDNA. Sc pDNA, due to negative supercoiling, contains structure deformations that expose their bases, unlike oc pDNA, interacting more with ligand 2/1 under both binding conditions, and being retained for longer time than oc isoform [17,85].

Ligand 2/1 comprises two distinct substituents: 1,5-diaminopentane mimicking amino acid lysine and L-alanine, analogue of alanine and glycine. Lysine is a positively charged amino acid able to mediate a large number of interactions with nucleic acids, mainly electrostatic interactions between charged amines and negative charges from phosphate groups but also hydrogen bonding with phosphate groups or N atoms that are in the outer surface of nucleic acids or located in DNA grooves, in double stranded molecules [22,77,84,85]. These types of interactions may be the main driven force in hydrophilic environment, interacting strongly with nucleic acids. On the other hand, this substituent contains an aliphatic chain that promotes hydrophobic interactions under proper hydrophobic environment [115]. Additionally to amine 2, also L-alanine is involved in hydrophobic, van der Waals and hydrogen bonding, and can be involved in interactions with nucleic acids at hydrophobic environment. This substituent has a short side chain and it is probable that substituents with larger side chains (e.g. 1,5-diaminopentane) may hinder the access of this substituent to nucleic acids. Thus, in this case it is possible that the interactions between ligand and nucleic acid species may be mainly due to the presence of amine 2 and therefore ligand 2/1 acts in a similar way as natural lysine affinity ligand [84,85].

Despite the low yield in supercoiled isoform in fractions rich in this isoform for the assays under hydrophilic environment, ligand 2/1 can isolate supercoiled pDNA in some fractions under both binding conditions, recovering a significant amount supercoiled pDNA from the feed loaded under hydrophobic environment (79.9%). Thus, this ligand was selected as the second lead ligand chosen for further studies, together with ligand 8/1, in ÄKTA purifier system, using a Tricorn™10/50 column.

4.2.4. Global Results

In this section, the results obtained from the screening of the selected ligands in terms of ability for plasmid purification, are summarized in Table 8 to Table 10.

4.2.4.1. Ligands predominantly hydrophobic

Table 8: General results obtained in the chromatographic studies with ligands with hydrophobic profile selected for screening. These chromatographic approaches were performed under hydrophobic binding conditions (except for ligand 6/5 that was also evaluated under hydrophilic conditions), as described in topic 3.8.1.

Ligand tested (R1/R2)	**Substituent R1** (Structure/Name/analogue amino acid)	**Substituent R2** (Structure/Name/analogue amino acid)	**Conditions used**	**Results**
6/5	**Isoamylamine (Leucine- Leu)**	**Phenethylamine (Phenylalanine- Phe)**	Hydrophobic (ÄKTA)	Oc pDNA isolated eluted around 1.25 M $(NH_4)_2SO_4$ followed by co-elution of pDNA and RNA
			Hydrophilic (ÄKTA)	pDNA isoforms and RNA are co-eluted during elution with NaCl (~0,25 M)
3/5	**Tyramine (Tyrosine - Tyr)**	**Phenethylamine (Phenylalanine – Phe)**	Hydrophobic	Between 0.5 and 1.0 M $(NH_4)_2SO_4$ this ligand can separate pDNA isoforms from RNA in the initial fractions, where fractions rich in oc pDNA are detected before fractions rich in sc pDNA, followed by co-elution of pDNA and RNA, till the end of washing and during elution.
11/8	**2-methylbutylamine (Isoleucine – Ile)**	**4-aminobenzamide (Asparagine – Asn- and glutamine - Gln)**	Hydrophobic	At 1.0 M $(NH_4)_2SO_4$, both plasmid isoforms are isolated from RNA, followed by co-elution of pDNA and RNA under elution with Tris-HCl 20 mM.
8/11	**4-aminobenzamide (Asparagine – Asn- and glutamine - Gln)**	**2-methylbutylamine (Isoleucine – Ile)**	Hydrophobic	Ligand 8/11 can isolate part of pDNA isoforms from RNA at 0.5 M ammonium sulphate and in some fractions eluting with Tris-HCl 20 mM.
8/1	**4-aminobenzamide (Asparagine – Asn- and glutamine - Gln)**	**L-alanine (Alanine – Ala- and Glycine- Gly)**	Hydrophobic	Around 0.5 M $(NH_4)_2SO_4$, sc pDNA rich-fractions are obtained followed by co-elution of both isoforms and RNA during elution.

4.2.4.2. Ligands with mixed behaviour (hydrophobic + charged substituent)

Table 9: General results obtained in the chromatographic studies with ligands with mixed behavior (hydrophobic and charge substituents), selected for screening. These chromatographic approaches were performed under hydrophobic and hydrophilic binding conditions, as described in topic 3.8.1.

Ligand tested (R1/R2)	**Substituent R1** (Structure/Name/analogue amino acid)	**Substituent R2** (Structure/Name/analogue amino acid)	**Conditions used**	**Results**
2/1	**1,5-diaminopentane (Lysine- Lys)**	**L-alanine (Alanine -Ala and Glycine - Gly)**	Hydrophobic	pDNA isoforms isolated from RNA around 1.0 M $(NH_4)_2SO_4$. Fractions rich in sc pDNA (93.3 % over oc isoform) are obtained between 1.0 and 0.5 M $(NH_4)_2SO_4$, recovering about 79.9 % supercoiled isoform loaded to the column. RNA eluted in small amount after elution withTris-HCl 20 mM.
			Hydrophilic	A significant amount of sc and oc pDNA are washed out in the beginning of the assay (with Tris-HCl 20 mM), followed by fractions rich in sc pDNA (81.4 %over oc isoform) and co-elution of all nucleic acid molecules at 0.5 M NaCl, during elution
3/4	**Tyramine (Tyrosine – Tyr)**	**m-xylylenediamine (Lysine - Lys)**	Hydrophobic	No RNA is detected during the assay. Oc pDNA rich-fractions are detected between 1.0 and 0.5 M $(NH_4)_2SO_4$, followed by the co-elution of both isoforms till the end of the assay.
			Hydrophilic	During washing with Tris-HCl 20 mM, fractions containing pDNA isoforms oc and sc and isolated from RNA are obtained in low amount. Both isoforms and RNA are then co-eluted around 0.5 M NaCl, followed by RNA with traces of sc pDNA.
4/11	**m-xylylenediamine (Lysine - Lys)**	**2-methylbutylamine (Isoleucine – Ile)**	Hydrophobic	Fractions containing mainly oc pDNA are eluted at 1.0 M $(NH_4)_2SO_4$, followed by the co-elution of both isoforms without RNA and fractions with both isoforms and traces of RNA during elution.
			Hydrophilic	Traces of isoforms are washed-out using Tris-HCl 20 mM, followed by the co-elution of pDNA and RNA from 0.5 M to 2 M NaCl

4.2.4.3. Resins used as Controls

Table 10: General results obtained in the chromatographic studies with resins used as controls for these chromatographic studies. These chromatographic approaches were performed under hydrophobic and hydrophilic binding conditions, as described in topic 3.8.1.

Resins tested	Conditions used	Results
Sepharose CL-6B	Hydrophobic	Apparently, pDNA is partially separated from RNA in the beginning of the assay with initial fractions rich in ocpDNA, followed by the co-elution of both isoforms and several fractions rich in sc pDNA till elution of RNA, in washing. Apparently, all nucleic acids molecules are eluted during washing.
	Hydrophilic	Both pDNA and RNA are eluted in the beginning of the assay without selective elution. Traces of oc+sc pDNA are co-eluted till the end of washing and beginning of elution
Aminated Sepharose	Hydrophobic	All nucleic acid molecules are apparently eluted in the washing with fractions with oc and sc PDNA (3rd and 4th fractions) at a concentration of 2.5M, followed by the elution of remaining pDNA at 2.0 M, and fractions containing RNA, initially contaminated by sc pDNA.
	Hydrophilic	pDNA and RNA are retained in the washing and co-eluted during elution.
Sepharose with cyanuric chloride derivatized	Hydrophobic	pDNA isoforms are co-eluted and separated from RNA, which is retained for longer time.
	Hydrophilic	All nucleic acids are co-eluted in the beginning of washing step.
Ligand 0/0 (newly derivatized)	Hydrophobic	Co-elution of oc and sc pDNA in the beginning of washing, followed by RNA elution in the washing (between 1.5 M and 1.0 M ammonium sulphate) and traces of sc pDNA during elution step.
	Hydrophilic	Significant part of pDNA and RNA are co-eluted in the beginning of washing, which is maintained till the of washing and beginning of elution.
EDA CIM®monolithic disk	Hydrophobic	All nucleic acids are co-eluted in the beginning of washing step.
	Hydrophilic	RNA is separated from plasmid DNA

4.3. Chromatographic studies with lead ligands 8/1 and 2/1 in AKTA purifier system

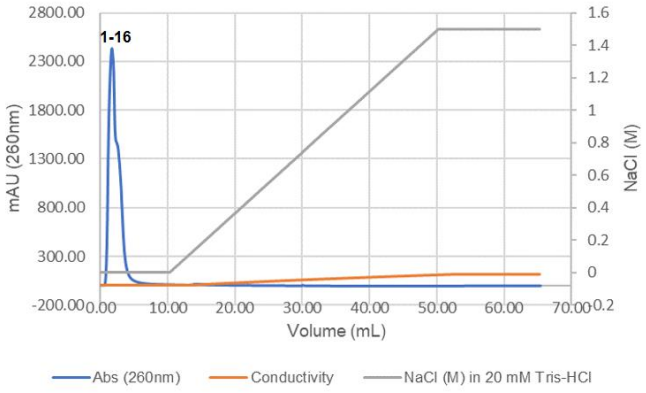
Ligands 2/1 and 8/1 were chosen as lead ligands to proceed to chromatographic studies using AKTA purifier system, due to promising results in terms of supercoiled isoform isolation form contaminants, as discussed in topic 4.2.2 and 4.2.3.

The chromatographic assays were performed using a Tricorn™10/50 column containing ligand 2/1 or 8/1 immobilized in Sepharose CL-6B. Those assays were performed under hydrophobic conditions, applying a decreasing gradient with $(NH_4)_2SO_4$ (between 1.5 or 1.0 M and 0 M, for ligand 2/1 and 8/1, respectively) in 20 mM Tris-HCl, pH 8.0 followed by Tris-HCl 20 mM, pH 8.0 as elution buffer; and under hydrophilic conditions, using Tris-HCl 20 mM, pH 8.0 as equilibration and washing buffers and a gradient with NaCl (between 0 and 1.05 or 1.5 M, for ligand 2/1 and 8/1, respectively) in the same buffer for elution, as described in topic 3.8.3. The results obtained for both ligands are discussed in topics 4.3.1, and 4.3.2.

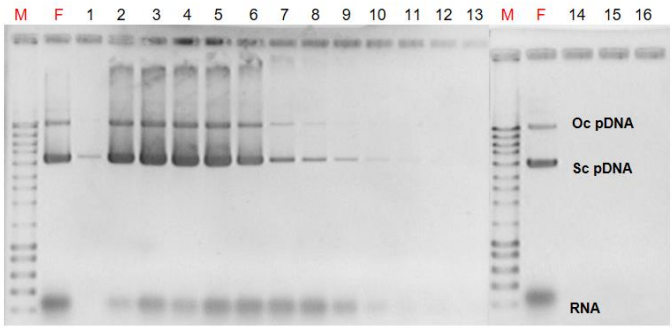
4.3.1. Chromatographic assays in ÄKTA purifier system (ÄKTA 10) using ligand 8/1 synthesized in Sepharose CL-6B

The results obtained from affinity chromatography assays with ligand 8/1 in AKTA purifier system are presented in Figures 60 to 62.

a)



c)



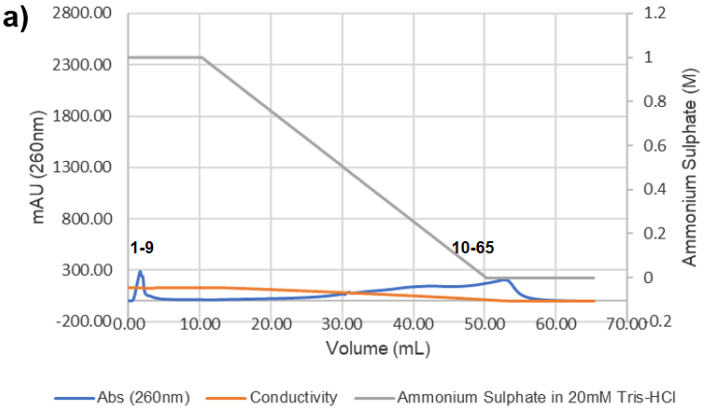
b)

Peak	Maximum mAU (260nm)	Volume(mL)/ [NaCl] (M)
	2344.08	1.54 mL /0 M

Figure 60: a) Chromatography in 2 mL Sepharose CL-6B resin derivatized with ligand 8/1 packed in an TRICORN ™ 10/50 column performed under hydrophilic conditions (using a gradient from 0 M to 1.5 M NaCl in 20 mM Tris-HCl pH 8.0 as elution buffer and Tris-HCl 20 mM as washing buffer. 1000 µL of a solution containing 500 µL of a clarified *E. coli* lysate was injected after column equilibration with Tris-HCl 20 mM, pH 8.0.From this chromatography, fractions (1 to 16), corresponding to the peak detected, were collected and analyzed by gel electrophoresis .b) Volume and concentration at which, the maximum absorbance of the peak, at 260 nm, was achieved and the respective fractions collected on those points.c) Gel electrophoresis of 30 µL of the fractions collected from the peak detected in chromatogram in a). M- DNA ladder III (NZYtech); F-Feed; oc pDNA – open-circular; sc- supercoiled. Complete band size description of DNA ladder is presented in appendix II.

In sepharose Cl-6B containing ligand 8/1 tested under hydrophilic conditions (Figure 60), a single and intense peak, with 2344.08 mAU, is detected in the beginning of washing (Figure 60a and 60b). From this peak, fractions 1 to 16 were collected and analyzed by gel electrophoresis and apparently all nucleic acid molecules loaded, namely open-circular, supercoiled pDNA and RNA, are eluted in this peak. Direct elution of all nucleic acids present in the feed indicates that those molecules are not retained by ligand 8/1 under hydrophilic conditions, which suggests that this support is not selective for any of these species when hydrophilic binding conditions are applied, explaining the absence of nucleic acid separation. This result can be associated to ligand properties. As mentioned before, ligand 8/1, comprises 4-aminobenzamide (amine 8), which contains an aromatic ring that confers hydrophobicity to the structure and L-alanine (amine 1) which is analogue to alanine and glycine, two amino acids with small side chains that are able to perform hydrophobic, van der Waals and hydrogen bonding [22,77,79]. It is expected that the combination of both substituents will yield a ligand with strong hydrophobic profile. Thus, under hydrophilic binding environment, hydrophobic interactions between the hydrophobic ligand and nucleic acids are not favored, explaining the lower retention of nucleic acids by the ligand and direct elution in the flow through.

a)



b)

Peak	Maximum mAU (260nm)	Volume(mL)/ $[(NH_4)_2SO_4]$ (M)
1st	287.4	1.61 mL/ 1 M
Beginning of 2nd	39.8	26.70 mL /0.58 M
2nd	204.1	52.77 mL/ 0 M

Figure 61: a) Chromatography in 2 mL Sepharose CL-6B derivatized with ligand 8/1 packed in an TRICORN ™ 10/50 column performed under hydrophobic conditions (using a gradient from 1 M to 0 M ammonium sulphate in 20 mM Tris-HCl pH 8.0 in washing and Tris-HCl 20bmM pH 8.0 as elution buffer. 1000 µL of a solution containing 500 µL of a clarified *E. coli* lysate was injected after column equilibration with 1.0 M ammonium sulphate in Tris-HCl 20mM, pH 8.0. From this chromatography, fractions (1 to 9 and 10 to 65), corresponding to the peaks detected, were collected and analyzed by gel electrophoresis. b) Volume and concentration at which, the maximum absorbance of the peaks, at 260 nm, was achieved and the respective fractions collected on those points.

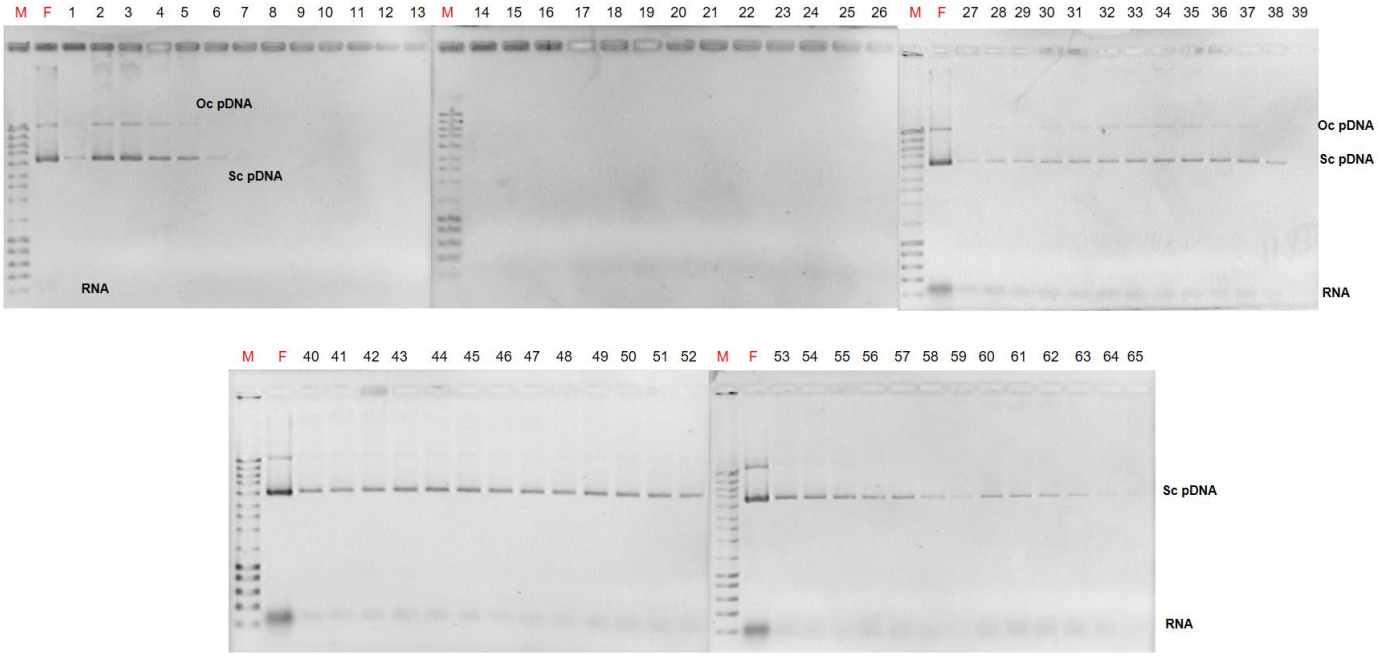


Figure 62: Gel electrophoresis of 30µL of each fraction collected from the peaks detected in chromatography, in figure 61; M- DNA ladder III (NZYtech); F-Feed; oc pDNA – open-circular; sc- supercoiled.. b) Gel electrophoresis of 20 µL of each fraction collected in a) that was concentrated; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

In sepharose Cl-6B containing ligand 8/1 tested under hydrophobic conditions (Figure 61a), a small peak is detected in the beginning of the assay, while a second broad base peak is detected during gradient washing/elution of $(NH_4)_2SO_4$.

Regarding the first peak (Figure 61), containing an UV absorbance of 287.4 mAU, fractions 1 to 9 were collected analyzed by gel electrophoresis and apparently part of plasmid isoforms (oc and sc) are eluted in this peak (Figure 62). By densitometry analysis, 43.7 % of plasmid DNA is recovered in this peak. The second peak is a broader peak, with maximum UV absorbance of 204.1 mAU, that starts to be detected at concentration of 0.58 M ammonium sulphate. From this peak, fractions 10 to 65 were collected and analyzed by gel electrophoresis. As can be seen in Figure 62, the fractions collected from this peak include traces of open-circular pDNA but also supercoiled pDNA and RNA. This washing and elution patterns are similar to the assays with ligand 8/1 using bench chromatographic columns, namely partial pDNA separation from RNA (Figure 43 to 47). Nevertheless, supercoiled plasmid isolation is not achieved.

Direct washing out of part of plasmid DNA isoforms, its associated not only to the hydrophobic properties of ligand 8/1, but also to the degree of hydrophobic bases exposure in different nucleic acid molecules, already discussed in topics 4.2.2.2. and 4.2.2.5. As mentioned before, double-stranded molecules such as plasmid isoforms, have their hydrophobic bases shielded and packed inside the double helix, thus having low or no interaction with ligand 8/1 and are not retained by the latter, being eluted in the flow through. On the other hand, RNA have higher number of hydrophobic bases exposed, interacting more with the hydrophobic ligand and is retained for longer time in the column

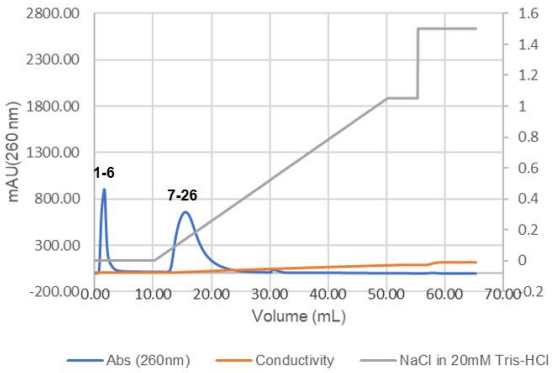
[109,112]. However, RNA is co-eluted with sc pDNA that is also partly retained in the column. This partial retention is not easily explained but may be due to some variability in sc conformations in the sample, with different degree of bases exposure and therefore different hydrophobicity [16].

Although ligand 8/1 is able to separate part of plasmid DNA from RNA at 1 M $(NH_4)_2SO_4$ concentration, it did not allow any selective separation of sc and oc plasmid DNA under the conditions tested.

4.3.2. Chromatographic assays in ÄKTA purifier system (ÄKTA 10) using ligand 2/1 synthesized in Sepharose CL-6B

The results obtained in the assays with ligand 2/1 in ÄKTA purifier system are presented in Figures 63 to 68.

a)



c)

Peak	Maximum mAU (260nm)	Volume(mL)/ [NaCl] (M)
1st	850.3	1.47 mL / 0 M
2nd	658.5	15.45 mL/ 0.136 M

b)

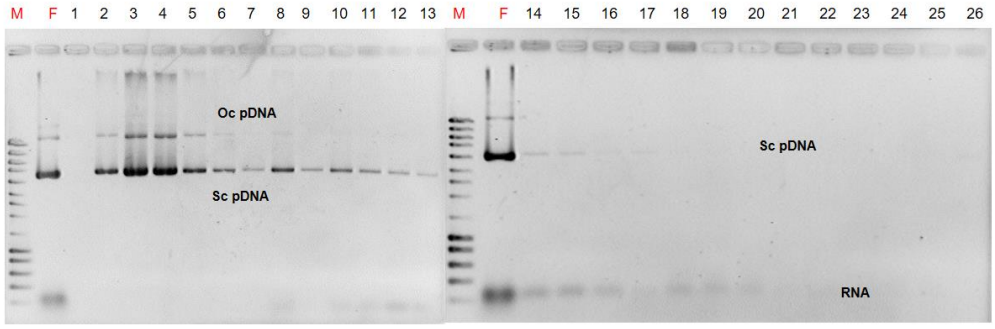


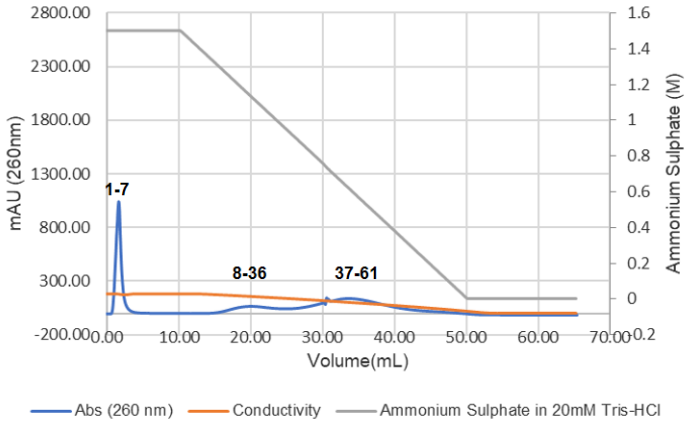
Figure 63: a) Chromatography in 2 mL Sepharose CL-6B derivatized with ligand 2/1 packed in an TRICORN ™ 10/50 column performed under hydrophilic conditions (using a gradient from 0 to 1.05 M NaCl in 20 mM Tris-HCl, pH 8.0 and Tris-HCl 20 mM, pH 8.0 as washing buffer. 1000 µL of a solution containing 500 µL of a clarified *E. coli* lysate was injected after column equilibration with Tris-HCl 20mM, pH 8.0. From this chromatography, fractions (1 to 6 and 7 to 26), corresponding to the peaks detected in the chromatogram, were collected and analyzed by gel electrophoresis.b) Gel electrophoresis of 30 µL of each fraction collected, corresponding to the peaks detected in the chromatogram in a). M- DNA ladder III (NZYtech); F-Feed; oc pDNA – open-circular; sc- supercoiled. Complete band size description of DNA ladder is presented in appendix II.c) Maximum absorbance, at 260 nm, obtained in each peak and the respective valor and concentration of NaCl.

In Sepharose CL-6B containing ligand 2/1 tested under hydrophilic conditions (Figure 63a), a small peak is detected in the beginning of the assay, while a second peak is detected during elution.

Regarding the first peak, containing an UV absorbance of 850.3 mAU, fractions 1 to 6 were collected to be analyzed by gel electrophoresis (Figure 63b), and, apparently part of plasmid isoforms (oc and sc) are eluted in this peak. By densitometry analysis, 82.5% of plasmid DNA is recovered in this peak (whose value was obtained by densitometry analysis of band intensities). On the other hand, samples collected from the feed and first peak were analyzed by analytical hydrophobic interaction chromatography (HIC)-HPLC to quantify pDNA, following a protocol described in topic 3.11. Using this approach, only 64.6% of supercoiled isoform is recovered from this peak (Table 11).

The second peak is broader peak, with maximum UV absorbance of 658.5 mAU. From this peak, fractions 7 to 26 were collected and analyzed by gel electrophoresis. As can be seen in figure 63, fractions 7-13 seem to contain mainly sc plasmid DNA while in fractions 14-26 RNA is also detected. This elution pattern can be associated to the properties of the ligand. Ligand 2/1 acts in a similar way as lysine affinity-ligand as discussed in topic 4.2.3.3., being able to perform electrostatic interactions and H-bonding [22,77,84]. In double stranded molecules such as plasmid DNA, part of groups involved in hydrogen bonding are shielded in DNA grooves (Figure 7) and thus those molecules interact less by hydrogen-bonding with amino acids compared to single-stranded molecules (e.g. RNA) which may explain their lower retention by the ligand [78].

a)



b)

Peak	Maximum mAU (260nm)	Volume(mL)/ $[(NH_4)_2SO_4]$ (M)
1st	819.6	1.47mL/1.5M
2nd	69.1	19.96mL/ 1.137 M
3rd	142.0	34.04mL/ 0.606 M

Figure 64: a) Chromatography in 2 mL Sepharose CL-6B derivatized with ligand 2/1 packed in an TRICORN ™ 10/50 column performed under hydrophobic conditions, using a gradient from 1.5 M to 0 M ammonium sulphate in 20 mM Tris-HCl pH 8.0 in washing and Tris-HCl 20 mM, pH 8.0 as elution buffer. 1000 µL of a solution containing 500 µL of a clarified *E. coli* lysate was injected after column equilibration with 1.5 M $(NH_4)_2SO_4$ in Tris-HCl 20 mM, Ph 8.0. From this chromatography, fractions (1 to 7, 8 to 36 and 37 to 61), corresponding to the peaks detected in the chromatogram, were collected and analyzed by gel electrophoresis. b) Maximum absorbance, at 260 nm, obtained in each peak and the respective valor of chromatographic volume and concentration of $(NH_4)_2SO_4$.

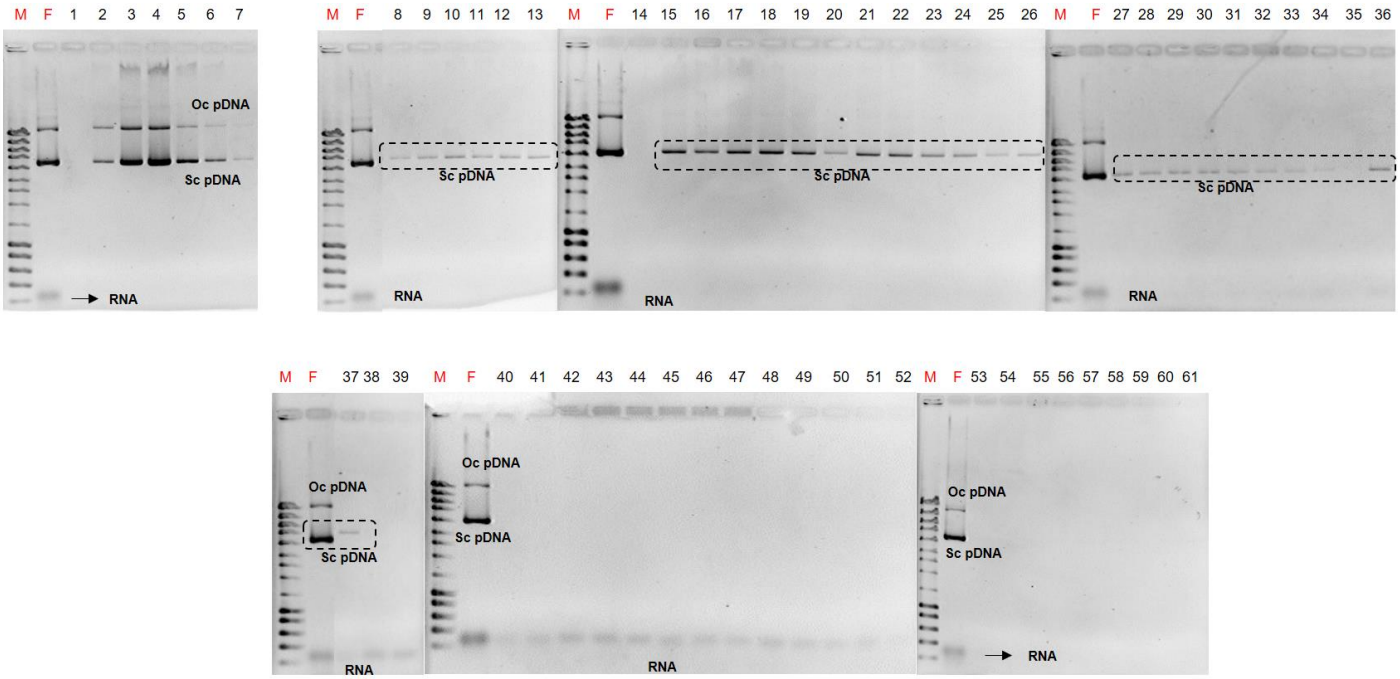


Figure 65: Gel electrophoresis of 30 µL of each fraction collected, corresponding to the peaks detected in the chromatogram in figure 61. M- DNA ladder III (NZYtech); F-Feed; oc pDNA – open-circular; sc- supercoiled. Complete band size description of DNA ladder is presented in appendix II

Under hydrophobic conditions, where the resin containing ligand 2/1 is subjected to a gradient elution between 1.5M and 0M $(NH_4)_2SO_4$ in 20mM Tris-HCl, pH 8.0 (Figure 64a), three different peaks are detected; an intense peak detected in the beginning of the assay, at maximum ammonium sulphate concentration, and two smaller peaks that appear during decreasing gradient of salt.

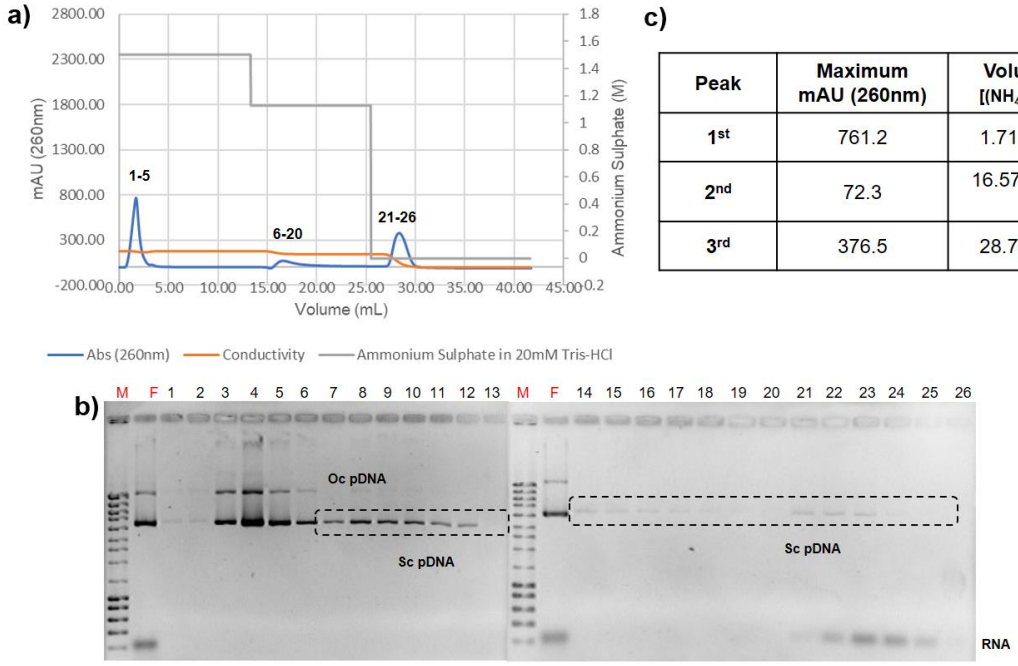
Regarding the first peak, it is an intense peak with an UV absorbance around 820 mAU (Figure 64b). From this peak, fractions 1 to 7 were collected and analyzed by gel electrophoresis (Figure 65) and it is observed that this peak elutes sc and oc isoform at high concentration and isolated from RNA.

The second peak, is less intense than the first one and apparently it is not fully separated from the third peak (Figure 64a), being detected around 1.1 M ammonium sulphate. Fractions collected from the second peak, namely fractions 8 to 36, were analyzed by gel electrophoresis, showing that this peak contains mainly supercoiled isoform isolated from other isoforms and RNA. By densitometry analysis of band intensities from feed and fractions from this peak, 59.5 % of supercoiled isoform is recovered in this peak, whereas HIC-HPLC analytical quantification reveal a supercoiled pDNA recovery around 47.3 % (Table 11).

The third peak is more intense than the second one, with a maximum UV signal of 142 mAU (Figure 64). Gel electrophoresis analysis of fractions collected at this point (37-61) detect traces of supercoiled isoform only in the first fraction and RNA elution from the column (Figure 65). Detection of sc isoform

in one single fraction from 3rd peak beginning can be associated to low resolution between the second and third peak. There, it may be assumed that the third peak is mainly composed of RNA.

After this elution pattern observed for gradient elution under hydrophobic conditions, a stepwise elution method was developed in order to optimize the resolution of each peak detected in gradient elution chromatographic assay. The stepwise approach was based on the concentration of ammonium sulphate that promoted the elution of nucleic acids in each peak. As such, this method included a step at 1.5 M $(NH_4)_2SO_4$, for the washing of unbound oc and sc pDNA, a step with 1.125 M $(NH_4)_2SO_4$ for sc pDNA elution (peak of interest) and a decrease to 0 M $(NH_4)_2SO_4$ for RNA removal (Figure 66). Additionally, the feed was loaded with a dilution of 1:3 and 1:6 to evaluate if the initial peak was associated to exceedance of column capacity.



c)

Peak	Maximum mAU (260nm)	Volume(mL)/ [$(NH_4)_2SO_4$] (M)
1st	761.2	1.71 mL/1.5 M
2nd	72.3	16.57 mL/ 1.125 M
3rd	376.5	28.75 mL/ 0 M

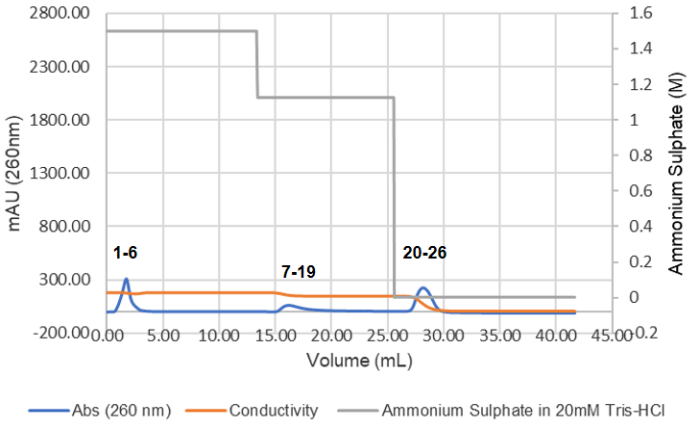
Figure 66: a) Chromatography in 2 mL Sepharose CL-6B derivatized with ligand 2/1, using a stepwise washing/elution, starting with a step with 1.5 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl pH 8.0, followed by a decrease to 1.125 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl pH 8.0, for elution of supercoiled isoform, and final decrease to 0 M, with Tris-HCl 20mM, pH 8.0 to remove the final retained molecules. A solution of 1000 µL containing about 333,3 µL of a clarified E. coli lysate (which correspond to a dilution 1:3) was injected after column equilibration with 1.5 M $(NH_4)_2SO_4$ in Tris-HCl 20 mM, pH 8.0. From this chromatography, fractions (1 to 5, 6 to 20 and 21 to 26), corresponding to the peaks detected in the chromatogram, were collected and analyzed by gel electrophoresis b) Gel electrophoresis of 30 µL of each fraction collected, corresponding to the peaks detected in the chromatogram in a). M- DNA ladder III (NZYtech); F-Feed; oc pDNA – open-circular; sc- supercoiled. Complete band size description of DNA ladder is presented in appendix II. c) Maximum absorbance, at 260 nm, obtained in each peak and the respective valor of chromatographic volume and concentration of $(NH_4)_2SO_4$

In the chromatographic assays applying stepwise elution and loading feed diluted 1:3, three peaks are detected as expected, namely a first peak more intense, with 761.2 mAU, at 1.5M $(NH_4)_2SO_4$, a second peak after a change from 1.5 to 1.125M $(NH_4)_2SO_4$, and a third peak more intense than the second (376.5 mAU) at 0M $(NH_4)_2SO_4$. Gel electrophoresis analysis of the fractions collected during this assay confirm that: i) the first peak (fractions 1-5), contains a significant amount of oc and sc pDNA isolated from RNA; ii) the second peak (fraction 6-20) is composed mainly by sc pDNA and iii) the third peak (fractions 21-26) comprises mainly RNA, but also sc isoform in the first

four fractions collected in this peak (Figure 66b). In this assay, apparently higher peak resolution is observed between the second and third peak. Nevertheless, it is not enough to obtain RNA completely isolated from sc isoform (Figure 66b). The presence of supercoiled pDNA isoform in some fractions from third peak can be related, as already discussed to ligand 8/1 (topic 4.3.1.), to some variability in sc conformations in the sample, containing different degree of bases exposure and therefore different hydrophobicity, explaining different retention times inside the column[16].

By densitometry analysis of band intensities from the fractions collected in the second peak, 65.1% of supercoiled isoform is recovered in this peak, whereas 44.9% is obtained by HIC-HPLC analytical quantification (Table 11). Moreover, this peak is composed 96.9% supercoiled isoform over open-circular pDNA, which was also determined by densitometry analysis.

a)



b)

Peak	Maximum mAU (260nm)	Volume(mL)/ [$(NH_4)_2SO_4$] (M)
1st	308.3	1.72 mL/1.5 M
2nd	61.1	16.07mL/ 1.125 M
3rd	219.2	28.01 mL/ 0 M

Figure 67: a) Chromatography in 2 mL Sepharose CL-6B resin derivatized with ligand 2/1 , using a stepwise elution, starting with a step with 1.5 M $(NH_4)_2SO_4$ in 20mM Tris-HCl pH 8.0, followed by a decrease to 1.125 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl pH 8.0, for elution of supercoiled isoform, and final decrease to 0M, with Tris-HCl 20mM, pH 8.0 to remove the final retained molecules. A solution of 1000 µL containing about 166,7 µL of a clarified *E. coli* lysate (which correspond to a dilution 1:6) was injected after column equilibration with 1.5 M $(NH_4)_2SO_4$ in Tris-HCl 20 mM, pH 8.0. From this chromatography, fractions (1 to 6, 7 to 19 and 20 to 26), corresponding to the peaks detected in the chromatogram, were collected and analyzed by gel electrophoresis. b) Maximum absorbance, at 260 nm, obtained in each peak and the respective valor of chromatographic volume and concentration of $(NH_4)_2SO_4$.

In the chromatographic assays applying stepwise elution and loading feed diluted 1:6, three peaks are detected in the chromatogram, namely a first peak, with 308.3 mAU, at 1.5 M $(NH_4)_2SO_4$, a second small peak at 1.125 M $(NH_4)_2SO_4$, and a third peak more intense than the second (376.5 mAU) when $(NH_4)_2SO_4$ concentration is decreased to zero (Figure 67). Gel electrophoresis analysis of the fractions collected during this assay reveal that: i) the first peak (fractions 1-6), contain a significant amount of oc and sc pDNA isolated from RNA; ii) the second peak (fraction 7-19) is composed mainly by sc pDNA (about 96.5 % over oc isoform – Table 11) and iii) the third peak (fractions 20-26) comprises mainly RNA, but also sc isoform in the first four fractions collected in this peak, which was confirmed by sample concentration (Figure 68b). The elution pattern is similar to the one observed for the previous chromatographic assay (Figure66a), including the presence of supercoiled isoform in the initial fractions from the third peak. Also, plasmid DNA yield seems to be higher in peaks containing sc pDNA isolated when the feed is diluted 1:6 compared to assay with feed diluted to 1:3, in both

quantification approaches, while sc pDNA yield is significantly higher compared in the assay with lower feed amount (1:6), obtained by densitometry analysis (Table 10). These results may indicate that, for lower amounts of feed loaded more pDNA is recovered in fractions containing sc pDNA isolated, suggesting that less pDNA is washed out in the flow through compared to assays using higher amounts of feed sample. Thus, these results seem to point out that initial plasmid elution can be partially due to exceedance of the capacity of the column and the amount of feed loaded will influence plasmid yield. This behavior was already reported in a previous work, in which the increase of pDNA load tended to decrease the recovery of purified sc isoform in chromatographic approach [57].

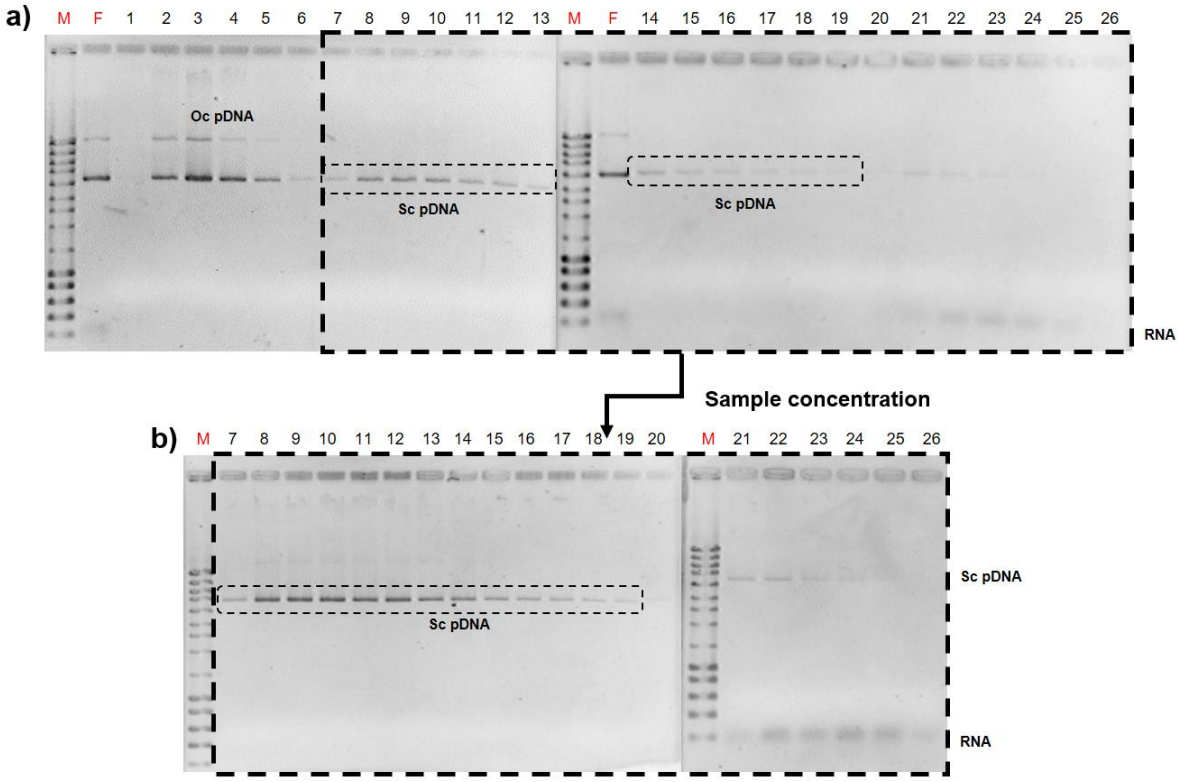


Figure 68: Gel electrophoresis of 30 µL of each fraction collected, corresponding to the peaks detected in the chromatogram in figure 67. b) Gel electrophoresis of 20 µL of each fraction collected in a) that was concentrated; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

In all assays performed with ligand 2/1 under hydrophobic conditions, plasmid DNA is directly eluted in the flow through, followed by supercoiled isoform elution and RNA at lower salt concentrations. This elution pattern indicates that ligand 2/1 recognizes differently pDNA isoforms and RNA, which is associated to different bases exposition of different nucleic acids, higher for RNA, followed by sc pDNA and oc pDNA, already discussed in topic 4.2.3.3. Also, ligand properties, namely aliphatic chain form 1,5-diaminopentane substituent or the ability to perform multiple interactions with nucleic acids under hydrophobic conditions, potentially explains the more selective affinity of this ligand for nucleic acids [84,85].

Thus, in general it is possible to isolate supercoiled pDNA from other impurities using ligand 2/1 under hydrophobic conditions, as well as obtain some fractions containing only RNA absent from other impurities.

4.3.2.1. Quantitative analysis of the fractions collected during chromatographic assays with ligand 2/1 in ÄKTA purifier system

Samples collected during the chromatographic assays with Sepharose CL-6B derivatized with ligand 2/1 in a Tricorn™10/50 column, namely the feed loaded and fractions collected in the peaks detected in the chromatogram, were analysed by analytical hydrophobic interaction chromatography (HIC)-HPLC to quantify pDNA, following the protocol described in topic 3.11. This analysis enables estimation of pDNA yield and, more specifically sc pDNA yield from the chromatographic step in the fractions collected in peak eluting isolated sc pDNA (under hydrophobic conditions) or from peaks eluting unbound plasmid isoforms (under hydrophilic conditions) (table 11). In parallel, densitometry analysis of band intensities from feed and from fractions from each peak was also performed, as described in topic 3.10, allowing the determination of pDNA and sc pDNA yields and of the relative amount of sc isoform over oc isoform in the peaks containing mainly sc isoform (Table 11).

Table 11: Chromatographic step pDNA and sc pDNA yield. Samples were collected throughout the process and analysed by HIC-HPLC and densitometry analysis to determine total pDNA (oc + sc) amounts and sc pDNA

Type of elution	Binding Conditions	Feed Dilution	pDNA (oc+sc) yield (%)		sc pDNA yield (%)[b]		sc pDNA over oc pDNA (%)[b]
			Densitometry analysis	(HIC)-HPLC quantification	Densitometry analysis	(HIC)-HPLC quantification	Densitometry analysis
Gradient	Hydrophilic	1:2	82.5[a]	64.6[a]	-	-	-
Gradient	Hydrophobic		55.8[b]	46.0[b]	59.5	47.3	-
Stepwise		1:3	62.9[b]	43.2[b]	65.1	44.9	96.9
Stepwise		1:6	69.0[b]	64.3[b]	70.1	75.1	96.5

[a.] yield determined by collection of samples collected from the first peak of hydrophilic assay (Figure 63).
[b.]value determined by collection of samples collected from the peak containing isolated supercoiled-isoform

The pDNA and sc pDNA yields obtained from the chromatographic step using ligand 2/1 are summarized in table 11, are have been already discussed in the text (see 4.3.2.). Regarding the values obtained for sc pDNA amount over oc pDNA in the peaks containing sc pDNA isolated a range between 96.5 and 96.9 % was obtained by densitometry analysis, almost reaching the range of acceptance by regulatory agencies for a final plasmid product (>97 %) (Table 1).

As observed in table 10, densitometry analysis and HIC-HPLC quantification results in yield values that are different between each other, except for stepwise assay under hydrophobic conditions using feed diluted 1:6. Densitometry analysis requires clear images with well-defined bands; otherwise it will not measure correctly the bands intensities and it may influence the measure of bands intensity. Then, the bands analyzed can be originated from fractions that were partially denatured or degraded during

manipulation of the sample, namely sample collection, concentration and loading in agarose gel, therefore affecting the final band intensity and plasmid yield determination.

Additionally to samples collected in the chromatographic process, samples were also collected during the course of the downstream process, namely after alkaline lysis, after isopropanol overnight precipitation and ammonium sulphate precipitation (topic 3.4.), to estimate plasmid DNA yield along the whole recovery and initial purification processes, which are presented in Table 12. Plasmid DNA yield from the chromatographic step was determined separately from the yield from recovery and primary isolation processes since different *E. coli* cellular batches were used to perform both steps, which may compromise an accurate comparison of the results.

Table 12: Step pDNA yield in downstream processing from *E. coli* cell extract. Samples were collected throughout the process and analyzed by HIC-HPLC to determine total pDNA (oc + sc) amounts. In this case, the process started with 1L of *E. coli* broth ($O.D._{600nm}$=3.145)

Downstream Step	**$pDNA_{total}$(µg)**	**Step pDNA yield (%)**
Alkaline lysis	2124.5	-
Isopropanol Precipitation	-	-[a]
Ammonium Sulphate precipitation	497.2	23.4[b]

[a.] A low value was obtained at this step (1.4%), not being considered in the present work.

[b.] Step yield was determined in relation to alkaline lysis.

From HIC-HPLC quantification, a step pDNA yield around 1.4 % was obtained after isopropanol precipitate of *E. coli* cell lysate (data not shown). This value is lower than expected since recovery yields around 80 % are usually reported for this step [57]. Several factors can be associated to this value. As described in topic 3.4., the pellets originated from isopropanol precipitation are subjected to resuspension with Tris-HCl 20 mM pH 8.0. Insufficient resuspension can lead to incomplete pellet dissolution and therefore nucleic acid losses can occur during this process. Additionally, this sample has high viscosity, mainly if it is kept at lower temperatures. Despite previous dilution (1:2), its viscosity may have affected HPLC analysis, due to introduction of pressure drops in the chromatographic system [116]. In this case, this sample should be even more diluted before quantification to assure that the viscosity does not influence HPLC quantification.

Regarding the step of $(NH_4)_2SO_4$ precipitation, it was obtained a plasmid DNA recovery of 23.4 %, in relation to the alkaline lysate (Table 12). This value is lower compared to other works, in which it was reported recovery yields around 70 % in relation to the initial alkaline lysate and a recovery around 80 % regarding the preceding step (isopropanol precipitation) [57]. *E. coli* cellular batch used for plasmid DNA quantification in the steps included in Table 12 was prepared after and independently from those used in the cycles of production and primary isolation of pDNA performed during the present work. As such, this quantification does not necessarily represent the downstream processing of all cellular batches produced. Higher nucleic acids losses are associated probably due to errors or incorrect processing of this specific batch, which significantly decreased the amount of plasmid DNA.

To have an idea of pDNA mass recovered after primary isolation of pDNA (after precition with $(NH_4)_2SO_4$)) in a different cellular batch, the feed used in chromatographic assay using ligand 2/1 under hydrophobic conditions (presented in figure 64) was considered as example, and the amount of pDNA present in the corresponding original lysate was extrapolated knowing the initial volume of *E. coli* lysate after ammonium sulphate precipitation (Table 13). A extrapolated mass of pDNA of 147.9 μg was obtained which is even lower than the mass determined for the lysate after ammonium sulphate precipitation (about 3 times), suggesting that different amounts of plasmid DNA may be obtained in different *E. coli* cellular batches. Thus, a correct pDNA quantification of the overall downstream processing requires the quantification of all batches using during work. Nevertheless, the results obtained or, at least, predicted, indicate that a significant part of pDNA present in the initial *E. coli* lysate is lost during downstream processing, which difficults plasmid DNA recovery and purification using affinity chromatography as final step.

Table 13: Extrapolation of the amount of total pDNA (oc + sc isoform) that is expected to be present in the lysate after ammonium sulphate precipitation step, which is obtained knowing the initial volume of *E. coli* lysate after this step (900 μL) and using as example the amount of pDNA that was determined in the feed applied in chromatographic assay using ligand 2/1 under hydrophobic conditions (Figure 64).

Sample under analysis	**$pDNA_{total}$ (μg)**
Feed [a]	94.5
Extrapolated value of $(NH_4)_2SO_4$ precipitated lysate	147.9
Value obtained for $(NH_4)_2SO_4$ precipitated lysate	497.2

[a] Feed that was used in chromatographic assay with ligand 2/1 under hydrophobic conditions, applying a gradient washing between 1.5 and 0 M $(NH_4)_2SO_4$ (in which a volume of 575 μL of this sample was applied).

5. Conclusions

Ligands from a 22-membered combinatorial library of triazine-scaffolded synthetic affinity ligands were selected according to their properties (hydrophobic and mixed-ligands) and screened in terms of binding to nucleic acids and potentially purify plasmid DNA (pVAX1/lacz) (Tables 8 to 10).

Before evaluating those ligands, several resins considered as controls for the affinity assays were evaluated, namely Sepharose CL-6B, aminated Sepharose, Aminated Sepharose with cyanuric chloride, ligand 0/0 and EDA CIM®monolithic disk. In general, those resins were not able to efficiently bind to nucleic acids or to separate either pDNA isoforms or pDNA from RNA. However, some exceptions were observed. Sepharose CL-6B resin, under hydrophobic conditions, led to the isolation of some fractions with sc pDNA and oc pDNA. This separation is likely due to molecular sieving effects.

Aminated Sepharose also retained nucleic acids, under hydrophilic conditions, due to the presence of free amino groups, suggesting that those groups, present in significant amount, can influence nucleic acid binding.

Aminated Sepharose with cyanuric chloride and Ligand 0/0 can separate pDNA from RNA under hydrophobic conditions, highlighting the contribution of the triazine ring structure in binding to nucleic acids.

EDA CIM® monolithic disk, a weak anionic exchanger, was able to completely separate RNA from pDNA.

Ligand 6/5 has been previously synthesized in an EDA CIM monolithic disk, by a solid-phase synthesis protocol well-established for agarose, and evaluated in terms of plasmid purification in AKTA purifying system. Results indicated that sc pDNA was apparently isolated and plasmid separation from RNA was achieved under hydrophobic conditions, but the results were not fully conclusive [92]. In the present work, this derivatized CIM disk was re-evaluated, in the same system, in terms of plasmid purification either in hydrophobic and hydrophilic binding environment. Under these conditions, ligand 6/5 was not able to separate supercoiled pDNA from other isoforms or to achieve pDNA separation from RNA, unlike had been reported [92]. However, the different behavior observed for non-derivatized and derivatized EDA CIM® disk, under both hydrophilic and hydrophobic conditions allowed to conclude that the derivatization of the monolithic disk with ligand 6/5 was successfully achieved. This is an important result that was not proven in preliminary work.

In general, the remaining solid-phase ligands selected with predominant hydrophobic profile (11/8, 8/11, 3/5 and 8/1) when tested in bench chromatographic columns, could separate, at least, part of pDNA from RNA, under hydrophobic conditions. From these ligands, ligand 8/1 was the only one able to isolate supercoiled isoform in some fractions, with significant amount of supercoiled isoform over open-circular isoform (around 95. 4%) being selected as one of lead ligands to proceed to chromatographic studies in ÄKTA purifier system.

On the other hand, ligands with mixed profile, namely 4/11, 3/4 and 2/1, were tested in bench chromatographic columns under either hydrophobic and hydrophilic conditions. In general, ligands acted differently under hydrophobic and hydrophilic binding conditions, highlighting the versatility of those ligands, compared to natural amino acids. From these ligands, 4/11 and 3/4 are promising candidates for plasmid isolation from RNA. Moreover, ligand 2/1 was able to isolate supercoiled isoform in some fractions, under hydrophilic and hydrophobic conditions. Under a hydrophobic binding environment, a recovery of 79.9 % of supercoiled isoform was obtained in those fractions. For this reason, ligand 2/1 was selected as the second lead ligand to proceed to chromatographic studies in ÄKTA purifier system.

In bench chromatographic assays, sample concentration revealed to be a useful and key-method to confirm elution patterns in gel electrophoresis performed, especially in diluted samples, optimizing bands visualization and avoiding incorrect interpretation of agarose gels.

Regarding the chromatographic assays performed in AKTA purifier system with ligand 8/1 immobilized in Sepharose CL-6B resin, part of plasmid DNA (about 43.7 %) was isolated from RNA under hydrophobic condition, but this ligand was not able to separate supercoiled from open-circular isoforms, under these conditions.

In the assays with Sepharose CL-6B derivatized with ligand 2/1 in the ÄKTA purifier system, pDNA separation from RNA was achieved (between 64.6 and 82.5 % pDNA recovery) under hydrophilic conditions. Under hydrophobic conditions, using a decreasing gradient of ammonium sulphate, isolated sc pDNA is recovered (between 47.3 % and 59.5 %, depending on quantification method). A step-wise washing/elution was developed to obtain better peak resolution, namely 1.5M $(NH_4)_2SO_4$ to elute unbound pDNA molecules, 1.125M $(NH_4)_2SO_4$ for sc pDNA elution and 0M $(NH_4)_2SO_4$ for RNA removal. Testing this method, the recovery of sc pDNA was between 44.9 %and 75.1 % (depending on quantification method) in fractions, having more than 96.5% sc pDNA over oc pDNA. This purity is close to the range of acceptance by regulatory agencies for a final plasmid product (>97 %). Thus, above-mentioned results seem to point out that ligand 2/1 is a promising ligand to isolate sc pDNA from other impurities under hydrophobic conditions, with considerable yields.

6. Further work

Further work to complement results presented in this thesis include, the evaluation of the presence of contaminants in the final sc pDNA product recovered from affinity chromatography with ligand 2/1. Those contaminants include proteins, which can be detected by micro-BCA analysis, or endotoxins, measured with LAL (Limulus Amoebocyte Lysate) test, for example [84].

Alternative buffers to promote hydrophobic and hydrophilic conditions such as sodium citrate or ammonium citrate may also be tested to surmount problems with ammonium sulphate waste treatment [57,70]. Moreover, ligand 2/1 can be also evaluated in terms of plasmid purification with plasmids with different size of pVAX1/lacz, to study the influence of plasmid size in plasmid retention.

Regarding the advantages of CIM monolithic disk over agarose resins, immobilization of ligand 2/1 in monolithic CIM disk, applying a similar derivatization approach of ligand 6/5 and further evaluation under hydrophobic and hydrophilic conditions can yield promising results[97]. Alternatively, a scale-up affinity chromatography with ligand 2/1, using higher volumes of derivatized Sepharose CL-6B, can be done.

To finalize, other ligands, such as 1/2, 4/1 or 1/4, containing substituents mimetics of lysine (Table 4) should be tested. Theoretically it is expected that such ligands behave in a similar way of ligand 2/1 under similar chromatographic conditions.

7. References

[1] F. Sousa, L. Passarinha, and J. A. Queiroz, “Biomedical application of plasmid DNA in gene therapy: a new challenge for chromatography.,” *Biotechnol. Genet. Eng. Rev.*, vol. 26, pp. 83–116, 2009.

[2] D. L. Nelson and M. M. Cox, “DNA cloning: the basics,” in *Lehninger - Principles of Biochemistry*, 5th ed., pp. 307–308, 2008.

[3] G. M. Cooper and R. E. Hausman, “Recombinant DNA,” in *The cell- A molecular Approach*, 5th ed., pp. 120–121, 2009.

[4] H. L. Robinson, “DNA vaccines,” *Clin. Microbiol. Newsl.*, vol. 22, no. 3, pp. 17–22, 2000.

[5] F. Sousa, D. M. F. Prazeres, and J. A. Queiroz, “Improvement of transfection efficiency by using supercoiled plasmid DNA purified with arginine affinity chromatography,” *J. Gene Med.*, vol. 11, no. 1, pp. 79–88, 2009.

[6] S. S. A. A. Hasson, J. K. Z. Al-Busaidi, and T. A. Sallam, “The past, current and future trends in DNA vaccine immunisations,” *Asian Pac. J. Trop. Biomed.*, vol. 5, no. 5, pp. 344–353, 2015.

[7] J. B. Alarcon, G. W. Waine, and D. P. McManus, “DNA vaccines: technology and application as anti-parasite and anti-microbial agents.,” *Adv. Parasitol.*, vol. 42, pp. 343–410, 1999.

[8] H. L. Robinson and T. M. Pertmer, “DNA vaccines for viral infections: basic studies and applications.,” *Adv. Virus Res.*, vol. 55, pp. 1–74, 2000.

[9] A. Ghanem, R. Healey, and F. G. Adly, “Current trends in separation of plasmid DNA vaccines: A review,” *Anal. Chim. Acta*, vol. 760, pp. 1–15, 2013.

[10] T. Kindt, R. Goldsby, and B. Osborn, “Vaccines Chapter 19,” in *Kuby Immunology*, Sixth., W.H. Freeman & Co, pp. 475–492, 2007.

[11] D. M. Bower and K. L. J. Prather, “Engineering of bacterial strains and vectors for the production of plasmid DNA,” *Appl. Microbiol. Biotechnol.*, vol. 82, no. 5, pp. 805–813, 2009.

[12] J. Stadler, R. Lemmens, and T. Nyhammar, “Plasmid DNA purification,” *J. Gene Med.*, vol. 6, no. S1, pp. S54–S66, 2004.

[13] D. M. Klinman, S. Klaschik, D. Tross, H. Shirota, and F. Steinhagen, “FDA guidance on prophylactic DNA vaccines: Analysis and recommendations,” *Vaccine*, vol. 28, no. 16, pp. 2801–2805, 2010.

[14] FDA, “Vaccines Guidances - Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications,” Center for Biologics Evaluation and Research, 2007.

[15] D. R. Latulippe and A. L. Zydney, “Size exclusion chromatography of plasmid DNA isoforms,” *J. Chromatogr. A*, vol. 1216, no. 35, pp. 6295–6302, 2009.

[16] F. Sousa and J. A. Queiroz, “Circular dichroism investigation of the effect of plasmid DNA structure on retention in histidine chromatography,” vol. 467, pp. 154–162, 2007.

[17] F. Sousa, D. M. F. Prazeres, and J. A. Queiroz, “Affinity chromatography approaches to overcome the challenges of purifying plasmid DNA,” *Trends Biotechnol.*, vol. 26, no. 9, pp. 518–525, 2008.

[18] V. B. Pillai, M. Hellerstein, T. Yu, R. R. Amara, and H. L. Robinson, “Comparative studies on in vitro expression and in vivo immunogenicity of supercoiled and open circular forms of plasmid DNA vaccines.,” *Vaccine*, vol. 26, no. 8, pp. 1136–41, 2008.

[19] L. Cupillard, V. Juillard, S. Latour, G. Colombet, N. Cachet, S. Richard, S. Blanchard, and L. Fischer, “Impact of plasmid supercoiling on the efficacy of a rabies DNA vaccine to protect cats,” *Vaccine*, vol. 23, no. 16, pp. 1910–1916, 2005.

[20] J. Adamčík, V. Víglasky´, F. Valle, M. Antalík, D. Podhradsky´, and G. Dietler, “Effect of bacteria growth temperature on the distribution of supercoiled DNA and its thermal stability,”

Electrophoresis, vol. 23, no. 19, pp. 3300–3309, 2002.

[21] ThermoFisher, “Guidelines for Plasmid DNA Transfection,” *ThermoFisher Scientific*, 2016. .

[22] D. L. Nelson and M. M. Cox, *Lehninger - Principles of Biochemistry*, 5th ed. Freeman, 2008.

[23] U. Černigoj, U. Martinuč, S. Cardoso, R. Sekirnik, N. L. Krajnc, and A. Štrancar, “Sample displacement chromatography of plasmid DNA isoforms,” *J. Chromatogr. A*, vol. 1414, pp. 103–109, 2015.

[24] P. A. Shamlou, “Scaleable processes for the manufacture of therapeutic quantities of plasmid DNA,” *Biotechnol. Appl. Biochem.*, vol. 37, no. 3, p. 207, 2003.

[25] U. Černigoj, U. Vidic, M. Barut, A. Podgornik, M. Peterka, and A. Štrancar, “A multimodal histamine ligand for chromatographic purification of plasmid DNA.,” *J. Chromatogr. A*, vol. 1281, pp. 87–93, 2013.

[26] Â. Sousa, F. Sousa, and J. A. Queiroz, “Advances in chromatographic supports for pharmaceutical-grade plasmid DNA purification,” *J. Sep. Sci.*, vol. 35, no. 22, pp. 3046–3058, 2012.

[27] J. Robertson, J. Ackland, and A. Holm, “Guidelines for assuring the quality and nonclinical safety evaluation of DNA vaccines,” 2007.

[28] N. Casali, “Escherichia coli Host Strains,” in *E. coli Plasmid Vectors*, New Jersey: Humana Press, pp. 27–48, 2003.

[29] National Institutes of Health, “NIH guidelines for research involving recombinant or synthetic nucleic acid molecules (NIH guidelines),” 2016.

[30] J. A. Williams, A. E. Carnes, and C. P. Hodgson, “Plasmid DNA vaccine vector design: impact on efficacy, safety and upstream production.,” *Biotechnol. Adv.*, vol. 27, no. 4, pp. 353–70, 2009.

[31] Thermo Fisher Scientific, “MAX Efficiency DH5 Competent Cells - Thermo Fisher Scientific,” *Thermo Fisher Scientific* , 2016. [Online]. Available: https://www.thermofisher.com/order/catalog/product/18258012. [Accessed: 20-Jan-2017].

[32] A. E. Carnes, C. P. Hodgson, and J. A. Williams, “Inducible Escherichia coli fermentation for increased plasmid DNA production,” *Biotechnol. Appl. Biochem.*, vol. 45, no. 3, p. 155, 2006.

[33] W. Saltzman, H. Shen, and J. Brandsma, *DNA Vaccines: Methods And Protocols*. Humana Press, 2006.

[34] Invitrogen, “pVAX1™,” V260-20, 2012.

[35] G. A. Gomes, A. M. Azevedo, R. M. Aires-Barros, and D. M. F. Prazeres, “Purification of plasmid DNA with aqueous two phase systems of PEG 600 and sodium citrate/ammonium sulfate,” *Sep. Purif. Technol.*, vol. 65, no. 1, pp. 22–30, 2009.

[36] J. Urthaler, W. Buchinger, and R. Necina, “Improved downstream process for the production of plasmid DNA for gene therapy.,” *Acta Biochim. Pol.*, vol. 52, no. 3, pp. 703–11, 2005.

[37] R. Reames, M. Gelinske, and S. Thapa, “Optimization of Plasmid DNA Production in Escherichia coli Utilizing the Thermo Scientific MaxQ 8000 Incubated Stackable Shakers,” 2016.

[38] K. J. Prather, S. Sagar, J. Murphy, and M. Chartrain, “Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification,” *Enzyme Microb. Technol.*, vol. 33, no. 7, pp. 865–883, 2003.

[39] S. Selvarasu, D. S.-W. Ow, S. Y. Lee, M. M. Lee, S. K.-W. Oh, I. A. Karimi, and D.-Y. Lee, “Characterizing *Escherichia coli* DH5α growth and metabolism in a complex medium using genome-scale flux analysis,” *Biotechnol. Bioeng.*, vol. 102, no. 3, pp. 923–934, 2009.

[40] K. E. Kram and S. E. Finkel, “Rich Medium Composition Affects Escherichia coli Survival,

Glycation, and Mutation Frequency during Long-Term Batch Culture.," *Appl. Environ. Microbiol.*, vol. 81, no. 13, pp. 4442–50, 2015.

[41] M. T. Madigan, J. M. Martinko, D. A. Stahl, and C. P. David, "I. Nutrition and culture of microorganisms," in *Brock - Biology of Microorganisms*, 13th ed., Pearson Education Inc., pp. 88–92, 2011.

[42] L. Yee and H. W. Blanch, "Defined media optimization for growth of recombinantEscherichia coli X90," *Biotechnol. Bioeng.*, vol. 41, no. 2, pp. 221–230, 1993.

[43] R. O'Kennedy, "Effects of growth medium selection on plasmid DNA production and initial processing steps," *J. Biotechnol.*, vol. 76, no. 2–3, pp. 175–183, 2000.

[44] M. V. Baev, D. Baev, A. J. Radek, and J. W. Campbell, "Growth of Escherichia coli MG1655 on LB medium: monitoring utilization of sugars, alcohols, and organic acids with transcriptional microarrays," *Appl. Microbiol. Biotechnol.*, vol. 71, no. 3, pp. 310–316, 2006.

[45] G. Sezonov, D. Joseleau-Petit, and R. D'Ari, "Escherichia coli physiology in Luria-Bertani broth.," *J. Bacteriol.*, vol. 189, no. 23, pp. 8746–9, 2007.

[46] M. Szostková, D. Horáková, and M. Němec, "The influence of the growth phase of enteric bacteria on electrotransformation with plasmid DNA.," *Folia Microbiol. (Praha).*, vol. 44, no. 2, pp. 177–80, 1999.

[47] A. P. J. Middleberg, "Process-scale disruption of microorganisms," *Biotechnol. Adv.*, vol. 13, no. 3, pp. 491–551, 1995.

[48] P. A. S. M.S. Levy, L.A.S. Ciccolini, S.S.S. Yim, J.T. Tsai, N. Titchener-Hooker and P. Dunnill, "The effects of material properties and fluid flow intensity on plasmid DNA recovery during cell lysis," *Chem. Eng. Sci.*, vol. 54, pp. 3171–3178, 1999.

[49] I. Feliciello and G. Chinali, "A Modified Alkaline Lysis Method for the Preparation of Highly Purified Plasmid DNA from Escherichia Coli," *Anal. Biochem.*, vol. 212, no. 2, pp. 394–401, 1993.

[50] C. for F. S. and A. Nutrition, "Generally Recognized as Safe (GRAS)," *U.S. Food and Drug Admnistration*, 2017. [Online]. Available: https://www.fda.gov/food/ingredientspackaginglabeling/gras/. [Accessed: 06-Oct-2017].

[51] H. C. Birnboim and J. Doly, "A rapid alkaline extraction procedure for screening recombinant plasmid DNA.," *Nucleic Acids Res.*, vol. 7, no. 6, pp. 1513–23, 1979.

[52] J. Schorr, P. Moritz, A. Breul, and M. Scheef, "Production of Plasmid DNA in Industrial Quantities According to cGMP Guidelines," in *DNA Vaccines*, vol. 127, New Jersey: Humana Press, pp. 339–350, 2006.

[53] M. M. Diogo, J. A. Queiroz, and D. M. Prazeres, "Studies on the retention of plasmid DNA and Escherichia coli nucleic acids by hydrophobic interaction chromatography.," *Bioseparation*, vol. 10, no. 4–5, pp. 211–20, 2001.

[54] NEB, "DNA Isolation | NEB," *New England Biolabs*, 2017. [Online]. Available: https://www.neb.com/applications/cloning-and-synthetic-biology/dna-isolation. [Accessed: 22-Jan-2017].

[55] S. S. Freitas, A. R. Azzoni, J. A. L. Santos, G. A. Monteiro, and D. M. F. Prazeres, "On the stability of plasmid DNA vectors during cell culture and purification," *Mol. Biotechnol.*, vol. 36, no. 2, pp. 151–158, 2007.

[56] M. M. Diogo, J. A. Queiroz, G. A. Monteiro, and D. M. F. Prazeres, "Separation and Analysis of Plasmid Denatured Forms Using Hydrophobic Interaction Chromatography," *Anal. Biochem.*, vol. 275, no. 1, pp. 122–124, 1999.

[57] A. R. Silva-Santos, C. P. A. Alves, D. M. F. Prazeres, and A. M. Azevedo, "A process for supercoiled plasmid DNA purification based on multimodal chromatography," *Sep. Purif. Technol.*, vol. 182, pp. 94–100, 2017.

[58] N. A. Horn, J. A. Meek, G. Budahazi, and M. Marquet, “Cancer Gene Therapy Using Plasmid DNA: Purification of DNA for Human Clinical Trials,” *Hum. Gene Ther.*, vol. 6, no. 5, pp. 565–573, 1995.

[59] A. Eon-Duval, R. H. MacDuff, C. A. Fisher, M. J. Harris, and C. Brook, “Removal of RNA impurities by tangential flow filtration in an RNase-free plasmid DNA purification process,” *Anal. Biochem.*, vol. 316, no. 1, pp. 66–73, 2003.

[60] C. Haber, J. Skupsky, A. Lee, and R. Lander, “Membrane chromatography of DNA: Conformation-induced capacity and selectivity,” *Biotechnol. Bioeng.*, vol. 88, no. 1, pp. 26–34, 2004.

[61] L.-Z. Li, Y. Liu, M.-S. Sun, and Y.-M. Shao, “Effect of salt on purification of plasmid DNA using size-exclusion chromatography,” *J. Chromatogr. A*, vol. 1139, no. 2, pp. 228–235, 2007.

[62] M. D. Costioli, I. Fisch, F. Garret-Flaudy, F. Hilbrig, and R. Freitag, “DNA purification by triple-helix affinity precipitation,” *Biotechnol. Bioeng.*, vol. 81, no. 5, pp. 535–545, 2003.

[63] S. Balan, J. Murphy, I. Galaev, A. Kumar, G. E. Fox, B. Mattiasson, and R. C. Willson, “Metal chelate affinity precipitation of RNA and purification of plasmid DNA,” *Biotechnol. Lett.*, vol. 25, no. 13, pp. 1111–1116, 2003.

[64] S. C. Ribeiro, G. A. Monteiro, J. M. S. Cabral, and D. M. F. Prazeres, “Isolation of plasmid DNA from cell lysates by aqueous two-phase systems,” *Biotechnol. Bioeng.*, vol. 78, no. 4, pp. 376–384, 2002.

[65] J. A. Asenjo and B. A. Andrews, “Aqueous two-phase systems for protein separation: A perspective,” *J. Chromatogr. A*, vol. 1218, no. 49, pp. 8826–8835, 2011.

[66] R. F. Boyer, “Separation and purification of biomolecules by chromatography,” in *Modern Experimental Biochemistry*, 2nd ed., Benjamin Cummings, pp. 102–110, 1993.

[67] Bio-Rad Laboratories, “Liquid Chromatography Principles.” [Online]. Available: http://www.bio-rad.com/en-pt/applications-technologies/liquid-chromatography-principles. [Accessed: 11-Oct-2017].

[68] B. Separations, “BIA Separations - CIM® EDA Disk (Ethylene diamino) (Pores 1.3 um),” *BIA Separations*. [Online]. Available: http://www.biaseparations.com/component/hikashop/product/177-cim-eda-0-34-ml. [Accessed: 18-Jan-2017].

[69] I. Molnár and C. Horváth, “Reverse-phase chromatography of polar biological substances: separation of catechol compounds by high-performance liquid chromatography.,” *Clin. Chem.*, vol. 22, no. 9, pp. 1497–502, 1976.

[70] S. S. Freitas, J. A.L. Santos, and D. M.F. Prazeres, “Plasmid purification by hydrophobic interaction chromatography using sodium citrate in the mobile phase,” *Sep. Purif. Technol.*, vol. 65, no. 1, pp. 95–104, 2009.

[71] P. Guerrero-Germán, R. M. Montesinos-Cisneros, D. M. F. Prazeres, and A. Tejeda-Mansir, “Purification of plasmid DNA from Escherichia coli ferments using anion-exchange membrane and hydrophobic chromatography,” *Biotechnol. Appl. Biochem.*, vol. 58, no. 1, pp. 68–74, 2011.

[72] M. Wilchek and I. Chaiken, *Affinity Chromatography*. Humana Press, 2000.

[73] T. Santos, J. Carvalho, M. C. Corvo, E. J. Cabrita, J. A. Queiroz, and C. Cruz, “l-tryptophan and dipeptide derivatives for supercoiled plasmid DNA purification,” *Int. J. Biol. Macromol.*, vol. 87, pp. 385–396, 2016.

[74] A. C. A. Roque, C. S. O. Silva, and M. Â. Taipa, “Affinity-based methodologies and ligands for antibody purification: Advances and perspectives,” *J. Chromatogr. A*, vol. 1160, no. 1–2, pp. 44–55, 2007.

[75] A. C. A. Roque, M. Â. Taipa, and C. R. Lowe, “Synthesis and screening of a rationally designed combinatorial library of affinity ligands mimicking protein L fromPeptostreptococcus magnus,” *J. Mol. Recognit.*, vol. 18, no. 3, pp. 213–224, 2005.

[76] C. R. Lowe, S. J. Burton, N. P. Burton, W. K. Alderton, J. M. Pitts, and J. A. Thomas, “Designer dyes: ‘biomimetic’ ligands for the purification of pharmaceutical proteins by affinity chromatography.,” *Trends Biotechnol.*, vol. 10, no. 12, pp. 442–8, 1992.

[77] F. Sousa, C. Cruz, and J. A. Queiroz, “Amino acids-nucleotides biomolecular recognition: from biological occurrence to affinity chromatography,” *J. Mol. Recognit.*, vol. 23, no. 6, pp. 505–518, 2010.

[78] A. Sousa, F. Sousa, and J. A. Queiroz, “Differential interactions of plasmid DNA, RNA and genomic DNA with amino acid-based affinity matrices,” *J. Sep. Sci.*, vol. 33, no. 17–18, pp. 2610–2618, 2010.

[79] N. M. Luscombe, R. A. Laskowski, and J. M. Thornton, “Amino acid-base interactions: a three-dimensional analysis of protein-DNA interactions at an atomic level.,” *Nucleic Acids Res.*, vol. 29, no. 13, pp. 2860–74, 2001.

[80] M. Stewart, T. Dunlap, E. Dourlain, B. Grant, and L. McFail-Isom, “Cations form sequence selective motifs within DNA grooves via a combination of cation-pi and ion-dipole/hydrogen bond interactions.,” *PLoS One*, vol. 8, no. 8, 2013.

[81] G. B. McGaughey, M. Gagné, and A. K. Rappé, “pi-Stacking interactions. Alive and well in proteins.,” *J. Biol. Chem.*, vol. 273, no. 25, pp. 15458–63, 1998.

[82] F. Sousa, S. Freitas, A. R. Azzoni, D. M. F. Prazeres, and J. Queiroz, “Selective purification of supercoiled plasmid DNA from clarified cell lysates with a single histidine–agarose chromatography step,” *Biotechnol. Appl. Biochem.*, vol. 45, no. 3, p. 131, 2006.

[83] J. F. A. Valente, A. Sousa, J. A. Queiroz, and F. Sousa, “Selective purification of supercoiled p53-encoding pDNA with l-methionine–agarose matrix,” *Anal. Biochem.*, vol. 459, pp. 61–69, 2014.

[84] A. Sousa, F. Sousa, and J. A. Queiroz, “Impact of lysine-affinity chromatography on supercoiled plasmid DNA purification,” *J. Chromatogr. B*, vol. 879, no. 30, pp. 3507–3515, 2011.

[85] A. Sousa, F. Sousa, and J. A. Queiroz, “Biorecognition of supercoiled plasmid DNA isoform in lysine-affinity chromatography,” *J. Chromatogr. B*, vol. 877, no. 27, pp. 3257–3260, 2009.

[86] V. M. Gaspar, I. J. Correia, Â. Sousa, F. Silva, C. M. Paquete, J. A. Queiroz, and F. Sousa, “Nanoparticle mediated delivery of pure P53 supercoiled plasmid DNA for gene therapy,” *J. Control. Release*, vol. 156, no. 2, pp. 212–222, 2011.

[87] M. A. Vijayalakshmi, “Pseudo-Biospecific Affinity Ligand Chromatography,” in *Molecular Interactions in Bioseparations*, Boston, MA: Springer US, pp. 257–275, 1993.

[88] A. C. A. Roque, M. Â. Taipa, and C. R. Lowe, “A new method for the screening of solid-phase combinatorial libraries for affinity chromatography,” *J. Mol. Recognit.*, vol. 17, no. 3, pp. 262–267, 2004.

[89] J. C. Murphy, D. L. Jewell, K. I. White, G. E. Fox, and R. C. Willson, “Nucleic acid separations utilizing immobilized metal affinity chromatography.,” *Biotechnol. Prog.*, vol. 19, no. 3, pp. 982–6, 2003.

[90] L. Tan, W.-B. Lai, C. T. Lee, D. S. Kim, and W.-S. Choe, “Differential interactions of plasmid DNA, RNA and endotoxin with immobilised and free metal ions,” *J. Chromatogr. A*, vol. 1141, no. 2, pp. 226–234, 2007.

[91] D. S. Hage and J. Cazes, *Handbook of Affinity Chromatography*, 2th ed. CRC, 2005.

[92] C. Jorge, “Development of novel biomimetic affinity adsorbents for plasmid DNA purification: Preliminary results,” MSc Thesis in Biotechnology - Instituto Superior Técnico - Universidade de Lisboa, 2014.

[93] L. Ruiu, A. C. A. Roque, M. A. Taipa, and C. R. Lowe, “De novo design, synthesis and screening of a combinatorial library of complementary ligands directed towards the surface of cutinase fromFusarium solani pisi,” *J. Mol. Recognit.*, vol. 19, no. 4, pp. 372–378, 2006.

[94] Â. Sousa, F. Sousa, and J. A. Queiroz, “Advances in chromatographic supports for pharmaceutical-grade plasmid DNA purification,” *J. Sep. Sci.*, vol. 35, no. 22, pp. 3046–3058, 2012.

[95] GE Healthcare, “Epoxy-activated Sepharose™ 6B,” 2010.

[96] C. A. Costa and J. S. Cabral, *Chromatographic and Membrane Processes in Biotechnology*. Springer Science+Business Media, B.V., 1990.

[97] A. Podgornik, J. Vidič, J. Jančar, N. Lendero, V. Frankovič, and A. Štrancar, “Noninvasive methods for characterization of large-volume monolithic chromatographic columns,” *Chem. Eng. Technol.*, vol. 28, no. 11, pp. 1435–1441, 2005.

[98] B. Separations, “Product Specific Information Sheet - CIM Disk for Ion Exchange Chromatography,” 210.5116, 2015.

[99] M. Ângela Taipa, “AFFINITY SEPARATION | Rational Design, Synthesis, and Evaluation: Affinity Ligands,” in *Reference Module in Chemistry, Molecular Sciences and Chemical Engineering*, Elsevier, 2014.

[100] I. T. Sousa and M. Â. Taipa, “Biomimetic Affinity Ligands for Protein Purification,” in *Methods in molecular biology (Clifton, N.J.)*, vol. 1129, Springer Science MIMB Series, pp. 231–262, 2014.

[101] J. F. Belchior, “Screening and evaluation of synthetic protein-mimic affinity ligands for the purification of plasmid DNA,” MSc Thesis in Biotechnology - Instituto Superior Técnico - Universidade de Lisboa, 2013.

[102] S. F. Teng, K. Sproule, A. Hussain, and C. R. Lowe, “A strategy for the generation of biomimetic ligands for affinity chromatography. Combinatorial synthesis and biological evaluation of an IgG binding ligand,” *J. Mol. Recognit.*, vol. 12, no. 1, pp. 67–75, 1999.

[103] U. D. Palanisamy, A. Hussain, S. Iqbal, K. Sproule, and C. R. Lowe, “Design, synthesis and characterisation of affinity ligands for glycoproteins,” *J. Mol. Recognit.*, vol. 12, no. 1, pp. 57–66, 1999.

[104] H. Filippusson, L. S. Erlendsson, and C. R. Lowe, “Design, synthesis and evaluation of biomimetic affinity ligands for elastases,” *J. Mol. Recognit.*, vol. 13, no. 6, pp. 370–381, 2000.

[105] I. T. Sousa, N. M. T. Lourenço, C. A. M. Afonso, and M. A. Taipa, “Protein stabilization with a dipeptide-mimic triazine-scaffolded synthetic affinity ligand,” *J. Mol. Recognit.*, vol. 26, no. 2, pp. 104–112, 2013.

[106] G. N. Ferreira, G. A. Monteiro, D. M. Prazeres, and J. M. Cabral, “Downstream processing of plasmid DNA for gene therapy and DNA vaccine applications.,” *Trends Biotechnol.*, vol. 18, no. 9, pp. 380–8, 2000.

[107] I. Matsumoto, N. Seno, A. M. Golovtchenko-Matsumoto, and T. Osawa, “Amination and subsequent derivatization of epoxy-activated agarose for the preparation of new affinity adsorbents.,” *J. Biochem.*, vol. 87, no. 2, pp. 535–40, 1980.

[108] S. Yoshiki, T. Ueno, T. Akita, and M. Yamanouchi, “Improved procedure for histological identification of osteoid matrix in decalcified bone.,” *Stain Technol.*, vol. 58, no. 2, pp. 85–9, 1983.

[109] R. J. F. Branco, A. M. G. C. Dias, and A. C. A. Roque, “Understanding the molecular recognition between antibody fragments and protein A biomimetic ligand,” *J. Chromatogr. A*, vol. 1244, pp. 106–115, 2012.

[110] A. E. Carnes and J. A. Williams, “Plasmid DNA manufacturing technology.,” *Recent Pat. Biotechnol.*, vol. 1, no. 2, pp. 151–66, 2007.

[111] D. Bicho, B. F. Santos, C. Caramelo-Nunes, A. Sousa, F. Sousa, J. A. Queiroz, and C. T. Tomaz, “Application of ethylenediamine monolith to purify a hemagglutinin influenza deoxyribonucleic acid-based vaccine,” *Sep. Purif. Technol.*, vol. 154, pp. 320–327, 2015.

[112] N. Lendero, J. Vidič, P. Brne, V. Frankovič, A. Štrancar, and A. Podgornik, “Characterization of

ion exchange stationary phases via pH transition profiles," *J. Chromatogr. A*, vol. 1185, no. 1, pp. 59–70, 2008.

[113] I. T. Sousa, L. Ruiu, C. R. Lowe, and M. A. Taipa, "Synthetic affinity ligands as a novel tool to improve protein stability," *J. Mol. Recognit.*, vol. 22, no. 2, pp. 83–90, 2009.

[114] L. R. Pereira, D. M. F. Prazeres, and M. Mateus, "Hydrophobic interaction membrane chromatography for plasmid DNA purification: Design and optimization," *J. Sep. Sci.*, vol. 33, no. 9, 2010.

[115] P. Pereira, Â. Sousa, J. Queiroz, A. Figueiras, and F. Sousa, "New approach for purification of pre-miR-29 using lysine-affinity chromatography," *J. Chromatogr. A*, vol. 1331, pp. 129–132, 2014.

[116] L. R. Snyder, J. J. Kirkland, and J. L. Glajch, *Practical HPLC Method Development*, 2nd ed. John Wiley & Sons, Inc., 1997.

8. Appendix

Appendix I – Information about plasmid pVAX/lacZ (Invitrogen)

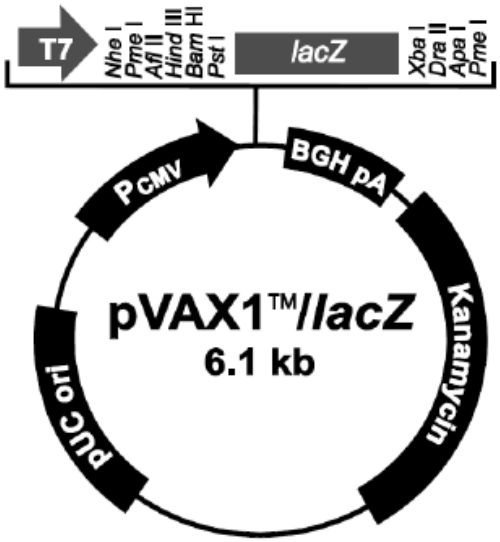


Figure A1: Scheme of the structure and features of plasmid pVAX1/lacZ.

Size: 6050bp

CMV promoter: bases 137-724

T7 promoter/priming sites: bases 664-683

LacZ ORF: bases 773-3829

BGH reverse priming site: bases 3874-3891

BGH polyadenylation signal: bases 3880-4104

Kanamycin resistance gene: bases 4277-5071

pUC origin: bases 5371-6044

Appendix II – DNA ladder applied in gel electrophoresis – DNA ladder III (NZYtech)

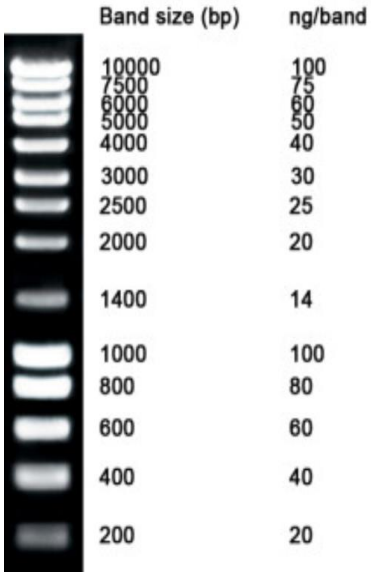


Figure A2: DNA ladder applied in gel electrophoresis; bp- base pairs.

Appendix III – Cell growths performed during this study

As mentioned, several cell fermentations were performed during the present study. After cell lysis and primary isolation, cell lysates were analyzed by gel electrophoresis to evaluate their content in nucleic acids, namely supercoiled, open-circular isoform and RNA.

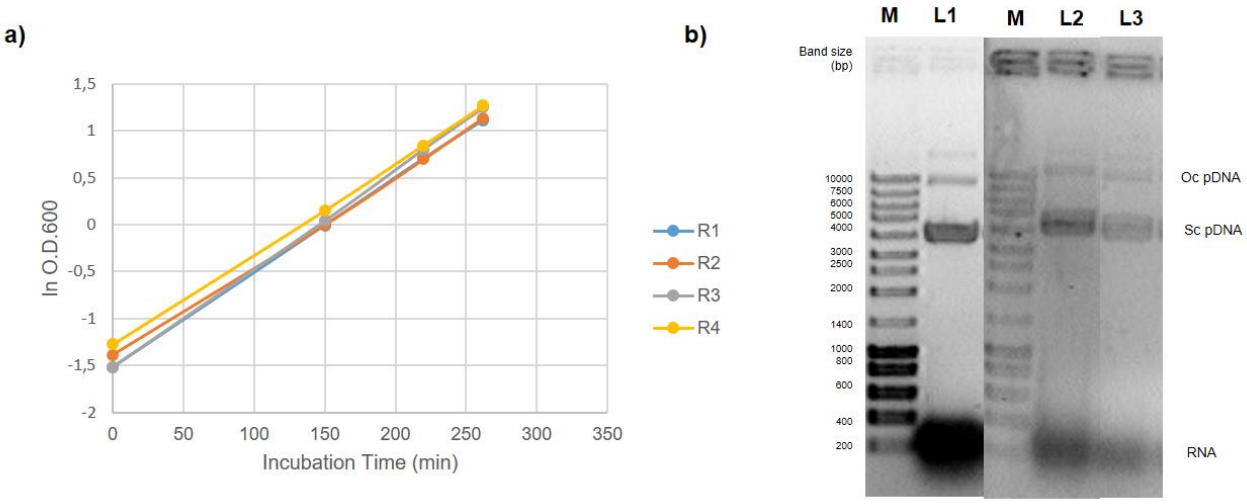


Figure A3: a) Growth of *E. coli* DH5α cells containing the plasmid pVAX1/lacZ, in 500 mL LB medium, over incubation time (in minutes) performed in 14th February 2017. R1 to R4 – replicates containing 500 mL of cell broth each. b) Gel electrophoresis obtained after alkaline lysis and primary isolation to evaluate nucleic acid content; M- DNA Ladder III (NZYtech); L1, L2 and L3- lysates resultant from cell lysis and plasmid primary isolation of cell broth in a) performed in different days.

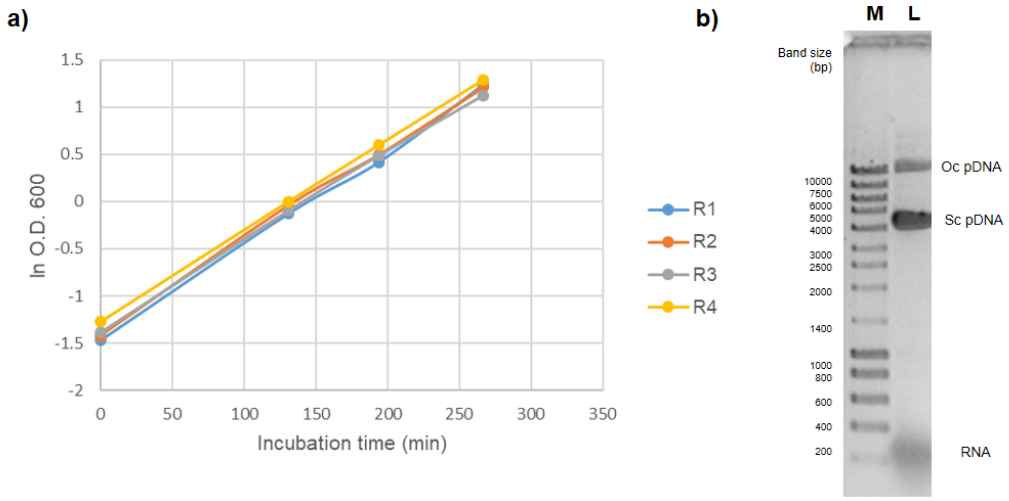


Figure A4: a) Growth of *E. coli* DH5α cells containing the plasmid pVAX1/lacZ, in 500 mL LB medium, over incubation time (in minutes) performed in 4th April 2017. R1 to R4 – replicates containing 500 mL of cell broth each. b) Gel electrophoresis obtained after alkaline lysis and primary isolation to evaluate nucleic acid content; M- DNA Ladder III (NZYtech); L - lysate resultant from cell lysis and plasmid primary isolation of cell broth in a).

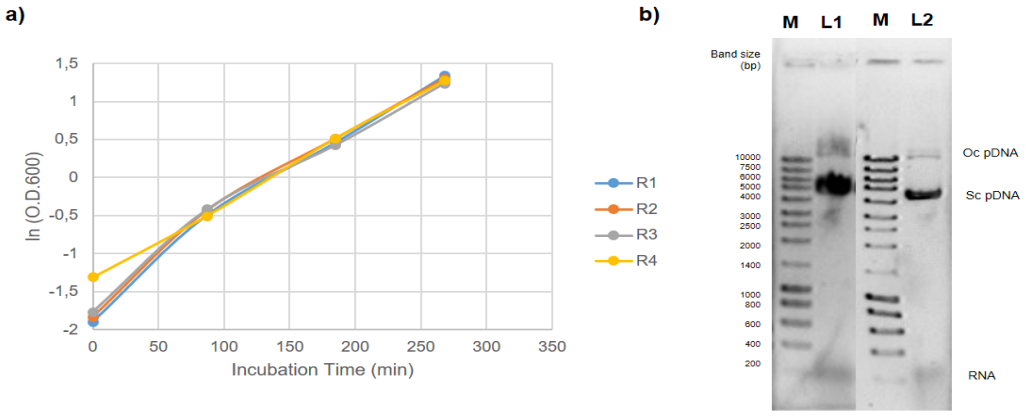


Figure A5: a) Growth of *E. coli* DH5α cells containing the plasmid pVAX1/lacZ, in 500 mL LB medium, over incubation time (in minutes) performed in 11st April 2017. R1 to R4 – replicates containing 500 mL of cell broth each. b) Gel electrophoresis obtained after alkaline lysis and primary isolation to evaluate nucleic acid content; M- DNA Ladder III (NZYtech); L1 and L2 - lysate resultant from cell lysis and plasmid primary isolation of cell broth in a) performed in different days.

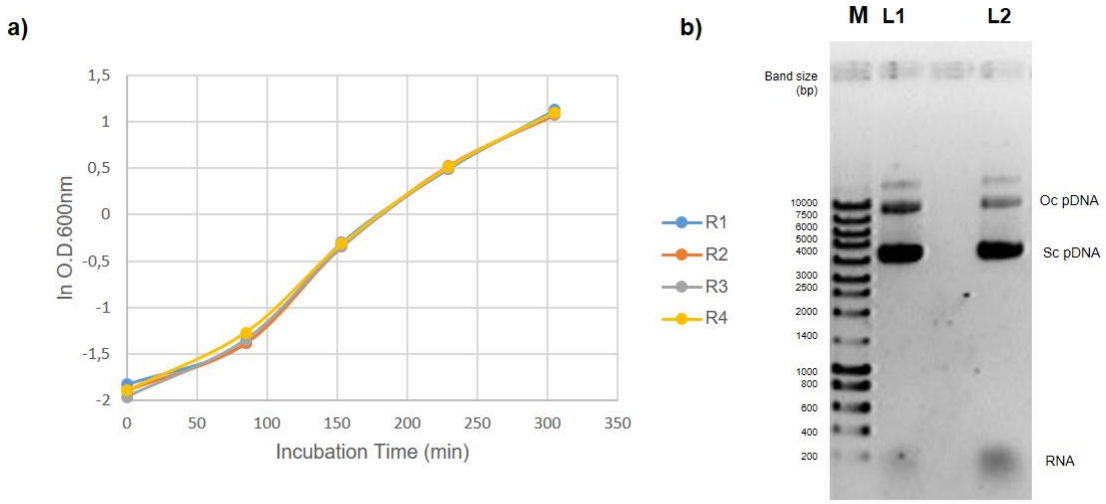


Figure A6: a) Growth of *E. coli* DH5α cells containing the plasmid pVAX1/lacZ, in 500 mL LB medium, over incubation time (in minutes) performed in 21st April 2017. R1 to R4 – replicates containing 500 mL of cell broth each. b) Gel electrophoresis obtained after alkaline lysis and primary isolation to evaluate nucleic acid content; M- DNA Ladder III (NZYtech); L1 and L2 - lysate resultant from cell lysis and plasmid primary isolation of cell broth in a) performed in different days.

As observed in Figure A5b, lysate 1 (L1) presents a low content in RNA. Since it was required to have cell lysates with similar pattern to proceed to chromatographic studies, this sample was discarded for further studies.

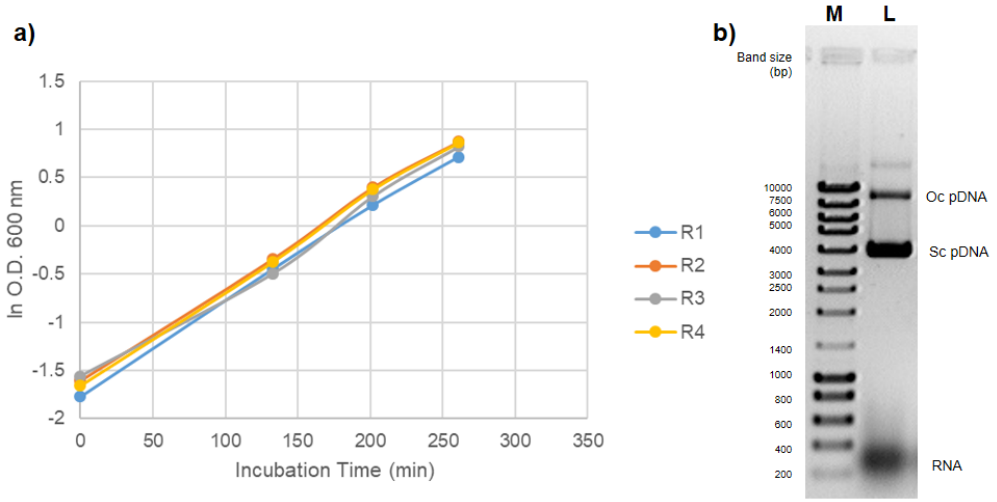


Figure A7: a) Growth of *E. coli* DH5α cells containing the plasmid pVAX1/lacZ, in 500 mL LB medium, over incubation time (in minutes) performed in 11st July 2017. R1 to R4 – replicates containing 500 mL of cell broth each. b) Gel electrophoresis obtained after alkaline lysis and primary isolation to evaluate nucleic acid content; M- DNA Ladder III (NZYtech); L- lysate resultant from cell lysis and plasmid primary isolation of cell broth in a).

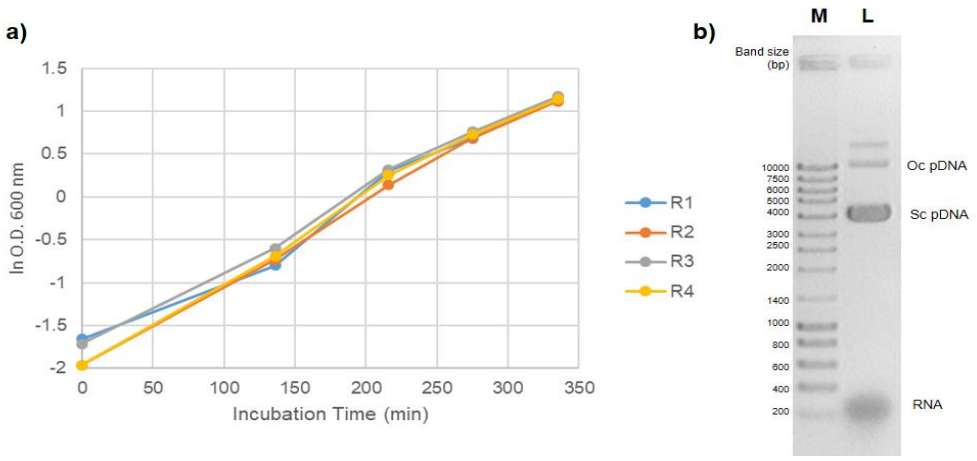


Figure A8: a) Growth of *E. coli* DH5α cells containing the plasmid pVAX1/lacZ, in 500 mL LB medium, over incubation time (in minutes) performed in 1st September 2017. R1 to R4 – replicates containing 500 mL of cell broth each. b) Gel electrophoresis obtained after alkaline lysis and primary isolation to evaluate nucleic acid content; M- DNA Ladder III (NZYtech); L- lysate resultant from cell lysis and plasmid primary isolation of cell broth in a).

Appendix IV – Bench chromatographic assays using ligand 0/0 previously synthesized in Sepharose CL-6B

A newly synthesized resin (Sepharose CL-6B) containing ligand 0/0 was evaluated in terms of selective elution of nucleic acids (see 4.2.1.4.) and compared with the results obtained by chromatographic assays in the same conditions with another resin derivatized with ligand 0/0 already available on the lab from a previous preliminary work [101]. The results obtained are presented in figures A9 to A12.

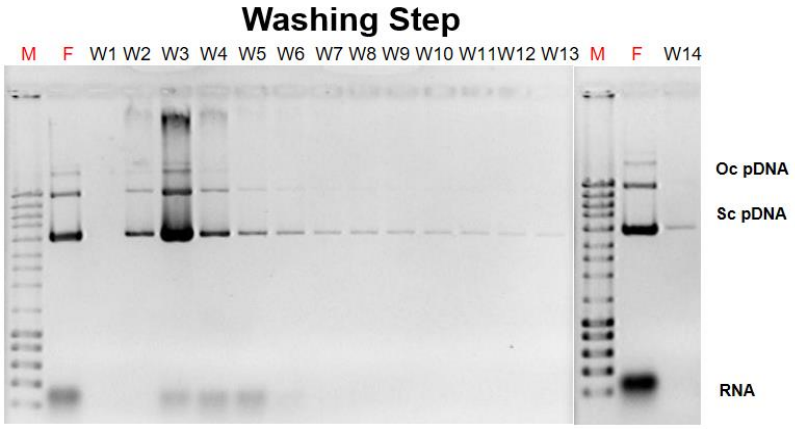


Figure A9: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 0/0 packed in a 4 mL column and evaluated in hydrophilic conditions. W1 to W14- Fractions collected, in increasing order, during washing, using Tris-HCl 20 mM, pH 8.0; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc-open-circular. Complete band size description of DNA ladder is presented in appendix II.

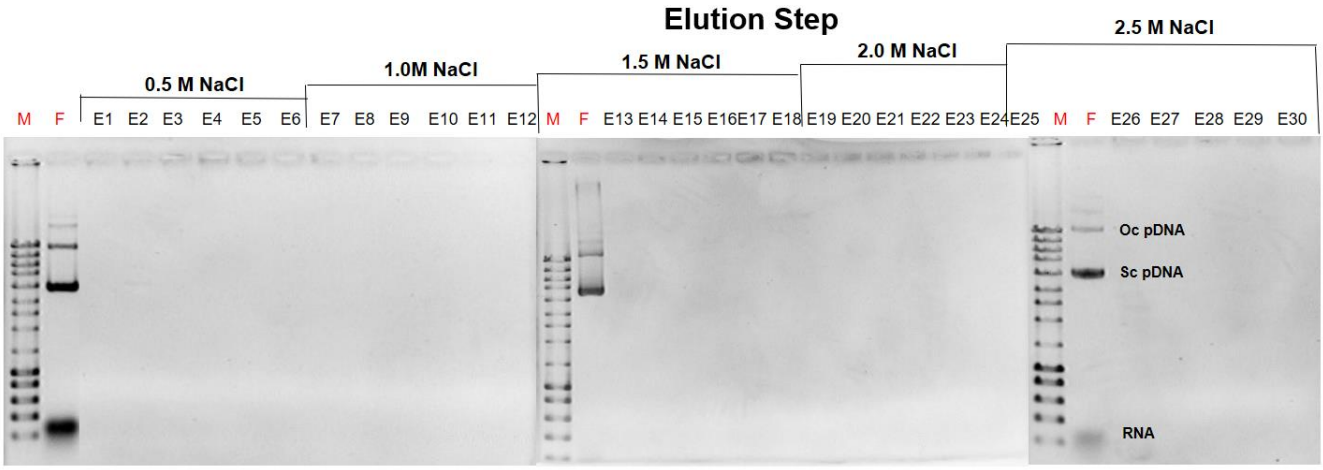


Figure A10: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 0/0 packed in a 4 mL column in hydrophilic conditions (stepwise elution from 0.5 to 2.5M NaCl). a) Fractions collected during elution; E1 to E30- Fractions collected, in increasing order, during elution, where the concentration of elution buffer was increased from 0.5 M to 2.5 M NaCl; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophilic conditions, co-elution of a significant part of pDNA and RNA is observed in the beginning of the washing, followed by elution of traces of oc and sc isoforms during the washing. Apparently, all nucleic acid molecules are eluted in the washing since no bands are detected in the fractions collected during this step. Co-elution of a significant part of pDNA isoforms and RNA in the washing indicates that these molecules are not retained by the ligand, suggesting that this ligand is not selective for any of these nucleic acid molecules and a selective separation does not occur. This behavior is similar to the one observed in the chromatographic assays using ligand 0/0 newly synthesized and derivatized under hydrophilic conditions as well as to binding results obtained for ligand 0/0 in a previous work [101].

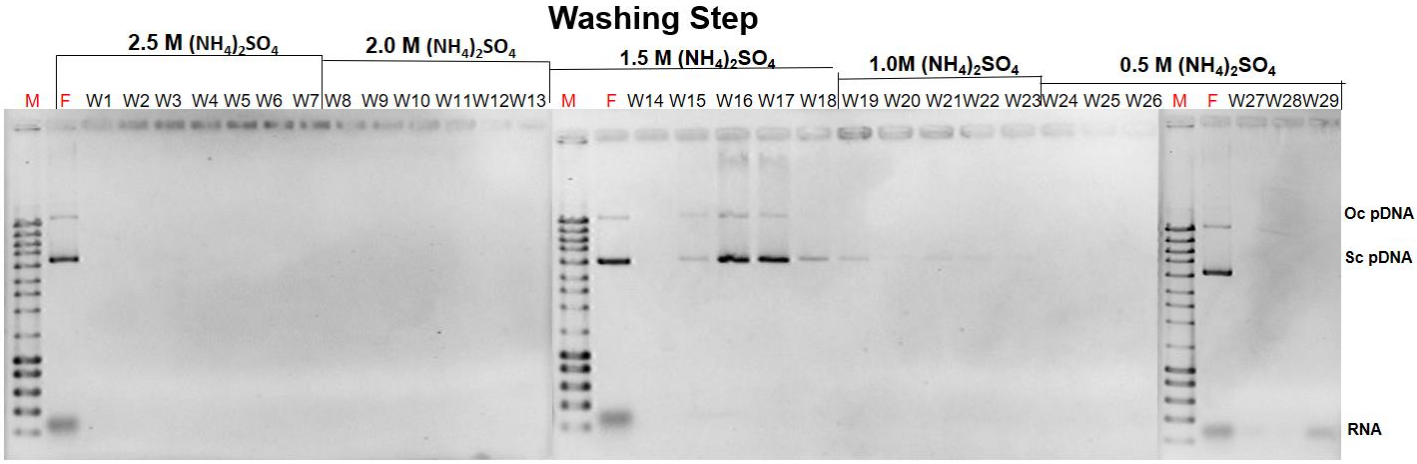


Figure A11: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 0/0 packed in a column in 4 mL hydrophobic conditions (stepwise washing/elution from 0.5 M to 2.5 M $(NH_4)_2SO_4$). a) Fractions collected during washing step; W1 to W29- Fractions collected, in increasing order, during washing step, where the concentration of elution buffer was decreased from 2.5 M to 0.5 M $(NH_4)_2SO_4$;.M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc- open circular. Complete band size description of DNA ladder is presented in appendix II.

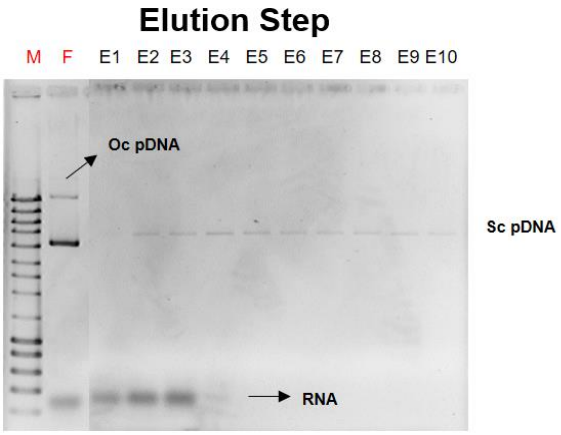


Figure A12: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 0/0, packed in a 4 mL column in hydrophobic conditions. E1 to E10- Fractions collected, in increasing order, during the elution step, where the 20 mM Tris-HCl pH 8.0 was used as elution buffer; M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophobic conditions, nucleic acid molecules are retained inside the column till a concentration of ammonium sulphate of 1.5 M, in which sc and oc pDNA are co-eluted, followed by several fractions containing traces of supercoiled pDNA. At 0.5M $(NH_4)_2SO_4$, RNA starts to be eluted, containing a contamination of supercoiled isoform, being also detected during the elution step in the first four fractions collected. Traces of supercoiled pDNA are detected along elution. The concentration of the final fractions containing apparently isolated supercoiled isoform could have been done to confirm that there is no contamination with other nucleic acid molecules.

The retention of plasmid, mainly sc isoform, and RNA as well as different retention times between species indicates that this ligand may be selective for the supercoiled pDNA and RNA under hydrophobic binding environment, allowing selective separation. In a previous preliminary work, ligand 0/0 did not bind to oligonucleotides in hydrophilic conditions (20 mM Tris-HCl, pH 8.0) but binding under hydrophobic environment was not tested [101].

Appendix V – Bench Chromatographic assays using ligand 6/5 synthesized in Sepharose CL-6B

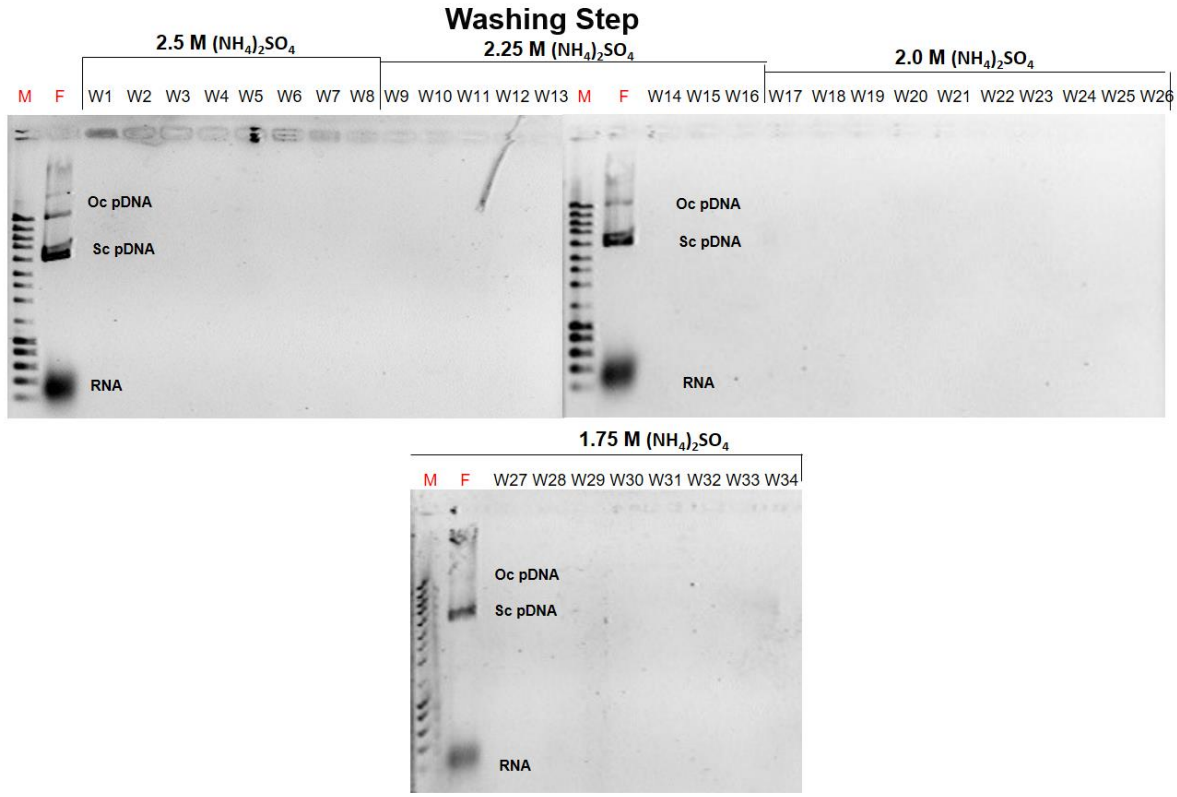


Figure A13: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 6/5, packed in a 4 mL column in hydrophobic conditions (stepwise washing/elution from 2.5 M to 1.75 M $(NH_4)_2SO_4$). W1 to W34- Fractions collected, in increasing order, during washing step, where the concentration of washing buffer was decreased from 2.5 M to 1.75 M $(NH_4)_2SO_4$;.M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; Oc-open circular. Complete band size description of DNA ladder is presented in appendix II.

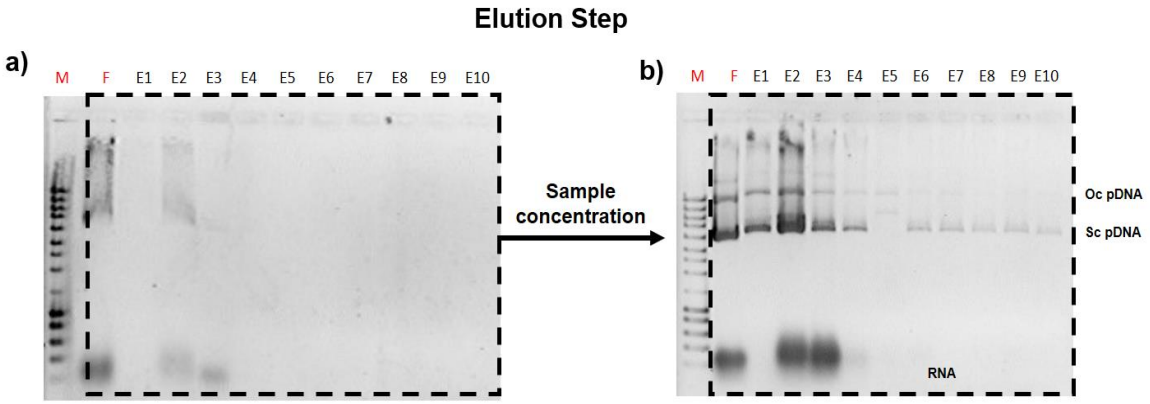


Figure A14: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 6/5, packed in a 4 mL column in hydrophobic conditions. a) Fractions collected during elution. Fractions included in dashed area were the one selected for further concentration and analysis by gel electrophoresis. E1 to E10- Fractions collected, in increasing order, during the elution step, where the 20 mM Tris-HCl pH 8.0 was used as elution buffer. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

As it can be observed in figures A12 and A13, ligand 6/5 retains nucleic acid molecules between 2.5 M to 1.75 M $(NH_4)_2SO_4$ (Figure A13). In the beginning of elution, when the concentration of ammonium sulphate is decreased to zero, co-elution of pDNA isoforms (oc and sc) and RNA is detected, followed by the co-elution of open-circular and super-coiled pDNA at lower amount (Figure A14).

Nucleic acid molecules retention between 2.5 M and 1.75 M $(NH_4)_2SO_4$ indicates that these species bound to the matrix under this range of salt concentrations. Ligand 6/5 contains substituents that mimic Leucine and Phenylalanine (Table 4), amino acids that are involved in hydrophobic interactions, thus this ligand is expected to have a predominant hydrophobic character [77]. As such, this ligand may interact with nucleic acid by hydrophobic interactions, favored by the hydrophobic binding environment promoted by the concentration of ammonium sulphate applied. When the concentration of ammonium sulphate is decreased to zero, hydrophobic interactions between ligand and nucleic acid molecule are no longer promoted and the species are eluted.

Appendix VI- Bench Chromatographic assays with the predominantly hydrophobic ligands

The ligands with a predominant hydrophobic character selected for screening were initially evaluated performing chromatographic studies applying a stepwise elution with decreasing concentrations of $(NH_4)_2SO_4$ (from 2.5 to 2.0, 1.5, 1.0 and 0.5 M), as described in topic 3.8.1. In parallel, additional chromatographic studies were performed applying a single washing step with specific $(NH_4)_2SO_4$ concentrations, like 1.0 or 0.5 M, as described in 3.8.1., to evaluate possible plasmid isoform separation. Only the assays with the hydrophobic ligands yielding relevant results were considered in the main part of this work. The remaining affinity chromatographic assays are presented in this appendix in figures A15 to A17.

A- Ligand 11/8

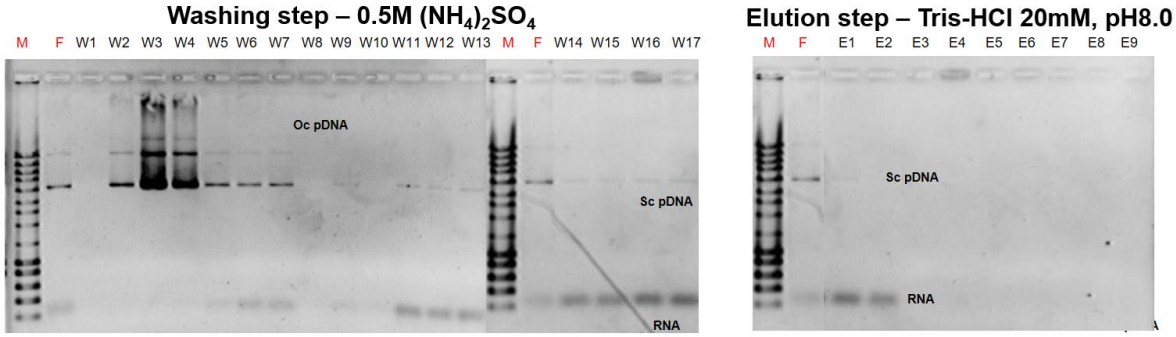


Figure A15: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL Sepharose CL-6B derivatized with ligand 11/8 packed in a 4 mL column in hydrophobic conditions (single washing step with 0.5 M $(NH_4)_2SO_4$). W1 to W17- Fractions collected, in increasing order, during washing, using 0.5 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl, pH 8.0 as washing buffer ; E1 to E9- Fractions collected, in increasing order, during the elution step, using 20 mMTris-HCl, pH 8.0 as elution buffer. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophobic conditions, at 0.5 M $(NH_4)_2SO_4$, a significant part of pDNA isoforms are co-eluted since the beginning of the assay, apparently isolated from RNA, while RNA is retained till the 4th fraction, with sc isoform contamination along the washing and in the first 2 fractions of elution. RNA delayed elution indicates that this ligand retains this molecule inside the column (Figure A15)

Nevertheless, the concentration used is not enough to maintain the interactions between ligand and RNA, which is eluted in the washing. Under these conditions, only part of pDNA is separated from RNA, and no selective separation of plasmid isoforms is achieved.

B- Ligand 8/11

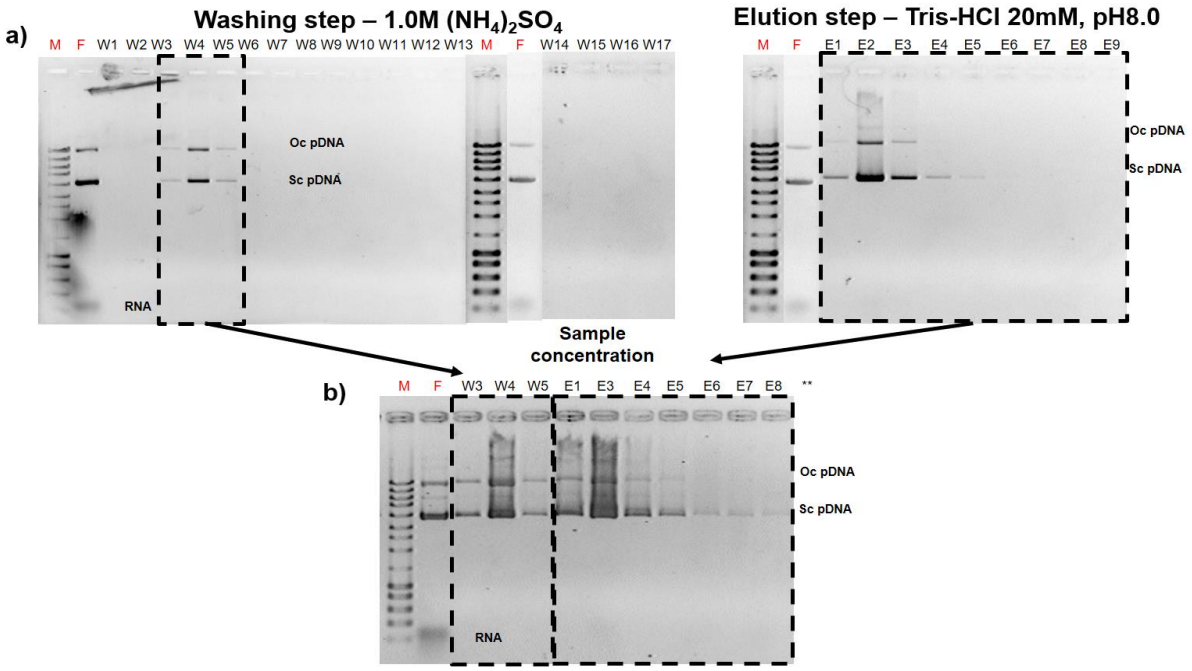


Figure A16: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 8/11 packed in a 4 mL column in hydrophobic conditions (single washing step with 1.0 M $(NH_4)_2SO_4$). a) fractions collected during washing, using 1.0 M $(NH_4)_2SO_4$) in 20 mM Tris-HCl, pH 8.0 as washing buffers and elution step, applying Tris-HCl 20mM, pH 8.0 as elution buffer. Fractions included in dashed area were the one selected for further concentration and analysis by gel electrophoresis. W1 to W17- Fractions collected, in increasing order, during washing; E1 to E9- Fractions collected, in increasing order, during the elution step. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. ** fractions E2 and E9 were lost during processing. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophobic conditions, at 1.0 M $(NH_4)_2SO_4$, a significant part of pDNA isoforms are co-eluted in the beginning of the assay, apparently isolated from RNA, in fractions w3,w4 and w5. The elution of the remaining plasmid isoforms is apparently eluted in elution after ammonium sulphate removal (Figure A16a). Apparently, even after sample concentration, no RNA is detected in the fractions collected during the assay (Figure A16b).

which may indicate that this ligand retains RNA even without promotion of hydrophobic binding environment (in elution). Under these conditions, ligand 8/11 is able to obtain plasmid DNA isolated from RNA but is not selective for pDNA, and selective plasmid isoforms separation is not achieved.

C- Ligand 3/5

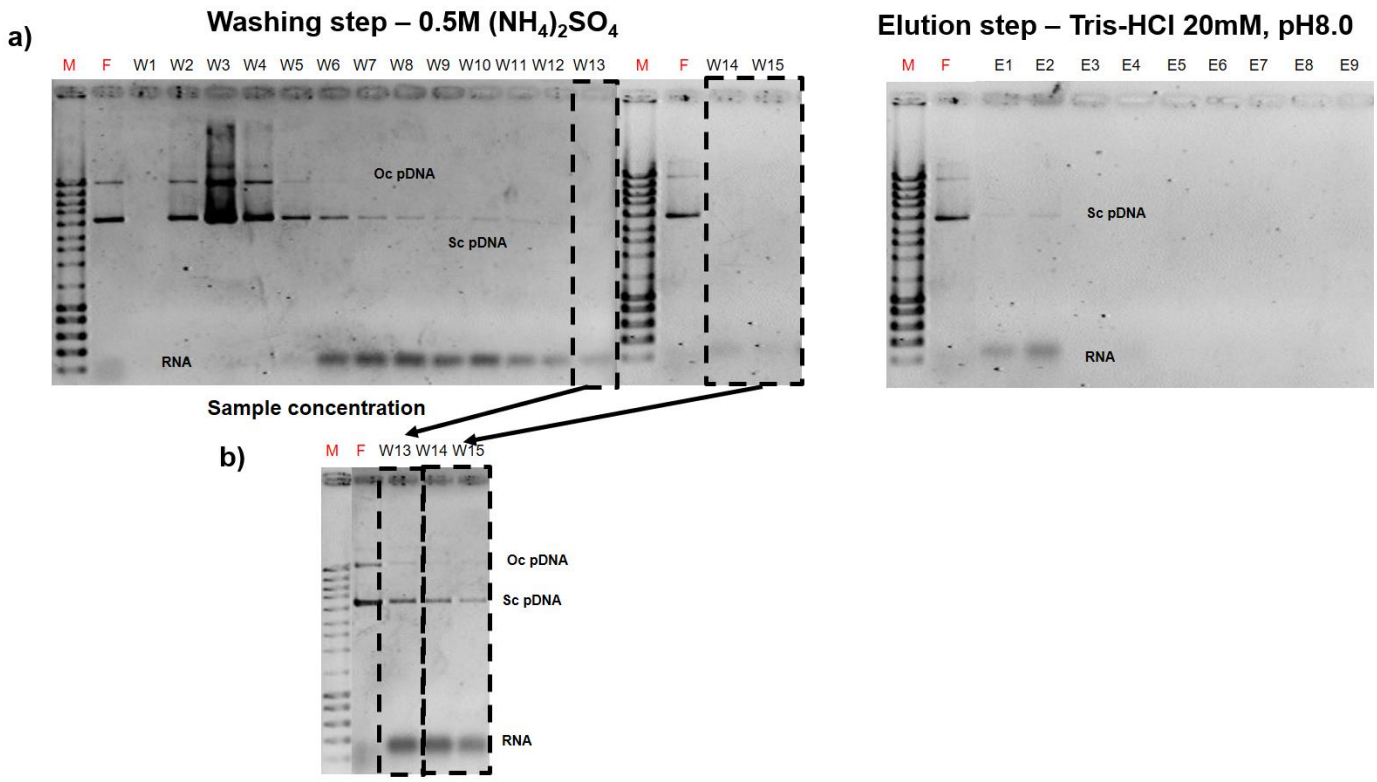


Figure A17: Gel electrophoresis obtained from the bench chromatographic assay using 1 mL of Sepharose CL-6B derivatized with ligand 3/5 packed in a 4 mL column in hydrophobic conditions (single washing step with 0.5 M $(NH_4)_2SO_4$). a) fractions collected during washing, using 0.5 M $(NH_4)_2SO_4$) in 20 mM Tris-HCl, pH 8.0 as washing buffers and elution step, applying Tris-HCl 20 mM, pH 8.0 as elution buffer. Fractions included in dashed area were the one selected for further concentration and analysis by gel electrophoresis. W1 to W15- Fractions collected, in increasing order, during washing; E1 to E9- Fractions collected, in increasing order, during the elution step. b) Fractions collected from chromatography in a) that were concentrated and re-analyzed by gel electrophoresis. M- DNA Ladder III (NZYtech); F- Feed (clarified lysate added to the column); sc- Supercoiled; oc- open-circular. Complete band size description of DNA ladder is presented in appendix II.

Under hydrophobic conditions, at 0.5 M $(NH_4)_2SO_4$, a significant part of pDNA isoforms are co-eluted in the beginning of the assay, apparently isolated from RNA, followed by RNA elution with supercoiled pDNA contamination till the end of washing and beginning of elution (Figure A17). Under these conditions, ligand 3/5 is able to obtain plasmid DNA isolated from RNA in some fractions but is not selective for pDNA, and selective isoform separation is not achieved.

Appendix VII – Pure plasmid DNA standard curve for analysis by (HIC)- HPLC system

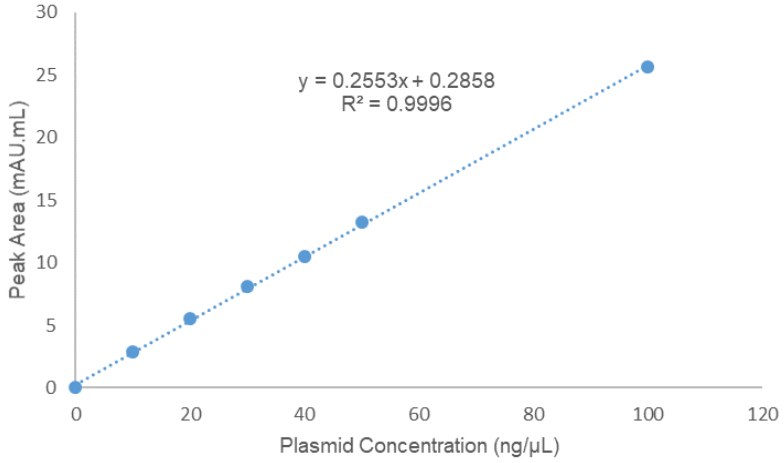


Figure A 18: Calibration curve obtained by HIC in a HPLC system, using standard plasmid (pVAX1-GFP) concentrations.