Development of a method for the purification of minicircles

Cláudia Alves

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Abstract

Gene therapy and DNA vaccination are fields of study in which researchers have a high interest, being registered an ongoing increase in reported clinical trials. Plasmid DNA has been widely applied as a non-viral delivery vector in this context, but with the disadvantage of low transfection efficiency and transgene expression. The pursue of safer and more efficient delivery vectors resulted in the development of the minicircle DNA production system, an *in vivo* recombination process in *Escherichia coli* in which a vector comprising only an eukaryotic sequence is obtained by excision from a replicative and prokaryotic sequence-containing plasmid. This results in the formation, during bacterial cellular growth, of a therapeutically valuable DNA molecule – the minicircle – and of a miniplasmid comprised of the prokaryotic sequence. The high similarity in physicochemical properties of miniplasmids and minicircles hinders the development of an efficient purification method for separation and recovery of minicircle molecules.

The main focus of this work was the development of an efficient, simple, reproducible and scalable method for minicircle purification. With this purpose, differences in hydrophobicity were explored by modifying the structure of miniplasmids by resorting to a nicking enzyme. As a consequence, supercoiled miniplasmids were converted into their open circular forms and a single hydrophobic interaction chromatography method was successfully employed to isolate the therapeutically valuable supercoiled minicircle molecules from miniplasmid and RNA. Method robustness and reproducibility were verified. Future work must focus on process monitoring and product quality control.

Keywords: gene therapy, minicircle, enzymatic restriction, DNA-nicking enzyme, hydrophobic interaction chromatography

Abbreviations: bp – base pair; CV – column volume; HIC – hydrophobic interaction chromatography; MC – minicircle; MP – miniplasmid; oc – open circular; pDNA – plasmid DNA; PP – parental plasmid; sc – supercoiled.

1. Introduction

The concept of gene therapy emerged around 1970, along with the successful isolation of specific bacterial genes from DNA and complete synthesis of the gene for yeast alanine transfer RNA [1]. Currently, gene therapy is defined as the introduction of nucleic acids into cells, by utilization of a delivery vector in an *in vivo* or *ex vivo* approach, with the aim of treating, curing or preventing a disorder by modification of endogenous gene expression [2, 3].

The vectors developed with this objective – viral and non-viral vectors – should be able to safely deliver the gene of interest into cells for transcription, while preserving the gene from degradation [4].

Comparatively to their viral counterparts, non-viral vectors present several advantages, especially in terms of safety. The direct injection of naked plasmid DNA (pDNA) into tissues (e.g. liver, skeletal and heart muscles) or into a vessel of the systemic circuit is the safest and simplest method of non-viral transfection [5].

Minicircles (MC) are a type of non-viral, double-stranded DNA vectors, which comprise only a eukaryotic expression cassette. Minicircles are produced *in vivo* in a bacterial host upon induction of intramolecular site-specific recombination of a parental plasmid (PP), that also originates a molecule containing all the prokaryotic backbone – the miniplasmid (MP) [6, 7, 8]. Thus, during the recombination event, two molecules of almost identical size are produced – a minicircle (MC) and a miniplasmid (MP) – being both obtained in a supercoiled state. This renders the separation of these molecules a very difficult process [9].

The first methods for minicircle purification relied on *in vitro* restriction for miniplasmid linearization followed by caesium chloride gradient centrifugation [6, 10, 11] or agarose gel electrophoresis [11] for MC isolation. However, these methods are neither suitable for large-scale production or purification of clinical grade material [9], for which several guidelines are recommended

by regulatory agencies, such as the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [12].

Several approaches have been proposed to obtain pure MCs, namely *in vivo* restriction [13, 14] and affinity-based chromatography [8].

The purification method developed in this work relies on the application of hydrophobic interaction chromatography (HIC). This type of chromatography explores differences in the hydrophobic characteristics of biomolecules to be separated and purified. When applied to purify plasmid DNA, HIC is typically conducted at 1.5 M ammonium sulfate to separate pDNA molecules from more hydrophobic impurities (RNA, denatured gDNA, oligonucleotides and endotoxins) [15]. The higher hydrophobic character of these impurities when compared to pDNA is due to the single stranded nature of RNA, denatured gDNA and oligonucleotides, which results from a higher exposure of the hydrophobic bases. This results in a stronger interaction of impurities with the hydrophobic matrix. As for endotoxins, its hydrophobicity is explained by the presence of lipid A [12]. Although pDNA is naturally a hydrophilic molecule due to shielding of hydrophobic bases inside the double helix and exposure of sugar-phosphate chains that are available to establish hydrogen bonds with surrounding water molecules, this character can be to some extent modified by an increase on ionic strength of a kosmotropic salt (such as ammonium sulfate) on the mobile phase [12]. These salts are strongly hydrated and present stabilizing and salting-out effect on proteins and macromolecules [16]. Several reports have attested the feasibility of using hydrophobic interaction chromatography (HIC) with a mobile phase with high content of a kosmotropic salt (>2 M) to separate supercoiled (sc) pDNA from open circular (oc) forms [15-17]. Experience has shown that recovery of sc pDNA is favored by an increase of kosmotropic salt concentration in the mobile phase [17].

The goal of this work was to establish an efficient, simple, reproducible and scalable method for MC purification by exploring differences in hydrophobicity. To facilitate HIC purification of sc MC, the topology of the MP species was enzymatically modified. In particular, the nicking enzyme Nb.BbvCI [18] was used to convert sc MP molecules to the respective oc isoform by nicking one of the DNA strands. This enzyme, obtained by mutation of one catalytic subunit (R2) of the heterodimeric Rb.BbvCI from *B. brevis*, acts on DNA substrates within a seven-base pair asymmetric recognition sequence by introducing a single cut in one of the DNA chains (CCTCAGC/GCTGAGG→CCTCAGC/GC^TGAGG) rather than cleaving the duplex [18]. As MC species do not contain the recognition site for Nb.BbvCI, sc and oc MC remain unaltered in the presence of the enzyme. The chromatographic conditions for HIC (flow-rate, step-wise length elution and the mobile phase salt concentration) were studied and optimized. Finally, method robustness and reproducibility were evaluated by purifying different MCs and pDNA, and performing a loading study.

2. Materials and methods

2.1 Materials

Phenyl Sepharose 6 Fast Flow (High Sub) was obtained from GE Healthcare. Restriction enzymes SacII and XhoI were from Promega. Restriction enzymes Bsp1407I (isoschizomer of BsrGI) and PvuII were from ThermoScientific. Nicking enzyme Nb.BbvCI was from New England Biolabs. All salts used were of analytical grade.

2.2 Bacterial strain and plasmid

The parental plasmid was produced in *E. coli* BW2P, a strain constructed by disrupting the endA gene via the insertion of a single copy of the P_{BAD}/*araC*-RBS-*parA* cassette, with an optimized ribosome binding site (RBS), into the commercial BW27783 *E. coli* strain chromosome (purchased from The Coli Genetic Stock Center at Yale). After successful cassette insertion, the construction of BW2P was finalized with knockout of recA gene [19]. The 4563 bp parental plasmid (pMINILi-CMV-VEGF-GFP) was constructed ([20], in preparation) by a series of modifications of pVAX1GFP [19].

2.3 Cellular growth and minicircle production

A pre-inoculum was prepared in 15 mL Falcon tubes (VWR) with 5 mL of LB (Luria-Bertani, Sigma) supplemented with 30 µg/mL kanamycin (AMRESCO) and 0.5% (w/v) glucose, after which was inoculated with a loop of frozen BW2P cells (previously transformed by heat shock with the pMINILi-CMV-VEGF-GFP plasmid) and incubated overnight at 37°C and 250 rpm. Next, an inoculum was prepared with 30 mL of LB in 100 mL shake flasks, supplemented with 30 µg/mL kanamycin and 0.5% (w/v) glucose, and seeded with the appropriate volume of pre-inoculum for OD_{600nm}=0.1. Cultures were incubated at 37°C and 250 rpm until an $OD_{600nm} \approx 2.5$ was reached. Cellular growth was performed at 37°C and 250 rpm with 250 mL of LB in 2 L shake flasks, supplemented with 30 µg/mL of kanamycin, after

inoculation up to an OD_{600nm} of 0.1. Cells were incubated for about 3 hours to reach late exponential phase ($OD_{600nm} \approx 2.5$). At this moment, recombination was induced by addition of 0.01% (w/v) L-(+)arabinose (Merck) directly into the medium. Samples of 2 mL of culture were collected at 0, 1 and 2 hours of recombination, centrifuged and stored at -20°C until further analysis of recombination efficiency. After 2 hours of recombination, cell growth was suspended and the medium was centrifuged for 15 min at 6000 g and 4°C in a Sorvall RC 6 centrifuge. Cell pellets were stored at -20°C until further processing.

2.4 Gel electrophoresis

Agarose gels were prepared with 1% (w/v) agarose (SeaKem LE agarose) in TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8) and loaded with samples mixed with a 6X loading buffer (40% (w/v) sucrose, 0.25% (w/v) bromophenol blue), using NZYDNA ladder III (NZYTech) as molecular weight marker. Horizontal gel electrophoresis was performed with 1% TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8) at 100 V for 60 minutes and 120 V for 90 minutes for small or larger gels, respectively. Gels were stained in an ethidium bromide solution (0.4 µg/mL) and images were obtained with an Eagle Eye II gel documentation system (Stratagene).

2.5 Analysis of recombination efficiency by densitometry

Samples of 2 mL of culture, collected after 2 hours of recombination, were processed with the High Pure Plasmid Isolation Kit (Roche) following the manufacture's protocol. A volume equivalent to 500 ng of plasmid samples was digested for 2 hours at 37°C with 0.2 µL of SacII– a restriction enzyme for which only the parental plasmid and miniplasmid present a recognition site (localized in the 3.9 kbp region of the parental plasmid). Digested samples were subjected to gel electrophoresis, as described in section 2.4. Recombination efficiency (Re) was determined by densitometry analysis of the band intensities of parental plasmid and linearized miniplasmid, using the ImageJ software and equation 1, where A_{PP} and A_{MP} are, respectively, the areas of the band intensities of parental plasmid and linearized miniplasmid and r_m is the molar ratio of parental plasmid to miniplasmid [8].

$$\mathrm{Re}\ (\%) = \left(1 - \frac{\mathrm{A_{PP}}}{\mathrm{A_{PP}} + r_m \mathrm{A_{MP}}}\right) \times 100 \qquad \textbf{(1)}$$

2.6 Primary purification

Cells were lysed using a modification of the alkaline method described by Birnboim *et al.* [21]. The volume of buffer P1 was calculated to concentrate the solution to an OD_{600nm}=60, taking into account the final OD_{600nm} and volume of the respective cellular growth (V_{cg}), following the equation 2. In the subsequent steps an equal volume of buffers P2 and P3 was used.

$$\mathrm{V_{P1}} = \mathrm{V_{P2}} = \mathrm{V_{P3}} = \frac{\mathrm{OD_{600nm}} \times \mathrm{V_{cg}}}{60} \qquad \textbf{(2)}$$

The cells harvested at the end of cellular growth were resuspended in P1 buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA base, pH 8) resorting to vortex. Alkaline lysis was performed adding the appropriate volume of P2 buffer (0.2 N NaOH, 1%(m/v) SDS). The mixture was gently homogenized and left to rest at room temperature for 10 minutes. To stop cell lysis and neutralize the mixture, the appropriate volume of P3 (5 M potassium acetate, acetic acid) was added. After gentle homogenization, the tubes were placed on ice and left to rest for 10 minutes. The mixture was then centrifuged at 4°C for 30 min at 13000 rpm in a Sorvall RC 6 centrifuge (SS-34 rotor) to remove cell debris, genomic DNA and proteins. The supernatant was transferred into new tubes and subjected to an additional centrifugation. Plasmid in the supernatant was precipitated after a 2 hours incubation period at -20°C with 0.7 volumes of pure isopropanol in 50 mL Falcon tubes (VWR). After this period, the mixture was centrifuged at 12000 rpm and 4°C for 30 min in an Eppendorf centrifuge 5810R. After centrifugation, the supernatant was discharged and the pellets were left to dry overnight at 4°C. Next morning, the pellets were resuspended in 10 mM Tris-HCl, pH 8 (1 mL for each tube) and pooled in a single 50 mL Falcon tube. The DNA rich solution was conditioned to 2.5 M of ammonium sulfate by dissolution of appropriate amount of salt, homogenized and left to rest for 15 min on ice. Precipitated proteins were removed by centrifugation in an Eppendorf centrifuge 5417R for 30 min at 13000 rpm and 4°C. The supernatant was collected into a 15 mL Falcon tube and diluted with an equal volume of MilliQ water.

2.7 Diafiltration and concentration

To perform desalting, solutions obtained from 1 litter of cellular growth. were subjected to centrifugation at 4000 g and 4°C (Eppendorf centrifuge 5810R) in a single 2 mL Amicon with a membrane cut-off of 30kDa (Amicon Ultra-2 30K from Millipore). Retained nucleic acids were

diafiltered with MilliQ water, following manufacturer instructions for diafiltration and concentration procedures. To guarantee maximum recovery of DNA, a final step of washing with 2 mL MilliQ water and concentrate recovery were performed.

2.8 Miniplasmid enzymatic digestion

Concentrated salt-free nucleic acids were digested with Nb.BbvCI from New England Biolabs (10 U per 700 µg of nucleic acids) to convert sc MP in its oc form. The mixtures were incubated at 37°C overnight.

2.9 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography was performed using a column with inner diameter of 10 mm (Tricorn 10/100, GE Healthcare) packed with 10 mL of Phenyl Sepharose 6 Fast Flow resin (GE Healthcare) connected to an ÄKTApurifier100 system (GE Amersham) under the control of UNICORN 5.11 software (GE). The mobile phase consisted on mixtures of buffer A (2.2 M Ammonium Sulfate in 10 mM Tris-HCl pH8) and buffer B (10 mM Tris-HCl, pH 8) prepared by the system on a 2 mL Mixer M-925 Mixing Chamber (GE Amersham). The absorbance of the eluate was continuously measured at 254 nm by a UV detector positioned after the column outlet.

The purification of MCs was performed using a step gradient at 2 mL/min. The column was equilibrated with 3 column volumes (CV) of buffer 17% B (≈ 204 mS/cm) and 1 mL of Nb.BbvCI digested samples were conditioned with 2.5 M ammonium sulfate by dissolution of the appropriate amount of salt and manually injected to a 1 mL loop on the system. After column equilibration, the sample was injected into the column by washing the loop with 3 mL of buffer 17% B (≈ 204 mS/cm). All unbound material was washed out of the column in 4 CV at 17% B (≈ 204 mS/cm). The first and second steps were performed at, respectively, 35% B (≈ 173 mS/cm) and 100% B (≈ 2 mS/cm) during 2 CV each. The eluate was collected along all chromatographic run in 1.5 mL fractions to 2 mL eppendorf tubes positioned on a Frac-920 collector (GE Amersham). In between runs, the column was washed with MilliQ water until conductivity inferior to 0.08 mS/cm was reached and no variation on controlled parameters was observed.

A cleaning in place was performed after each 5 runs with 2 CV of 1 M NaOH followed by 3 CV of MilliQ water, 2 CV of ethanol 20% (v/v) and 3 CV of MilliQ water.

2.10 Micro-dialysis

For identification of the purified samples, 200 µL of peak fractions were collected into 0.5 mL eppendorf tubes. The tube caps were removed and the top of each tube was covered with a dialysis membrane (OrDial D14, Orange Scientific) that was kept in place with a rubber band. The tubes were inverted and placed floating in a beaker with 2 L of MilliQ water for desalting for 48 hours at 4°C with agitation.

2.11 Restriction enzyme mapping for DNA identification

To confirm the identity of purified forms, 15 µL of desalted samples were digested with 0.3 µL of XhoI (a restriction enzyme with one restriction site on the MC segment), BsrGI (a restriction enzyme with two restriction sites on the MC segment) and PvuII (a restriction enzyme with six restriction sites on the MP segment) in total reaction volumes of 20 µL for 2 hours at 37°C and analyzed by gel electrophoresis.

3. Results

3.1 Cell growth and recombination

For cellular growth and PP replication, BW2P cells harboring the pMINILi-CMV-VEGF-GFP plasmid were cultured in the presence of 0.5% (w/v) glucose [8]. The inclusion of glucose at this phase of the production process is critical to repress the P_{BAD}/*araC* arabinose promoter by reduction of 3',5'-cyclic AMP, thus preventing leaky expression of ParA resolvase and premature recombination of the PP before arabinose induction [8, 22]. These cultures were then used to inoculate 250 mL of LB in 2 L shake flasks, with a starting OD_{600nm} of 0.1, and cellular growth was allowed to proceed until late exponential phase was reached (OD_{600nm}≈2.5). At this point, ParA resolvase expression was induced by addition of 0.01% (w/v) of L-arabinose directly to the growth medium. The ParA resolvase catalyzes the intramolecular recombination between the two MRS on the pMINILi-CMV-VEGF-GFP PP, resulting in two circular DNA molecules: a MP containing the bacterial backbone and a MC containing the eukaryotic expression cassette with a multimer resolution site of the *parA* resolvase system (MRS), the cytomegalovirus immediate early promoter (CMV), the vascular endothelial growth factor and green fluorescent protein genes (VEGF and GFP, respectively) and the bovine growth hormone polyadenylation signal (BGH).

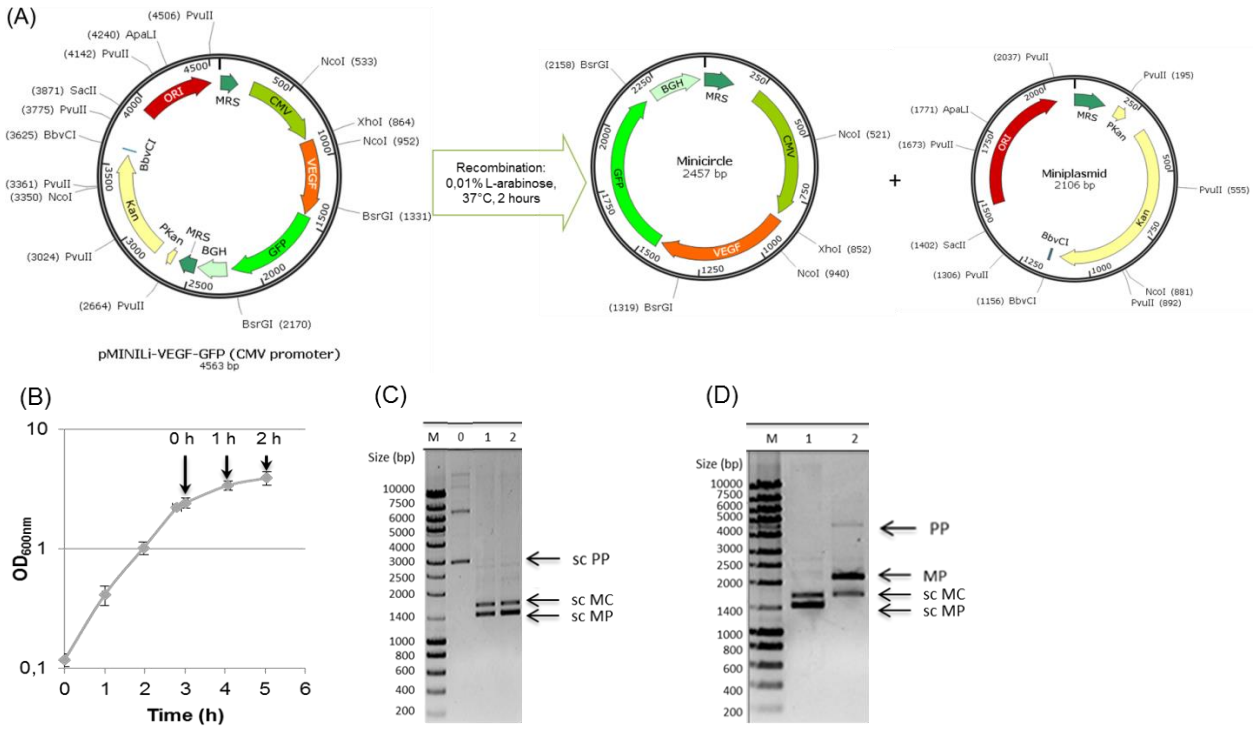

Figure 1: (A) Schematic representation of the *in vivo* recombination event mediated by expression of ParA resolvase upon induction with 0.01% of L-arabinose. **(B)** Average growth curve of *E. coli* BW2P harboring pMINILi-CMV-VEGF-GFP in LB (37°C, 250 rpm). Black arrows indicate time of recombination after induction with 0.01%(w/v) L-arabinose. Average growth curve was obtained from eight independent cell cultures, by calculation of the mean time and mean OD_{600nm} at which each point was collected. Error bars are indicative of the standard deviation obtained for the mean time (horizontal bars) and mean OD_{600nm} (vertical bars). **(C)** Gel electrophoresis analysis of 500 ng DNA samples obtained after 0 hours (lane 0), 1 hour (lane 1) and 2 hours (lane 2) of recombination. Lane M – molecular weight marker. sc PP – supercoiled parental plasmid (4563 bp); sc MC – supercoiled minicircle (2457 bp); sc MP – supercoiled miniplasmid (2106 bp). **(D)** Agarose gel electrophoresis analysis of pDNA samples collected after 2 hours of recombination. Lane M – molecular weight marker; lane 1 – non-digested sample (500 ng); lane 2 – 500 ng sample digested with SacII. PP – linearized parental plasmid; MP – linearized miniplasmid; sc MC – supercoiled minicircle; sc MP – supercoiled miniplasmid.

Cells were allowed to grow for two additional hours after induction (Figure 1, (B)) to promote complete recombination of PP, originating a 2457 bp minicircle that carries the eukaryotic cassette and a 2106 bp miniplasmid with the prokaryotic backbone (Figure 1, (A)).

Samples collected at 0, 1 and 2 hours of recombination were purified with a commercial pDNA purification kit (High Pure Plasmid Isolation Kit, Roche) and analyzed by gel electrophoresis (Figure 1, (C)). Observing lane 0 (0 hours of recombination) it is clear that PP did not undergo recombination before arabinose induction. In lanes 1 and 2 (after 1 and 2 hours of recombination induction, respectively), production of MP and MC is detected by the presence of the respective bands of sc isoforms (sc MP at 1.5 kbp and sc MC at 1.7 kbp). Recombination efficiency was determined by densitometry analysis of samples with 2 h of recombination digested with SacII (Figure 1, (D)) – a restriction enzyme that only has a restriction site in the MP segment of PP (at 3871 bp). Analysis of 30 independent cell cultures showed that the efficiency of recombination after 2 hours was 96.6% ± 2.6%. Considering samples collected after 2 hours of recombination of 20 independent cultures and purified with the same kit, the mass of plasmid DNA determined to be produced was 6.6 ± 1.7 mg per liter of bacterial growth. A decrease on the MC to MP ratio from 0.7 ± 0.2 after 1 hour of recombination to approximately half of this value (0.4 ± 0.1) at the end of cellular growth was also determined.

3.2 Hydrophobic interaction chromatography

The purification method developed explores differences of hydrophobicity between sc MC and oc MP. To allow the desired separation, samples of DNA species (MC, MP and un-recombined PP) pre-purified by alkaline lysis and precipitation with

isopropanol and ammonium sulfate were digested with Nb.BbvCI [18] before column loading. This DNA-nicking enzyme acts on the MP sequence (at 3625 bp of the PP, Figure 1 (A)), modifying its topology by creation of a nick in one of the strains and thus converting sc MP and any un-recombined sc PP into the corresponding oc isoforms.

3.2.1 Screening of chromatographic parameters

Method establishment involved a first study to determine optimal flow-rate and buffer conditions, which would result in successful separation of oc and sc DNA molecules. This was accomplished by applying linear gradients with different lengths (5 and 15 column volumes), three flow-rates (1.5, 2 and 2.5 mL/min) and initial concentrations of an ammonium sulfate containing buffer (2 M and 2.2 M ammonium sulfate in 10 mM Tris-HCl pH 8). Samples were injected into the column (1 mL) at 2.5 M ammonium sulfate without previous digestion with Nb.BbvCI. The strategy that resulted in the best separation and isolation of sc MC, and which was used for method optimization, employed a 15 column volumes (CV) linear gradient at 2 mL/min, using as buffers 2.2 M ammonium sulfate in 10 mM Tris-HCl pH8 (buffer A) and 10 mM Tris-HCl pH 8 (buffer B). Chromatographic runs using this linear gradient resulted in four peaks: oc isoforms were eluted in the first two peaks, sc DNA molecules in the third peak and finally RNA in the fourth peak. A stepwise method was then constructed by selecting percentages of buffer B at beginning of second peak (14% B) and at the maximum of third peak (28% B). A third step at 100% B was used to elute all strongly bounded species, including RNA. At this stage, samples were digested with Nb.BbvCI before loading into the column. This digestion was performed to convert all sc MP and un-recombined sc PP in the corresponding oc forms and thus allow the exploration of differences on hydrophobicity between sc MC, and oc MP and PP species. For successful digestion, samples had to be diafiltered and concentrated in MilliQ water after ammonium sulfate precipitation. After digestion, samples were again conditioned up to 2.5 M ammonium sulfate by salt dissolution and 1 mL was injected into the column.

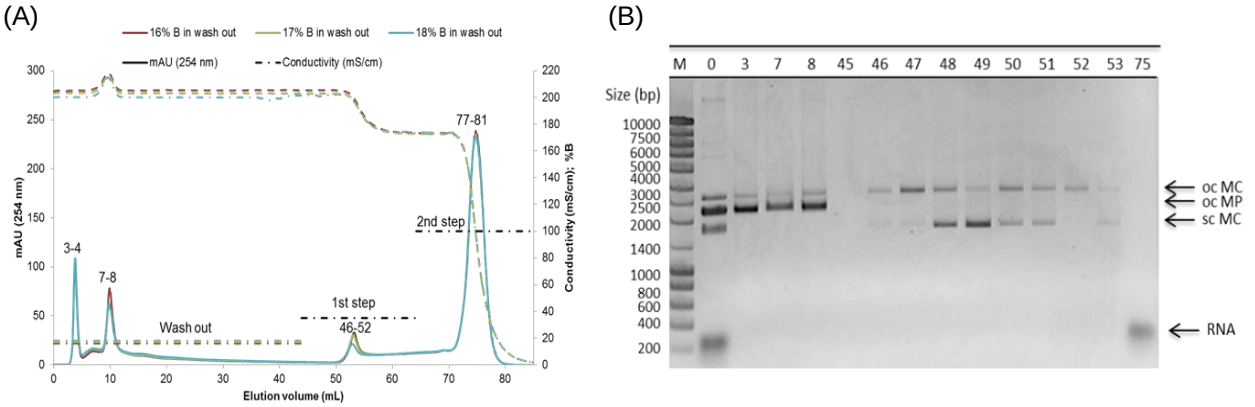

Figure 2: (A) Optimization of hydrophobic interaction chromatography separation of covalently-closed DNA isoforms using a stepwise gradient elution scheme. 1 mL of DNA samples obtained after recombination, alkaline lysis and pre-purification with isopropanol and ammonium sulfate precipitation were digested with Nb.BbvCI and loaded onto a phenyl-Sepharose column (100 mm x 10 mm). Buffer A: 2.2 M ammonium sulfate in 10 mM Tris-HCl pH 8. Buffer B: 10 mM Tris-HCl pH 8. Washing of unbound material was performed at 2 mL/min with three different percentages of buffer B for 4 CV, followed by a step-wise elution with a first step at 35% B (≈ 173 mS/cm) for 2 CV and a second step at 100% B (≈ 2 mS/cm) for 2 CV. Three percentages of buffer B during washing were studied: 16% B (red), 17% (green), 18% B (blue). Numbers over peaks are indicative of the respective peak range fractions. Continuous line – mAU (254 nm); dashed line – conductivity (mS/cm); dashed dotted line – percentage of buffer B. **(B)** Agarose gel analysis of 24 µL fractions collected during the chromatographic run with washing of unbound material at 17% B (Figure 2 (A), green). Lane M – molecular weight marker; lane 0 – column feed; numbered lanes correspond to fractions collected during chromatographic run (lane 3 – first peak; lanes 7 to 8 – second peak; lanes 46 to 52 – third peak; lane 75 – fourth peak).oc MC – open circular minicircle; oc MP – open circular miniplasmid; sc MC – supercoiled minicircle; sc MP – supercoiled miniplasmid.

Method was optimized by screening the appropriate percentages of buffer B that would allow best DNA isoforms separation. As oc and sc DNA isoforms were eluted at, respectively, 14% and 28% B, for method optimization, three percentages of buffer B were tested for washing unbound material (16, 17 and 18% B) and for first step (32, 35 and 40% B) in chromatographic runs

conducted at 2 mL/min using buffers A and B as mobile phase. Analyzing eluted fractions, it was concluded that best MC isolation was achieved when using a first step at 35% B for 2 CV (for MC elution) and a final step at 100% B for 2 CV in which RNA was eluted. Percentage of buffer B during washing of unbound material was set at 17% as no difference on elution peaks were observed between all three percentages tested (see Figure 2, (A)). At 17% B, elution of sc MC was not observed (see agarose gel analysis in Figure 2, lanes 3 to 8 in gel (B)). However, when the percentage of B was increased to 35%, MC was eluted free from MP and RNA molecules (lanes 46 to 52). A higher percentage of B during washing (18% B) resulted in loss of sc MC in the flowthrough (results not shown).

An interesting aspect is the high similarity between the chromatograms – all peaks are superimposed in the three cases studied with minimum differences in the maximum absorbance (see Figure 2, (A)). These results suggest a strong robustness of the method developed. In view of the results obtained, the method used from this stage on involved an initial washing of unbound material at 17% of buffer B (≈ 204 mS/cm), followed by a first step at 35% B (≈ 173 mS/cm) for MC elution and a final step at 100% B (≈ 2 mS/cm) to remove RNA and other strongly bound molecules from the matrix.

3.2.2 Confirmation of eluted DNA forms

To confirm if the species eluted in each peak were the desired DNA forms, samples collected during HIC were dialyzed and digested with restriction enzymes chosen according to the MC and MP maps (Figure 1, (A)). With this purpose, three enzymes were chosen: (1) PvuII, which has no restriction sites on the MC and cuts the MP into six fragments (264, 337, 360, 364. 367 and 414 bp), (2) XhoI, which only has one restriction site on the MC and results in its linearization (2457 bp) and (3) BsrGI, which has restriction sites only on the MC that result in two fragments with 839 bp and 1618 bp. Samples from column feed sample, the first chromatographic peak (at 17% B) and from the peak in which sc MC is eluted (at 35% B) were digested. The results obtained are presented in Figure 3.

Observation of the lanes corresponding to the column feed (1 to 4) shows that PvuII only digests the band appointed as oc MP (lane 2 at ≈2.4 kbp) resulting in fragments of low molecular weight, whilst the other two bands (1.7 and 2.6 kbp, approximately), previously identified as oc and sc MC, remain unchanged. Regarding digestions with XhoI and BsrGI (lanes 3 and 4, respectively), it is possible to confirm that there is no enzymatic activity on the MP (≈2.4 kbp) and that the bands at 1.7 and 2.6 kbp are digested. In the case of digestion with XhoI (lane 3), a new band at approximately 2.5 kbp appears which corresponds to linearized MC. In the case of digestion with BsrGI, two new bands appear at 843 and 1618 bp which correspond to the expected MC fragments. The bands at 200 bp in lanes 1 to 4 correspond to the presence of RNA. Similar observations are made when digesting DNA species from fractions of the first (lanes 5 to 8) and third (lanes 9 to 12) peaks: digestions with PvuII only affect the MP, whereas digestions with XhoI and BsrGI only affect oc and sc MC. These results confirm that only MC is being eluted in the step at 35% B.

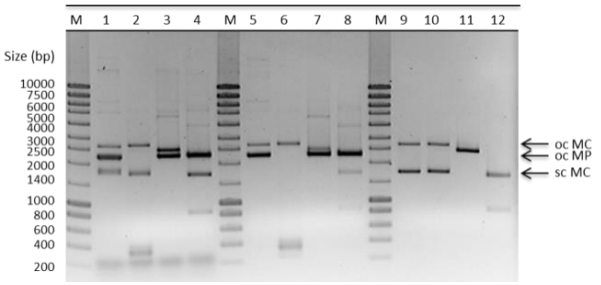

Figure 3: Restriction analysis of DNA species present in the column feed and in eluted fractions from the first (oc MC and oc MP) and third peaks (MC) obtained with the HIC method developed. Column feed (lane 1) was digested with PvuII (lane 2), XhoI (lane 3) and BsrGI (lane 4). A fraction from the first peak (lane 5) was digested with PvuII (lane 6), XhoI (lane 7) and BsrGI (lane 8). A fraction from the third peak (lane 9) was digested with PvuII (lane 10), XhoI (lane 11) and BsrGI (lane 12). Lanes M – molecular weight marker.

3.2.3 Method robustness evaluation

The ability of the method developed to separate oc and sc forms was studied with two other MCs (produced and pre-purified by Brito, L. [20], in preparation) and with the plasmid pVAX-LacZ (6.05 kbp).

Similarly to pMINILi-CMV-VEGF-GFP, both MCs used at this stage harbor a restriction site for Nb.BbvCI. After recombination, pMINILi-hEF1α-VEGF-GFP (4476 bp) originates a MP with 2106 bp and a MC with 2370 bp, while pMINILi-hEF1α(CpGfree)-VEGF-GFP (4125 bp) originates a MP with 2106 bp and a MC with 2019 bp. These MCs were recovered and pre-purified before digestion with Nb.BbvCI, with a commercial kit (NucleoBond Xtra Midi EF, MACHEREY-NAGEL) which relies on alkaline lysis for cell disruption and on an anion-exchange column for MC

pre-purification. Furthermore, this kit makes use of a RNase containing buffer, thus resulting in samples containing both oc and sc isoforms of MP and MC that are free from RNA. The use of RNase explains the absence of a peak during the step at 100% B in the respective chromatograms (red and blue lines in Figure 4 (A)). Regarding the plasmid pVAX-LacZ (green line in Figure 4 (A)), a sample containing oc and sc pDNA isoforms was obtained by alkaline lysis and precipitation with isopropanol and ammonium sulfate. Digestion with Nb.BbvCI was not performed on this sample.

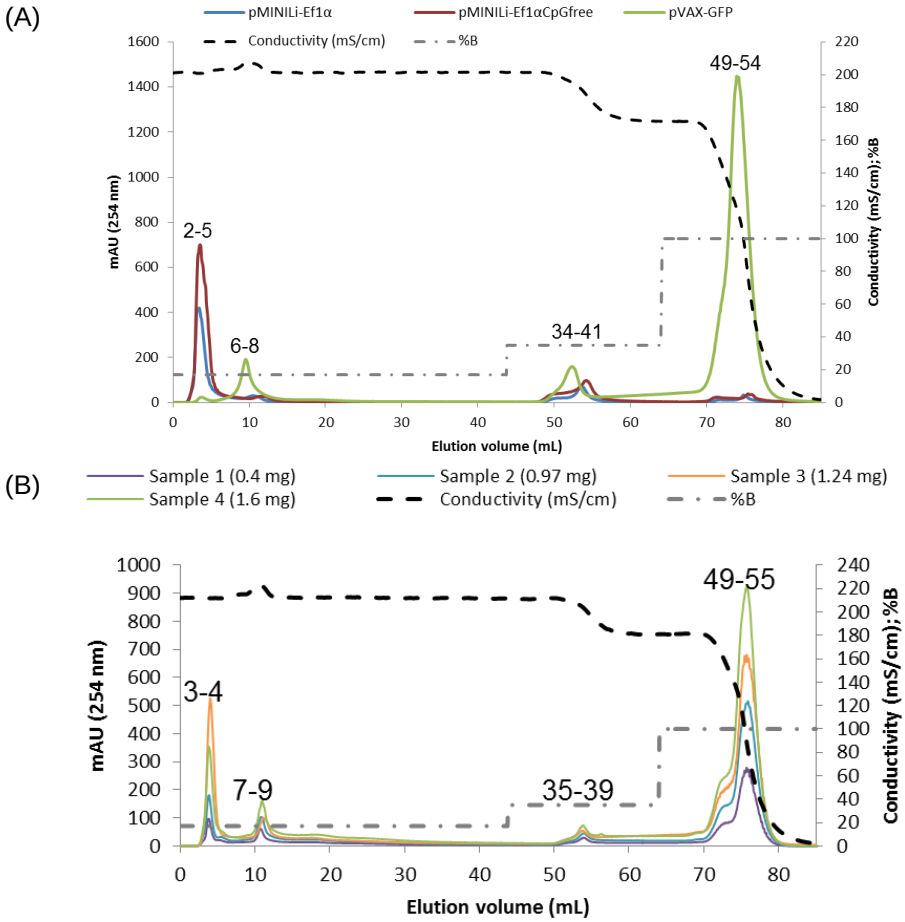

Figure 4: Hydrophobic interaction chromatography separation of covalently-closed DNA isoforms using a stepwise gradient elution scheme. 1 mL of DNA samples obtained after pre-purification was loaded onto a phenyl-Sepharose column (100 mm x 10 mm). Buffer A: 2.2 M ammonium sulfate in 10 mM Tris HCl pH 8. Buffer B: 10 mM Tris-HCl pH 8. Washing of unbound material was performed at 2 mL/min with 17% B (≈ 204 mS/cm) for 4 CV, followed by a step-wise elution with a first step at 35% B (≈ 173 mS/cm) for 2 CV and a second step at 100% B (≈ 2 mS/cm) for 2 CV. **(A)** Three different samples were studied: pMINILi-hEF1α-VEGF-GFP (blue), pMINILi-hEF1α(CpGfree)-VEGF-GFP (red), both previously digested with Nb.BbvCI, and pVAX-LacZ (green). **(B)** Chromatographic runs of samples containing pMINILi-CMV-VEGF-GFP recombination products, obtained by alkaline lysis and precipitation with isopropanol and ammonium sulfate. Four different amounts of nucleic acids were digested with Nb.BbvCI and loaded into the column: sample 1 – 0.4 mg (purple line), sample 2 – 0.97 mg (blue line), sample 3 – 1.24 mg (orange line) and sample 4 – 1.6 mg (green line). Numbers over peaks are indicative of the respective peak range fractions. Continuous line – mAU (254 nm); dashed line – conductivity (mS/cm); dashed dotted line – percentage of buffer B.

Analysis of chromatograms in Figure 4 (A) suggests that separation of oc and sc DNA forms is occurring for all the three samples studied at the desired elution volume. Gel electrophoresis analysis of eluted fractions (results not shown) confirmed these observations. It is thus possible to conclude that the method developed is applicable to the separation of oc and sc DNA forms others than the ones for which it was originally developed, proving the method robustness and reproducibility. Regarding the molecules eluted at 35% B, densitometry analysis on agarose gels images of collected fractions showed that it was possible to recover pVAX-LacZ with a sc homogeneity over 90%. The values obtained for the other two MC tested were however lower (69% of sc pMINILi-

hEF1α-VEGF-GFP and 50% of sc pMINILi-hEF1α(CpGfree)-VEGF-GFP).

3.2.4 Column loading capacity evaluation

To test column loading capacity, samples containing the recombination products of pMINILi-CMV-VEGF-GFP were obtained by alkaline lysis and precipitation with isopropanol and ammonium sulfate. Four 1 mL samples of different nucleic acid content (ranging from 0.4 to 1.6 mg of total nucleic acids) were digested with Nb.BbvCI and, after conditioning to 2.5 M ammonium sulfate, were injected into the column. The resulting chromatograms present a highly similar elution pattern, being the main difference registered in peak intensities, as is possible to observe in Figure 4 (B). The peak superimposition confirms method reproducibility. Agarose gel analysis of eluted fractions from all chromatographic runs (results not shown) confirmed separation of MC, being observed that oc MP and RNA are not eluted at 35% B, similarly to what was obtained in Figure 2 (B). Percentage of sc MC in fractions recovered with elution at 35% B was also calculated for a pool of fractions corresponding to three chromatographic runs with a global injection of 2.08 mg of total nucleic acids. The desalted and concentrated pool obtained (100 µL) presented a recovery of 1.4% MC from the initial mass of nucleic acids injected into the column in the three runs. Densitometry analysis of this sample confirmed that 79.6% of MC recovered were in supercoiled state, which is close to the minimum of 80% recommended by FDA [23].

4. Conclusions

The present work shows that the development of a robust and reproducible chromatographic method for minicircle purification was successfully achieved. The process developed involves bacterial cell culture and recombination of parental plasmid into target minicircle, cell harvesting and alkaline lysis, precipitation with isopropanol and ammonium sulfate and diafiltration/concentration by microfiltration. Then, the key step in the process is performed which involves the selective modification of the structure of miniplasmid impurities by resorting to a nicking enzyme that recognizes a specific sequence in the molecule. As a consequence, supercoiled miniplasmids are converted into their open circular forms, whereas minicircles are unaffected. Finally, supercoiled and open circular minicircle species are isolated from miniplasmid and RNA by hydrophobic interaction chromatography using a downward step elution with high concentration (2.2 M) of ammonium sulfate. Agarose gel electrophoresis analysis confirmed that the method is able to produce minicircles that are virtually free from miniplasmid, parental plasmid and RNA.

Co-elution of oc and sc MCs in the target fractions (eluted at 35% B) can be attributed either to interaction of oc MC with the ligands on the chromatographic matrix or to relaxation of sc MC during or after the chromatographic run. A high content of oc MC was also detected after alkaline lysis (results not shown), indicating that special attention should be given to determine if lysis conditions are having a negative effect on sc MC leading to its relaxation. Provided that optimization of the lysis step is unsuccessful to increase the homogeneity of the final product, addition of a final polishing step, such as size-exclusion chromatography, should be considered.

For environmental concerns the study of alternative elution buffers, such as sodium citrate, for the hydrophobic chromatographic method here developed should be evaluated. This salt is more environmentally friendly and its use has already been reported as successfully in hydrophobic interaction chromatography [16]. Provided that method efficacy and reproducibility is maintained its use would be advisable, especially at large scale.

Method was also tested with a larger plasmid (pVAX-LacZ, 6.05 kbp) with good results, indicating that the method efficiency is not dependent on plasmid size.

Regarding the production stage, an optimization of the cell culture/recombination step is advised to increase MC production. Reduction of time allowed for the recombination process should be considered due to the significant decrease on MC/MP ratio during the last hour of cell growth. Although this alteration of the recombination strategy could lead to a lower recombination efficiency and thus to a higher content of un-recombined parental plasmid at the end of fermentation, preliminary studies indicate that the HIC method developed is also able to separate PP species from MCs.

Future work must focus on process monitoring and product quality control, being also relevant to determine the column binding capacity.

5. References

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