Plasmid DNA purification with synthetic protein-mimic affinity ligands

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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.

Introduction

Efficient therapeutics based on gene delivery and manipulation have been developed to treat a wide range of diseases including infections, genetic disorders or cancer [1]. Part of these therapeutics use non-viral systems like recombinant plasmid DNA for DNA based-vaccines [2]. These vaccines are based on the introduction of recombinant plasmids in host cells, where they replicate separately from host genome and express protein immunogens that promote an immune response, representing a safer alternative to viral vectors [3,4].

Plasmid DNA or pDNA can be presented in different topological isoforms: open circular (oc), linear, corresponding to more relaxed structures, and supercoiled pDNA (sc), a highly coiled and compact structure [5,6]. Sc isoform is the one preferred for gene therapy since it has the highest transfection efficiency and biological activity, being more resistant to enzymatic degradation and showing more effective immune response compared to open circular isoform when applied in DNA-vaccines [7,8]. Regarding these features, in order to be used in pharmaceutical purposes, it is mandatory that the final plasmid product has a high content in supercoiled isoform. Nevertheless,

purification of supercoiled DNA faces some challenges and limitations like the lower content of pDNA in cells, the mechanical lability of this isoform or the presence of similar properties with other macromolecules that are present in cell lysate as impurities -oc pDNA, gDNA, RNA or endotoxins- which includes negative charge, high molecular mass, bases exposition or hydrophobic profile. As such, efficient separation methods must be applied to overcome those limitations and maximize the amount of supercoiled pDNA recovered [9-11].

Liquid chromatography has been widely applied in plasmid purification and is considered the method with highest resolution, explaining its use for pDNA therapeutic-grade applications [12] There are several types of liquid chromatography whose plasmid separation is based on different molecular size, charge exposure or hydrophobic exposure. However, those techniques are associated to low-resolution, risk of gDNA and endotoxins contamination or co-elution of plasmid isoforms [13,14]. Affinity chromatography is an alternative chromatographic technique that may overcome those limitations. This technique allows the elimination of additional steps in downstream processing and is based on molecular recognition,

in which a target molecule have specific properties that can be recognized by ligands immobilized in a solid support, being one of the approaches with higher molecular specificity [15,16]. Ligands used in affinity chromatography can be classified as natural or biospecific ligands and pseudobiospecific ligands. Amino acids have been explored as natural ligands in affinity chromatography for plasmid purification, including histidine, lysine, arginine or L-tryptophan[17-21]. Amino acid affinity chromatography is based on the natural interactions between amino acids residues from proteins and nucleic acids [22]. Using this approach, it is possible to isolate considerable amounts supercoiled pDNA from other isoforms and impurities [18-21]. Pseudobiospecific synthetic molecules that are able to mimic interactions between amino acid residues and nucleic acids can represent an advantage to affinity chromatography. Those molecules, more specifically biomimetic ligands, are an improved version of natural ligands since they are chemically resistant to degradation, have high ligand specificity and scale-up potential and can be sterilized [17,23]. Triazine-based ligands are synthetic molecules that uses a triazine derivative, cyanuric chloride, as a scaffold molecule that allows the introduction of aromatic and aliphatic amine substituents that mimic the side chains of different amino acids, being able to simulate the natural interactions between amino acids residues from proteins and nucleic acids, in a similar way of amino acid affinity chromatography [24]. A large combinatorial library of triazine-based ligands was developed by Roque et al. and have been extensively explored for the purification of antibodies, Fab fragments or cutinase [23,24]. However, only few preliminary studies using these ligands are available in the field of plasmid purification, representing an innovative technique [25,26]. Those ligands are generally immobilized in Sepharose CL-6B resins, accomplishing the exigencies of solid-phase synthesis but its non-uniform pore distribution may affect the flow-rate of liquid phase [27]. CIM® monolithic resins are based on convective transport, allowing the mobile phase to flow through the pores without flow rate restrictions, overcoming Sepharose CL-6B limitations, being a promising solid support in affinity chromatography [28].

The main goal of this study was to evaluate the potential of several synthetic ligands from a 22-ligand library in terms of plasmid purification performing bench chromatographic assays. The best ligand candidates selected from these assays were evaluated in a larger scale chromatography (ÄKTA purifying system) to confirm the previous results.

Materials and Methods

Materials
All reagents were of the highest purity available unless otherwise stated.

Methods
Cell Fermentation
About 20 µL of a cell bank (*E. coli* DH5α with 6.1 kb pVAX1/lacZ in 17.5 %(v/v) glycerol stored at -80ºC), was pre-inoculated in 100 mL shake flasks containing 30 mL of autoclaved (121 ºC, 20 min) LB medium and 30 µL of kanamycin (30 µg/mL) being incubated overnight (37°C, 250 rpm). After this period, the volume of pre-inoculum to start the growth at O.D.$_{600nm}$ of 0.1 (at 600 nm) was determined (Equation 1), where O.D.$_P$ and O.D.$_I$ represent the O.D. (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.

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

The determined volume was centrifuged (6000 xg, 3 min) (Eppendorf 5810R centrifuge) and inoculated in 2 L shake-flasks with 500 mL of autoclaved LB medium and 500 µL of kanamycin (30 µg/ml). The inoculated cells were incubated at 37 °C and 250 rpm, until an O.D.$_{600nm}$ value around 3. The resultant broth was centrifuged at 6000xg at 15 min and 4°C (*Sorvall RC6* Ultracentrifuge), being the supernatant discarded and the pellet stored at -20°C for further work.

Cell lysis and Plasmid Primary Isolation
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. To start alkaline lysis, this suspension was divided in 50 mL centrifuge tubes, containing buffer P2 (0.2 M NaOH, 1 % SDS (m/v)) in a final ratio of 1:1 (v/v), being gently homogenized and placed to rest for 10 min at room temperature. Buffer P3 (5 M potassium acetate, acetic acid) was added to each centrifuge tube, followed by gentle and uniform homogenization and rest on ice for 10 min. The volume of P1, P2 and P3 was determined using equation 2, where O.D.$_{growth}$ and O.D.$_F$ represent the final O.D.$_{600nm}$ of the pellets resultant from cell growth and desired final optical density (O.D.$_F$=60), respectively. 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.

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

The resultant lysate was centrifuged at 13000 rpm, 30 min at 4°C (*Sorvall RC6* Ultracentrifuge), for the removal of cell debris, proteins and gDNA. The supernatants were placed in new tubes and centrifuged again in equal conditions, discarding

the pellets at the end. For primary plasmid DNA purification, a volume of isopropanol equal to 70 % of the total supernatant volume, was added to the supernatant and mixed. The mixture was divided into 50mL tubes and placed at -18°C for 2 h, for nucleic acid precipitation. After this period, the mixture was centrifuged at 13000 rpm for 30 min, 4°C (*SLC-3000* Sorvall RC6 Ultracentrifuge), being the supernatant discarded and the tubes inverted on adsorbent paper and placed overnight at 4°C, for isopropanol removal. Tris-HCl (20 mM; pH 8) buffer was then added to the tubes until total resuspension of the pellets and collected in 1.5mL Eppendorf tubes. This suspension was conditioned in $(NH_4)_2SO_4$, to obtain a concentration of this salt of 2.5 M, and left on ice for 15 min. Mixture was centrifuged (11000 rpm, 4°C, 35 min) in Eppendorf 5810R centrifuge, and the supernatant placed in *Eppendorf* tubes for dialysis.

Micro-dialysis of clarified *E. coli* Lysate ou fractions collected from chromatography
The samples for desalting were placed in *Eppendorf* Tubes with their caps removed. The top of each tube was covered with dialysis membranes, (MWCO=12-14 kDa; 23 µm, Orange Scientific), previously washed with distilled water, and tightened with a rubber band. The tubes were inverted and shaked firmly to ensure that all the solution was in contact with membrane, being placed overnight in a flask with 1 L of Tris-HCl (20 mM, pH 8) with gentle agitation. After incubation, the rubber bands and membranes were removed from the tubes and the samples were directly used or stored at -20ºC till further processing.

Concentration of clarified *E. coli* Lysate or fractions collected from chromatography
The samples requiring concentration were evaporated using a Vacuum Concentrator (Thermo Scientific™), until total evaporation, being analyzed by gel electrophoresis, by resuspension with 30 µL Tris-HCl (20 mM, pH 8), or stored at 4ºC.

Solid-Phase Synthesis of triazine-based ligands
For epoxy activation of Sepharose CL- 6B (Sigma), this resin was washed with distilled water and resuspended with 1 M NaOH (0.8 mL/ g gel). Epichlorohydrin was added (0.1 mL/g gel), followed by overnight incubation at 30°C at 100 rpm in a rotary shaker (*Heidolph, Titramax 1000*). For amination of epoxy-activated gel, it was washed with water and resuspended with ammonia (33 % v/v) (1.5 mL/g gel) and incubated overnight (30ºC,200 rpm). The suspension was washed with water, till a pH close to pH of distilled water. Part of aminated supports was used

immediately for cyanuric chloric activation or stored in 20 % ethanol at 4ºC. Aminated agarose (20 µmol amine groups/g gel) was suspended in acetone/water 50 %(v/v) (1 mL/g moist gel) and maintained in an ice bath at 0ºC with gentle agitation. 5 molar equivalent of cyanuric chloride (relative to the extent of amination) was dissolved in acetone (8.6 mL/g cyanuric chloride) and divided into 4 aliquots, each of them added to the aminated slurry in 30 minutes intervals, that was kept under agitation and at 0ºC. The pH was monitored and maintained around 7 with addition of NaOH 1 M. 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 water. From this gel, part was packed in bench chromatographic columns and the remaining gel was immediately used. For R1 substitution, this gel was divided into 2 aliquots, each of them for R1 substitution in triazine with L-alanine (1) (for ligand 1/2) and ammonia (0) (for ligand 0/0). 2 molar equivalent of each amine (relative to the extent of amination) was dissolved in water. For amine 1, 1 equivalent of $NaHCO_3$ was also added to the previous solution, resulting in a total volume of solvent of 1 mL /g of gel. Each aliquot of 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 washed with water. R2 nucleophilic substitution was performed using amine 2 (ligand 1/2) and amine 0 (ligand 0/0). 5-molar equivalent (related to the extent of amination) was dissolved in distilled water (3 mL /g gel) and the substitution was carried out at 83ºC for 72h in a hybridization oven/shaker (Amersham Pharmacia Biotech). After incubation, both aliquots were washed in water and stored in ethanol 20 % (v/v) at 4ºC.

Chromatographic assays using selected ligands in Sepharose CL-6B
For bench-scale chromatographic assays, 1 mL of Sepharose-CL 6B containing selected ligands were packed in a micro column. To regenerate the column, 8 mL of regeneration buffer (NaOH (0.1M) in 30% (v/v) isopropanol) were passed through the column, followed by 8 mL of distilled water. Under hydrophobic conditions, column equilibration was performed with 10 mL of washing buffer (2.5 M $(NH_4)_2SO_4$ in Tris-HCl (20 mM, pH 8) for multiple step washing assays; and 1.0 M or 0.5 M $(NH_4)_2SO_4$ in Tris-HCl (20 mM, pH 8) for single step-assays). After column equilibration, 250 µL of clarified *E. coli* lysate was conditioned with $(NH_4)_2SO_4$ to achieve the concentration of 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 with several concentrations of

(NH$_4$)$_2$SO$_4$ in Tris-HCl (20mM, pH8) in the following order: 2.5, 2.0, 1.5, 1.0 and 0.5 M, whereas 200 µL fractions were collected. For elution, 5 mL of Tris-HCl (20 mM, pH 8) were applied to the column while fractions of 500 µL were collected. In single-step assays, washing was performed by applying 3 mL of 0.5 M or 1.0 M (NH$_4$)$_2$SO$_4$ in Tris-HCl (20 mM, pH8), with collection of 200 µL fractions, followed by 5 mL of Tris-HCl (20 mM, pH8) being collected 500 µL-fractions during this process. For hydrophilic chromatographic assays, column equilibration was performed with 10 mL of Tris-HCL (20 mM,pH 8). After column equilibration, 250 µL of clarified *E. coli* lysate, was loaded to the column. After lysate loading, washing was performed by applying 3 mL of Tris-HCl (20 mM, pH 8), while fractions of 200 µL were collected, followed by elution step, with 1.5 mL of each buffer with increasing concentrations of NaCl applied in the following order: 0.5, 1.0, 1.5, 2.0 and 2.5M. During elution, fractions of 500 µL were collected. For column cleaning and storage, 5 mL of distilled water was applied followed by 2 mL of ethanol 20 % (v/v), being stored at 4°C. Ligand 2/1 and 8/1 were selected for chromatographic assays in ÄKTA 10 purifying system, packing 2 mL Sepharose CL-6B (= 1 CV) with those ligands in a Tricorn™10/50 column. Under hydrophobic conditions, the column was regenerated with 8 CV of regeneration buffer, washed with distilled water and equilibrated with 1.5 M or 1.05 M (NH$_4$)$_2$SO$_4$ in Tris-HCl, (20 mM, pH8) (for 2/1 and 8/1 respectively). 1 mL of clarified *E. coli* lysate (diluted 1:2; 1:3 or 1:6 and conditioned with (NH$_4$)$_2$SO$_4$ concentration of washing buffer) was loaded, the column was equilibrated with 10 CV equilibration buffer. Washing was performed with decreasing 20 CV gradient starting with 1.5 M (for 2/1) or 1.05 M (for 8/1) (NH$_4$)$_2$SO$_4$ in Tris-HCl (20 mM, pH8) followed by 5 CV of Tris-HCl (20 mM, pH8) for elution. Column and ÄKTA system were washed with water and 3 CV of ethanol 20% (v/v). Under hydrophilic conditions, the column was regenerated with 8 CV of regeneration buffer, washed with distilled water and equilibrated with Tris-HCl (20 mM, pH8). The lysate was injected (1 mL, diluted 1:2), the column was equilibrated with 10 CV equilibration buffer, washing was performed with 5CV Tris-HCl (20 mM, pH8) and elution with of 20 CV gradient with final concentration of 1.5 M (for 8/1) or 1.05 M NaCl (for 2/1). Column and channels were washed with water and 3 CV of ethanol 20 % (v/v). Fractions of 250 µL were collected and examined by gel electrophoresis and the chromatograms were analyzed using UNICORN™ software (GE Healthcare).

Chromatographic assays using 6/5 in CIM monolithic disks

EDA CIM® monolithic disks with 0.34 mL (= 1 CV) (Bia Separations), were connected to ÄKTA 10 purifying system for the chromatographic assays. One of the monolithic disks, was derivatized with ligand 6/5 and the other was not subjected to derivatization. Under hydrophobic conditions, the disk was equilibrated with 15 CV of 2.5 M (NH$_4$)$_2$SO$_4$ in Tris-HCl (20 mM, pH8). 100 µL of clarified *E. coli* lysate (conditioned with (NH$_4$)$_2$SO$_4$ concentration of washing buffer) was loaded, and the column was washed with decreasing 100 CV gradient between 2.5 and 0 M (NH$_4$)$_2$SO$_4$ in Tris-HCl (20 mM, pH8) and elution was performed with 20 CV of Tris-HCl (20 mM, pH8). ÄKTA system and disk were washed with water and regenerated with 20 CV of 2 M NaCl, followed by washing with water and 30 CV of ethanol 20 % (v/v). Under hydrophilic conditions, the channels and column were equilibrated with 15 CV Tris-HCl (20 mM, pH 8). 100 µL lysate was loaded, followed by washing of unbound molecules with 30 CV Tris-HCl (20 mM, pH8). Elution was carried out with 100 CV gradient between 0 to 1 M NaCl in 20 Tris-HCl (20 mM, pH8). Channels and monolith were washed with distilled water and regenerated with 20 CV of 2 M NaCl, washed with distilled water and 30 CV of ethanol 20 % (v/v). Fractions of 250 µL were collected and examined by gel electrophoresis and the chromatograms were analyzed using UNICORN™ software.

Agarose Gel Electrophoresis
Samples were analyzed by electrophoresis in 1% (v/v) of agarose horizontal gel in 1x TAE buffer (40 mM Tris base, 1 mM EDTA, 20 mM acetic acid), running at a voltage of 120 V, for 90 min. 4 µL of NZYDNA DNA ladder III (NZYTECH) was used as DNA weight marker and 40% (w/v) sucrose, 0.25% (w/v) bromophenol blue was used as loading buffer(LB). Sample loading was performed with the following volumes: 5µL lysate (+ 1 µL LB), 30 µL fractions collected from chromatography (+ 5 µL LB) and 20 µL concentrated samples (+ 5 µL LB). The samples were stained in 0.4 mg/mL ethidium bromide and analyzed in Stratagene EagleEye II Video Imaging System.

Densitometry analysis
Plasmid and supercoiled pDNA yield were determined by densitometry analysis of the band intensities, using the ImageJ software and using 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 of bands of plasmid isoforms in feed. FVC stands for the factor of volume correction regarding the lower amount of feed loaded compared to 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, only applied for samples that were previously concentrated.

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

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

(HIC)-HPLC analysis for pDNA quantification

To quantify the amount of plasmid DNA, a 15 PHE-PE column (GE Healthcare) was connected to ÄKTA purifier system (ÄKTA 10). The column was firstly equilibrated at 1 mL/min with 1.5 M $(NH_4)_2SO_4$ 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 1 ml/min in a step mode with Tris-HCl (10 mM, pH8). The absorbance during the process was recorded at 260 nm. A calibration curve was constructed with standard plasmid concentrations, prepared in a concentration range between 0 and 100 µg, to quantify plasmid DNA. Equation 5 and 6 were used to obtain step yield and sc pDNA yield, respectively.

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

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

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

$m_{pDNA-peak2}$ and $m_{pDNA-feed}$, correspond to the mass 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.

Results and discussion

Chromatographic assays with ligand 6/5 immobilized in EDA CIM® monolithic disk using ÄKTA 10

In a previous preliminary work it was reported that sc pDNA was isolated from other isoforms in some fractions using EDA CIM disk with ligand 6/5 under hydrophobic conditions [26]. Since the results were not fully conclusive, this disk was evaluated under hydrophobic and hydrophilic conditions to confirm the previous results.
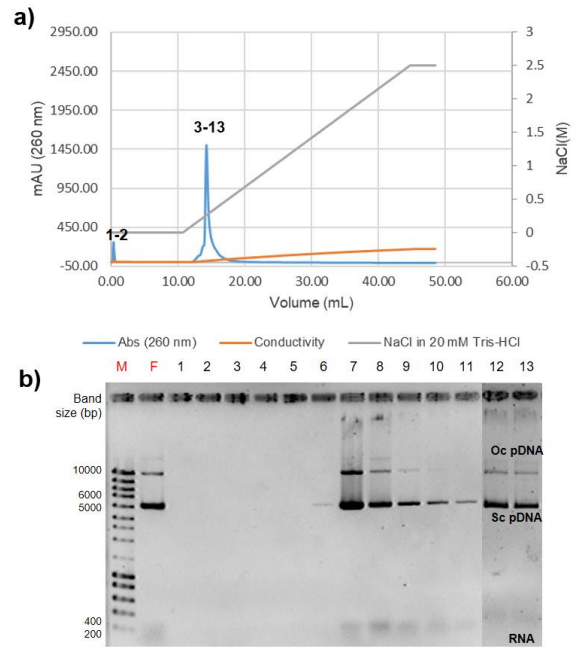

Figure 1: a) Chromatography in EDA CIM® monolithic disk with 6/5 immobilized, under hydrophilic conditions (Tris-HCl (20mM; pH8) in washing; 0-2.5M NaCl gradient in elution). In the chromatogram, the conductivity scale (in mS/cm) is presented in left vertical axis. b) Gel electrophoresis of the fractions collected from a) (1 to 13); fractions 12 and 13 correspond to concentrated fractions; M- DNA ladder III (NZYtech); F-Feed; oc- open circular; sc supercoiled.
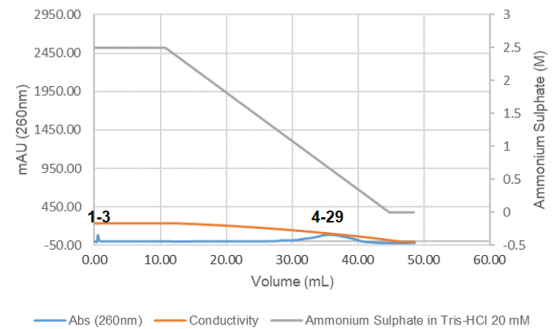

Figure 2: Chromatography in EDA CIM® monolithic disk with 6/5 immobilized, under hydrophobic conditions (2.5 - 0M $(NH_4)_2SO_4$ gradient in washing; Tris-HCl (20mM; pH8) in elution). In the chromatogram, the conductivity scale (in mS/cm) is presented in left vertical axis; 1-29: fractions collected from the peaks.
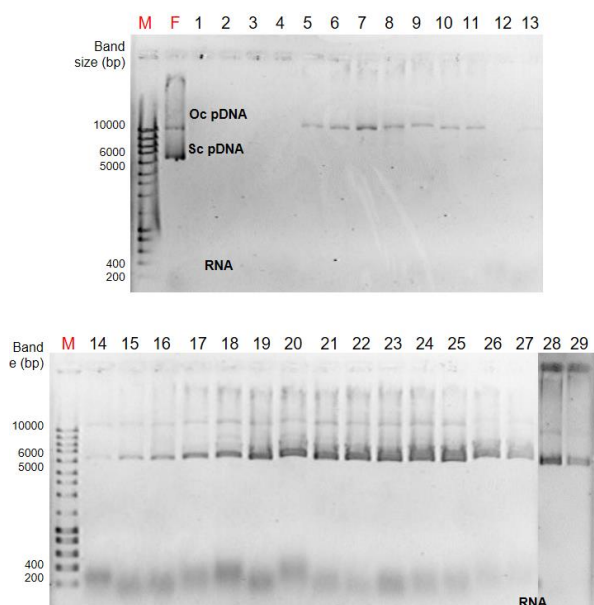
Figure 3: Gel electrophoresis of the fractions collected from chromatography using ligand 6/5 (Fig.2) (1 to 29) corresponding to concentrated fractions; M- DNA ladder III (NZYtech); F-Feed; oc- open circular; sc supercoiled.

As observed in Figure 1 to 3, supercoiled isoform is not isolated using EDA CIM® disk immobilized with ligand 6/5 under both conditions tested, unlike previously reported. At hydrophobic conditions, some fractions contain oc pDNA isolated but in low amount. Co-elution of a significant part of nucleic acids suggests that this ligand, in fact, is not selective for any of nucleic acids and no selective separation is achieved.

Chromatographic assays with ligands selected in Sepharose CL-6B bench columns

Before evaluating the potential of selected ligands in terms of plasmid purification, several resins considered as controls – Sepharose CL-6B, aminated Sepharose, Aminated Sepharose with cyanuric chloride, ligand 0/0 and EDA CIM® monolithic disk – were tested in terms of nucleic acid binding under hydrophobic and hydrophilic binding environment in bench chromatographic columns. In general, those resins are not able to efficiently bind nucleic acids. Nevertheless, Ligand 0/0 and aminated resin with cyanuric chloride can separate pDNA from RNA under hydrophobic conditions, which can be associated to the aromatic ring in cyanuric chloride that perform interactions with nucleic acids, suggesting that the presence of this group can influence *a priori* nucleic acid binding [29]. On the other hand, aminated sepharose can retain nucleic acids under hydrophilic conditions, probably related to free charged amino groups present in the solid support, suggesting that the presence of those groups may influence nucleic acid binding in ligands assays.

Beside ligand 6/5, other ligands (namely 11/8, 8/11, 3/5, 8/1, 4/11, 3/4 and 2/1) were selected to perform bench chromatographic assays under hydrophobic and hydrophilic conditions (for ligands with both hydrophobic and hydrophilic properties). In general, the hydrophobic ligands tested - 11/8, 8/11, 3/5, 8/1 - are able to separate, at least, part of plasmid isoforms from RNA. On the other hand, ligands with mixed profile (hydrophobic and hydrophilic) – 4/11, 3/4 and 2/1 – act differently under hydrophobic and hydrophilic conditions, which highlights the versatility of those ligands. From this group, ligands 4/11 and 3/4 apparently are able to totally separate pDNA from RNA.

Ligands 8/1 and 2/1 were the only ones able to isolate sc pDNA in some fractions (Figure 4 to 6).
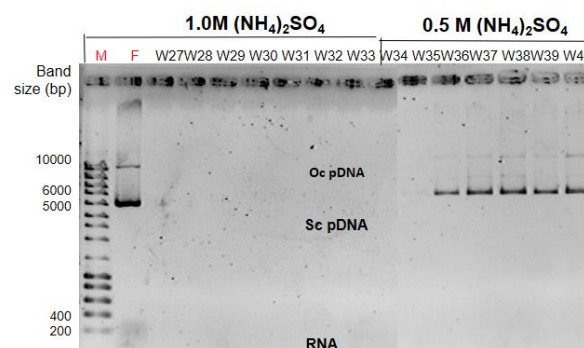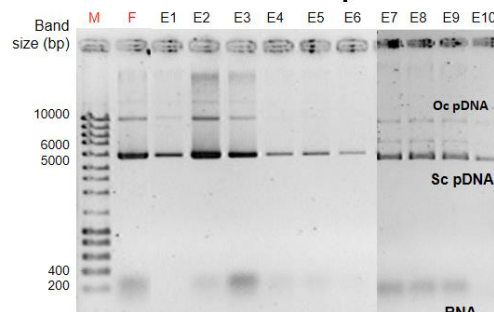

Figure 4: Gel electrophoresis of the fractions collected in bench chromatographic assay using ligand 8/1 immobilized in Sepharose CL-6B, under hydrophobic conditions (2.5 - 0M $(NH_4)_2SO_4$ stepwise washing; Tris-HCl (20mM; pH8) in elution), where nucleic acids start to be detected; W27 to W40 correspond to fractions from washing while E1 and E10 correspond to fractions from elution; w35 to w40 and E7 to E10 correspond to concentrated fractions; M- DNA ladder III (NZYtech); F-Feed; oc- open circular; sc supercoiled.

Testing ligand 8/1 under hydrophobic environment (Figure 4), it is possible to obtain some fractions rich in supercoiled isoform around 0.5 M $(NH_4)_2SO_4$, followed by co-elution of plasmid isoforms and RNA. Nucleic acid retention can be related to amine 8 (or 4-aminobenzamide) properties, which contains an aromatic ring able to perform hydrophobic interactions with

hydrophobic bases of nucleic acids. Sc pDNA is retained for shorter time than RNA since it contains its hydrophobic bases shielded inside the double helix unlike RNA, interacting less with ligand 8/1 [30,31].
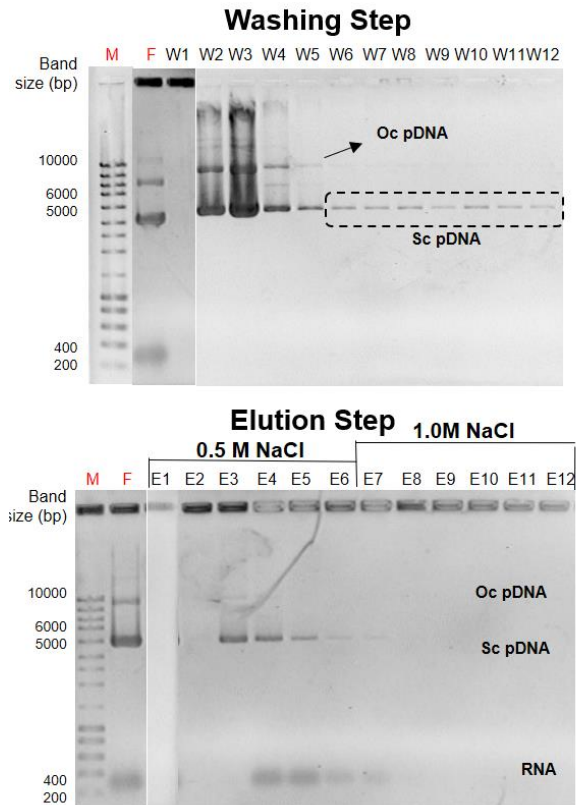
Washing Step

Elution Step

Figure 5: Gel electrophoresis of the fractions collected in bench chromatographic assay using ligand 2/1 immobilized in Sepharose CL-6B, under hydrophilic conditions (Tris-HCl (20mM; pH8) in washing; 0-2.5M NaCl stepwise elution), where nucleic acids were detected; W1 to W12 correspond to fractions from washing while E1 and E12 correspond to fractions from elution; The fractions presented were concentrated before analysis; M- DNA ladder III (NZYtech); F-Feed; oc- open circular; sc supercoiled.

On the other hand, ligand 2/1 can isolate part of sc isoform in washing under hydrophilic (Figure 5) and hydrophobic conditions (around 0.5M $(NH_4)_2SO_4$) (Figure 6). Sc isolation can be related to ligand properties, specifically to amine 2 (or 1,5-diaminopentane) since it is an analogue of lysine, an amino acid reported to have high selectivity for supercoiled isoform [19].

From this preliminary study, regarding their apparent ability to isolate supercoiled isoform from other isoforms and RNA, ligand 8/1 and 2/1 were considered as lead ligands to proceed to larger scale chromatography (ÄKTA purifying system).
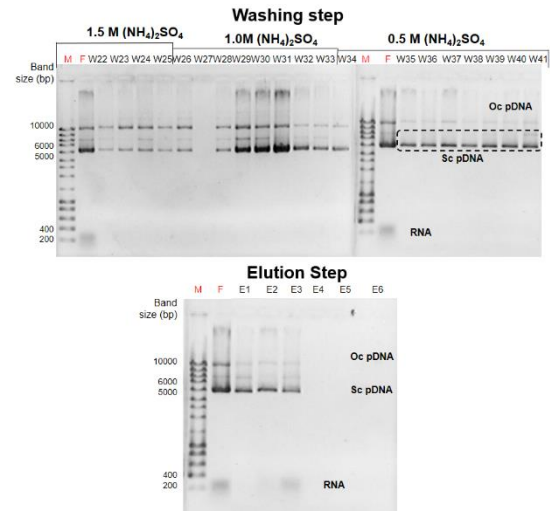
Washing step

Elution Step

Figure 6: Gel electrophoresis of the fractions collected in bench chromatographic assay using ligand 2/1 immobilized in Sepharose CL-6B, under hydrophobic conditions (2.5 - 0M $(NH_4)_2SO_4$ stepwise washing; Tris-HCl (20mM; pH8) in elution), in which nucleic acids were detected; W27 to W40 correspond to fractions from washing while E1 and E10 correspond to fractions from elution; The fractions presented were concentrated before analysis M- DNA ladder III (NZYtech); F-Feed; oc- open circular; sc supercoiled.

Chromatographic assays with ligand 8/1 and 2/1 in Sepharose CL-6B using ÄKTA 10

Ligand 8/1 and 2/1 were immobilized in Sepharose CL-6B and packed in a Tricorn 10/50 column to be evaluated in AKTA purifying system in terms of plasmid purification.
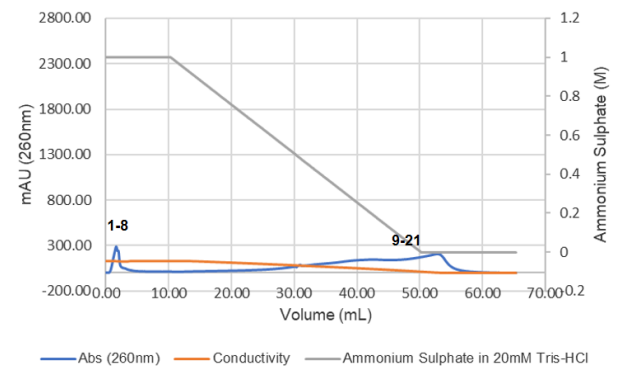
Figure 7: Chromatography in Sepharose CL-6B with 8/1 immobilized, performed in ÄKTA purifying system under hydrophobic conditions (1.05 - 0M $(NH_4)_2SO_4$ gradient in washing; Tris-HCl (20mM; pH8) in elution). In the chromatogram, the conductivity scale (in mS/cm) is presented in left vertical axis. 1-21: fractions collected from the peaks
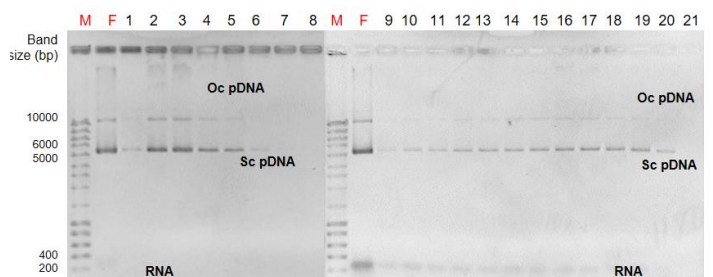
Under hydrophobic conditions, ligand 8/1 is able to perform a partial separation of plasmid sc and oc isoforms from RNA, followed by co-elution of sc pDNA and RNA (Figure 7 and 8). In this case, sc pDNA is not isolated unlike it was reported in bench chromatography (Figure 4), suggesting that this ligand is not able to selective separation of plasmid isoforms.
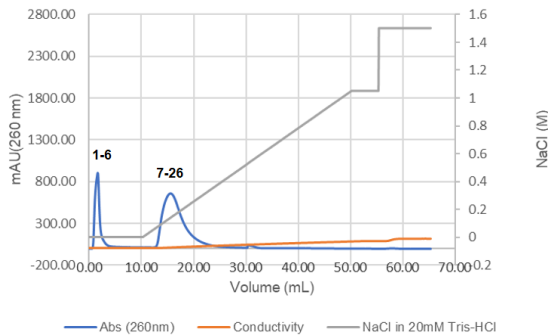

Figure 9: Chromatography in Sepharose CL-6B with 2/1 immobilized, performed in ÄKTA purifying system under hydrophilic conditions (Tris-HCl (20mM; pH8) in washing; 0-1.05M NaCl gradient in elution). In the chromatogram, the conductivity scale (in mS/cm) is presented in left vertical axis. 1-26: fractions collected from the peaks.

Regarding ligand 2/1, when tested under hydrophilic conditions, with a gradient elution between 0 and 1.05 M NaCl (Figure 9 and 10), plasmid DNA is isolated from RNA in the flow through and fractions with RNA isolated are obtained, but is not selective for different isoforms and thus selective isoform separation is not achieved (Figure 10).
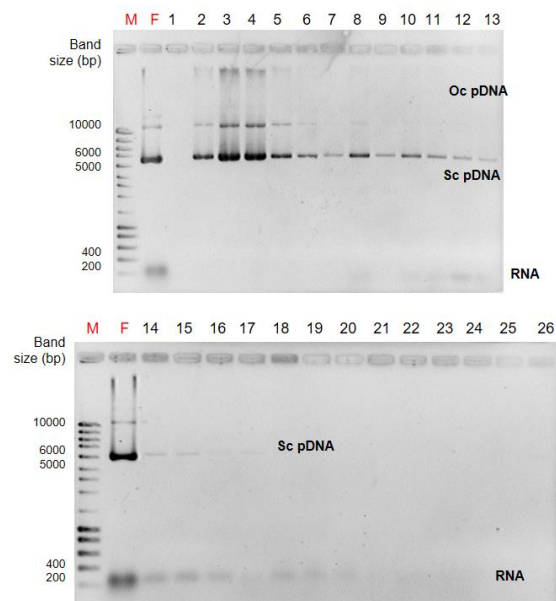

Figure 10: Gel electrophoresis of the fractions collected from chromatography using ligand 2/1 (Fig.9) (1 to 26); M- DNA ladder III (NZYtech); F-Feed; oc- open circular; sc supercoiled.
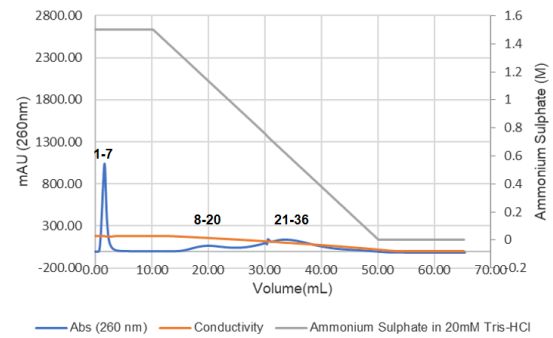

Figure 11: Chromatography in Sepharose CL-6B with 2/1 immobilized, performed in ÄKTA purifying system under hydrophobic conditions (1.5 - 0M $(NH_4)_2SO_4$ gradient in washing; Tris-HCl (20mM; pH8) in elution). In the chromatogram, the conductivity scale (in mS/cm) is presented in left vertical axis. 1-36: fractions collected from the peaks.

Performing a gradient between 1.5 and 0 M $(NH_4)_2SO_4$ (Figure 11 and 12), 3 peaks are detected: the 1st one around 1.5 M with pDNA isolated (Figure 12a); followed by isolated sc pDNA (Figure 12b) and the 3rd peak comprising RNA with sc pDNA contamination (Figure 12b). The 2nd and 3rd peaks seems to be overlapped, maybe explaining sc pDNA contamination in the third peak. From the 2nd peak, it was possible to recover between 47.3 and 59.5 % of sc pDNA, obtained respectively from (HIC)-HPLC quantification and densitometry analysis (Table 1).
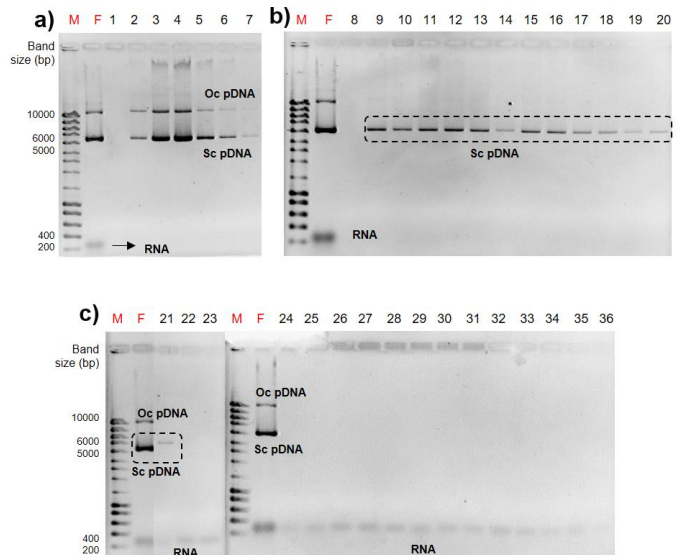

Figure 12: Gel electrophoresis of the fractions collected from chromatography using ligand 2/1 (Fig.11) (1 to 36); M- DNA ladder III (NZYtech); F-Feed; oc- open circular; sc supercoiled.

After chromatographic assay under hydrophobic conditions using gradient elution, a stepwise elution method was developed to optimize peak resolution in the chromatogram, which includes an initial 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$_4$)$_2$SO$_4$ for sc pDNA elution and a decrease to 0 M (NH$_4$)$_2$SO$_4$ for RNA removal (Figure 13).
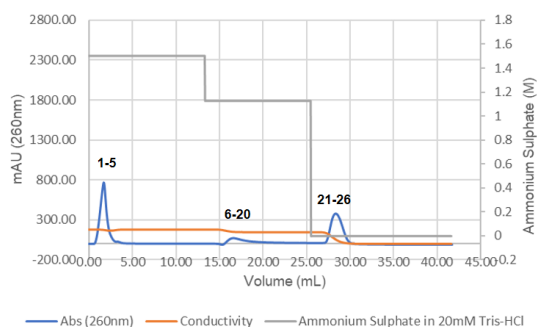

Figure 13: Chromatography in Sepharose CL-6B with 2/1 immobilized, performed in ÄKTA purifying system under hydrophobic conditions (feed diluted 1:3; step with1.5 M (NH$_4$)$_2$SO$_4$; step with 1.125 M (NH$_4$)$_2$SO$_4$; Tris-HCl (20mM; pH8) in elution). In the chromatogram, the conductivity scale (in mS/cm) is presented in left vertical axis. 1-26: fractions collected from the peaks.

In Figure 13, 3 peaks are detected, as expected, including oc and sc pDNA in the 1st peak, sc pDNA isolated in the 2nd peak and RNA with sc pDNA contamination in the 3rd peak (Figures 13 and 14). From the 2nd peak, it was possible to recovered between 44.9 and 65.1 % of sc pDNA, obtained respectively from (HIC)-HPLC quantification and densitometry analysis, being composed of 96.9 % over oc isoform, almost reaching the range of acceptance for the final plasmid product to be used in pharmaceutical purposes (Table 1). An additional assay was performed under similar conditions, but the feed was diluted 1:6. In this assay, a similar elution pattern was observed, where sc pDNA was isolated with a recovery yield between 70.1 and 75.1 % (data not shown).
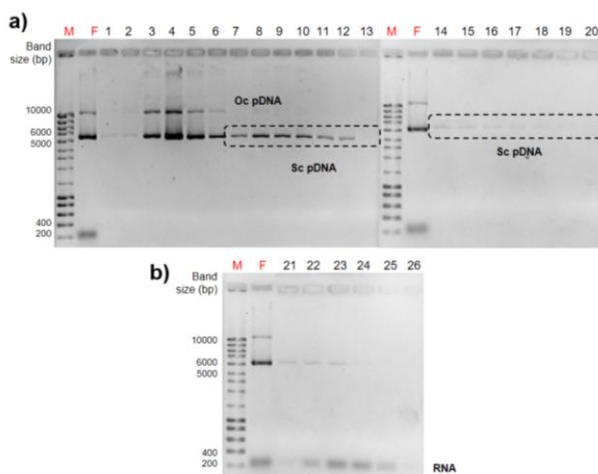

Figure 14: Gel electrophoresis of the fractions collected a) from the 1st and 2nd peaks (1 to 20) and b) from the 3rd peak (21-26) of the chromatogram using ligand 2/1 (Fig.13). f M- DNA ladder III (NZYtech); F-Feed; oc- open circular; sc supercoiled.

Table 1: pDNA and sc pDNA yield and sc amount over oc, from chromatography assays. Samples were analysed by HIC-HPLC (HH) and densitometry analysis (DA) a..value determined by collection of samples collected from the peak containing isolated supercoiled-isoform.b. value obtained from the 1st peak of the chromatogram.

Elution type	Binding conditions	pDNA yield (%)		Sc pDNA yield (%)[a]		Sc over oc (%)[a]
		DA	HH	DA	HH	
Gradient	Hydrophilic	82.5 b	64.6 b	-	-	-
Gradient	Hydrophobic	55.8 a	46.0 a	59.5	47.3	-
Stepwise		62.9 a	43.2 a	65.1	44.9	96.9

Differences between the values obtained from densitometry and (HIC)-HPLC quantification may be related to the presence of gel with undefined or bands, which influence intensity quantification in densitometry analysis or due to incorrect sample's volume collection during HIC-HPLC quantification.

Concluding Remarks

This works results from an extensive screening of selected ligands in terms of plasmid purification.

Ligand 6/5 is not able to separate sc pDNA from other isoforms and RNA under hydrophobic conditions, unlike previously reported [26].

The remaining hydrophobic ligands tested in bench chromatographic columns, in general can separate partially pDNA from RNA without selective isoform separation, except for 8/1 that is the only one able to isolate sc isoform from other impurities. Regarding ligands with mixed profile, different behavior is observed in different binding environments. 4/11 and 3/4 can totally separate pDNA from RNA and 2/1 is the only ligand from this group able to separate sc isoform from other isoforms and RNA either in hydrophobic and hydrophilic conditions. Regarding the ability to isolate sc isoform, 2/1 and 8/1 were considered as lead ligands for larger scale assays in ÄKTA 10.

Ligand 8/1 tested in ÄKTA 10 separates pDNA from RNA and is not able to isolate sc pDNA under hydrophobic conditions, unlike what was observed in bench-scale chromatography.

A similar behavior is observed for ligand 2/1 under hydrophilic conditions, where 64.6 to 82.5 % of pDNA is recovered in the peak containing isolated pDNA. On the other hand, 2/1 can isolate supercoiled isoform under hydrophobic conditions, applying a decreasing gradient between 1.5 and 0 M (NH$_4$)$_2$SO$_4$ where is possible to recover between 47.3 and 59.5 % of sc pDNA. Developing a stepwise method, it is possible to elute unbound pDNA at 1.5M (NH$_4$)$_2$SO$_4$, obtain part of sc pDNA isolated at 1.125 M (NH$_4$)$_2$SO$_4$ and elute the remaining bound species with total (NH$_4$)$_2$SO$_4$ removal, recovering between 44.9% and 75.1% of sc pDNA, and 96.9% sc over oc isoform in the fractions with sc isolated. Thus, the above-

mentioned results seem to point that 2/1 can be a promising ligand to isolate sc pDNA from other impurities under hydrophobic conditions, with considerable yields.

Further work includes the evaluation of the presence of contaminants – endotoxins, protein - in the final sc pDNA product recovered from 2/1 affinity chromatography; the use of alternative buffers to promote binding environment such as sodium citrate or ammonium citrate; evaluation of 2/1 with plasmid with different size of pVAX1/lacZ or the immobilization of 2/1 in EDA CIM® disk in a similar protocol performed to ligand 6/5 [32,33]. Also, other ligands such as 1/2, 4/1 or 1/4, which comprise substituents mimetics of lysine, are expected to behave in a similar way of ligand 2/1 and should be also evaluated using similar chromatographic conditions.

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