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# Purification of supercoiled G-quadruplex pDNA for *in vitro* transcription

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ABSTRACT

The formation of G-quadruplex in G-rich regions of DNA can be induced by transcription and is formed within plasmid transcribed in *Escherichia coli*. The G-loops are more evidenced on supercoiled (sc) topology than on relaxed (oc) or linearized (ln) plasmid isoforms. Thus, the present work reports different purification strategies to efficiently purify the pPH600 sc isoform from other plasmid topologies and host contaminants present in a clarified *E. coli* lysate. To accomplish this purpose, two affinity supports, L-tyrosine-Sepharose and L-tryptophan-Sepharose were prepared by linking L-tyrosine and L-tryptophan onto epoxy-activated Sepharose CL-6B and were further characterized. The commercial support L-arginine Sepharose was also used to purify sc pPH600 since it has already been efficiently applied to separate sc isoforms of other plasmids using mild binding and elution conditions.

A first screen was performed to select the best support that allows to obtain highly pure sc pPH600 from a native sample (sc + oc). By comparing the binding/elution conditions of the three supports, L-tyrosine support showed the preeminent result in separation of two isoforms, allowing the total recovery of sc pPH600, using a decreasing $(NH_4)_2SO_4$ gradient in HEPES 100 mM at 10 °C. The purification of sc pPH600 directly from clarified *E. coli* lysate was achieved with the support L-tyrosine-Sepharose and the quality control analysis revealed that the level of *E. coli* impurities (other pDNA topologies, proteins, endotoxins, gDNA and RNA) present in the final sc pPH600 sample was in accordance with the guidelines of regulatory agencies. *In vitro* transcription was performed using the purified sc pDNA to induce G-quadruplex formation and it was confirmed by circular dichroism (CD) that the transcript adopted parallel G-quadruplex topology.

Overall, this work showed that sc pPH600 can be purified using L-tyrosine support and the transcript adopted parallel G-quadruplex topology.



## 1. Introduction

DNA has the potential to form a number of secondary structures, namely, G-quadruplex, which is a four-stranded structure stabilized by G-quartets planar arrays of four hydrogen-bonded guanines and coordinated with cations (especially $K^+$) within the central cavity [1]. Thus, G4 DNA is very stable once formed. These structures have been found in telomere ends and in promoter regions of oncogenes and some of these G4 structures have been identified as repressor elements to regulate gene transcription and translation in a wide variety of genes [2]. G-rich sequences are also found in immunoglobulin switch regions and are involved in the regulation of *Escherichia coli* (*E. coli*) plasmid replication [3]. It was found the formation of G-loops within a plasmid upon transcription of the S regions in living cells, in which one strand contains a RNA/DNA hybrid and the other strand contains G-quadruplex DNA [4]. G-loop formation depends on plasmid topology and it is more efficient on supercoiled (sc) templates [4]. The pPH600 plasmid (3562 bp) is derivate from the commercial plasmid pBluescript KS+ and encodes a sequence of a G-C rich sequence of immunoglobulin switch region Sγ3 of murine that can form G-quadruplex [3]. To assess G-loop formation on sc isoform, the pPH600 containing a G-rich coding strand needs to be transcribed *in vitro*. After biosynthesis of pPH600 in *E. coli*, the isolation and purification of sc isoform from lysate is still a huge challenge. Indeed, a special downstream processing is required since most of the molecules present in the lysate share analogous chemical, physical, and structural properties with plasmid: negative charge (RNA, genomic DNA (gDNA), and endotoxins) and molecular mass (gDNA and endotoxins) [5]. In what concerns to the preparation of a plasmid DNA (pDNA) product, the requirements of the regulatory agencies establish that host proteins and RNA must be undetectable, the level of gDNA should be lower than 2 ng gDNA/μg pDNA and endotoxins should not exceed 0.1 EU/μg pDNA, in the

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final product [6,7]. Moreover, the purification method should not comprise the use of organic reagents, mutagenic and toxic compounds and animal derived enzymes [8]. Also, during the manufacturing and recovery process, pDNA is subjected to several stresses that damage the sc pDNA, resulting in relaxed or open circular (oc) forms, and other variants such as linear, denatured or dimeric conformations [9]. Thus, for its final purification it is necessary to have a highly selective chromatographic process. Affinity chromatography is one of the most powerful techniques employed in the selective isolation of target molecules [10]. Chromatographic supports take advantage of the small differences between the sc pDNA and its natural impurities, on properties such as charge, size, hydrophobicity, accessibility of the nucleotide bases, and topological constraints imposed by supercoiling and/or affinity, permitting the design of selective pDNA purification strategies [10]. Several amino acids have already been efficiently applied as chromatographic ligands to separate plasmid isoforms, revealing the presence of a particular recognition of sc isoform [11–13]. The aromatic amino acids show unique and important properties due to its side chain [14]. Therefore, the properties of phenol and indole groups in L-tyrosine and L-tryptophan, respectively, can be used to evaluate the separation of sc pDNA from contaminants.

Here, taking advantage from these properties, we prepared two affinity matrices by immobilizing L-tyrosine and L-tryptophan onto epoxy-activated Sepharose CL-6B (see Fig. 1). The commercial support L-arginine-Sepharose was also used in the strategy to purify sc pPH600 since it has already been efficiently applied to separate this isoform using mild binding and elution conditions. Then, we investigated the retention behavior of pPH600 isoforms on these affinity matrices and discussed the factors that influence the separation efficiency. Also, purification of sc pPH600 directly from clarified *E. coli* lysate was achieved with the support L-tyrosine-Sepharose, and its quality and yield was further evaluated. Finally, the G-quadruplex formation after *in vitro* transcription of the purified sc pPH600 was assessed by circular dichroism (CD).

## 2. Material and methods

### 2.1. Materials

All solutions were freshly prepared with ultra-pure grade deionized water purified in MilliQ system from Millipore (Billerica, MA, USA). Solutions were filtered through a 0.20 μm pore size membrane (Whatman, Dassel, Germany) and degassed before use. Sodium chloride (NaCl) and HEPES Acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium sulfate ($(NH_4)_2SO_4$) and sodium carbonate were obtained from Panreac (Barcelona, Spain). Hyper Ladder I (Bioline, London, UK) was used as DNA molecular weight marker. GreenSafe Premium and NZY Maxiprep Kit were purchased from NZYTech (Lisbon, Portugal). The Maxima® SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher Scientific Inc.) was used for gDNA quantification.

### 2.2. Plasmid production and isolation

The plasmid pPH600 (3562 bp), kindly provided by Dr. N. Maizels, was amplified by a cell culture of *E. coli* DH5α. Growth was carried out overnight in shake flasks (250 rpm) at 37 °C using a terrific broth medium (12 g/L Tryptone, 24 g/L Yeast extract, 4 ml/L glycerol, 0.017 M $KH_2PO_4$ and 0.072 M $K_2HPO_4$) supplemented with 100 μg/mL of ampicillin. pDNA was purified using NZYTech Plasmid Maxiprep kit according to the supplier's protocol to obtain the native pDNA (sc and oc isoforms). The protocol is based on alkaline lysis procedure followed by the binding of pDNA to the NZYTech anion-exchange resin under appropriate low salt and pH conditions. After that, the impurities were removed by a medium salt wash. Finally, plasmid was eluted with high salt conditions and then is concentrated and desalted by isopropanol precipitation. The resulting pDNA preparations were dissolved in 10 mM Tris–HCl (pH 8.0) buffer. These samples were used for initial evaluation of chromatographic retention behavior. Crude extract of pDNA with all of contaminants (gDNA, RNA, proteins, endotoxins) was obtained through a modified alkaline lysis method described by Diogo and collaborators [15]. The pDNA samples concentration was measured with a NANOPhotometer (Implen). All the samples were stored at −20 °C.

### 2.3. Immobilization of L-tryptophan and L-tyrosine on Sepharose CL-6B

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 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 L-tyrosine and L-tryptophan. Each ligand was dissolved (L-tyrosine, 8.27 mmol, 1.5 g; L-tryptophan, 14.7 mmol, 3 g) 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. After that, the supports were washed extensively with a mixture of acetone-milli-Q water (1:9; 3:7; 5:5; 8:2 v/v) followed by washing with milli-Q water (3 × 100 mL).

### 2.4. HR-MAS NMR spectroscopy and sample preparation

Approximately 10 mg of the support was placed in a 4-mm MAS zirconia rotor (50 μL). 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 hardware. Spectra were processed using Bruker Topspin 3.1. 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 matrix, experiments were acquired in 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. 2D TOCSY experiments were acquired by MLEV-17 pulse sequence with 75 ms mixing time in 8 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.

### 2.5. Preparative chromatography

The three affinity supports, L-arginine-Sepharose 4B gel (commercial), L-tryptophan-Sepharose CL-6B and L-tyrosine-Sepharose CL-6B were packed individually using 2.5 mL of each support in 10 mm diameter × 35 mm long/bed height columns. All preparative chromatographic experiments using the three supports were performed in Akta Pure 25 L controlled by UNICORN software, version 6.3, at a flow-rate of 1 mL/min. The absorbance was continuously measured at 260 nm. According to each experiment the desirable temperature of column was maintained (4, 10, 15 and 25 °C), connecting a water-jacket column to circulate water bath. After purification, the fractions were pooled, according to the obtained chromatograms, concentrated and desalted with Vivaspin