Separation and Purification Technology 188 (2017) 81–89

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Plasmid purification by using a new naphthalene tripodal support

T. Santos, Z. Proença, J.A. Queiroz, C. Tomaz, C. Cruz *

CICS-UBI - Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal

ARTICLE INFO

Article history:
Received 23 February 2017
Received in revised form 24 June 2017
Accepted 29 June 2017
Available online 30 June 2017

Keywords:
Naphthalene tripodal support
HR-MAS NMR spectroscopy
Affinity chromatography
Supercoiled plasmid purification

ABSTRACT

The aim of this work was to employ a new naphthalene tripodal support for the isolation of supercoiled (sc) isoform of plasmid (pDNA) from a native sample. This support is for the first time synthesized and used in pDNA purification.

The naphthalene tripodal ligand was synthesized and characterized to assess its purity and subsequently immobilized onto an epoxy-activated Sepharose CL-6B, using mild conditions and resulting in a ligand density of 0.32 mmol naphthalene tripodal/g derivatized Sepharose CL-6B. The complete characterization of naphthalene tripodal Sepharose CL-6B support was performed by High Resolution Magic Angle Spinning (HR-MAS) NMR spectroscopy, scanning electron microscopy (SEM) and elemental analysis. The affinity was measured by SPR biosensor between naphthalene tripodal ligand immobilized on the surface and sc pVAX1-*LacZ* and the K_D was $8.65 \times 10^{-8} \pm 1.0 \times 10^{-8}$ M in 10 mM Tris-HCl pH 8.0, at T = 25 °C, indicating a high affinity. For comparison reasons, the affinity ligand 3,8-diamino-6-phenylphenanthridine (DAPP) was also immobilized on the chip surface and the K_D for sc pVAX1-*LacZ* is lower than with naphthalene tripodal. Saturation transfer difference-nuclear magnetic resonance spectroscopy (STD-NMR) experiments showed that the interactions between the naphthalene tripodal–Sepharose CL-6B and DAPP-Sepharose supports and the 5′-mononucleotides are mainly hydrophobic and π-π stacking. The isolation of sc pDNA isoform was achieved with low salt concentrations, using 95 mM NaCl in binding step and 550 mM NaCl in elution step at T = 4 °C and pH 8, thus reducing the economic and environmental impact.

1. Introduction

In last decades, gene therapy and DNA vaccination arise as potential methods for treating several gene-related disorders, infectious diseases, AIDS and cancer [1]. However, non-viral gene delivery requires plasmid DNA (pDNA) with high degree of purity, potency, safety and efficacy [2]. pDNA used in gene therapy and DNA vaccination is usually produced in *Escherichia coli* (*E. coli*) host by fermentation and purified by chromatography to accomplish the requirements of regulatory agencies [3]. The use of pDNA for therapeutics relies on methods that purify the most biologically active and effective topology, the supercoiled (sc) isoform with a long, thin and branched shape [4]. Unlike, open circular (oc) and linear (ln) isoforms are considered contaminants because are less efficient and actively biologically. Affinity chromatography allows the recovery of sc pDNA with high purity [5]. This technique exploits the multiple non-covalent interactions between pDNA and a specific ligand immobilized on a matrix, making possible the separation of sc pDNA from contaminants [5]. Ligands are the key component of adsorption process and more compounds should be studied to make the process more efficiently and economically sustainable [6]. Aromatic ligands are promisor molecules in sc pDNA purification due to the unique features derived from the presence of the aromatic ring [7]. Recently, several studies report the use of aromatic amino acids (L-tyrosine [8] and L-tryptophan [9]) and synthetized aromatic compounds (DAPP [10] and berenil [11,12]) for pDNA purification.

The structural and binding information of interaction between aromatic ligands and pDNA is of utmost importance because allow a fully understanding about the underlying mechanisms of biorecognition [13]. Surface plasmon resonance (SPR) and Nuclear magnetic resonance (NMR) are powerful techniques, that can be employed in a fast screening of chromatography ligands [14]. Despite proved importance of ligands, process questions related to parameters of chromatographic process, such as, buffer type, salt concentration, temperature and pH have not fully addressed so far [15]. These parameters are intrinsically linked to features of ligands and need to be optimized for each case [15].

Here we report for the first time the synthesis, characterization and application of the naphthalene tripodal, as affinity ligand for

* Corresponding author.
E-mail address: carlacruz@fcsaude.ubi.pt (C. Cruz).

http://dx.doi.org/10.1016/j.seppur.2017.06.072
1383-5866/

the separation of sc and oc pDNA isoforms. The synthesis of support (Fig. 1) was performed by coupling the ligand through amino via a spacer to epoxy-activated Sepharose CL-6B, followed by characterization by High Resolution Magic Angle Spinning (HR-MAS) NMR spectroscopy, scanning electron microscopy (SEM) and elemental analysis. SPR biosensor was used to measure the binding strength of the DAPP and naphthalene tripodal to sc pVAX1-*LacZ*. STD-NMR analysis was also used to characterize the interactions between the nucleotides and the supports DAPP-Sepharose and naphthalene tripodal-Sepharose CL-6B. Finally, chromatographic experiments were performed using the naphthalene tripodal-Sepharose CL-6B support to purify sc pVAX1-*LacZ*.

2. Material and methods

2.1. Materials

Sepharose CL-6B was obtained from GE Healthcare (Uppsala, Sweden). Sodium carbonate was purchased from Panreac (Barcelona, Spain), tris(hydroxymethyl) aminomethane (Tris) from Merck (Darmstadt, Germany) and sodium chloride from Fisher Scientific (Fair Lawn, NJ, USA). All buffers used for the chromatographic experiments were freshly prepared with deionized ultra-pure grade water, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and analytical grade reagents. DAAP and lyophilized 5′-mononucleotides were purchased from Sigma-Aldrich (St. Louis, MO, USA). The GreenSafe Premium and the NZYMaxiprep commercial kit used in the pDNA pre-purification were obtained from NZYTech (Lisbon, Portugal).

2.2. Synthesis of naphthalene tripodal ligand

Naphthalene tripodal ligand was synthesized by the reaction of naphtalene-1-carbaldehyde (1.00 g, 6.4 mmol) and tripodal tris(2-aminoethyl)amine (2.80 g, 19.2 mmol) dissolved in 100 mL of EtOH-CH_3CN (1:1). The resulting solution was stirred for 2 h and then the solvent was evaporated. The obtained residue was dissolved in EtOH, and $NaBH_4$ (2.24 g, 59.0 mmol) was added portion wise. After 24 h at room temperature, the excess of $NaBH_4$ was filtered off and the solvent was evaporated to dryness. The resultant residue was treated with deionized water and extracted with CH_2Cl_2 (3×50 mL). The organic phase was evaporated to dryness to give an oil. The organic phase was then dried with anhydrous sodium sulfate and the solvent evaporated to yield the free amine as a yellowish oil. The oil was then taken in a minimum amount of EtOH and precipitated with aqueous HCl as its hydrochloride salt. Mp: 203–205 °C. ^{1}H NMR (D_2O, 400 MHz): 2.85 (t, 4H, J) 6 Hz, 2.93 (t, 2H, J) 7 Hz, 3.09 (t, 4H, J) 6 Hz, 3.32 (t, 2H, J) 7 Hz, 4.82 (s, 2H), 7.59–7.76 (m, 4H), 8.06–8.16 ppm (m, 3H). ^{13}C NMR (D_2O, 100 MHz): 36.7, 44.3, 48.5, 48.8, 50.1, 122.9, 126.0, 127.1, 127.9, 129.5, 130.1, 131.0, 131.3 ppm. ES-MS *m*/*z* calcd for $C_{17}H_{26}N_4$, 286.22; found 287.22 $[M+H]^+$.

2.3. Synthesis of naphthalene tripodal support

Sepharose CL-6B was activated and naphthalene tripodal ligand immobilized using a method described by Sundberg and Porath [16]. Briefly, Sepharose CL-6B was washed with Milli-Q water (1 L) on a vacuum system filtration. Then 20 g of washed Sepharose CL-6B were mixed with 40 mL of 0.6 M NaOH solution. After an incubation period of 4 h at 25 °C, 30 mL of 1,4-butanediol diglycidyl ether and 160 mg of $NaBH_4$ were added. The suspension was mixed by rotation at 25 °C overnight and the reaction was stopped by washing the gel with large volumes of deionized water. The gel was then suction-filtered to near dryness. The epoxy-activated Sepharose CL-6B matrix was used to couple naphthalene tripodal ligand. Briefly, 2 g of epoxy-activated Sepharose CL-6B was mixed with 11 mL of Na_2CO_3 solution (2 M). This mixture was incubated at 25 °C with agitation (110 rpm) in an orbital shaker for 6 h. Then naphthalene tripodal ligand was mixed in 50 mL of a Na_2CO_3 solution (2 M, pH 8.1) and added to epoxy-activated Sepharose CL-6B solution. The mixture was incubated at 55 °C with agitation overnight. The reaction was stopped by washing the gel with large amounts of Milli-Q water.

2.4. Characterization of the naphthalene tripodal support

2.4.1. By HR-MAS NMR spectroscopy

All NMR experiments were performed at room temperature using a Bruker Avance III 400 operating at 400.15 MHz for protons, equipped with a 4-mm triple resonance (HNC) HR-MAS probehead. Approximately 12 mg of the naphthalene tripodal support (lyophilized) was placed in a 4-mm MAS zirconia rotor (50 μL). The sample was spun at the magic angle at a rate of 4.0 kHz, and all spectra were acquired under field-frequency locked conditions using that probe channel with the spectrometer's lock hardware. Spectra were processed using Bruker Topspin 3.1. Unless otherwise stated, all ^{1}H NMR spectra were referenced internally to the residual ^{1}H signal of DMSO-d_6, which also serves as the swelling agent for the polymer beads (~0.05 mL). Carr-Purcell-Meiboom-Gill (CPMG) sequence with an echo time of 1.5 ms was used to suppress the broad signals of the polymer and experiments were acquired in

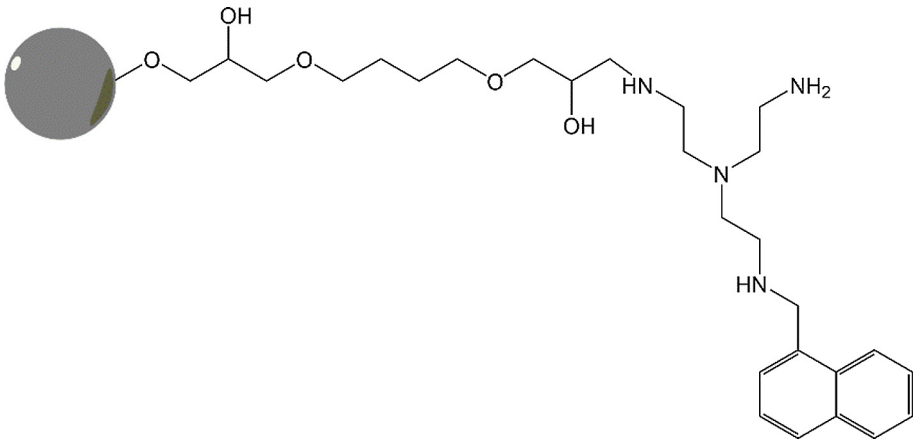

Fig. 1. Chemical structure of the naphthalene tripodal support.

256 transients. NOESY experiments were acquired with 150 ms mixing time in 16 transients with a relaxation delay of 2.0 s and a spectral width of ca 6000 Hz in a total of 2 K data points in F2 and 256 data points in F1.

2.4.2. By scanning electron microscopy and elemental analysis

The micrographs of epoxy-activated Sepharose CL-6B and naphthalene tripodal-Sepharose CL-6B were obtained using SEM. The samples were mounted on an aluminum board using double-sided adhesive tape and then made electrically conductive by coating with gold using an Emitech K550 sputter coater (London, UK). Then, the samples were analyzed on a Hitachi S-2700 with a UHV Dewar detector (Rontec EDX) (Tokyo, Japan) operating at an accelerating voltage of 20 kV at 200×, 500× and 3000× magnifications.

2.5. Surface plasmon resonance (SPR) biosensor

Surface plasmon resonance analyses were carried out on Biacore T200 system using carboxymethylated dextran-coated sensor chip CM5. The carboxylic groups on the surface of each flow cell were activated using a mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 0.1 M *N*-hydroxysuccinimide (NHS) at a flow rate of 10 μL/min and at 25 °C, to create reactive esters. The naphthalene tripodal ligand (2.3 mM, pH 9.64) and the phenanthridine derivative 3,8-diamino-6-phenylphenanthridine (DAPP) (16.6 mM, pH 5.19) were dissolved in 100 mM sodium borate and then injected at flow rate of 2 μL/min in respective flow cells. All the surfaces, with unreacted esters, were blocked with injection of 1 M ethanolamine. The immobilized level for flow cell 2 was 392.3 RU and for flow cell 3 was 364.9 RU. Flow cell 1 was left in blank, without ligand immobilization, to be used as a reference surface. Immediately before analysis, dilutions of sc pVAX1-*LacZ* were prepared in two different solutions: 10 mM Tris-HCl pH 8 and 10 mM sodium acetate pH 5 and injected at flow rate of 5 μL/min and contact time 420 s. The concentrations injected for sc pVAX1-*LacZ* range between 0.1 μM and 0.98 nM and were injected in triplicate. The concentrations were chosen considering the saturation of ligand surface in each flow cell. To collect affinity binding data, the experiments were performed at 10 °C and 25 °C. No regeneration solution was required since the plasmid isoforms were dissociated after 7 min from the sensor surfaces. Subtraction of the response of the blank injections from flow cells 2 and 3 removed unspecific binding and systematic imperfections in the response curve. In all responses in sensorgram the complex associate and dissociate rapidly making calculations of kinetics unfeasible and only steady state was possible to estimated.

The dissociation constants (K_D) were obtained by fitting plots of RU versus concentration of analyte in plateau region of the sensorgrams over a 300–400 s. The equation to calculate the K_D was: $R_{eq} = R_{max} - (1/(1 + K_D/[A]))$, where R_{eq} is the amount of pDNA bound to the ligand, R_{max} is the maximum binding capacity pDNA, and [A] is the concentration of pDNA. The K_D and affinity is inversed related. All data processing was performed using BIAevaluation software v. 4.1.

2.6. Saturation transfer difference- nuclear magnetic resonance spectroscopy (STD-NMR)

All the STD-NMR experiments were recorded on a Bruker Avance III 600 MHz spectrometer equipped with a cryoprobe and conducted at 25 °C.

The mononucleotides resonances were assigned using $^1H—^1H$ COSY, $^1H—^1H$ TOCSY and $^1H—^{13}C$ HSQC and $^1H—^{13}C$ HMBC. The supports naphthalene tripodal and DAPP were prepared as described previously [16]. The mononucleotides 5′-AMP, 5′-CMP, 5′-GMP and 5′-TMP as well as the supports, were suspended in 10 mM potassium phosphate buffer, pH 8.0, prepared in 90% H_2O and 10% (v/v) D_2O. The ^1H STD-NMR experiment was performed with a suspension of supports 0.80 mM and 5′-mononucleotides 7.5 mM being the spectra acquired at proton frequency of 600.13 MHz with 512 scans and a spectral width of 7211.5 Hz, centered at 2801 Hz. Sample volume was 600 μL in a 5 mm NMR tube. The selective saturation of the supports was performed using EBURP-shaped pulses (50 ms, 1-ms delay between pulses) for a total saturation time of 2.04 s. The selective irradiation of the support resonances was between 0.0 and 3.3 ppm (on-resonance) and 36 ppm (off-resonance). The on- and off-resonance spectra were acquired simultaneously with the same number of scans. The relative STD effect was calculated by dividing the STD signal intensities by the intensities of the corresponding signals in a reference spectrum of the same sample $[(I_0 - I_{STD})/I_0]$, expressing the signal intensity in the STD spectrum as a fraction of the intensity of an unsaturated reference spectrum [17]. The STD intensity of the largest STD effect was set to 100% as a reference and the relative intensities I_{STD} were determined [18]. Reference experiments using only the free mononucleotides were performed under the same experimental conditions to verify true ligand binding, demonstrating that the effects in the presence of the supports were only due to true saturation transfer as no signal was observed in STD spectra in the reference experiments. The resulting spectra were processed with TOPSPIN 3.1 software (Bruker).

2.7. Purification of sc pVAX1-LacZ using naphtalene tripodal support

Chromatographic experiments were performed at different temperatures (4, 10, 15 and 20 °C) and pH values (6, 7 and 8). A standard 10 mm diameter × 20 mm length column was packed with about 2.5 mL of naphthalene tripodal support, previously synthesized in order to evaluate the separation of pVAX1- *LacZ* isoforms. All affinity chromatography experiments were performed in an ÄKTA Avant system controlled by UNICORN 6.1 software (GE Healthcare Biosciences, Uppsala, Sweden). The solutions used in chromatographic runs were filtered through a 0.20 μm pore size membrane (Whatman, Dassel, Germany) and degassed before use. The water-jacket column was connected to a circulating water bath to maintain the desirable temperature of each experiment. Unless otherwise stated, the column was first equilibrated with 95 mM NaCl in 10 mM Tris-HCl (pH 8). The pVAX1- *LacZ* solution (25 μg pDNA/mL) was loaded onto column using a 100 μL loop at flow rate 1 mL/min. After elution of unbound species, the ionic strength of mobile phase was increased to 550 mM of NaCl in 10 mM Tris-HCl (pH 8). The absorbance of each run was continuously measured at 260 nm. Fractions were pooled according to the chromatograms and were concentrated and desalted with Vivaspin concentrators (Vivascience) for further analysis by electrophoresis. The fractions recovered in each chromatographic experiment were analyzed by horizontal electrophoresis using 15-cm-long 0.8% agarose gels (Hoefer, San Francisco, CA, USA), stained with GreenSafe Premium (1 μg/mL). Electrophoresis was performed at 120 V, for 40 min, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) and revealed under UV radiation in a transilluminator system (ILC Lda, Lisbon, Portugal).

3. Results and discussion

3.1. Synthesis of naphthalene tripodal support

The naphthalene tripodal ligand was designed to allow multiple non-covalent interactions (hydrophobic, electrostatic and hydrogen bonds) with pDNA, as reported for other similar ligands such

as DAPP [10], berenil [11,12], L-tryptophan [9] and L-tyrosine [8]. DAPP is a groove binder, and the interaction with pDNA occurs mainly through π-π stacking and hydrophobic interactions [10]. However, DAPP is dependent of pH, and when protonated binds to pDNA through electrostatic interactions between amine groups and pDNA backbone [10]. Berenil support was also successful applied for pDNA purification and the binding preferences are similar to DAPP [12].

Amino acids aromatics such as, L-tryptophan [9] and L-tyrosine [8] were also used as ligands in purification of pDNA. They interact with pDNA mainly through π-π stacking and hydrophobic interactions, however hydrogen bonds with specific nucleic acid bases and electrostatic interactions with pDNA backbone cannot be neglected since purifications of sc isoform from oc isoform occurs at 10 °C [8,9].

The structure of naphthalene tripodal is similar to the ligands reported above; however, the presence of the three amine arms provide it some flexibility to create an equilibrium between several interactions involved in binding of ligand to pDNA. Therefore, the versatility of this new support can increase yields and scale up by eliminating steps, and thereby reducing the economic and environmental impact.

Naphthalene tripodal ligand was synthesized by the condensation of naphthalene-1-carbaldehyde with the tripodal tris(2-aminoethyl)amine, in the presence of ethanol and acetonitrile in 1:1 proportion. This reaction leads to the formation of Schiff base, which was reduced with sodium borohydride to give the amine. The pure ligand was precipitated as its hydrochloride salt in a minimum amount of ethanol with aqueous HCl. The immobilization of the ligand on Sepharose CL-6B was achieved by the formation of

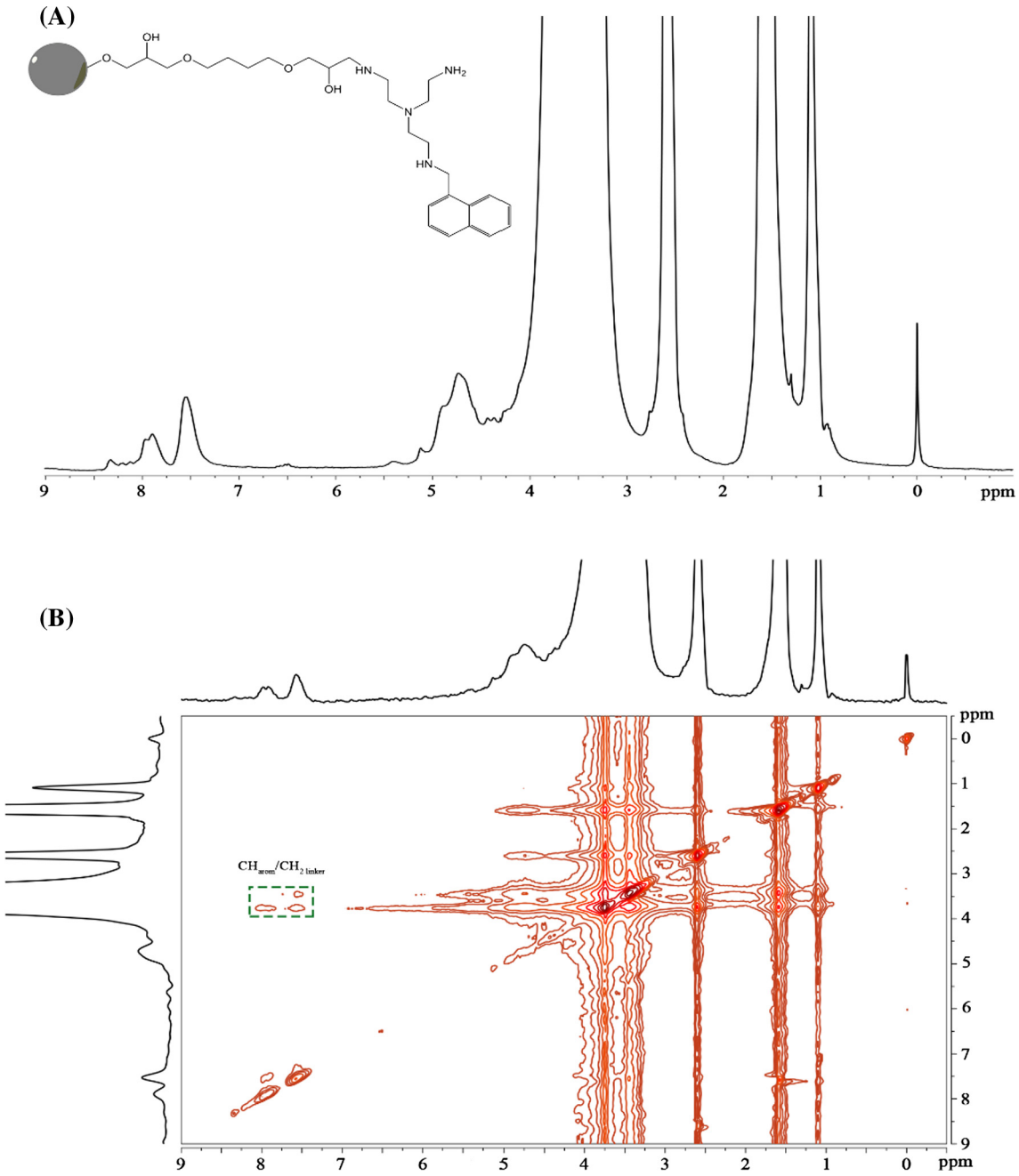

Fig. 2. (A) CPMG ^{1}H HR-MAS spectrum of Sepharose CL-6B with linker bound to naphthalene tripodal. (B) ^{1}H ^{1}H-NOESY HR-MAS spectrum of Sepharose CL-6B with linker bound to naphthalene tripodal.

epoxy groups, followed by ligand immobilization by the amine groups. The support was then characterized by HR-MAS NMR spectroscopy in order to ascertain if the ligand was immobilized onto the matrix.

3.2. Caracterization of the naphthalene tripodal support

3.2.1. By HR-MAS NMR spectroscopy

The high-resolution NMR spectroscopy combined with magic angle spinning (HR-MAS) reduces the broad Sepharose CL-6B background signals thus resulting in better resolved spectra, possibly contributing to the identification and quantification of compounds linked to the matrix [19]. The background signal was removed by using a CPMG pulse sequence with duration of 1.5 ms to abolish both transverse and longitudinal magnetization of the Sepharose CL-6B that allows the relatively immobile spins to relax and provides a high-resolution spectrum of the more mobile molecules [20].

The chemical shifts of the naphthalene tripodal on the Sepharose CL-6B are identified in the resonances between 8.4 and 7.5 ppm from the naphthalene protons and also in the methylene resonances of the tris(2-aminoethyl)amine between 3.0 and 2.8 ppm. The linker chemical shifts present distinct resonances upfield from CH and CH_2 at 1.1 ppm and 1.6 ppm, respectively, which are common to both spectra. These chemical shifts allow for the evaluation of ligand density in the support sample. Comparing the integration at 1.6 ppm due to two CH_2 units from the linker, with the resonances at 7.53 ppm from the ligand's aromatic protons, we obtained 26% of ligand density immobilized in the Sepharose CL-6B.

The coupling of L_1 to the matrix was identified in the MAS NOESY spectrum (Fig. 2) by the cross-correlation peaks between the aromatic protons at 7.53 ppm from ligand and methylene resonances from the linker at 1.6 and 1.1 ppm.

3.2.2. By SEM and elemental analysis

The efficiency of chromatographic processes is affected by bead morphology; by this way is important the characterization of the matrix prior and after immobilization of the ligand. To accomplish the characterization of support, SEM micrographs of 200, 500 and 3000× magnifications were obtained from Sepharose CL-6B, epoxy-activated Sepharose CL-6B and naphthalene tripodal-Sepharose CL-6B. Representative micrographs in Fig. 3 clearly show that bead morphology remains unaffected, maintaining their physical properties after exposition of beads to high temperatures and pH values during the immobilization procedure. Therefore, from SEM micrographs it is possible guarantees the efficiency of chromatographic process concerning that the flow properties are maintained, and the separation of pDNA is only influenced by the ligand, which is of the utmost importance for the purification.

Elemental analysis of naphthalene tripodal support was performed to obtain the ligand density. Eq. (1) was used to determine the amount of naphthalene tripodal immobilized on Sepharose CL-6B (Q, mmol of ligand immobilized/g Sepharose CL-6B), considering that all nitrogen (%N) in the sample comes exclusively from ligand [21].

$$Q = \frac{\%N}{(1.4 \times 4)} \quad (1)$$

The ligand density of the naphthalene support was 0.32 mmol per gram of Sepharose CL-6B. This value is significantly higher than in DAPP support, in which the ligand density was 0.15 mmol per gram of Sepharose. High values of ligand density can be favorable to bind pDNA to support with high efficiency allowing the separation of isoforms.

3.3. SPR analysis between pVAX1-LacZ isoforms and ligands

Surface plasmon resonance (SPR) is a technique that was used to measure interactions between the immobilized ligands, naphthalene tripodal and DAPP, on the sensor surface and sample solutions of pVAX1-*LacZ* isoforms that flows across the SPR surface. The DAPP-Sepharose support was used for the purification of pharmaceutical grade sc pDNA in previous studies [10,22]. Due to chemical similarities (condensed aromatic moiety) to naphthalene tripodal ligand, we chosen DAPP to immobilize it in the SPR surface for comparing the binding constants with our ligand. The SPR experiments were performed with two running buffers, Tris-HCl 10 mM

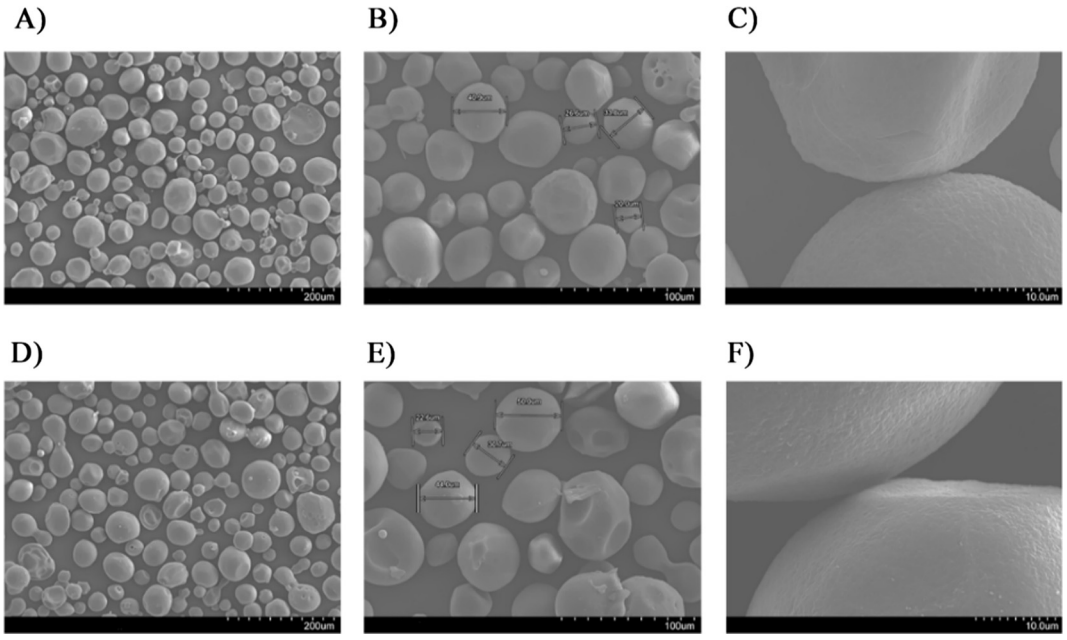

Fig. 3. SEM micrographs of Sepharose CL-6B beads. (A–C) Epoxy-activated Sepharose CL-6B at 200×, 500× with measurement of particle diameter and 3000× magnification. (D–F) naphthalene tripodal Sepharose CL-6B at 200×, 500× with measurement of particle diameter and 3000× magnification.

pH 8 and sodium acetate 10 mM pH 5, at two different temperatures, 10 °C and 25 °C. The distinct running buffers affected interaction strength, with an increase in response for sodium acetate 10 mM pH 5 with DAPP and no response for 10 mM Tris-HCl (pH 8.0), whereas with naphthalene tripodal the behavior is the opposite. We also compared the effect of temperature on the binding and no responses were found for DAPP at T = 25 °C and for naphthalene tripodal at T = 10 °C.

There are differences in equilibrium dissociation constants (K_D) values determined for naphthalene tripodal and DAPP. The equilibrium dissociation constant for sc pVAX1-*LacZ* and DAPP in sodium acetate 10 mM pH 5 at T = 10 °C is $1.73 \times 10^{-7} \pm 5.7 \times 10^{-8}$ M and for naphthalene tripodal in 10 mM Tris-HCl pH 8.0 at T = 25 °C is $8.65 \times 10^{-8} \pm 1.0 \times 10^{-8}$ M.

For both ligands and under these experimental conditions, the affinity is high, especially for naphthalene tripodal.

3.4. Molecular recognition studies using 5′-mononucleotides and the DAPP and naphthalene tripodal supports by STD-NMR

The structural aspects of the binding in solution of the 5′-AMP, 5′-GMP, 5′-CMP, 5′-TMP to the supports DAPP-Sepharose and naphthalene tripodal-Sepharose CL-6B were evaluated by STD-NMR spectroscopy. The 5′-mononucleotides were used to explore and understand which nucleotides contribute to the interaction with the ligands on the support. These data indicated the involvement of the nucleotide protons in the interaction with the supports.

3.4.1. 5′-mononucleotides binding to support DAPP-Sepharose

The STD spectra of 5′-CMP (Fig. S1(b) of Supplementary Material) and 5′-TMP (Fig. S3(h) of Supplementary Material) showed the strongest signals with protons from of the base, namely, proton H_5 (100% of saturation) for cytosine and CH_3 (100% of saturation) for thymine. The remaining contacts are found with ribose protons $H_{1'}$ (58% of saturation) for 5′-CMP and H'_2/H''_2 (10% of saturation) for 5′-TMP. No STD contacts were found for 5′-AMP. The STD percentages found for 5′GMP (Fig. S2(e) of Supplementary Material) showed the strongest STD signal for proton H'_2 (100% of saturation) followed by H'_1 (76% saturation), indicating that ribose is more intimate with the support than guanine or protons near the phosphate groups.

3.4.2. 5′-mononucleotides binding to support naphthalene tripodal-Sepharose CL-6B

In the presence of this support the 5′-AMP (Fig. S4(f) of Supplementary Material) showed STD signals only with protons

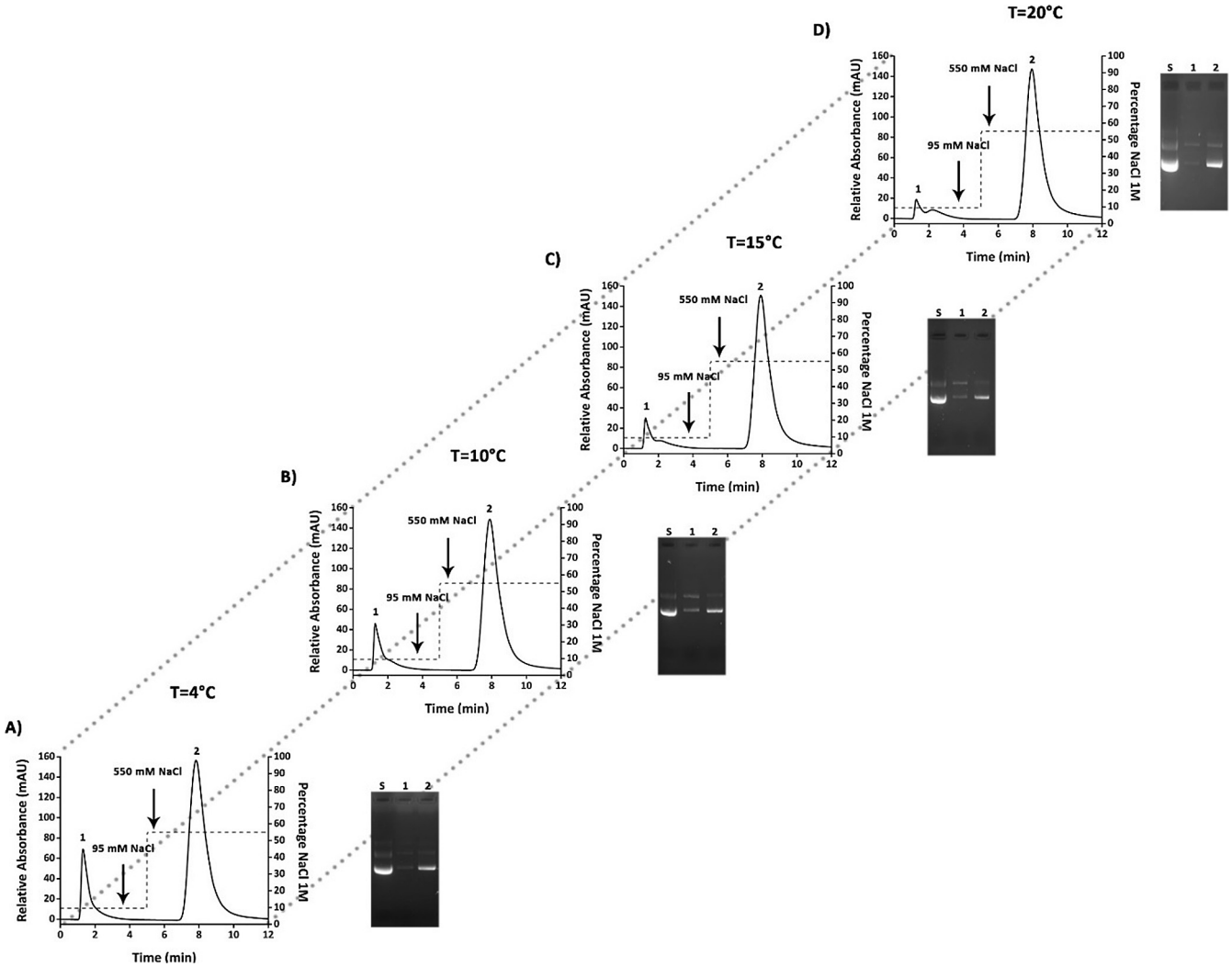

Fig. 4. Chromatographic profile and gel agarose electrophoresis of pVAX1-*LacZ* isoforms separation from a native sample (oc + sc) on naphthalene tripodal support. The elution was performed at flow rate of 1 mL/min, pH 8 and different temperatures: (A) 4 °C, (B) 10 °C, (C) 15 °C and (D) 20 °C. The dashed line represents NaCl concentration. Lane S — pVAX1-*LacZ* native sample (oc + sc) injected onto column; Lane 1 – pVAX1-*LacZ* recovered from peak 1; Lane 2 – pVAX1-*LacZ* recovered from peak 2.

H_8 (100% of saturation) and H_2 (49% of saturation), indicating that adenine is closer to support naphthalene tripodal Sepharose CL-6B.

Like above results, the STD spectra of 5′-CMP (Fig. S1(c) of Supplementary Material) and 5′-TMP (Fig. S3(i) of Supplementary Material) showed the strongest signals for H_6 and CH_3 (100% of saturation), respectively, suggesting that the base is involved in the interaction to the support. Similar result was found for 5′GMP (Fig. S2(f) of Supplementary Material) in which the only STD signal was for H_8 (100% of saturation).

In summary, the results from STD-NMR experiments provided a nucleotide epitope mapping through identification of their binding sites involved in interaction with the DAPP and naphthalene tripodal supports. The binding preferences of most the 5′-mononucleotides with the two supports are mainly through the base, meaning that H-bonds or π-π stacking can prevail with the aromatic moieties of the DAPP and naphthalene tripodal ligands.

3.5. Sc pVAX1–LacZ purification using the support naphthalene tripodal-Sepharose CL-6B

The data obtained in previous techniques provided useful information about the conditions to purify sc pVAX1-*LacZ* from native sample. SPR analysis show high affinity of naphthalene tripodal to sc pVAX1-*LacZ* at 25 °C in Tris-HCl pH 8 and data from STD-NMR experiments demonstrated that the interaction with naphthalene tripodal ligand was mainly through H-bonds and π-π stacking with the majority of the 5′-mononucleotides. Taking this into account, series of chromatographic experiments were performed to check the influence of salt concentration, temperature and pH. NaCl was used to promote the binding and elution of sc pVAX1-*LacZ* on naphthalene tripodal support using low salt concentrations reducing the costs and the environmental impact of high salt concentrations. Moreover, NaCl increase electrostatic interactions and has proved results in purification of sc pVAX1-*LacZ* with DAPP support [10] and amino acids such as, L-arginine [23] and L-lysine [24].

An initial screening for the evaluation of retention/elution profile of pVAX1-*LacZ* on naphthalene tripodal support was performed by manipulating the ionic strength between 90 mM and 1 M NaCl. For this preliminary assay, the temperature and pH was maintained at 4 °C and 8, respectively. After injecting the native pVAX1-*LacZ* sample containing both isoforms (sc and oc), the separation was achieved in a two-step process with an increase of salt in eluent buffer. The first step promoted the binding to support of sc isoform, which is more compacted than oc isoform, that eluted on the flowthrough and has a less degree of pDNA base exposition [25]. The second step was the elution of sc isoform, by increasing the NaCl concentration in eluent buffer. The most prominent result

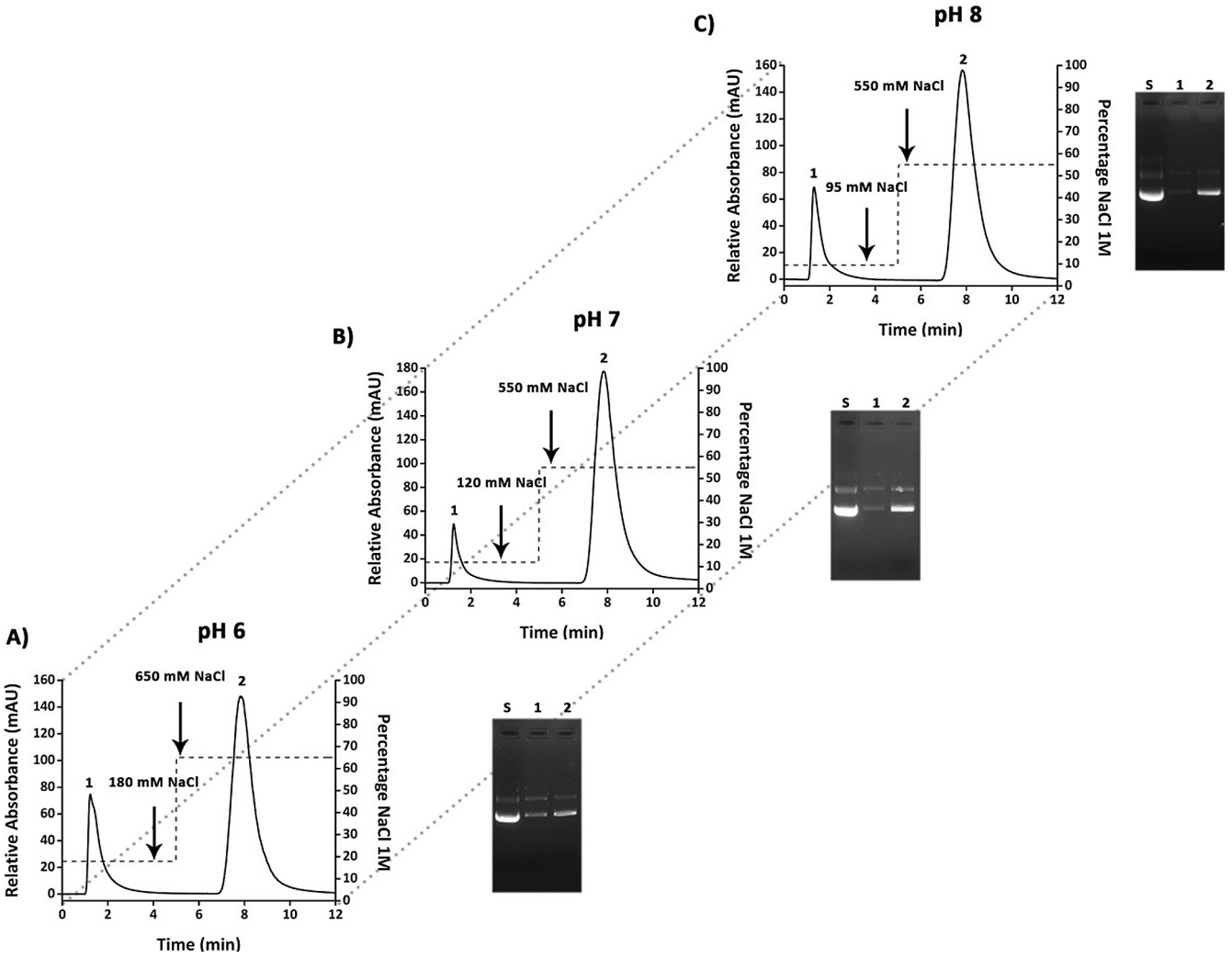

Fig. 5. Chromatographic profile and gel agarose electrophoresis of pVAX1-*LacZ* isoforms separation from a native sample (oc + sc) on naphthalene tripodal support. The elution was performed at flow rate of 1 mL/min, temperature of 4 °C and different pH values: (A) 6, (B) 7 and (C) 8. The dashed line represents NaCl concentration. Lane S — pVAX1-*LacZ* native sample (oc + sc) injected onto column; Lane 1 – pVAX1-*LacZ* recovered from peak 1; Lane 2 – pVAX1-*LacZ* recovered from peak 2.

was achieved when the concentrations of the binding and elution steps were 95 mM and 550 mM of NaCl, respectively. In fact, these salt concentrations were significantly lower comparing with previously described for DAPP [10] and berenil [12] supports. The separation of pVAX1-*LacZ* isoforms with DAPP was performed in sodium acetate buffer pH 5 with 220 mM NaCl in the first step and 1 M of NaCl in the second step at room temperature [10]. In purification of pVAX1-*LacZ* with berenil support the conditions were 1.3, 0.6 and 0 M ammonium sulfate in Tris-HCl pH 8 at room temperature [12].Taking this into account, naphthalene tripodal support presents an advantage over these chromatographic supports previously described [10,12].

To improve the isolation of sc pVAX1-*LacZ*, the temperature was increased to 10 °C, 15 °C and 20 °C and the effects on retention/elution profile are discussed. Fig. 4 shows the chromatographic profile and gel agarose electrophoresis at T = 4, 10, 15 and 20 °C. The ionic strength was maintained at 95 mM NaCl for binding step and 550 mM NaCl for elution step, resulting in poor isolation at high temperatures due to low specificity and selectivity for sc pVAX1-*LacZ*. In fact, the increase in temperature from 4 to 20 °C disrupt the electrostatic interactions and hydrogen bonds, that are stablished between protonated naphthalene tripodal and negatively charged phosphate groups in pDNA, while increases hydrophobic and π-π stacking interactions between naphthalene aromatic ring and pDNA bases [23]. This results in an equilibrium between multiple non-covalent interactions [23]. The better selectivity, specificity and recuperation of sc pVAX1-*LacZ* were achieved at 4 °C, mainly due to the electrostatic contributions and hydrogen bonds.

In the following experiments, the influence of pH at different temperatures was checked. The chromatographic profile and gel agarose electrophoresis at 4 °C and pH 6, 7 and 8 (Fig. 5) showed a significant increase of the retention of sc pVAX1-*LacZ* with a decrease in pH. Considering that this behavior could be correlated with the pK_a of naphthalene tripodal ligand that is around 9.2, the electrostatic interactions become stronger at low temperatures and pH, increasing the binding to sc pVAX1-*LacZ*. Also, we noticed that the decrease of pH affects the salt concentration required to promote the separation of the isoforms. Indeed, the concentration of NaCl used in the experiment at 4 °C and pH 6 was almost two times higher (180 mM NaCl) than salt concentration used in the first step of the experiments performed at 4 °C and pH 8. The pH manipulation effects were also studied at 10, 15 and 20 °C and have similar results to 4 °C (data not shown).

Unlike naphthalene tripodal ligand, the DAPP has pK_a of 5.8 [10]. Total retention of sc pVAX1-*LacZ* on this support was only observed when the pH was decreased to a value below DAPP's pK_a. Being protonated, DAPP can more easily bind to the negatively charged phosphate groups of pVAX1-*LacZ* [10].

In summary, the purification of sc pVAX1-*LacZ* was achieved using an ionic gradient with different concentrations of NaCl from 95 mM to 550 mM at T = 4 °C and pH 8.

4. Conclusions

The main goal of this work was to achieve the purification of sc pVAX1-*LacZ* isoform by employing for the first time the new naphthalene tripodal support. The Sepharose CL-6B derivatized with naphthalene tripodal was prepared through epoxide with a long spacer arm, resulting in a ligand density of 0.32 mmol naphthalene tripodal/g derivatized Sepharose CL-6B. Moreover, HR-MAS NMR spectroscopy showed the presence of naphthalene tripodal linked to Sepharose CL-6B and SEM analysis showed the preservation of the original beaded Sepharose CL-6B morphology after the derivatization process. Furthermore, the K_D value of $8.65 \times 10^{-8} \pm 1.0 \times 10^{-8}$ M determined by SPR biosensor with sc pVAX1-*LacZ* showed that naphthalene tripodal-Sepharose CL-6B can be applied as affinity chromatographic support. The STD-NMR experiments showed that the interaction between the naphthalene tripodal-Sepharose CL-6B is established mainly through H-bonds and π-π stacking, with the most of the 5′-mononucleotides.

This new support was used to purify sc isoform of pVAX1-*LacZ*, at temperature 4 °C, using Tris-HCl pH 8 with 95 mM NaCl, followed by 550 mM NaCl in the same buffer. The overall process has the advantage of using low amounts of salt in the eluent, providing the separation of the biologically active sc isoform in a two-step chromatographic process.

Acknowledgements

C. Cruz acknowledges the FCT project "Projeto de Investigação Exploratória" reference IF/00959/2015 supported by Fundo Social Europeu e ao Programa Operacional Potencial Humano.

T. Santos acknowledges a fellowship from Santander/Totta-UBI (Ref BID/ICI-FCS/CICS/Santander Universidades-UBI/2016).

This work was supported by FCT - Foundation for Science and Technology (project FCOMP-01-0124-FEDER-041068 and EXPL/QEQ-MED/1068/2013) and FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi/00709/2013).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2017.06.072.

References

[1] T. Wirth, N. Parker, S. Ylä-Herttuala, History of gene therapy, Gene 525 (2013) 162–169.
[2] J. Mairhofer, R. Grabherr, Rational vector design for efficient non-viral gene delivery: challenges facing the use of plasmid DNA, Mol. Biotechnol. 39 (2008) 97–104.
[3] H. Li, H. Bo, J. Wang, H. Shao, S. Huang, Separation of supercoiled from open circular forms of plasmid DNA, and biological activity detection, Cytotechnology 63 (2011) 7–12.
[4] D.M.F. Prazeres, T. Schluep, C. Cooney, Preparative purification of supercoiled plasmid DNA using anion-exchange chromatography, J. Chromatogr. A 806 (1998) 31–45.
[5] F. Sousa, D.M. Prazeres, J.A. Queiroz, Affinity chromatography approaches to overcome the challenges of purifying plasmid DNA, Trends Biotechnol. 26 (2008) 518–525.
[6] D.S. Hage, Affinity chromatography: a review of clinical applications, Clin. Chem. 45 (1999) 593–615.
[7] C. Caramelo-Nunes, P. Almeida, J.C. Marcos, C.T. Tomaz, Aromatic ligands for plasmid deoxyribonucleic acid chromatographic analysis and purification: an overview, J. Chromatogr. A 1327 (2014) 1–13.
[8] T. Santos, P. Pereira, F. Sousa, J.A. Queiroz, C. Cruz, Purification of supercoiled G-quadruplex pDNA for in vitro transcription, Sep. Purif. Technol. 163 (2016) 59–71.
[9] T. Santos, J. Carvalho, M.C. Corvo, E.J. Cabrita, J.A. Queiroz, C. Cruz, l-tryptophan and dipeptide derivatives for supercoiled plasmid DNA purification, Int. J. Biol. Macromol. 87 (2016) 385–396.
[10] C. Caramelo-Nunes, P. Almeida, J.C. Marcos, C.T. Tomaz, Specific recognition of supercoiled plasmid DNA by affinity chromatography using the intercalator DAPP as ligand, J. Chromatogr.B, Anal. Technol. Biomed. Life Sci. 928 (2013) 121–124.
[11] C. Caramelo-Nunes, M.F. Gabriel, P. Almeida, J.C. Marcos, C.T. Tomaz, Purification of plasmid DNA from clarified and non-clarified *Escherichia coli* lysates by berenil pseudo-affinity chromatography, J. Chromatogr.B, Anal. Technol. Biomed. Life Sci. 904 (2012) 81–87.
[12] C. Caramelo-Nunes, T. Tente, P. Almeida, J.C. Marcos, C.T. Tomaz, Specific berenil-DNA interactions: an approach for separation of plasmid isoforms by pseudo-affinity chromatography, Anal. Biochem. 412 (2011) 153–158.
[13] F. Sousa, C. Cruz, J.A. Queiroz, Amino acids-nucleotides biomolecular recognition: from biological occurrence to affinity chromatography, J. Mol. Recognit.: JMR 23 (2010) 505–518.

[14] C. Cruz, S.D. Santos, E.J. Cabrita, J.A. Queiroz, Binding analysis between L-histidine immobilized and oligonucleotides by SPR and NMR, Int. J. Biol. Macromol. 56 (2013) 175–180.
[15] A. Sousa, F. Sousa, D.M. Prazeres, J.A. Queiroz, Histidine affinity chromatography of homo-oligonucleotides. Role of multiple interactions on retention, Biomed. Chromatogr.: BMC 23 (2009) 745–753.
[16] L. Sundberg, J. Porath, Preparation of adsorbents for biospecific affinity chromatography, J. Chromatogr. A 90 (1974) 87–98.
[17] A. Viegas, J. Manso, F.L. Nobrega, E.J. Cabrita, Saturation-transfer difference (STD) NMR: a simple and fast method for ligand screening and characterization of protein binding, J. Chem. Educ. 88 (2011) 990–994.
[18] M. Mayer, B. Meyer, Group epitope mapping by saturation transfer difference NMR to identify segments of a ligand in direct contact with a protein receptor, J. Am. Chem. Soc. 123 (2001) 6108–6117.
[19] R. Warrass, J.M. Wieruszeski, C. Boutillon, G. Lippens, High-resolution magic angle spinning NMR study of resin-bound polyalanine peptides, J. Am. Chem. Soc. 122 (2000) 1789–1795.
[20] L.R. Carvalho, M.C. Corvo, R. Enugala, M.M. Marques, E.J. Cabrita, Application of HR-MAS NMR in the solid-phase synthesis of a glycopeptide using Sieber amide resin, Mag. Reson. Chem.: MRC 48 (2010) 323–330.
[21] A. Silva, R.E.F. Boto, R.M. El-Shishtawy, P. Almeida, Rhodamine B as ligand for affinity chromatography. Fixation studies onto cellulose by a curing method, Eur. Polym. J. 42 (2006) 2270–2282.
[22] C. Caramelo-Nunes, D. Bicho, P. Almeida, J.C. Marcos, C.T. Tomaz, Dynamic binding capacity and specificity of 3,8-diamino-6-phenylphenanthridine-Sepharose support for purification of supercoiled plasmid deoxyribonucleic acid, J. Chromatogr. A 1307 (2013) 91–98.
[23] F. Sousa, T. Matos, D.M. Prazeres, J.A. Queiroz, Specific recognition of supercoiled plasmid DNA in arginine affinity chromatography, Anal. biochem. 374 (2008) 432–434.
[24] A. Sousa, F. Sousa, J.A. Queiroz, Biorecognition of supercoiled plasmid DNA isoform in lysine-affinity chromatography, J. Chromatogr.B, Anal. Technol. Biomed. Life Sci. 877 (2009) 3257–3260.
[25] A. Sousa, F. Sousa, J.A. Queiroz, Differential interactions of plasmid DNA RNA and genomic DNA with amino acid-based affinity matrices, J. Sep. Sci. 33 (2010) 2610–2618.