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Affinity analysis and application of dipeptides derived from L-tyrosine in plasmid purification

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ABSTRACT

The developments in the use of plasmid DNA (pDNA) in gene therapy and vaccines have motivated the search and improvement of optimized purification processes. In this context, dipeptides L-tyrosine-L-tyrosine and L-tyrosine-L-arginine are synthetized to explore their application as affinity ligands for supercoiled (sc) plasmid DNA (pDNA) purification. The synthesis is based on the protection of *N*-Boc-L-tyrosine, followed by condensation with L-tyrosine or L-arginine methyl esters in the presence of dicyclohexylcarbodiimide (DCC), which after hydrolysis and acidification give the afforded dipeptides. The supports are then obtained by coupling L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine-L-arginine to epoxy-activated Sepharose and are characterized by high resolution magic angle spinning (HR-MAS) NMR and Fourier transform infrared spectroscopy (FTIR). Surface plasmon resonance (SPR) biosensor is used to establish the promising ligand to be used in the chromatographic experiments and ascertain experimental conditions. Sc isoform showed the highest affinity to the dipeptides, followed by linear (ln) pDNA, being the open circular (oc) the one that promoted the lowest affinity to L-tyrosine-L-arginine. Saturation transfer difference (STD)-NMR experiments show that the interaction is mainly hydrophobic with the majority of the 5′-mononucleotides, except for 5′-GMP with L-tyrosine-L-arginine Sepharose that is mainly electrostatic. The support L-tyrosine Sepharose used in chromatographic experiments promotes the separation of native pVAX1-*LacZ* and pcDNA3-FLAG-p53 samples (oc+sc) by decreasing the salt concentration. The results suggest that it is possible to purify different plasmids with the L-tyrosine Sepharose, with slight adjustments in the gradient conditions.

1. Introduction

The development of nucleic acid-based therapies, such as gene therapy and DNA vaccination, which require large quantity and high purity of these molecules led to the improvement of DNA purification processes, namely plasmid DNA (pDNA) purification [1]. The super-coiled (sc) pDNA recovery and purification from a lysate is extremely important due to the effectiveness of this isoform in the transfection experiments and in the subsequent expression of the target gene [2]. For this purpose, affinity chromatography has been widely used to isolate the sc plasmid isoform in a single chromatographic step, whereas RNA, gDNA, proteins and endotoxins are efficiently eliminated [3–5]. Exploiting the multiple non-covalent interactions involved in affinity chromatography it is possible to induce a selective biorecognition of the target biomolecules relying on a strong but reversible interaction between the pDNA and the immobilized ligand of the matrix [6].

Amino acids have been used as affinity ligands since they act as electron acceptors, have high physical and chemical stability and are easy to be obtained in large quantities at a relatively low cost [7]. Numerous amino acid have been recently employed as ligands in affinity chromatography matrices in order to purify sc pDNA such as L-histidine and L-arginine, used to purify sc pDNA from a clarified *Escherichia coli* lysate and fully separate sc pDNA from oc isoform [5–8]. The underlying mechanisms of biorecognition and interactions were also studied by techniques such as surface plasmon resonance (SPR) and nuclear magnetic resonance (NMR) [9,10]. The dipeptides have been poorly investigated as affinity ligands for purification of plasmid DNA. This can be attributed to the low availability of dipeptides due to the lack of an efficient and cost-effective process for dipeptide production [11]. Dipeptide syn-

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thesis can be accomplished either by using chemical or enzymatic methods [12].

Based on this, other amino acids such as L-tyrosine and its dipeptide derivatives are potential and versatile affinity ligands for sc plasmid purification. The polar nature of L-tyrosine favours the interaction with the DNA through amphipathic interactions (imino group hydrogen bonding, specific dipolar interactions, cation-π and hydrophobic interactions) [13]. Moreover, L-tyrosine has been described to interact with DNA through intercalative modes [14]. The current study focus on development of new supports based on dipeptides derived from L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine- L-arginine (see Fig. 1), followed by the screening of their affinity to pVAX1-*LacZ* and pcDNA3-FLAG-p53 isoforms using SPR-biosensor and amino acid-nucleotide interactions using STD-NMR spectroscopy. Finally, the retention behaviour of pVAX1-*LacZ* and pcDNA3-FLAG-p53 was also evaluated by chromatography using the L-tyrosine support.

2. Material and methods

All the water used to prepare solutions was ultra-pure grade, purified with a Milli-Q system from Millipore. L-tyrosine and L-arginine methyl ester were purchased from Sigma–Aldrich. NZY Maxi Prep Kit was purchased from NZYTech (Lisbon, Portugal). The 6.07 kbp pcDNA3-FLAG-p53 addgene plasmid 10838 [15] was from Addgene (Cambridge, MA, USA), the 6.05 kpb pVAX1-*LacZ* was purchased to Invitrogen (Carsland, CA, USA) and all the reagents used in bacterial growth were obtained from Sigma–Aldrich. The DNA ladder was obtained from Bioline (London, UK).

2.1. Synthesis of dipeptides L-tyrosine-L-tyrosine and L-tyrosine-L-arginine

The *N*-tertbutoxycarbonyltyrosine and L-tyrosine methyl ester were synthesized according to following procedures.

2.1.1. L-tyrosine methyl ester

Thionyl chloride (0.15 mol, 1.14 mL) was added dropwise to methanol (10 mL) at −10 °C whilst stirring. The mixture was stirred for a further 0.5 h at −10 °C. L-tyrosine (0.013 mol, 2.4 g) was added to the reaction solution and stirred for 1 h at −10 °C. The ice bath was removed and the reaction mixture was allowed to stir for 2 h. The solution was heated to reflux at 50 °C for 30 min. After that the mixture was concentrated in vacuum to half of the volume and precipitated with 10 mL of ethyl acetate. The precipitate was recrystallized with ethyl acetate and dried at 55 °C for 24 h to obtain the pure compound as a white solid.

Yield: 97%

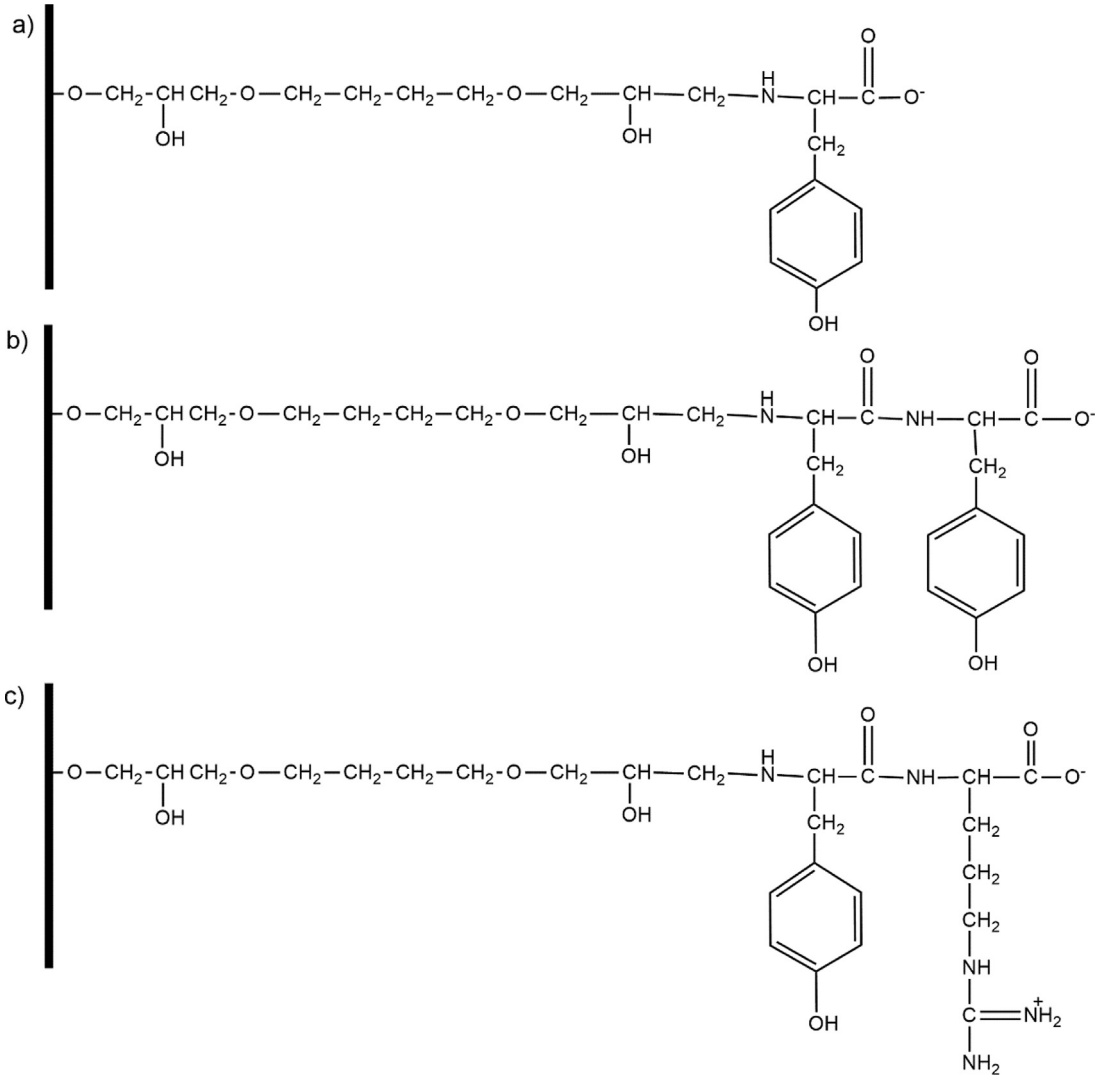

Fig. 1. Chemical structures of the supports (a) L-tyrosine Sepharose (b) L-tyrosine-L-tyrosine Sepharose and (c) L-tyrosine- L-arginine Sepharose.

^{1}H RMN (400 MHz, DMSO-d_6, δ, ppm): 3.07 (2H, q, CH_2), 3.74 (3H, s, CH_3), 4.2 (1H, t, Hα), 6.7 and 7.06 (4H, m, ArH), 8.6 (2H, s, NH_2), 9.52 (1H, s, OH).

^{13}C RMN (400 MHz, DMSO-d_6, δ, ppm): 169.9, 157.2, 130.8, 124.7, 115.8 53.9, 52.9, 35.5.

ESI-MS: m/z: 196.09 $[L+H]^+$.

2.1.2. Boc-L-tyrosine

To a mixture of 1:1 dioxane:water (50 mL) L-tyrosine (13.8 mmol, 2.5 g) in water was added with stirring for 2 h. After that triethylamine (19.9 mmol, 2.9 mL) was added dropwise and stirring by 10 min. The reaction flask was cooled to 0 °C with an ice bath and di-*tert*-butyl dicarbonate (15.2 mmol, 3.3 g) was added in one batch and stirring for 1 h. After that, the cold bath was removed and the reaction mixture was stirred at ambient temperature for 42 h. After evaporation the residue was acidified to pH 3 with 1 M HCl and extracted with ethyl acetate (3 × 50 mL). The extract was washed with saturated NaCl (3 × 25 mL) dried over Na_2SO_4 and evaporated to give the protected amino acid, Boc-L-tyrosine as a white product.

Yield: 50%.

^{1}H RMN (400 MHz, $CDCl_3$, δ, ppm): 1.34 (9H, s, Boc), 2.95 (2H, m, CH_2), 4.47 (1H, m, Hα), 5.02 (1H, s, NH), 5.77 (1H, s, OH), 6.7 and 6.9 (4H, 2d, ArH).

^{13}C RMN (400 MHz, $CDCl_3$, δ, ppm): 175.2, 171.2, 155.2, 130.2, 127.3, 115.6, 60.5, 54.7, 37.1, 31.1, 28.3.

ESI-MS: m/z: 282.13 $[L+H]^+$.

2.1.3. Boc-L-tyrosine-L-tyrosine methyl ester

A solution of DCC (3.6 mmol, 0.74 g) in 10 mL of THF was added dropwise to a solution of Boc-L-tyrosine (3.6 mmol, 1 g) in 30 mL of THF and cooled to 0 °C. The reaction was stirred for 15 min and a solution of L-tyrosine methyl ester (3.6 mmol, 0.7 g) in 25 mL of THF was added. The mixture was stirred for 40 h at room temperature, followed by increasing the temperature to 65 °C and left stirring for 24 h. The precipitate was filtered off, the solvent was removed from the filtrate under reduced pressure and the residue was washed with hexane (2 × 50 mL), dried under reduced pressure, and extracted with diethyl ether (3 × 100 mL). Then the extract was filtered and evaporated under reduced pressure and the residue was washed with hexane and dried under reduced pressure. Chloroform was added to the mixture cooled to 0 °C for 2 h and the precipitate dicyclohexylurea (DCU) was then filtered off.

Yield: 33%.

^{1}H RMN (400 MHz, $CDCl_3$, δ, ppm): 1.14–1.34 (12H, unresolved m,CH_3 and Boc), 2.9 (2H, m, CH_2), 3.02 (2H, m, CH_2), 3.4 (1H, m, H-α), 3.69 (1H, m, H-α), 4.55 (1H, s, OH), 5.07 (1H, s, OH), 6.67, 6.74, 6.93 and 6.99 (8H, 4d, ArH).

^{13}C RMN (400 MHz, $CDCl_3$, δ, ppm): 173.5, 170.6, 153.9, 129.5, 114.6, 79.4, 66.9, 64.8, 51.4, 28.7, 35.9, 32.5.

ESI-MS: m/z: 459.21 $[L+H]^+$.

2.1.4. Boc-L-tyrosine-L-arginine methyl ester

The dipeptide was synthesized in a similar way as previously described for Boc-L-tyrosine-L-tyrosine methyl ester. Boc-L-tyrosine (3.6 mmol, 1 g), L-arginine methyl ester (3.6 mmol, 0.7 g) and DCC (3.6 mmol, 0.74 g).

Yield: 35%.

^{1}H RMN (400 MHz, $CDCl_3$, δ, ppm): 1.18–1.31 (12H, unresolved m, CH_3 and Boc), 1.52–1.67(4H, m, CH_2), 2.67 (2H, m, CH_2), 2.9 (2H, m, CH_2), 4.45 (1H, s, H-α), 5.0 (1H,s, OH), 6.9 and 6.7 (4H, 2d, ArH).

^{13}C RMN (400 MHz, $CDCl_3$, δ, ppm): 172.5, 170.6, 158.1, 155.9, 154.7, 130.4, 129.2, 115.8, 79.5, 58.9, 56.2, 51.6, 41.6, 37.3, 31.2, 28.4, 24.6.

ESI-MS: m/z: 458.35 $[L+H]^+$.

2.1.5. L-tyrosine-L-tyrosine

Boc-L-tyrosine-L-tyrosine methyl ester (1.2 mmol, 466 mg) was dissolved in 90% TFA and 10% CH_2Cl_2 (v = 3 mL). The mixture was stirred for 1 h at room temperature and then placed under reflux at 90 °C for 24 h. The mixture was basified with 5 M solution of NaOH and the residue was extracted with diethyl ether (3 × 100 mL) evaporated and dried under reduced pressure.

Yield: 36%.

^{1}H RMN (400 MHz, δ, D_2O) 2.9 (2H, m, CH_2), 3.1 (2H, m, CH), 3.9 (1H, m, H-α), 4.11 (1H, m, H-α), 5.46 (1H, s, OH), 6.8, 6.9, 7.1 e 7.2 (8H, 4d, ArH).

^{13}C RMN (400 MHz, δ, D_2O): 178.4, 171.7, 155.7, 130.2, 129.5, 115.8, 63.5, 56.1, 38.7, 37.2.

ESI-MS: m/z: 349.29 $[L+H]^+$.

2.1.6. L-tyrosine-L-arginine

The deprotection of the dipeptide was done in a similar way as previously described for L-tyrosine-L-tyrosine.

^{1}H RMN (400 MHz, δ, D_2O): 1.49 (2H, m, CH_2 Arg), 1.68 (2H, m, CH_2 Arg), 1.79 (2H, m, CH_2 Arg), 3.10 (2H, m, CH_2 Tyr), 4.15 (H, d, H-α Arg), 4.22 (H, d, H-α Tyr), 6.80 (2H, d, ArH), 7.11 (2H, d, ArH).

^{13}C RMN (400 MHz, δ, D_2O): 174.8, 168.9, 156.7, 155.1, 130.9, 125.4, 115.8, 54.3, 52.9, 40.4, 35.9, 27.8, 24.2.

ESI-MS: m/z: 338.18 $[L+H]^+$.

2.2. Immobilization of L-tyrosine, L-tyrosine-L-tyrosine and tyrosine-L-arginine on Sepharose CL-6B

Sepharose CL-6B was activated according to the method previously described by Sundberg and Porath [16]. Sepharose CL-6B was washed with 300 mL of milli-Q water and suspended in 0.6 M NaOH with 1.32 mM $NaBH_4$. After 15 min, 1.4-butanediol diglycidyl ether (5 mL) was added slowly with stirring at 25 °C for 6 h. The mixture was washed with distilled water and acetone (200 mL) to remove an oily from the surface of the gel (the remaining epoxy compound). The epoxy-activated Sepharose CL-6B matrix (3 g) was used to couple-tyrosine, L-tyrosine-L-tyrosine and tyrosine-L-arginine. Each ligand was dissolved (L-tyrosine (8.27 mmol, 1.5 g), L-tyrosine-L-tyrosine (0.2 mmol, 70 mg) and L-tyrosine-L-arginine (0.2 mmol, 70 mg) in a solution of sodium carbonate 2 M at pH approximately 9. The mixture was stirred on the orbital at 55 °C for 16 h. The reaction was stopped by washing the support with 250 mL of milli-Q water in a sintered glass funnel, followed by a thorough wash with different acetone–water solutions (100 mL) (1:9, 3:7, 5:5, 8:2 v/v) and finally with a large amount of milli-Q water (3 × 100 mL).

2.3. L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine-L-arginine supports characterization

2.3.1. Fourier transformed infrared spectroscopy (FT-IR)

Fourier transformed infrared spectroscopy (FT-IR) spectra of the epoxy-activated Sepharose and of the immobilized supports were acquired on a Thermo Scientific Nicolet iS10 FT-IR spectrometer (Thermo Scientific, Waltham, MA). Spectra were recorded at 4 cm^{-1} (128 scans) over the 400–4000 cm^{-1} range with baseline corrections. Bands were given in cm^{-1}. For all spectra three replicates of FT-IR analysis were performed.

2.3.2. HR-MAS NMR spectroscopy and sample preparation

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. Samples were 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

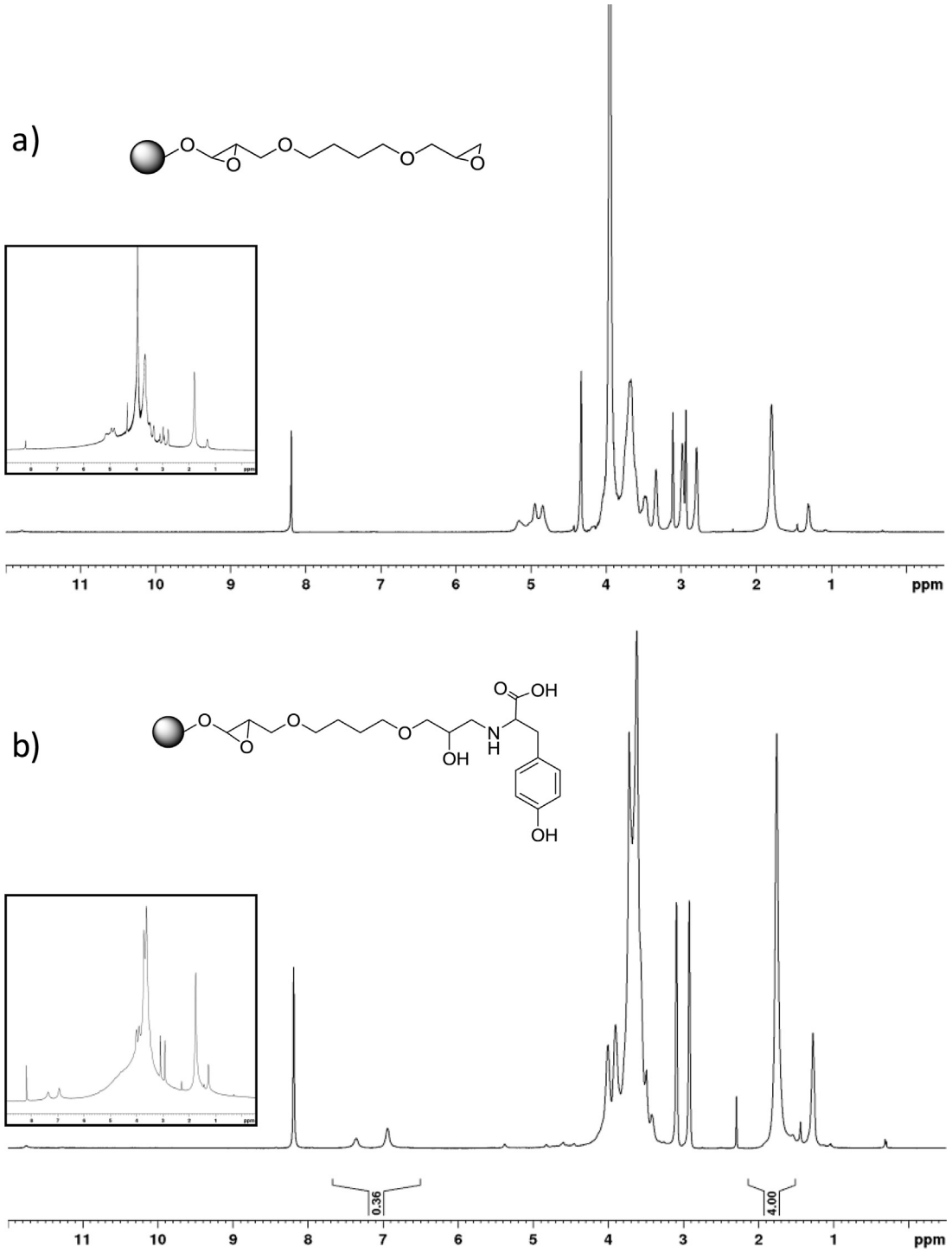

Fig. 2. CPMG ^{1}H HR-MAS spectra and inset with respective single pulse ^{1}H HR-MAS of agarose with activated linker (a) and agarose with linker bound to L-tyrosine (b) in DMF-d_7.

hardware. Spectra were processed using Bruker Topspin 3.2. All ^{1}H NMR spectra were referenced internally to the residual ^{1}H signal of DMF-d_7, which also serves as the swelling agent for the polymer beads (~0.05 mL), unless stated otherwise. Carr-Purcell-Meiboom-Gill (CPMG) sequence with an echo time of 1.5 ms was used to suppress the broad signals of the polymer, experiments were acquired in 256 transients. NOESY experiments were acquired with 300 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.

Approximately 10 mg of the polymer sample was placed in a 4-mm MAS zirconia rotor (50 μL).

2.4. Plasmid production and isolation

*E. coli*DH5α cell cultures were used to amplify the pcDNA3-FLAG-p53 and the pVAX1-*LacZ* plasmids. The growth was carried out at 37 °C, 250 rpm, in an erlenmeyer with 250 mL of Terrific Broth medium (20 g L^{-1} of tryptone, 24 g L^{-1} of yeast extract, 4 mL L^{-1} of glycerol, 0.017 M KH_2HPO_4, 0.072 M K_2HPO_4) supplemented with

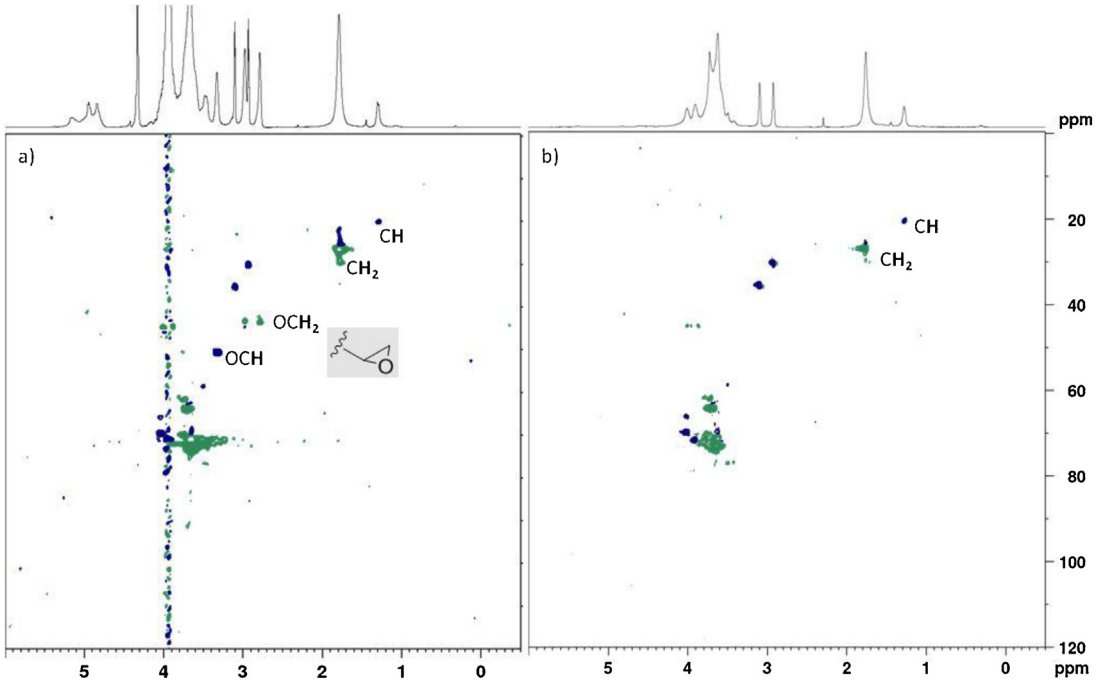

Fig. 3. $^{1}H,^{13}C$-DEPT-HSQC HR-MAS spectra of agarose with activated linker (a) and agarose with linker bound to L-tyrosine (b) in DMF-d_7.

30 μg mL^{-1} of ampicillin for amplification of pcDNA3-FLAG-p53 and with 30 μg mL^{-1} of kanamycin for pVAX1-*LacZ* production. The cells were grown until the log phase (OD600 nm ± 9) and then were collected by centrifugation and stored at −20 °C.

The plasmids were recovered using the NZYTech (Lisbon, Portugal) plasmid Maxiprep kit according to the manufacturer's protocol based on a modified alkaline lysis procedure. Briefly, after an alkaline lysis the pDNA was bound to the NZYTech anion exchange resin under appropriate low-salt and pH conditions. Then, the impurities were removed by a medium salt wash and finally the plasmid was eluted through the increase of ionic strength. Afterwards, the plasmid samples were concentrated via an isopropanol precipitation and the final pellet was resuspended in 1 mL of 10 mM Tris–HCl at pH 8.0. The pDNA samples concentration was measured with NANOPhotometer (Implen). Pure samples of oc and ln isoforms were prepared from the sc plasmid samples. Oc isoform was prepared by incubating sc samples at room temperature (25 °C) while the ln isoform was prepared by enzymatic digestion of plasmids with Hind III enzyme, during 1 h of incubation at 37 °C. The isoform conversion was monitored over time by hor-

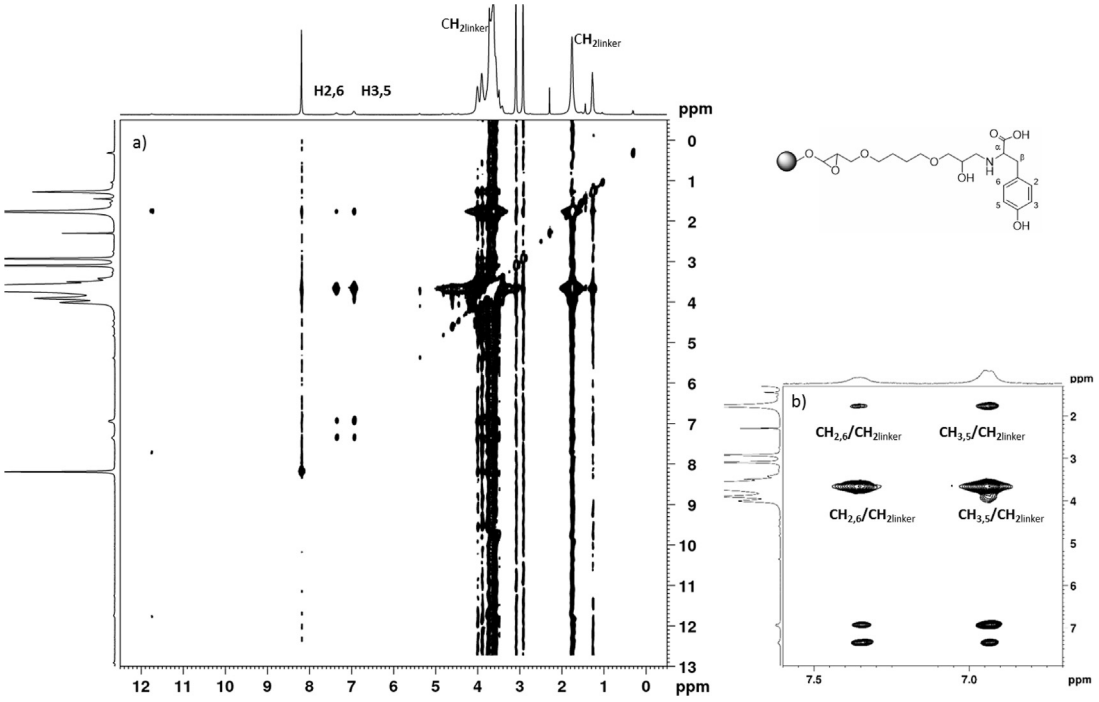

Fig. 4. $^{1}H,^{1}H$-NOESY HR-MAS spectrum of agarose with linker bound to L-tyrosine in DMF-d_7.

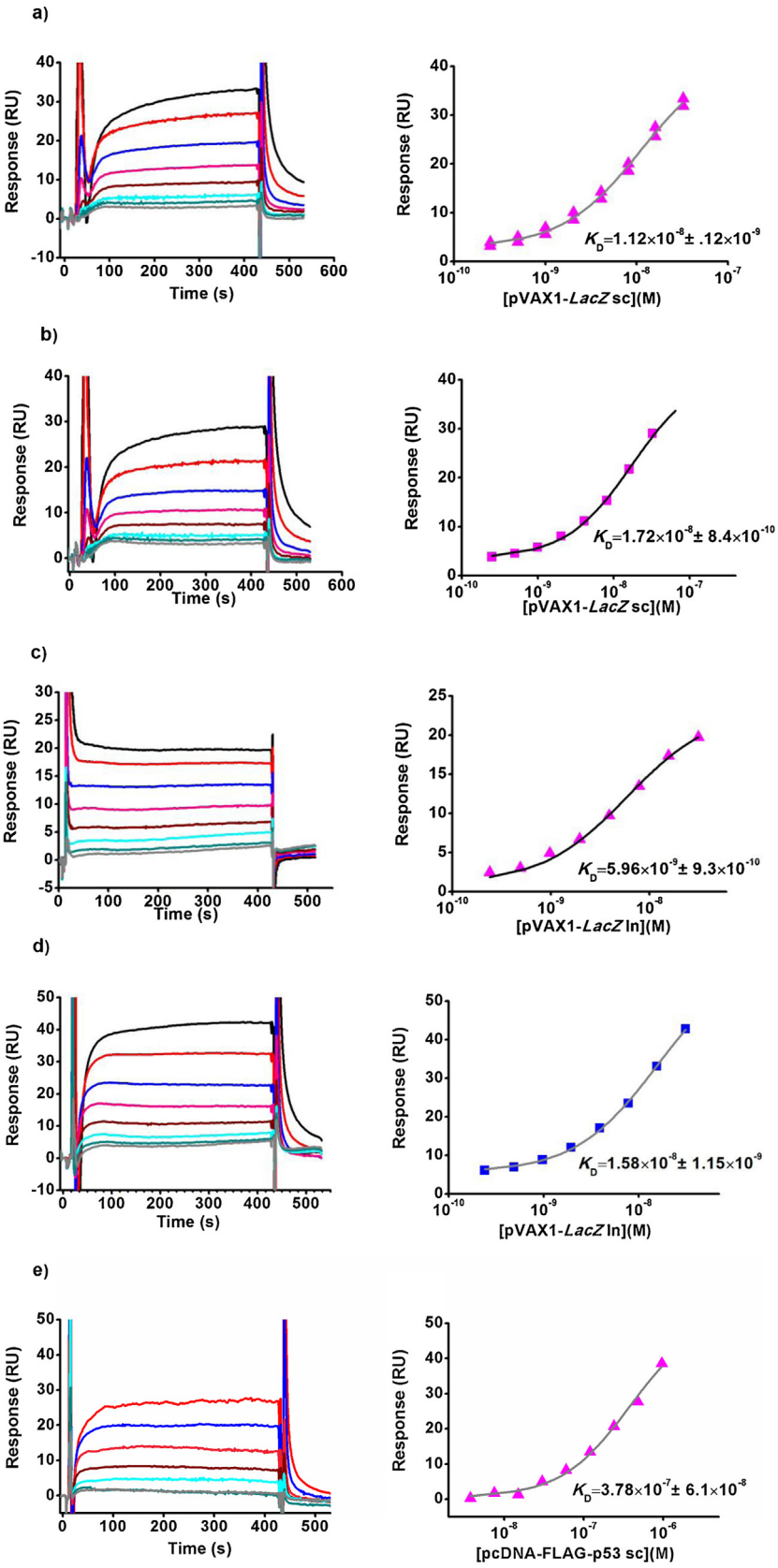

Fig. 5. Sensorgrams and equilibrium binding analysis at T = 10 °C of (a) pVAX1-*LacZ* sc with L-tyrosine-L-tyrosine, (b) pVAX1-*LacZ* sc with L-tyrosine-L-arginine; (c) pVAX1-*LacZ* ln with L-tyrosine and (d) pVAX1-*LacZ* ln with L-tyrosine-L-arginine; (e) pcDNA-FLAG-p53 sc with L-tyrosine-L-tyrosine.

izontal agarose gel electrophoresis until total sample conversion (about 3 days).

2.5. SPR measurements

2.5.1. Ligands immobilization

Experiments were carried out in a BIAcore T200 with a CM5 sensor chip. L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine-L-arginine were dissolved in 0.1 M borate buffer at pH >9.1 and immobilized using the method of amino-coupling [17] in running buffer HBS-N (0.1 M HEPES and 1.5 M NaCl). An unmodified reference flow cell 1 was treated as above, without immobilization of ligands. Each flow cell was activated for 7 min with a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.4 M *N*-ethyl-*N*-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC) at a flow rate of 5 μL/min and at 25 °C. The immobilized density averaging for L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine-L-arginine was 71.7, 103.5 and 89 RU, respectively. All the surfaces were blocked with a 7 min injection of 1 M ethanolamine, pH 8.0, followed by HBS-EP injection to stabilize the baseline.

2.5.2. Equilibrium binding experiments

The profile of association/dissociation between plasmid isoforms of pVAX1-*LacZ* and L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine-L-arginine was evaluated in two running buffers, 10 mM Tris–HCl and 10 mM HEPES acid, and at two temperatures, 10 °C and 25 °C. Duplicate injections of each plasmid isoform concentration were analyzed in random order at flow rate 2 μL/min and contact time of 420 s. In the case of sc isoform, the range of concentrations used was 0.3 μM to 0.25 mM. For ln, the concentrations used were 0.24 μM to 0.125 mM and for oc isoform was 0.48 μM to 0.5 mM.

Subtraction of the response of the reference cell from that of the flow cells with immobilized L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine-L-arginine allows to eliminate non-specific interactions with the matrix and artefacts of the detector. Each isoform of pDNA associates and dissociates rapidly from the ligands surface, making calculations of kinetics unfeasible. Only steady-state studies were possible to carry out by averaging the resonance unit values (RU) in the plateau region of the sensorgrams over a 300–400 s. Responses were fitted to a single-site bimolecular interaction model, yielding a single equilibrium dissociation constant, K_D ($R_{eq} = KA \times [\,] \times R_{max}/(1 + K_A \times [\,] \times n)$. All data processing and analyses were performed using BIAevaluation software v. 4.1.

2.6. STD-NMR spectroscopy

The residues of 5′-AMP, 5′-CMP, 5′-GMP and 5′-TMP involved in binding to the supports L-tyrosine Sepharose, L-tyrosine-L-tyrosine Sepharose and L-tyrosine-L-arginine Sepharose were evaluated by STD-NMR. The supports stock solution and the 5′-mononucleotides (200 mM) were prepared in 10 mM potassium phosphate buffer pH 8.0 in 90% H_2O and 10% (v/v) D_2O. Sample volume was 600 μL in a 5 mm NMR tube. All spectra were acquired at 25 °C on a Bruker Avance III 600 MHz spectrometer equipped with a cryoprobe. The selective irradiation of the support resonances were: for L-tyrosine Sepharose, 1157HZ to 5′-AMP, 1649 Hz to 5′-CMP, 4134 Hz to 5′-GMP and 436 Hz to 5′-TMP; for L-tyrosine-L-tyrosine Sepharose, 1048 Hz to 5′-AMP, 789 Hz for the 5′-CMP, 1814 Hz to 5′-GMP and 436 Hz for 5′-TMP. The L-tyrosine-L-arginine Sepharose support was irradiated at 800 Hz for 5′-AMP, 700 Hz to 5′-TMP and 1344 Hz to 5′-CMP and 5′-GMP. STD-NMR spectra were recorded with 512 scans in a spectral window with 12,019.2 Hz centered at 6194.03 Hz. The selective saturation of the supports was achieved by EBURP-shaped pulses of 50 ms each, truncated at 1%, and separated by a 1 ms for a total saturation time of 2.04 s. The STD spectrum was obtained by subtraction of the on-resonance from the off-resonance spectrum [18]. Reference experiments of samples containing only the free 5′-mononucleotides were performed in the same experimental conditions to verify true ligand binding. The STD effect was calculated by $(I_0 - I_{STD})/I_0$, in which $(I_0 - I_{STD})$ is the peak intensity in the STD spectrum and I_0 is the peak intensity in the off-resonance spectrum [19]. The STD intensity of the largest STD effect was set to 100% as a reference and the relative intensities (I_{STD}) were determined [20].

2.7. Preparative chromatography

The chromatographic experiments were performed in an AKTA pure system (GE Healthcare Life, USA) using a 10 × 50 mm (approximately 4 mL) column packed with the previously prepared L-tyrosine support. To control and maintain the temperature of the experiments a circulating water bath was also used. The samples containing the sc and oc isoforms were loaded onto the column using a 100 μL loop at 1 mL/min. The absorbance of the eluate was continuously monitored at 260 nm. The column was equilibrated with different concentrations of ammonium sulfate depending on the experiment and the elution of bound species was carried out using a decreasing ammonium sulfate stepwise gradient. To elute all remaining bound species, 100 mM of acid HEPES, pH 8.0 was used.

2.8. Agarose gel electrophoresis

To analyze the species eluting in each peak recovered from the chromatographic experiments, an agarose gel electrophoresis was performed. The fractions were pooled, according to the chromatograms obtained, concentrated and desalted with Vivaspin concentrators and analysed by gel electrophoresis using a 0.8% agarose gel (Hoefer, San Francisco, CA, USA), stained with GreenSafe Premium (0.01%) (NZYTech). Electrophoresis was carried out at 110 V with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). The gel was visualized in an Uvitec Cambridge system.

3. Results and discussion

3.1. Synthesis of dipeptides L-tyrosine-L-tyrosine and L-tyrosine-L-arginine

The dipeptide synthesis can be achieved by enzymatic and chemical procedures, being the chemical synthesis used in this work. The efficient combination of protecting groups and coupling reagents are the main issue. The coupling reagents commonly used are *N*,*N*′-dicyclohexyl carbodiimide (DCC), 1-ethyl-3-(3′-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC-HCl) and *N*,*N*′-diisopropylcarbodiimide (DIC) and the carbamate, trifluoroacetyl and tertbutoxycarbonyl (BOC) groups are the most used to protect amino groups in amino acids.

The synthesis begins with the protection of L-tyrosine with Boc to give the *N*-Boc derivative, followed by condensation of L-tyrosine or L-arginine methyl esters in the presence of DCC, which were subjected to hydrolysis by the action of aqueous sodium hydroxide; and subsequent acidification with trifluoroacetic acid: CH_2Cl_2 to remove the Boc group and afford target dipeptides. To remove the DCU, the final product was dissolved in the minimum of $CHCl_3$ and cooled to 0 °C. The crystalized DCU was removed by filtration. Diethyl ether was added to the filtrate at 0 °C to recrystallize the pure compound. The global yield of the reaction is low and allows for disposing of milligram quantities of the final product. The synthesis details were described in Section 2, as well as, the

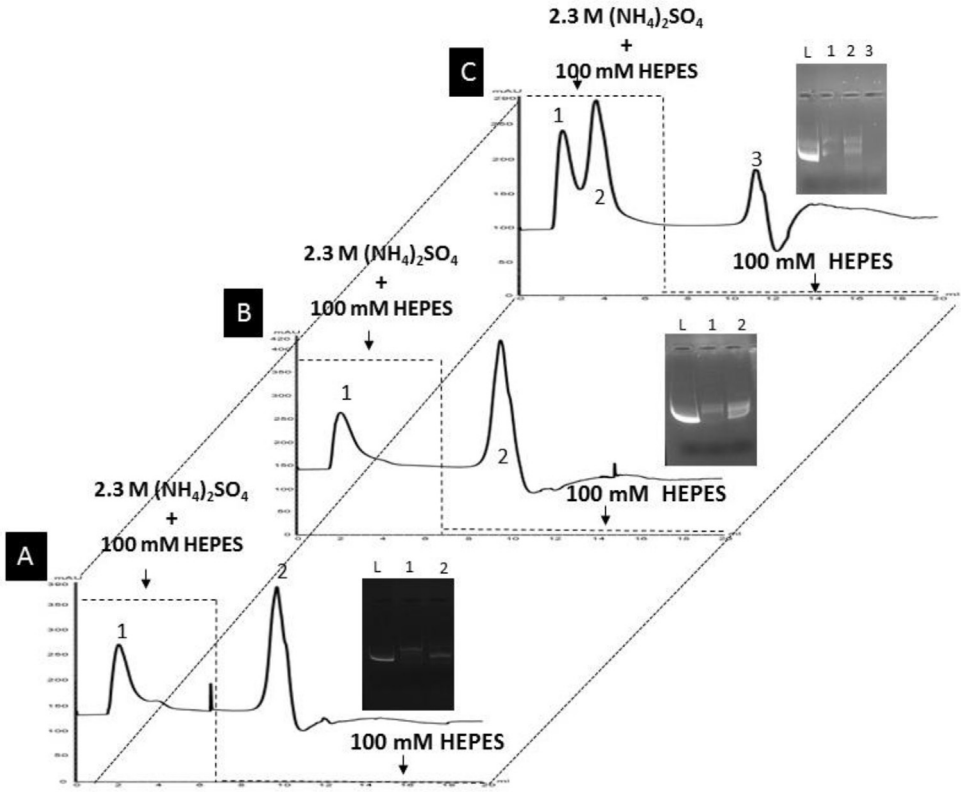

Fig. 6. Chromatographic profiles and respective agarose gel electrophoresis of plasmid isoforms separation from a pre-purified p53-encoding pDNA sample (sc+oc) in L-tyrosine Sepharose by using different temperatures: (A) 5 °C, (B) 10 °C and (C) 25 °C. The elution was performed by stepwise gradient of 2.3 M of $(NH_4)_2SO_4$ in 100 mM HEPES acid (pH 8.0) to 100 mM HEPES acid (pH 8.0), as represented by the dashed line. In the agarose gel electrophoresis, lane L represents the pre-purified plasmid sample and lanes 1–3 represent the respective peaks of each chromatogram.

characterization of all the compounds by NMR spectroscopy and mass spectrometry.

3.1.1. *Immobilization of* L-*tyrosine and dipeptides* L-*tyrosine*-L-*tyrosine and* L-*tyrosine*-L-*arginine on Sepharose CL-6B*

The epoxide-activated supports are produced by the immobilization of bioxiranes, such as, 1,4-butanediol diglycidyl ether onto Sepharose CL-6B. These epoxy-activated groups allow stabilization of the gel through introduction of hydrophilic spacer arm between the matrix and the ligand and covalent immobilization of $-NH_2$ of their amino acids. L-tyrosine and the dipeptides L-tyrosine-L-tyrosine and L-tyrosine-L-arginine are coupled to epoxy-activated Sepharose at alkaline pH (9–10) using a temperature between 55 and 70 °C. The structural characterization of the supports was achieved by FT-IR and HR-MAS NMR, which provide solution-like spectra for matrix-bound molecules.

3.2. *Characterization of the supports* L-*tyrosine Sepharose,* L-*tyrosine*-L-*tyrosine Sepharose and* L-*tyrosine*-L-*arginine Sepharose*

The supports were characterized by FT-IR and HR-MAS NMR. The derivatized Sepharose FT-IR spectra were compared with the epoxy-Sepharose spectrum in order to evaluate the immobilization of the affinity ligands on the matrix. FT-IR spectra of the (a) epoxy-Sepharose, (b) L-tyrosine support, (c) L-tyrosine-L-tyrosine support (d) and L-tyrosine-L-arginine support are presented in Figs. S1, S2, S3 and S4 of Supplementary material. All spectra revealed the existence of an additional weak adsorption band at 1516 cm^{-1} (Fig. S5) when compared with the epoxy-Sepharose spectrum, which could be due to the vibrations originated from the L-tyrosine side-chain, specifically the aromatic ring of L-tyrosine [21–22]. Since this band is inexistent in the epoxy-Sepharose spectrum it is a qualitative indication of an effective coupling between the affinity ligands and the Sepharose matrix. Other technique used to characterize the immobilization of the ligands onto the support was HR-MAS NMR which is a very useful technique for detection, identification and quantification of compounds bounded to a polymer support. The line broadening due to residual dipolar couplings and variations in bulk magnetic susceptibility is significantly reduced by the application of magic angle spinning combined with the swelling of the polymer [23–28].

Broad agarose background signals were attenuated by using a spin lock pulse with duration of 1.5 ms to destroy both transverse and longitudinal magnetization of the macromolecule (Fig. 2). The CPMG sequence allows the relatively immobile spins to relax and provides a high resolution spectrum of the more mobile components. The CPMG experiments for both Sepharose with linker (blank) and support of L-tyrosine (see Fig. 2a and b) show some attenuation in the broadening of polymer signals in comparison with the single pulse ^1H experiment (inset, respectively). In the bound sample the chemical shifts of the amino acid are clear, especially with the resonances between 7.37 and 6.95 ppm from tyrosine aromatic protons. The analysis of the DEPT-HSQC spectra for both the bound and unbound polymer samples allowed the identification in the unreacted sample of the terminal epoxide moiety with the methylene signals 2.98 and 2.79 ppm and the CH resonance at 3.33 ppm (Fig. 3). These resonances are absent from the spectrum of the tyrosine bound polymer. The CH and CH_2 from the linker resonate upfield at 1.30 and 1.79 ppm, respectively. These chemical shifts are common to both spectra and allow

Table 1
Equilibrium analysis data of pVAX1-*LacZ* isoforms in 10 mM HEPES acid at pH 7.4.

Isoforms	Ligands	$T = 10\,^{\circ}C$ K_D	$T = 25\,^{\circ}C$ K_D
sc	L-tyrosine	$1.89 \times 10^{-8} \pm 5.5 \times 10^{-9}$	–
	L-tyrosine-L-tyrosine	$1.12 \times 10^{-8} \pm 1.2 \times 10^{-9}$	$4.75 \times 10^{-9} \pm 3.58 \times 10^{-10}$
	L-tyrosine-L-arginine	$1.72 \times 10^{-8} \pm 8.4 \times 10^{-10}$	$1.97 \times 10^{-9} \pm 3.05 \times 10^{-10}$
ln	L-tyrosine	$5.96 \times 10^{-9} \pm 9.3 \times 10^{-10}$	$5.20 \times 10^{-9} \pm 4.6 \times 10^{-10}$
	L-tyrosine-L-tyrosine	$8.09 \times 10^{-9} \pm 7.6 \times 10^{-10}$	$4.44 \times 10^{-9} \pm 5.1 \times 10^{-10}$
	L-tyrosine-L-arginine	$1.58 \times 10^{-8} \pm 1.15 \times 10^{-9}$	$8.58 \times 10^{-9} \pm 5.83 \times 10^{-10}$
oc	L-tyrosine	–	–
	L-tyrosine-L-tyrosine	–	$1.18 \times 10^{-8} \pm 1.5 \times 10^{-7}$
	L-tyrosine-L-arginine	–	$6.9 \times 10^{-7} \pm 1.5 \times 10^{-6}$

the evaluation of the ligand density in the bound sample. Comparing the integration at 1.79 ppm due to two CH_2 units from the linker, with the resonances at 6.6–7.6 ppm from the ligand's aromatic protons (see Fig. S6 Supplementary material), we obtained 24% ligand density immobilized in the Sepharose support. The coupling between the amino acid and the polymer support was identified in the HR-MAS NOESY spectrum (Fig. 4), more specifically in the cross-correlation peaks between the aromatic protons from L-tyrosine, H2,6 and H3,5 at 7.38 and 6.96 ppm, respectively, and the methylene resonances from the linker at 3.69 ppm and 1.79. These experiments confirm that L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine-L-arginine are immobilized on Sepharose.

3.3. *SPR measurements of plasmids pVAX1-*LacZ *and pcDNA3-FLAG-p53 isoforms with immobilized* L-*tyrosine,* L-*tyrosine-*L-*tyrosine and* L-*tyrosine-*L-*arginine*

The apparent equilibrium binding constant (K_D) of each plasmid isoform to immobilized L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine-L-arginine were determined under conditions similar to those commonly used in chromatography experiments. The SPR binding profiles were square wave for all complexes indicating rapid association and dissociation rates making calculation of kinetics unfeasible (examples of sensorgrams and binding curves in Fig. 5. The SPR assays were conducted at two temperatures (10 °C and 25 °C) in order to evaluate the temperature effect on the binding of plasmid isoforms to amino acid surfaces. Also, to select the buffer that enhanced affinity, three solutions were analysed: 10 mM Tris–HCl pH 8, 10 mM and 100 mM HEPES acid at pH 7.4. This last concentration of HEPES acid was used to evaluate if increasing the concentration reduces the unspecific interactions. The experiments performed in Tris–HCl at 25 °C and 10 °C with both plasmids have not shown SPR signal. However, this result could be explored on chromatography assays, for example, to promote the elution of plasmid isoforms after retention in a column using these supports. Even with 100 mM of HEPES acid non-specific binding was detected with the reference surface (blocked dextran) with isoforms of both plasmids, making affinity calculations unfeasible. With 100 mM of HEPES acid, only binding responses were detected with ln pcDNA3-FLAG-p53 to L-tyrosine and L-tyrosine-L-tyrosine at both temperatures. The K_D values for L-tyrosine are $1.11 \times 10^{-8} \pm 6.4 \times 10^{-9}$ M and $1.02 \times 10^{-8} \pm 4.7 \times 10^{-9}$ M at 10 °C and 25 °C, respectively, and for L-tyrosine-L-tyrosine are $1.32 \times 10^{-8} \pm 3.8 \times 10^{-9}$ M and $1.49 \times 10^{-8} \pm 4.3 \times 10^{-9}$ M at 10 °C and 25 °C, respectively.

The optimal solution for the SPR experiments was 10 mM HEPES acid pH 7.4. The K_D values of pVAX1-*LacZ* and pcDNA3-FLAG-p53 to L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine-L-arginine are presented in Tables 1 and 2, respectively.

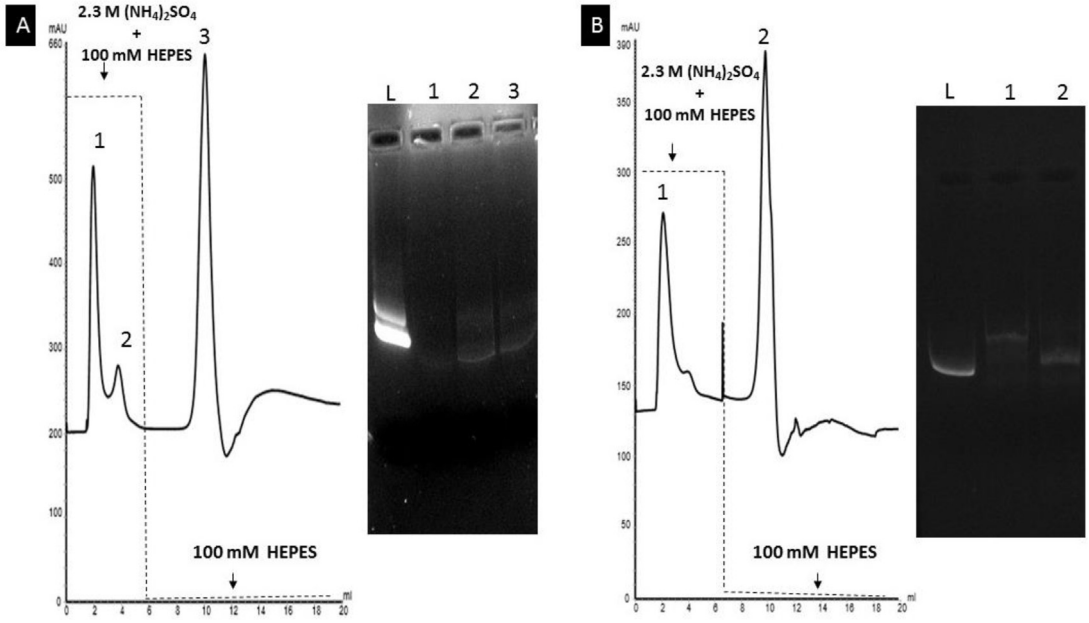

Fig. 7. Chromatographic profile and respective agarose gel electrophoresis of the plasmid isoforms separation from (A) pre-purified pVAX1-*LacZ* and (B) pre-purified p53 encoding samples (sc+oc) in the L-tyrosine Sepharose by using 5 °C. The elution was performed using a stepwise gradient of 2.3 M of $(NH_4)_2SO_4$ in 100 mM HEPES acid (pH 8.0) to 100 mM HEPES acid (pH 8.0), as represented by the dashed line. In the electrophoresis, lane L represents the pre-purified plasmid samples and lanes 1–3 represent the respective peaks of the chromatogram.

Table 2
Equilibrium analysis data of pcDNA3-FLAG-p53 isoforms in 10 mM HEPES acid at pH 7.4.

Isoforms	Ligands	$T = 10\,^{\circ}C$ K_D	$T = 25\,^{\circ}C$ K_D
sc	L-tyrosine	$3.53 \times 10^{-7} \pm 3.2 \times 10^{-8}$	$4.98 \times 10{-}7 \pm 9.5 \times 10^{-8}$
	L-tyrosine-L-tyrosine	$3.78 \times 10^{-7} \pm 6.0 \times 10^{-8}$	$3.74 \times 10^{-7} \pm 3.2 \times 10^{-8}$
	L-tyrosine-L-arginine	$5.39 \times 10^{-7} \pm 9.3 \times 10^{-8}$	$9.13 \times 10^{-7} \pm 9.3 \times 10^{-8}$
ln	L-tyrosine	–	–
	L-tyrosine-L-tyrosine	–	–
	L-tyrosine-L-arginine	–	–
oc	L-tyrosine	–	$3.5 \times 10^{-7} \pm 5.7 \times 10^{-8}$
	L-tyrosine-L-tyrosine	$7.66 \times 10^{-8} \pm 1.9 \times 10^{-8}$	$1.37 \times 10^{-7} \pm 5.1 \times 10^{-8}$
	L-tyrosine-L-arginine	$8.39 \times 10^{-8} \pm 2.3 \times 10^{-8}$	$2.75 \times 10^{-7} \pm 1.5 \times 10^{-7}$

Values for the equilibrium dissociation constants ranged from 1.97×10^{-9} to 6.9×10^{-7} M for pVAX1-*LacZ* isoforms and from 7.66×10^{-8} to 9.13×10^{-7} M for pcDNA3-FLAG-p53 both indicating high binding affinity. The highest affinity was found for sc pVAX1-*LacZ* with L-tyrosine-L-arginine at 25 °C ($K_D = 1.97 \times 10^{-9}$ M) while sc pcDNA3-FLAG-p53 showed the lowest binding affinity, suggesting that L-tyrosine-L-arginine could be a promising affinity ligand for the chromatographic assays. There were few differences in the K_D values for plasmid isoforms on amino acid and dipeptide surfaces. In general, the dipeptides had more affinity to both plasmid isoforms suggesting that the second amino acid improves the binding and decreases the unspecific interactions with the matrix. Supercoiled pVAX1-*LacZ* showed the highest affinity for the dipeptide surfaces, followed by ln isoform; oc had the lowest affinity for L-tyrosine-L-arginine surface. On the contrary, oc pcDNA3-FLAG-p53 showed the highest affinity with the dipeptide surfaces. For ln pcDNA3-FLAG-p53, no signal was detected as well as for oc pVAX1-*LacZ* at 10 °C.

We compared the effect of temperature on the binding and there were differences in equilibrium K_D values determined at 10 °C and 25 °C. Indeed by increasing the temperature to 25 °C, the binding of sc pVAX1-*LacZ* increased one order of magnitude, and oc did not have signal at 10 °C, suggesting that temperature can be manipulated in the chromatographic experiments to ascertain the best binding/elution conditions. Also, the K_D values determined at 25 °C are significantly different for sc pVAX1-*LacZ* (10^{-9} M) and oc (10^{-7} M) with the dipeptides (especially with L-tyrosine-L-arginine), indicating the stronger affinity of sc pDNA to these ligands. Moreover, for pcDNA3-FLAG-p53 there were differences in K_D magnitude order considering sc (10^{-7} M) and oc (10^{-8} M) at 10 °C, demonstrating a clear evidence of selectivity that could be favourable in the purification process.

The strongest interaction obtained between sc pVAX1-*LacZ* and L-tyrosine-L-arginine at $T = 25\,^{\circ}C$ could be attributed to the higher compactness degree that allow more interactions per surface area, inducing cooperative effects and increasing the stability of the interaction. Previous SPR studies also showed high affinity occurring between sc pVAX1-*LacZ* and L-arginine surface [9]. This can be explained by the fact that plasmid pVAX1-*LacZ* has higher guanine content when compared with pcDNA3-FLAG-p53, which could account for this favoured interaction.

In summary, this experimental data shows that sc pVAX1-*LacZ* could be discriminated by L-tyrosine-L-tyrosine and L-tyrosine-L-arginine by decreasing the temperature. Moreover the conditions tested in SPR biosensor revealed that HEPES acid can separate sc from oc and ln for both plasmids using L-tyrosine and L-tyrosine-L-tyrosine ligands. This data allowed us to get binding information to improve plasmid purification using the dipeptides derived from L-tyrosine as supports in chromatography.

3.4. STD experiments

The STD-NMR spectroscopy was used to distinguish and select which atoms of the 5′-mononucleotides are closer to the supports L-tyrosine Sepharose, L-tyrosine-L-tyrosine Sepharose and L-tyrosine-L-arginine Sepharose when the complex is formed (epitope mapping). The results can be interpreted as follows.

The 5′-TMP had the most prominent signal (100% saturation) with the three supports, followed by ribose protons $H_{2'}$ and $H_{4'}$ and $H_{5'}$ and $H_{5''}$ (≈ 50% saturation) with L-tyrosine Sepharose and L-tyrosine-L-tyrosine Sepharose. 5′GMP showed the strongest STD signal for H_8 (100% of saturation) and $H_{5'}$ and $H_{5''}$ protons (50 % saturation) with L-tyrosine-L-arginine, suggesting that L-arginine is involved in the interaction, which is in accordance with previous studies that describe L-arginine as "highly specific" for guanine over the other bases [29]. With the supports L-tyrosine and L-tyrosine-L-tyrosine only STD contacts were found for ribose protons $H_{1'}$ (100 % saturation), $H_{2'}$ and $H_{4'}$ (50%, 42% and 33%, respectively). STD results for 5′-CMP showed signals only with proton H_5 from cytosine with the three supports. Only moderate deoxyribose at position $H_{1'}$ was found with L-tyrosine; no response was detected for protons near the phosphate groups. Data for 5′-AMP showed the most intense signals for proton H_2 (100% saturation) of adenine with the three supports, followed by weaker contacts with H_8 and $H_{1'}$ from ribose.

3.5. Chromatographic experiments using L-tyrosine Sepharose

The ability of L-tyrosine to isolate the sc pDNA isoform from a p53 encoding plasmid native sample (sc + oc) was tested in order to evaluate the applicability of this new matrix to purify pDNA. To accomplish this, several buffers, salts and temperature conditions were tested and combined to achieve the best binding/elution conditions.

The first experiments were performed in order to understand the main interactions established between pDNA and L-tyrosine Sepharose. Thus, an increasing NaCl gradient was applied being verified that this gradient was not suitable to promote an effective retention of pDNA in the support (data not shown). Conversely, the pDNA retention was achieved by using ammonium sulfate in the binding buffer. The selective elution of the pDNA isoforms was then achieved by decreasing the salt concentration. Typically, the chromatographic experiments were performed by using a stepwise gradient starting with 2.3 M of $(NH_4)_2SO_4$ to elute unbound species and then removing the salt to recover the most retained species. These results also suggest that, despite the involvement of multiple non-covalent interactions, the hydrophobic interactions play an important role in pDNA retention on L-tyrosine Sepharose.

Moreover, and in accordance with the SPR experiments, different buffers were used in the chromatographic assays, namely 10 mM Tris–HCl and 100 mM of HEPES acid. In general, the results obtained were in agreement with the results achieved in the SPR experiments, since a more effective pDNA binding was achieved when using the HEPES acid.

The effect of temperature (5 °C, 10 °C and 25 °C) in pDNA retention and isoforms selectivity was also analyzed, as it is shown in Fig. 6. The results indicated that higher temperatures lead to

a decrease in the binding of pDNA to the L-tyrosine Sepharose. This result indicates that the pDNA binding is not exclusively due to hydrophobic interactions, which typically are favoured by increased temperatures. In fact, the biorecognition is achieved by the combination of multiple non-covalent elementary interactions, such as hydrogen bonds, van der Waals contacts and hydrophobic interactions.

Through the electrophoretic analysis of each peak it is noticed that sc pDNA is eluted when the ionic strength of the buffer is decreased. This result can be explained by the higher interaction of the sc pDNA isoform with the aromatic group of the L-tyrosine Sepharose, which will be mainly responsible for the hydrophobic interactions [30]. In addition, and although both pDNA isoforms are double-stranded, the sc p53-encoding plasmid can be specifically recognized by the L-tyrosine Sepharose due to the higher exposure degree of nucleotide bases, resultant from the torsion and supercoiling of this isoform [3]. As previously mentioned, the effect of the temperature on pDNA retention was also considered in the chromatographic studies. Actually, it was verified that this parameter has a significant influence on the pDNA binding behaviour. Regarding that, and using the same binding/elution gradient conditions, different temperatures were applied (5 °C, 10 °C and 25 °C) and, contrary to what is described to other chromatographic columns with a hydrophobic behaviour an increase of the temperature induced a decrease in the sc pDNA isoform retention and in the selectivity towards other contaminant pDNA conformations [4]. In order to evaluate the robustness of this support to purify different plasmids, the pVAX1-*LacZ* was also injected onto the column using the best binding/elution conditions achieved for the p53-encoding plasmid. The results obtained are shown in Fig. 7.

Comparing the results obtained from the purification of the pVAX1-*LacZ* and the p53-encoding plasmids, using the same conditions, it is possible to note that the selectivity and also the retention decrease in the experiment of pVAX1-*LacZ*. Although the similar molecular weight of both plasmids, the DNA sequence and base composition may significantly affect the interaction with chromatographic ligands. This result suggests that it is possible to purify different plasmids with the L-tyrosine Sepharose, but the gradient conditions should be slightly adjusted.

4. Conclusions

The dipeptides L-tyrosine-L-tyrosine and L-tyrosine-L-arginine were synthesised by protection of *N*-Boc-L-tyrosine and coupling with L-tyrosine or L-arginine methyl esters in the presence of DCC. The protected groups were removed by hydrolysis and acidification with TFA to give the afforded dipeptides. SPR biosensor was used to screen the promisor ligand for chromatographic assay and determine experimental conditions, like, buffer composition, concentration and temperature. The overall affinity with pVAX1-*LacZ* and pcDNA3-FLAG-p53 isoforms were high ($K_D \geq 10^{-7}$ M), namely for sc pVAX1-*LacZ* with L-tyrosine-L-arginine at $T = 25$ °C ($K_D = 1.97 \times 10^{-9}$ M) and oc pcDNA3-FLAG-p53 with the dipeptides ($K_D = 7.66 \times 10^{-8}$ with L-tyrosine-L-tyrosine and $8.39 \times 10^{-8} \pm 2.3 \times 10^{-8}$ with L-tyrosine-L-arginine) at $T = 10$ °C. Moreover the conditions tested in SPR biosensor suggested that HEPES 10 mM at pH 7.4 can separate sc from oc and ln by changing temperature and using L-tyrosine and L-tyrosine-L-tyrosine. The supports were then synthetized by coupling L-tyrosine, L-tyrosine-L-tyrosine and L-tyrosine-L-arginine to epoxy activated Sepharose and were characterized by HR-MAS NMR spectroscopy, demonstrating that the ligands are well immobilized on the matrix. The STD-NMR experiments showed that the interactions involved are non-covalent with prevalence of hydrophobic interactions with the majority of the 5′-mononucleotides. The overall yields of the synthesis were not high conditioning the preparation of the supports in large-scale for the chromatography experiments. Consequently the support selected for chromatographic assay was L-tyrosine Sepharose. The separation of native pVAX1-*LacZ* and pcDNA3-FLAG-p53 samples isoforms was achieved by decreasing the ammonium sulfate concentration in the eluent. The effect of temperature (5 °C, 10 °C and 25 °C) in plasmids retention and isoforms selectivity indicated that higher temperatures lead to a decrease in the binding of pDNA to the L-tyrosine Sepharose. These results suggest that it is possible to purify different plasmids with the L-tyrosine Sepharose, but the gradient conditions should be adjusted. Thus, the L-tyrosine derivatives supports can be a promising strategy to efficiently purify sc plasmid DNA with a therapeutic gene from a lysate sample.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.10.025.

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