Journal of Chromatography B, 904 (2012) 81–87

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Purification of plasmid DNA from clarified and non-clarified *Escherichia coli* lysates by berenil pseudo-affinity chromatography

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ARTICLE INFO

Article history:
Received 4 March 2012
Accepted 19 July 2012
Available online 31 July 2012

Keywords:
Plasmid purification
Small DNA ligands
Berenil
Pseudo-affinity chromatography

ABSTRACT

In this study, berenil was tested as a ligand, specifically to purify plasmids of different sizes pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp) from clarified *Escherichia coli* alkaline lysates. For this purpose, chromatographic experiments were performed using Sepharose derivatized with berenil. The results showed that both pDNA molecules are completely purified using lower amounts of salt in the eluent than those previously reported for other pseudo-affinity and hydrophobic interaction chromatography based processes. Total retention of all lysate components was achieved with 1.3 M ammonium sulphate in the eluent buffer and pDNA elution was obtained by decreasing the salt concentration to 0.55 M. All impurities were eluted after decreasing the concentration to 0 M. The recovery yield for pCAMBIA-1303 (45%) was lower than that obtained for pVAX1-LacZ (85%), however the larger pDNA showed a higher purity level. Purification of pVAX1-LacZ was also performed using non-clarified *E. coli* process streams, replacing the clarification step with a second chromatographic run on the berenil-Sepharose. Using the same binding and elution conditions as before, a pure plasmid sample was obtained with a 33% yield and with all host impurity levels in accordance with the requirements established by the regulatory agencies. These results suggest that this chromatographic method is a promising alternative to purify pDNA for therapeutic use.

1. Introduction

Molecular therapy approaches using non-viral vectors, such as plasmid DNA (pDNA) for the introduction of therapeutic genes, are fast becoming the preferred method to treat various types of diseases [1–3]. Thus, therapeutic pDNA is an emerging biotechnology product with great potential for use in human and animal healthcare. More than 400 clinical trials relating to gene therapy or DNA vaccines are currently being conducted worldwide using these vectors (http://www.wiley.com/legacy/wileychi/genmed/clinical/). In addition four DNA vaccine products have already been approved for veterinary application [4–6]. Accordingly, the expected far reaching application of these vectors in the future requires the large-scale production and purification of pDNA. In recent years, there has been an increased effort in the research and development of new methods for plasmid purification that meet strict quality criteria in terms of purity, efficacy and safety as required by the regulatory agencies. The critical contaminants of pDNA preparations are similar in size (genomic DNA (gDNA)), negatively charged (RNA, gDNA and endotoxins) and have similar hydrophobicity (endotoxins), which can complicate their separation [7]. The maximum levels of gDNA, host proteins and RNA in the final product should preferably be under 1% (w/w). The levels of endotoxins found on lysate solutions after disruption of the outer membrane of *Escherichia coli* by alkaline lysis should not exceed 40 EU/mg plasmid [8]. Moreover, the purification method should not comprise the use of organic reagents, mutagenic and toxic compounds and animal derived enzymes [9].

The process for pDNA preparation involves the production in *E. coli* cells by fermentation followed by an alkaline lysis step [10,11], a concentration with isopropanol and a pre-purification/clarification with ammonium sulphate [10]. The further downstream processing aims to eliminate impurities such as gDNA, low molecular weight RNA, residual proteins and endotoxins. Chromatography is the most suitable method for this purpose [10,12], however there are still several drawbacks that remain to be solved, like the poor selectivity of anion-exchange supports [13]. The use of affinity ligands can be a simple and efficient approach to overcome this problem [14,15]. Affinity and

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http://dx.doi.org/10.1016/j.jchromb.2012.07.023

pseudo-affinity chromatography use the specificity and biorecognition properties of the ligands to separate pDNA molecules from the impurities found in cell lysates [16].

Several molecules (certain antibiotics and anticancer agents, for example), bind DNA with high specificity [17] by different binding modes. Besides covalent binding there are several classes of specific and unspecific noncovalent binding modes, such as minor groove binding, intercalation between base pairs, bisintercalation, major groove binding and a combination of the above [18]. Berenil (1,3-bis(4-phenylamidinium) triazene) is used as an anti-trypanosomal agent in veterinary applications [19] and is a member of the aromatic diamidine class of DNA binding agents, which reversibly and preferentially binds to the DNA minor groove in the central AATT sequence [20,21]. Berenil was recently applied as ligand for the specific separation of the supercoiled (sc) plasmid isoform from the less active open circular (oc) isoform [15]. The DNA binding affinity of this ligand has been attributed to several factors: electrostatic interactions with the AT sequences, hydrophobic contacts between the phenyl rings and the hydrophobic regions of the DNA backbone and hydrogen bonds between the amidines and thymine and/or adenine acceptor groups of the bases at the floor of the groove [15].

This study reports a pseudo-affinity chromatographic technique to purify pDNA directly from clarified and non-clarified *E. coli* lysate solutions. This approach was tested for the purification of pDNA molecules with different sizes (pVAX1-LacZ and pCAMBIA-1303, with 6.05 Kbp and 12.361 Kbp, respectively).

2. Materials and methods

2.1. Materials

Sepharose CL-6B was obtained from Amersham Biosciences (Uppsala, Sweden). Berenil and 1,4-butanediol diglycidyl ether were purchased from Sigma–Aldrich (St. Louis, MO, USA). All salts were of analytical grade.

2.2. Bacterial culture

E. coli DH5α strain harbouring 6.05 Kbp plasmid pVAX1-LacZ (Invitrogen, Carlsband, CA, USA) and *E. coli* XL1 blue strain harbouring 12.361 Kbp plasmid pCAMBIA-1303 (Cambia, Brisbane, Australia) were cultured overnight in Luria Bertani agar (Lennox) medium (Laboratorios Conda, Madrid, Spain) supplemented with 30 μg/mL of kanamycin at 37 °C. *E. coli* DH5α strain was grown at the same temperature in an orbital shaker with Terrific Broth medium (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 30 μg/mL kanamycin. The XL1blue strain cells were grown in similar conditions using Luria Bertani medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0). Both cell strains were harvested by centrifugation at the end of the exponential growth phase and stored at −20 °C until use. Plasmid-free *E. coli* cells were also grown in the absence of antibiotic, under the same conditions as previously described.

2.3. Lysis and primary isolation

The plasmid harbouring cells were lysed using a modification of the alkaline method proposed by Sambrook et al. [22]. Centrifugation of a 250 mL sample of the cell broth was performed at 5445 × *g* for 30 min at 4 °C with a Sigma 3–18 K centrifuge. The supernatants were discarded and the bacterial pellets were resuspended in 20 mL of 50 mM glucose, 25 mM Tris–HCl and 10 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0). The cells were lysed by adding 20 mL of a 200 mM NaOH, 1% (w/v) sodium dodecyl sulphate solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated by gently adding and mixing 16 mL of prechilled 3 M potassium acetate (pH 5.0). The precipitate was removed by a double centrifugation at 20,000 × *g* for 30 min at 4 °C with a Beckman Allegra 25 R centrifuge. The plasmid in the supernatant was precipitated after the addition of 0.7 volumes of isopropanol and a 30 min incubation period on ice. The pDNA was recovered by centrifugation at 16,000 × g for 30 min at 4 °C. The pellets were then redissolved in 1 mL of 10 mM Tris–HCl buffer (pH 8.0). A fraction of this solution was subjected to a clarification step. For this purpose, and after optimization studies, solid ammonium sulphate was dissolved in the pDNA solutions up to a final concentration of 2.0 M, for the pVAX1-LacZ solution, and 2.5 M for the pCAMBIA-1303 solution, followed by a 15 min incubation period on ice. Precipitated proteins and RNA were then removed by centrifugation at 10,000 × *g* for 20 min at 4 °C. The supernatant was recovered and its nucleic acid concentration quantified by measuring the absorbance at 260 nm.

2.4. Preparation of berenil-Sepharose support

Sepharose CL-6B was epoxi-activated according to the method described by Sundberg and Porath [23] and coupled to berenil as previously described [15]. The orange derivatized gel thus obtained was stored at 4 °C in deionized water.

2.5. Preparative chromatography

Chromatographic studies were performed in a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden) at room temperature. A 10 cm × 10 mm column (Amersham Biosciences, Uppsala, Sweden) was packed with 2 mL berenil-derivatized (Fig. 1) gel and initially tested with different ammonium sulphate concentrations (0.2–1.5 M) in the mobile phase. Prior to sample application, and after achieving the optimal conditions of binding and elution, the column was equilibrated with 1.3 M ammonium sulphate in 10 mM Tris–HCl buffer (pH 8.0) at a flow rate of 1 mL/min.

2.5.1. Injection of clarified samples

Clarified samples (25 μL) were loaded onto the column in equilibration buffer at a flow rate of 1 mL/min. To promote the selective elution of bonded species, the salt concentration was first decreased to 0.55 M ammonium sulphate in 10 mM Tris–HCl buffer (pH 8.0) and then to 0 M. The absorbance was continuously monitored at 280 nm. Fractions were pooled according to the chromatograms obtained, concentrated and desalted using Vivaspin concentrators (Vivaproducts, Littleton, MA, USA) and kept for further analysis as described below.

2.5.2. Injection of non-clarified samples

Non-clarified samples (25 μL) with a nucleic acid concentration of approximately 600 μg/mL in equilibration buffer were loaded onto the column at 1 mL/min flow rate. The elution was performed as described above for clarified samples. A second chromatographic step was then performed in the same binding and elution conditions, injecting the pDNA fraction, pooled after the first run and concentrated to an approximate nucleic acid concentration of 600 μg/mL. Fractions were pooled according to the obtained chromatograms, then concentrated and desalted using Vivaspin concentrators (Vivaproducts, Littleton, MA, USA) and kept for further analysis as described below. After the chromatographic runs, the column was washed with at least 5 bed volumes of deionized water.

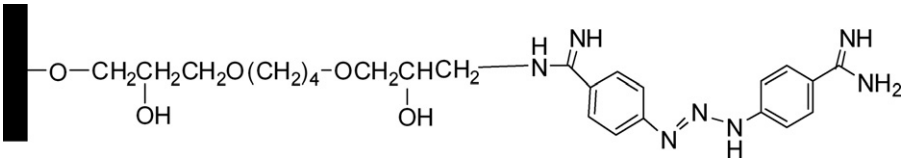

Fig. 1. Schematic representation of the berenil-Sepharose support. Berenil is covalently bonded to the epoxy arm.

2.6. Agarose gel electrophoresis

Pooled fractions were analyzed by horizontal electrophoresis (100 V for 40 min) using 1% and 0.8% agarose gel in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) in the presence of 0.5 μg/mL ethidium bromide. The gels were visualized in a UVITEC Cambridge system (UVITEC Limited, Cambridge, UK).

2.7. Analytical chromatography

The pDNA of feed samples were injected onto the berenil column and the fractions pooled after the chromatographic runs. The concentration and purity of the extracts was assessed by high-performance liquid chromatography (HPLC), according to the method described by Diogo et al. [24]. A 4.6/100 mm HIC (hydrophobic interaction chromatography) Source 15 PHE PE column (Amersham Biosciences, Uppsala, Sweden) was connected to a Waters HPLC system (Waters Corporation, Milford, MA, USA) and equilibrated with 1.5 M ammonium sulphate in 10 mM Tris–HCl buffer (pH 8.0). Samples (20 μL) were injected and eluted at a flow rate of 1 mL/min. After injection, the elution occurred with the equilibration buffer for 3 min, at which point the elution buffer was immediately changed to Tris–HCl 10 mM (pH 8.0) without ammonium sulphate. This condition was maintained for 6 min in order to elute bound species. The column was then re-equilibrated for 7 min with the equilibration buffer to prepare the column for the next run. The absorbance of the eluate was continuously recorded at 254 nm. The concentration of pDNA in each sample was calculated using a calibration curve constructed with pDNA standards (2.5–400 μg/mL), purified using the Qiagen plasmid midi kit (Hilden, Germany), according to the manufacturer's instructions. Plasmid quantification was achieved by measuring the absorbance at 260 nm, assuming an absorbance of 1.0 for a solution of 50 μg/mL. The degree of purity was defined as the percentage of the pDNA peak area in relation to the total area of all chromatographic peaks.

2.8. Protein analysis

Protein concentration of the samples was measured using the micro-bicinchoninic acid (BCA) assay from Pierce (Rockford, IL, USA), according to the manufacturer's instructions and 50 μL of each sample was added to 200 μL of BCA reagent in a microplate and incubated for 30 min at 60 °C. Absorbance was measured at 595 nm in a microplate reader. The calibration curve was prepared using bovine serum albumin standards (0.025–1 mg/mL).

2.9. Endotoxin analysis

Analysis of endotoxin contamination on both feed samples and pDNA fractions pooled after chromatography was performed using the ToxinSensor™ Chromogenic LAL Endotoxin Assay kit from GenScript (GenScript USA Inc., Piscataway, NJ, USA) which had a detection level of 0.005 EU/mL.

2.10. Genomic DNA analysis

Genomic DNA contamination in purified plasmid solutions and in feed samples was assessed using real-time polymerase chain reaction (PCR) in a iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), according to the method described by Martins et al. [25]. Sense (5′-ACACGGTCCAGAACTCCTACG-3′) and antisense (5′-GCCGGTGCTTCTTCTGCGGGTAACGTCA-3′) primers were used to amplify a 181-bp fragment of the 16S rRNA gene. PCR amplicons were quantified by following the change in fluorescence of the DNA binding dye Syber Green (Bio-Rad, Hercules, CA, USA). *E. coli* genomic DNA was purified with the Wizard gDNA purification kit (Promega, Madison, WI, USA) and used to generate a standard curve ranging from 0.005 to 50 μg/mL. Negative controls, with no template, were run at the same time as the standards.

3. Results and discussion

3.1. Clarification of pDNA lysates by salt precipitation

RNA is the main contaminant of non-clarified cell lysates, and since it is structurally similar to pDNA [7], its separation can represent a true challenge. One common procedure for RNA removal is the precipitation with high salt concentrations, using ammonium sulphate as the clarifying agent. This precipitation step is very effective in reducing the amounts of high molecular weight RNA and proteins, and equally effective in improving intermediate pDNA recovery, thus representing an excellent complement to any further purification step [26].

Standard ammonium sulphate precipitation uses 2.5 M of salt concentration to clarify crude cell lysates, which has a great environmental impact due to its high eutrophication potential [26]. However, Freitas et al. [27] concluded that precipitation with high salt concentration is not always needed. Based on these conclusions, optimization studies were performed to select the best conditions for high pDNA recovery and purity by analyzing the influence of salt concentration on RNA removal. Ammonium sulphate, in a concentration range from 1.3 M to 2.5 M, was added to the non-clarified pDNA suspensions and the precipitated proteins and RNA were then removed by centrifugation. The obtained samples were injected onto the berenil-Sepharose column (with the optimized conditions of binding and elution) and the purity of eluted pDNA fractions in terms of RNA contamination was evaluated by HPLC analysis.

The data obtained showed that for the 6.05 Kbp pDNA (pVAX1-LacZ), a concentration of 2.0 M ammonium sulphate in the precipitation step was enough to achieve a pure sample after the chromatographic step using berenil as ligand (Table 1). Precipitation with a salt concentration of 1.9 M and lower, led to a similar recovery yield, but resulted in impure pDNA samples. On the other hand, when the clarifying step was performed with ammonium sulphate concentrations above 2.0 M, all the pDNA samples were 100% pure, with similar recovery yields. In contrast, for pCAMBIA-1303 (12.361 Kbp) a higher ammonium sulphate concentration (2.5 M) was needed to obtain a 100% HPLC pure sample after the same chromatographic process (Table 1). Using a salt concentration of 2.0 M in the clarification step, the purity of pDNA samples was set

Table 1
Dependence of sample purity (HPLC) and recovery yield with ammonium sulphate concentration used in the clarification step.

Plasmid	$(NH_4)_2SO_4$ (M)	Sample purity (%)	Approximated yield (%)
pVAX1-LacZ	1.3	66	85
	1.9	80	
	2.0	100	
	2.5	100	
pCAMBIA-1303	2.0	20	45
	2.25	40	
	2.5	100	

around 20% increasing to more than 40% with 2.25 M of ammonium sulphate. It is known that large size plasmids are more susceptible to shear forces and, in general, smaller pDNA concentrations were obtained after alkaline lysis [28]. Thus, for pCAMBIA-1303, the pDNA/RNA ratio in non-clarified solutions is smaller and possibly because of that, higher ammonium sulphate concentrations are required to maximize plasmid purification and recovery from the highly RNA contaminated solutions.

3.2. Berenil-Sepharose pseudo-affinity chromatography

3.2.1. Injection of clarified pDNA solutions

Recently, berenil-Sepharose pseudo-affinity support (Fig. 1) was successfully applied for the separation of sc plasmid isoform from the less active oc isoform, showing a great affinity for pDNA [15]. Therefore, the ability of this support to purify pDNA directly from clarified *E. coli* cell lysates was exploited in the present study. Moreover, the applicability of this process to purify pDNA molecules with different sizes was also tested. For this purpose, solutions of pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp), obtained separately after clarification with ammonium sulphate, were used as feedstock for the pseudo-affinity chromatography studies. Several binding-elution experiments were performed to achieve optimal buffer conditions to separate pDNA from the lysate impurities, namely RNA. These experiments showed that the chromatographic conditions are the same for both plasmids: total retention of all lysate components was achieved using 1.3 M of ammonium sulphate in the binding buffer and elution of pDNA was obtained by a simple decrease of salt concentration to 0.55 M. The more hydrophobic impurities, such RNA, were eluted only when the concentration was decreased to 0 M. Fig. 2 shows the chromatograms after injection of pVAX1-LacZ and pCAMBIA-1303 samples onto the berenil-Sepharose column. Both plasmids showed a comparable separation performance represented by similar chromatograms, which demonstrates the reproducibility of the chromatographic process for plasmid molecules of different sizes. The chromatograms are characterized by a first small system peak, followed by a sharp higher peak of pDNA and a smaller one of strongly retained species such as RNA. As shown by agarose gel electrophoresis (Fig. 2) and confirmed by HPLC, RNA was completely separated from pDNA molecules (electrophoresis lane 1 in Fig. 2 for both plasmids) and was eluted in the peak 2. All cell impurities are retained in the column for longer when compared with pDNA (Fig. 2). In these double-stranded molecules, the hydrophobic bases are packed and shielded inside the helix and thus interaction with the support ligands is smaller, eluting first. On the other hand, the single-stranded RNA molecules are retained more strongly in the support because the hydrophobic bases are largely exposed [29].

The binding between the berenil ligand and the lysate components results not only from hydrophobic interactions between the phenyl rings of berenil and the hydrophobic regions of the backbone of the molecules, but also from other more specific interactions that are responsible for the great affinity that the support shows for those molecules [15].

An epoxy activated Sepharose gel obtained using the same experimental conditions minus the berenil ligand was used for the control experiments (results not shown). A distinct pattern from that which was originally observed with the berenil-Sepharose support was obtained: total retention was not observed at 1.3 M of ammonium sulphate and the lysate components were not separated by decreasing the salt concentration. Accordingly, these experiments unequivocally identified berenil bonded onto the activated Sepharose as the ligand responsible for the retention and separation of pDNA molecules. Moreover, the column cleaning and the high number of chromatographic runs did not cause any change in the chromatographic performance of the derivatized gel.

3.2.2. Injection of non-clarified pVAX1-LacZ solution

The ability of the berenil-Sepharose to separate and purify pDNA directly from non-clarified *E. coli* process streams was also tested. This was accomplished without the ammonium sulphate clarification step. In a first run, the feedstock sample was injected onto the berenil-Sepharose column with 1.3 M of ammonium sulphate in the eluent. The obtained chromatographic profile is shown in Fig. 3A, as is the agarose gel electrophoresis analysis of the pDNA eluted fractions. The electrophoresis shows that the pDNA fraction still contains a slight RNA contamination, even though the great majority of this contaminant clearly elutes in the second

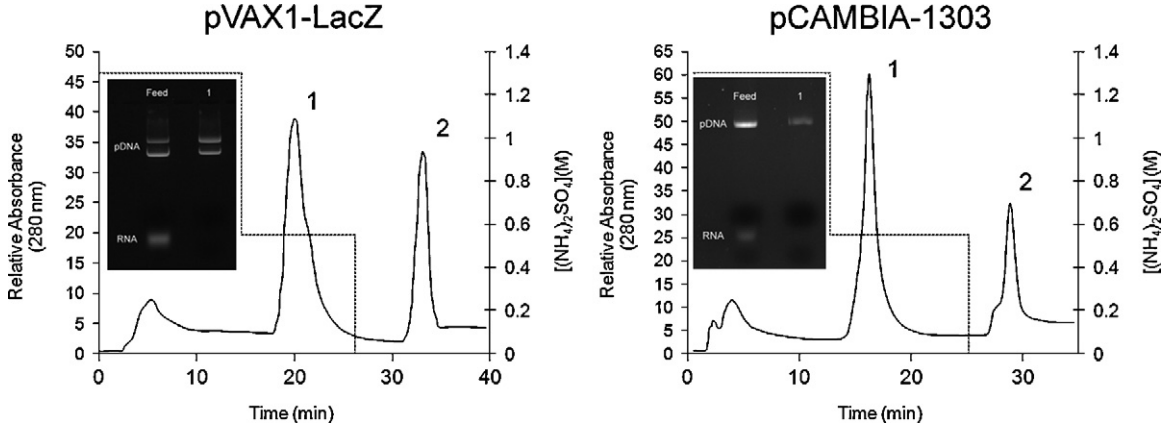

Fig. 2. Chromatographic separation of clarified feed solution (25 μL) of pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp) from the host cell impurities on berenil-Sepharose support. Agarose gel electrophoresis analysis of the pDNA fractions. Peak 1 and electrophoresis lane 1: pDNA fractions collected after elution with 0.55 M $(NH_4)_2SO_4$; Peak 2: impurities eluted with 0 M $(NH_4)_2SO_4$. The clarified lysate was also run in the agarose gel for comparative purposes (lane feed).

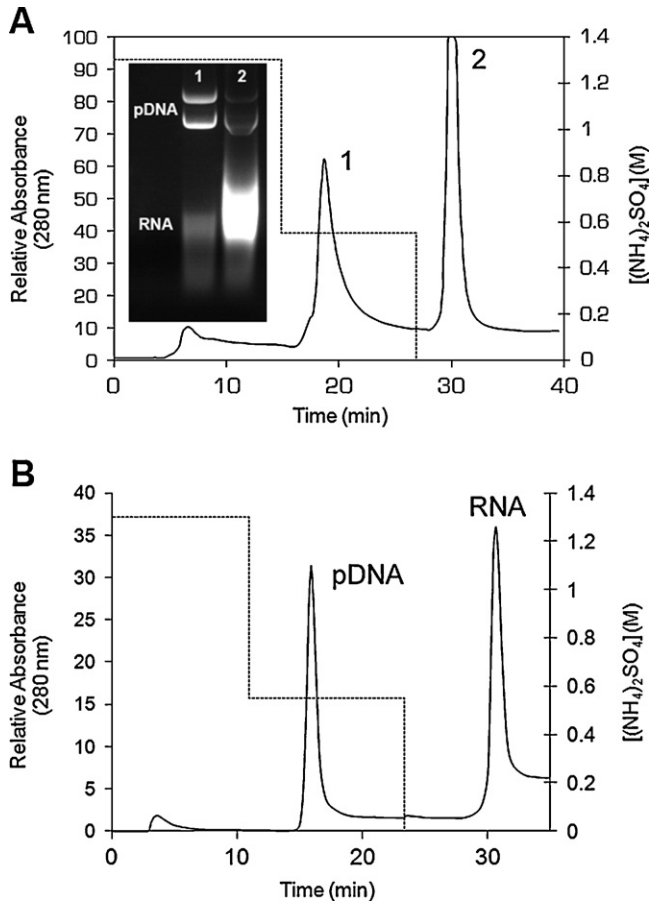

Fig. 3. Chromatographic separation of non-clarified pVAX1-LacZ samples on berenil-Sepharose column: (A) After one run through the support and agarose gel electrophoresis analysis of the eluted fractions: Peak 1 and electrophoresis lane 1: pDNA fraction eluted with 0.55 M $(NH_4)_2SO_4$; Peak 2 and electrophoresis lane 2: RNA eluted with 0 M $(NH_4)_2SO_4$. (B) After the second run through the berenil support.

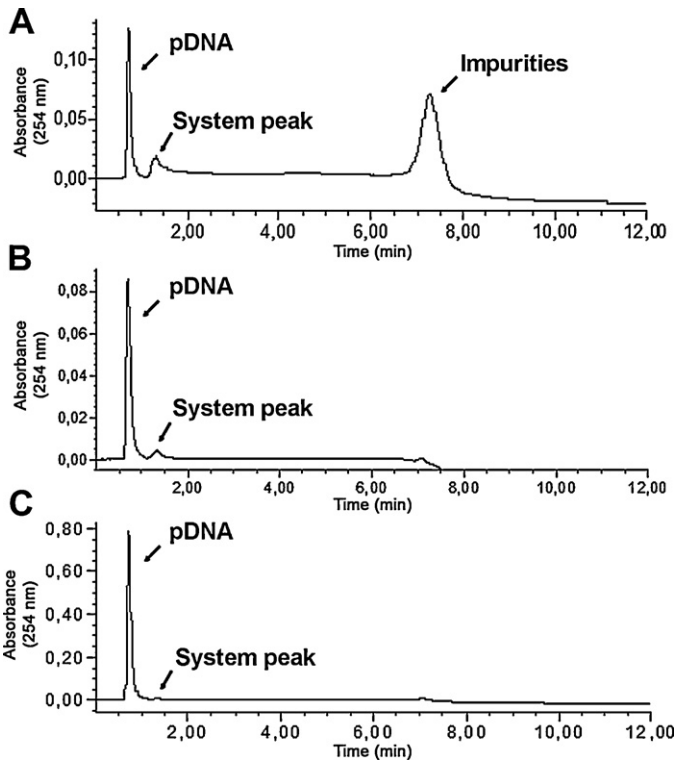

Fig. 4. HPLC analysis of different pVAX1-LacZ samples: (A) *E. coli* clarified lysate diluted 1:20. (B) pDNA fraction collected after the chromatographic run. (C) 400 μg/mL pVAX1-LacZ standard for comparative purposes. The chromatograms from HPLC analysis of pCAMBIA-1303 samples were similar to those here represented.

peak. In the second run, the pDNA fraction obtained after the first chromatographic step was concentrated and injected onto the berenil-Sepharose column using the same buffer conditions. The obtained chromatogram (Fig. 3B) showed two well defined peaks, the first one corresponding to the elution of pDNA after decreasing the salt concentration to 0.55 M and a second peak corresponding to the all contaminants, eluted after decreasing the salt concentration to 0 M.

3.3. Plasmid quality and purity assessment

The performance of pDNA purification processes using the berenil-Sepharose was examined by the determination of yield and purity (Table 2). Besides HPLC analysis of RNA contamination, the purity of the recovered plasmid fractions was also determined by quantification of proteins (BCA assay), endotoxins (Chromogenic Limulus amoebocyte lysate Endotoxin assay) and gDNA (real-time PCR) (Table 3).

3.3.1. Clarified pDNA solutions

For the clarified samples, agarose gel electrophoresis (Fig. 2) and HPLC analysis (Fig. 4) revealed that the plasmid pools were RNA free. The analytical chromatogram shown in Fig. 4A represents the clarified lysate injected onto the berenil-Sepharose column. Clearly a high percentage of the sample was constituted by impurities, namely RNA. In this feed sample, the HPLC pDNA purity was 23% (Table 2) for pVAX1-LacZ and 7% for pCAMBIA-1303, however after the chromatographic step, both plasmid samples were 100% pure (Table 2) and free of RNA. The analytical chromatogram for the obtained pDNA (Fig. 4B) shows a single plasmid peak, confirming the agarose gel electrophoresis results. The HPLC purification factors (Table 2) are higher than those described for similar chromatographic procedures [30,31], particularly for pCAMBIA-1303 which has an impressive value of 14.3. HPLC analysis also showed a 85% recovery of pVAX1-LacZ (Table 2). This value is similar to the 84% obtained with an affinity support by Sousa et al. [31], but slightly higher than the 70% yield obtained with a pure hydrophobic chromatographic matrix [29]. Nevertheless, the recovery yield of pCAMBIA-1303 was much lower than for the smaller pDNA molecule (Table 2). This may be due to the fact that larger plasmids are more unstable and degrade more easily during the extraction and purification procedures, since they are more sensible to shear forces [28] and more susceptible to losses during the chromatographic step.

The quantitative analysis of impurities in injected feeds and pDNA fractions is shown in Table 3. The BCA protein assay indicated that both plasmid pool solutions have undetectable levels of proteins. Real-time PCR analysis of pooled plasmid fractions showed a great reduction of gDNA content after the berenil-Sepharose chromatographic step. A gDNA decrease of 3653-fold was achieved for pVAX1-LacZ and an impressive 554,472-fold reduction was achieved for pCAMBIA-1303 (Table 3). Berenil-Sepharose support is thus extremely efficient to separate gDNA from heavily contaminated pDNA solutions. Genomic DNA from *E. coli* is double-stranded, but becomes mostly single-stranded during alkaline lysis. During this process, the complementary strands of gDNA are completely separated and partially cleaved. The resulting gDNA molecules show a high exposure of their hydrophobic bases

Table 2
HPLC analysis of purity and recovery yield of two plasmids with different sizes: pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp).

Plasmid	Process step	Purity (%)	Purification factor	Plasmid mass (μg)	Yield (%)
Clarified pVAX1-LacZ	Injected feed	23	–	11.1	–
	pDNA fraction	100	4.3	9.4	85
Clarified pCAMBIA-1303	Injected feed	7	–	11	–
	pDNA fraction	100	14.3	5	45
Non-clarified pVAX1-LacZ	Injected feed[a]	4.4	–	7.5	–
	pDNA fraction[b]	100	22.7	2.5	33

[a] Corresponding to the first run feed.
[b] pDNA fraction collected after two runs through the berenil-Sepharose column.

Table 3
Quantitative analysis of the pDNA, proteins, RNA, endotoxins and gDNA in injected feed and plasmid samples after berenil-Sepharose chromatography. For the non-clarified pVAX1-LacZ the feed corresponds to the initial non-clarified lysate solution and the pDNA fraction to the sample collected after the second chromatographic run.

Plasmid	Impurity	Feed	pDNA fraction[a]	Reduction factor (fold)
Clarified pVAX1-LacZ	pDNA (μg/mL)	1223.8	37.4	–
	Protein (μg/mL)	66	Undetectable	–
	RNA (mass%)	77	Undetectable	–
	Endotoxins (EU/mL)	2.71	0.136	20
	gDNA (μg/mL)	364.9	9.99×10^{-2}	3653
Clarified pCAMBIA-1303	pDNA (μg/mL)	278.9	25	–
	Protein (μg/mL)	Undetectable	Undetectable	–
	RNA (mass%)	93	Undetectable	–
	Endotoxins (EU/mL)	1.967	0.099	20
	gDNA (μg/mL)	6.82	1.23×10^{-5}	554,472
Non-Clarified pVAX1-LacZ	pDNA (μg/mL)	680	8.22	–
	Protein (μg/mL)	1170	Undetectable	–
	RNA (mass%)	95.6	Undetectable	–
	Endotoxins (EU/mL)	2.03	0.099	21
	gDNA (μg/mL)	726.7	2.4×10^{-3}	302,792

[a] Values extrapolated from results obtained with several injections.

[28] and can thus interact more strongly with berenil, eluting in the second peak (Fig. 2).

The chromatographic process described here was also very effective in reducing the endotoxin levels from plasmid solutions. A decrease of 20-fold was obtained for both types of plasmid molecules (Table 3) demonstrating that endotoxins bind to the berenil support more strongly than pDNA.

The quality analysis of pDNA showed that pVAX1-LacZ and pCAMBIA-1303, purified with the berenil-Sepharose support, meet the specifications of the regulatory agencies, namely FDA (Table 4) [8]. Both RNA and proteins (preferably <1%) are undetectable in the final plasmid solutions and the endotoxin levels are ten times lower than the maximum required by FDA (40 EU/mg pDNA). Genomic DNA content was the only parameter that greatly varies among pDNA feed solutions and plasmid pools, being much higher for pVAX1-LacZ. Nevertheless, both small and higher size plasmids are under FDA specifications (preferably <1%) (Table 4).

3.3.2. Non-clarified pVAX1-LacZ solution

Starting from a highly contaminated non-clarified lysate (Table 2) and after two consecutive chromatographic runs on the berenil-Sepharose column, the pVAX1-LacZ fraction became 100% free from RNA, with a purification factor of almost 23. Nevertheless, the yield (33%) is lower than expected. As before, the BCA protein assay showed that the pDNA pool had undetectable levels of proteins (Table 3), despite the feed solution being highly contaminated (1170 μg/mL).

Real-time PCR analysis of both feed and plasmid fraction showed a remarkable 302,792-fold decrease in gDNA contamination (Table 3). In regard to endotoxin contamination, a 21-fold reduction was achieved, an analogous value to that obtained with only one run through the berenil-Sepharose column. The comparison between FDA specifications and pDNA sample composition collected after the second chromatographic run shows that all values are in accordance to the requirements for molecular therapy products (Table 4).

In an overall analysis, the berenil-Sepharose chromatographic process meets all the requirements to be used as a pDNA purification step. This new chromatographic support enables pDNA purification using a lower salt concentration, compared with other processes that use hydrophobic or pseudo-affinity ligands [16,29], showing a better purification performance and yield for plasmids with similar size.

Table 4
Comparison of plasmid samples composition with FDA specifications.

Impurity	FDA specifications [8]	pVAX1-LacZ collected sample	pCAMBIA-1303 collected sample	pVAX1-LacZ collected sample after two runs
Protein (%)	Preferably <1	Undetectable	Undetectable	Undetectable
RNA (%)	Preferably <1	Undetectable	Undetectable	Undetectable
Endotoxins (EU/mg pDNA)	<40	4	4	12
gDNA (%)	Preferably <1	0.27	5×10^{-5}	0.03

4. Conclusions

In this study, a new chromatographic process using berenil as ligand was developed and applied for the purification of pDNA molecules with different sizes: pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp). Both types of plasmids were successfully separated from host impurities showing that the method can be applied to small plasmids as well as to larger ones.

An optimization of the clarification step was also performed. Precipitation with 2.0 M, a value lower than the 2.5 M usually used, is enough to achieve pVAX1-LacZ with a high recovery and purity. However pCAMBIA-1303 was only completely purified using 2.5 M of salt, perhaps due to its larger size. The purification of pCAMBIA-1303 showed a lower yield, however, its purity is superior to that obtained with pVAX1-LacZ. This could be interesting since future requirements for multigene vectors, including extensive control regions, may require the production of larger plasmids.

Despite the loss in yield, the replacement of the clarification step with ammonium sulphate by a second chromatographic run indicated real advantages such as reduction in salt usage and procedure steps. Even though gDNA decontamination is more efficient when applying two runs through the support, the association of a clarification step with a chromatographic run with berenil-Sepharose gel is more effective in removing endotoxins.

This pseudo-affinity chromatographic method showed to be very effective in purifying plasmid DNA from *E. coli* cell lysate impurities, using small amounts of salt in the overall process. The essential separation mechanism seems to involve not only differential hydrophobic interactions between the ligand, pDNA molecules and host impurities, but also other important and specific contributions for the affinity binding. This purification method seems to be more environmentally friendly and less costly, and can be used as a main purification process of pharmaceutical grade pDNA.

Acknowledgements

This work was supported by FCT, the Portuguese Foundation for Science and Technology PTDC/QUI-QUI/100896/2008. C. Caramelo-Nunes acknowledges a fellowship (SFRH/BD/64918/2009) from FCT.

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