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Sodium citrate and potassium phosphate as alternative adsorption buffers in hydrophobic and aromatic thiophilic chromatographic purification of plasmid DNA from neutralized lysate

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ABSTRACT

The number of studies on gene therapy using plasmid vectors (pDNA) has increased in recent years. As a result, the demand for preparations of pDNA in compliance with recommendations of regulatory agencies (EMEA, FDA) has also increased. Plasmid DNA is often obtained through fermentation of transformed *Escherichia coli* and purification by a series of unit operations, including chromatography. Hydrophobic interaction chromatography (HIC) and thiophilic aromatic chromatography (TAC), both using ammonium sulfate buffers, are commonly employed with success. This work was aimed at studying the feasibility of utilizing alternative salts in the purification of pDNA from neutralized lysate with phenyl-agarose (HIC) and mercaptopyrimidine-agarose (TAC) adsorbents. Their selectivity toward sc pDNA was evaluated through adsorption studies using 1.5 mol/L sodium citrate and 2.0 mol/L potassium phosphate as adsorption buffers. Chromatography with mercaptopyrimidine-agarose adsorbent and 1.5 mol/L sodium citrate was able to recover 91.1% of the pDNA with over 99.0% removal of gDNA and endotoxin. This represents a potential alternative for the primary recovery of sc pDNA. However, the most promising result was obtained using 2.0 mol/L potassium phosphate buffer and a mercaptopyrimidine-agarose column. In a single chromatographic step, this latter buffer/adsorbent system recovered 68.5% of the pDNA with 98.8% purity in accordance with the recommendations of regulatory agencies with regard to RNA and endotoxin impurity.

1. Introduction

Gene therapy and DNA vaccination are promising strategies for the treatment and prophylaxis of diseases. The former consists in modifying the genetics of the targeted cells or tissues through the introduction of one or more functional genes [1]. DNA vaccination is the insertion of genes encoding for antigens aiming at triggering immune responses [2]. Although viral vectors were the basis of the first-generation gene delivery systems and vaccines, they were associated with the risk of DNA integration into the host genome, activation of oncogenes, immunogenicity, and systemic inflammatory responses [3,4]. Therefore, nonviral vectors such as plasmid DNA (pDNA) are considered safer than viral vectors and have become increasingly desirable [4,5]. The drawback to the use of pDNA as the delivery vector is its low transfection efficiency, resulting in the need for large amounts of purified pDNA for efficient gene transfer *in vivo* [6]. Thus, the demand for processes capable of producing large amounts of high purity pDNA is continuously increasing [7].

Plasmid DNA is an intracellular molecule produced through fermentation in bacteria such as *Escherichia coli*. The first step in downstream processing for pDNA production is cell rupture, commonly performed by alkaline lysis, followed by neutralization with acidic buffer [8]. After centrifugation, the liquid-phase product (neutralized lysate) is composed of less than 3% (m/m) pDNA with a high content of impurities, mainly proteins, endotoxins, RNA, and genomic DNA (gDNA) [7]. One or more physicochemical characteristics of these impurities are similar to those of pDNA, thereby hindering the isolation of this molecule. In addition, the final

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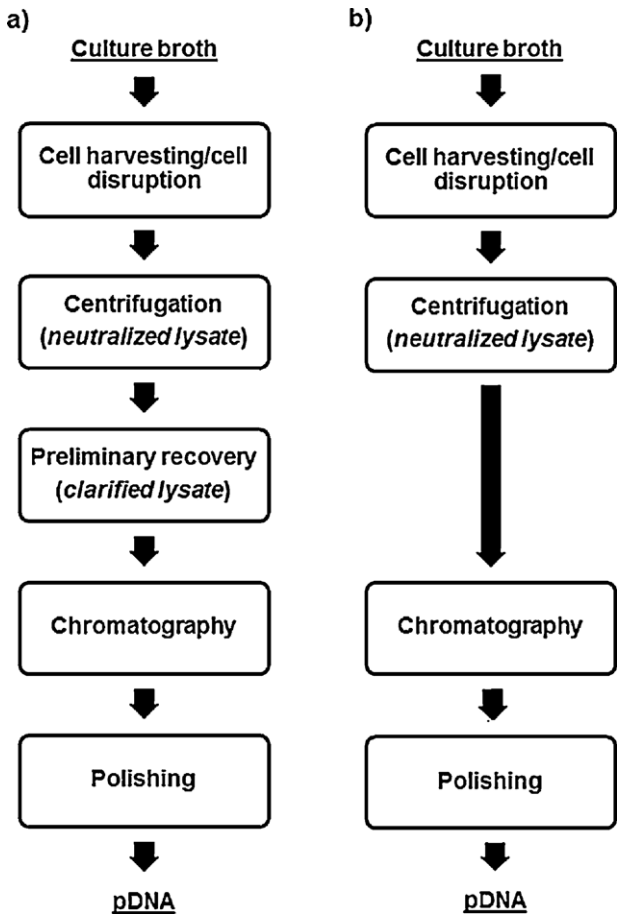

Fig. 1. Downstream processing of fermentation broth for the production of pharmaceutical grade pDNA: (a) steps commonly described in pDNA downstream processing and (b) pDNA purification process utilizing neutralized lysate directly in the chromatographic step.

product must be in accordance with the acceptance criteria established by the FDA (USA) and the European Medicines Agency (EMEA-EU). These criteria establish maximum amounts of different impurities such as proteins (<3 μg/mg pDNA), endotoxins (<10 EU/mg pDNA), RNA (undetectable by electrophoresis on 0.8% agarose gel), and gDNA (<2 μg/mg pDNA) besides homogeneity (>90% of the pDNA in the supercoiled isoform (sc)).

In the downstream processing of pDNA different chromatographic techniques, such as hydrophobic interaction (HIC) [9,10], aromatic thiophilic interaction (TAC) [11,12], size exclusion (SEC) [13], ion exchange (IEC) [14], interaction with immobilized metal ions (IMAC) [15], and pseudobiospecific affinity [16] are employed individually or together. In most of the published work that reports on purification by chromatography, including HIC and TAC, pDNA is purified in one or more preliminary recovery unit operations before being loaded into the chromatographic column. Usually these unit operations are salt, alcohol, or polymer precipitation; filtration; or separation in an aqueous two-phase system. Only a few studies available in the literature have proposed direct chromatographic purification of the neutralized lysate [17–20] (Fig. 1).

pDNA can be efficiently purified with HIC due to the difference in hydrophobicity between the plasmid and impurities, i.e., RNA, gDNA, endotoxins, and proteins [9]. Separation occurs through interaction between the hydrophobic ligand attached to the matrix and the nitrogen bases in the nucleotides [7] or other hydrophobic groups in side chains of the proteins. The application of HIC of pDNA in the purification of the gene therapy vector for cystic fibrosis [21] and the DNA vaccine against rabies [22,23] has been reported. HIC resin phenyl agarose is acclaimed for its high efficiency in purifying pDNA for therapeutic purposes [9,24].

Purification of pDNA with thiophilic aromatic chromatography (TAC) was first described by Lemmens et al. [11], who demonstrated that ligands containing an aromatic ring and at least one thioether motif are able to select the supercoiled isoform from the clarified lysate. It has been suggested that the mechanism of interaction involved in this purification is the hydrophobic forces (π–π) between the ligand and sc pDNA aromatic clusters, in addition to the electron donor–acceptor interactions between the sulfur atom and specific nucleotides [12].

However, the current technology available for pDNA purification with HIC and TAC has two main disadvantages: the requirement of a high salt concentration in the adsorption buffers and the need for one or more preliminary recovery steps. Both HIC and TAC require a high concentration of kosmotropic salts, usually ammonium sulfate ranging from 1.5 to 2.5 mol/L, to promote adsorption [7,9,11,12]. The disposal of substantial amounts of ammonium sulfate represents in large-scale processes is a drawback, due to its high eutrophication potential [25,26] and the impossibility of discharging more than 200 mg/L of ammonium in a biological wastewater treatment plant [27], which is the most common and efficient process for removing this pollutant [28]. Higher concentrations of ammonium in a wastewater stream must be lowered prior to biological treatment, and this can be accomplished by an air stripping step [27], but this operation alone results in a cost of € 6/kg of removed nitrogen [29]. Freitas and colleagues [9] reported the use of sodium citrate and potassium phosphate as potential alternatives to ammonium sulfate in the purification of pDNA with HIC. Sodium citrate is biodegradable and, non-toxic, and can be directly discharged into biological wastewater treatment [30–32]. Potassium phosphate can also be discharged for biological treatment and phosphate removal from wastewater costs in the order of € 4.3/kg [33].

Furthermore, according to the economical and environmental case study conducted by Freitas et al. [25] on the pDNA purification process proposed by Diogo et al. [22,23], the elimination of the alcohol precipitation step would benefit the process by reducing the overall cost of raw material, treatment and disposal of liquid waste, and environmental impact by 14%, 32%, and 70%, respectively. Thus, eliminating some preliminary recovery steps could represent economic and environmental benefits to the pDNA purification process.

In this work, sodium citrate and potassium phosphate were studied as alternative salts for the direct purification of sc pDNA from neutralized lysate by HIC and TAC using phenyl-agarose and mercaptopyrimidine-agarose adsorbents, respectively. For comparison purposes similar experiments were carried out with ammonium sulfate.

2. Materials and methods

2.1. Materials and equipment

Plasmid pVAX1GFP (3697 bp), described by Azzoni et al. [34], was kindly donated by Professor Miguel Prazeres (Instituto Superior Técnico, Lisbon, Portugal). Tris-(hydroxymethyl) aminomethane (Tris), ammonium sulfate, tri-sodium citrate, 2-propanol, ethylenediamine tetra-acetic acid (EDTA), sodium hydroxide, sodium chloride, acetic acid, glucose, and potassium acetate were purchased from Merck (Darmstadt, Germany). Yeast extract, tryptone, and potassium phosphate were purchased from Fluka (Buchs, Switzerland). All reagents were of analytical grade. Chromatographic experiments were performed in an ÄKTA Purifier FPLC system with a Frac-920 fraction collector provided with Unicorn 5.1 data acquisition and processing software (GE Healthcare,

Uppsala, Sweden). All chromatographic runs were monitored by absorbance at 260 nm. Sample desalinization for agarose gel electrophoresis and gDNA and protein quantification was performed using Microcon devices with a nominal membrane size cutoff of 10 kDa (Millipore, Bedford, USA). Genomic DNA quantification was done with PCR real time using an AB7300 System and sequence detection software SDS 1.3.1, both from Applied Biosystems (Foster City, USA) and Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Glen Burnie, USA).

2.2. Adsorbents

Phenyl Sepharose 6 Fast Flow (low sub) resin (GE Healthcare, Uppsala, Sweden) containing 25 mmol of phenyl per milliliter (according to the manufacturer) was the adsorbent used in the HIC. The adsorbent used for the TAC was divinyl sulfone (Sigma, St. Louis, USA) activated Sepharose 6B agarose (GE Healthcare, Uppsala, Sweden) to which the ligand 2-mercaptopyrimidine (Sigma, St. Louis, USA) was coupled in accordance with Hermanson et al. [35]. Ligand density in the derivatized agarose 18.5 mmol mercaptopyrimidine/mL media was determined indirectly through elemental analysis of nitrogen content at Central Analítica (Instituto de Química-Unicamp, Campinas, Brazil) using Perkin Elmer-Series II 2400 equipment.

2.3. Production of neutralized lysate

Cell lysate containing pDNA was produced for adsorption studies by growing competent DH5α *E. coli* cells transformed with pVAX1GFP plasmid. Fermentation was conducted for 16 h at 37 °C and 250 rpm in Erlenmeyer flasks containing 250 mL of LB medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) and kanamycin (30 mg/mL). At the end of the exponential phase, cells were harvested by centrifugation (3500 × *g* for 10 min) and disrupted in accordance with the alkaline lysis method described by Birnboim and Doly [36]. Then, the lysate was neutralized by the addition of chilled 3.0 mol/L potassium acetate at pH 5.5 and the resulting suspension was kept on ice for 10 min. A final centrifugation step was performed (12,000 × *g* at 4 °C for 30 min) for removal of precipitated cellular debris, gDNA, and proteins. To ensure the stability of the pDNA, the neutralized lysate was stored in aliquots at −70 °C for up to three months [9].

2.4. pDNA adsorption and desorption studies

Solutions of 1.5 mol/L sodium citrate buffer, 1.5 mol/L ammonium sulfate, and 2.0 mol/L potassium phosphate to be used as adsorption buffer were prepared in 10 mmol/L Tris–HCl at pH 8.0. All four solutions were filtered through a 0.22 μm filter (Millipore, Bedford, USA) and degassed under vacuum.

Chromatographic runs were performed in columns with 1 mL of adsorbent at a volumetric flow rate of 0.25 mL/min. The first chromatographic step consisted of matrix conditioning with five column volumes (CV) of adsorption buffer. A volume of 250 μL of neutralized lysate (preconditioned as adsorption buffer by the addition of salt crystals) was then injected into the column, followed by washing with 10 CV of the same adsorption buffer. Elution was carried out with a 10 CV linear gradient (0–100%) of 10 mmol/L Tris–HCl at pH 8.0. Fractions of 0.5 mL were collected and stored at −20 ° C for further analysis.

2.5. Analytical methods

The neutralized lysate and fractions from HIC and TAC that resulted in absorption peaks at 260 nm were analyzed by electrophoresis in 0.8% agarose gel (60 V for 4 h). The molecular mass standard kit used was the 1 Kb Plus DNA Ladder (Invitrogen, Carlsbad, USA) and gels were stained with ethidium bromide at a final concentration of 0.5 μg/mL.

pDNA was quantified with analytical HIC [30] with a Source 15 PHE column (GE Healthcare, Uppsala, Sweden) connected to an AKTA Purifier system. The concentration of plasmids was determined according to a calibration curve with standard pDNA solutions with concentrations ranging from 2.5 to 50 μg/mL. Purified pDNA was obtained according to the protocol described by Moreira et al. [24]. The concept of HPLC purity described by Diogo et al. [37] was used to quantify the purity of the samples. Briefly, the degree of HPLC purity was defined as the percentage of the plasmid peak area relative to the area of all peaks in the chromatogram after subtracting the corresponding blanks. A typical analytical chromatogram is shown in Fig. 2.

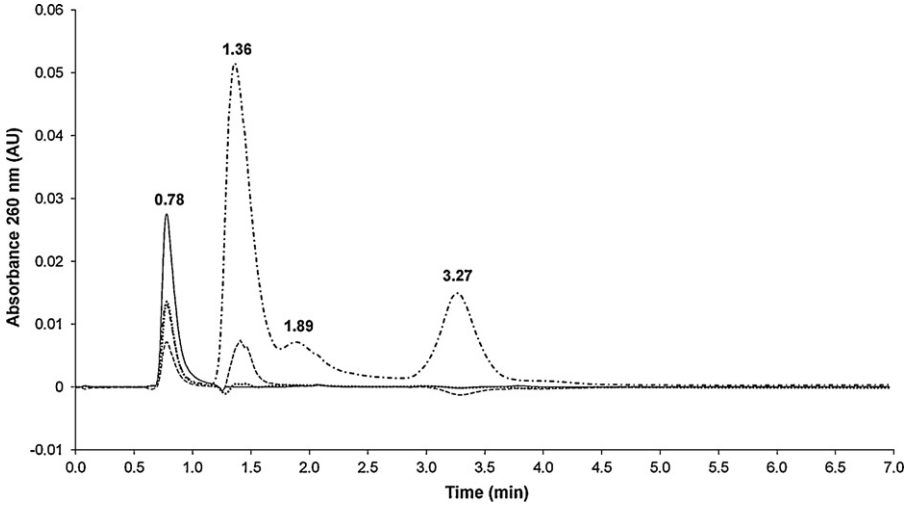

Fig. 2. Typical analytical HPLC for assessment of pDNA purification. Adsorption buffer, 2.0 mol/L potassium phosphate; adsorbent, mercaptopyrimidine agarose. The peak at 0.78 min corresponds to total plasmid DNA. The peak at 3.27 min corresponds to high molecular weight RNA and gDNA and the other peaks (1.36 min and 1.89 min) are attributed to low molecular weight RNA and remaining proteins [30,31]. Dotted and dashed line: neutralized lysate diluted ten times; dashed line: fraction 32; solid line: fraction 33; dotted line: fraction 34.

Total protein was quantified by the bicinchoninic acid method with a BCA kit in accordance with the manufacturer's instructions (Pierce, Rockford, USA). A calibration curve was built using bovine serum albumin as the standard protein (solutions of 5–40 μg/mL in 10 mmol/L Tris–HCl at pH 8.0).

Endotoxin was determined using a Limulus Amoebocyte Lysate (LAL) kit (Lonza, Walkersville, USA) in accordance with the manufacturer's instructions. Calibration curves were constructed utilizing solutions of *E. coli* endotoxin (supplied in the kit) with concentrations ranging from 0.1 to 1.0 EU/mL.

Genomic DNA quantification was accomplished with real-time PCR [38]. Calibration curves ranging from 1.7 to 140,000 ρg/mL of gDNA were constructed from serial dilutions. The standard *E. coli* gDNA was obtained by the protocol described by Ausubel et al. [39].

3. Results and discussion

3.1. Sodium citrate (1.5 mol/L) as adsorption buffer

3.1.1. HIC study

The HIC of neutralized lysate using phenyl agarose as the adsorbent and 1.5 mol/L sodium citrate as the mobile phase allowed the adsorption and desorption of pDNA and RNA (Fig. 3a). Partial adsorption of the pDNA took place since it was detected in the wash and elution chromatographic steps. The peak corresponding to fractions 28–30 contained only the supercoiled (sc) isoform of pDNA. The preferential interaction of sc pDNA and hydrophobic resins is stronger than the interaction of the open circular (oc) pDNA isoform and hydrophobic resins, as explained by Ferreira et al. [1] who suggested that the sc isoform can access ligands in the pores of the adsorbent particle because they are more compact than the oc isoform.

The high salt concentration buffer favored selectivity between RNA molecules and sc pDNA. Murphy et al. [40] and Bencina et al. [41] suggested that a high salt concentration can reduce the repulsion between the negatively charged groups in the nucleic acid chain, allowing its compact packaging and resulting in a higher adsorption capacity. The single-strand nature of the RNA molecules allows denser packing and stronger hydrophobic interactions in a high salt environment than pDNA [13].

The peak containing sc pDNA (fractions 28–30) showed no protein, gDNA, or endotoxins; given the sensitivity of the analytical methods employed, removal of these impurities was shown to be greater than 97.0%, 99.9%, and 99.2%, respectively (Table 1). Despite the absence of protein and endotoxin, the purity of the pDNA in this peak was low (41.6%), probably due to contamination with RNA, undetected by electrophoresis but detected by the analytical method utilized to assess purity (HPLC). RNA desorption occurred in the following fractions along the elution gradient (data not shown) as the ionic strength of the buffer and therefore the intensity of the hydrophobic interactions, decreased.

3.1.2. TAC study

TAC using mercaptopyrimidine agarose adsorbent and 1.5 mol/L sodium citrate adsorption buffer resulted in a chromatographic profile with five absorbance peaks (Fig. 3b). Electrophoretic analysis showed that fractions 2, 3, and 27 contained mainly oc pDNA. Fractions 28–32 were composed mostly of sc pDNA. Fractions 39–43 corresponded to the RNA molecules desorbed along the gradient elution.

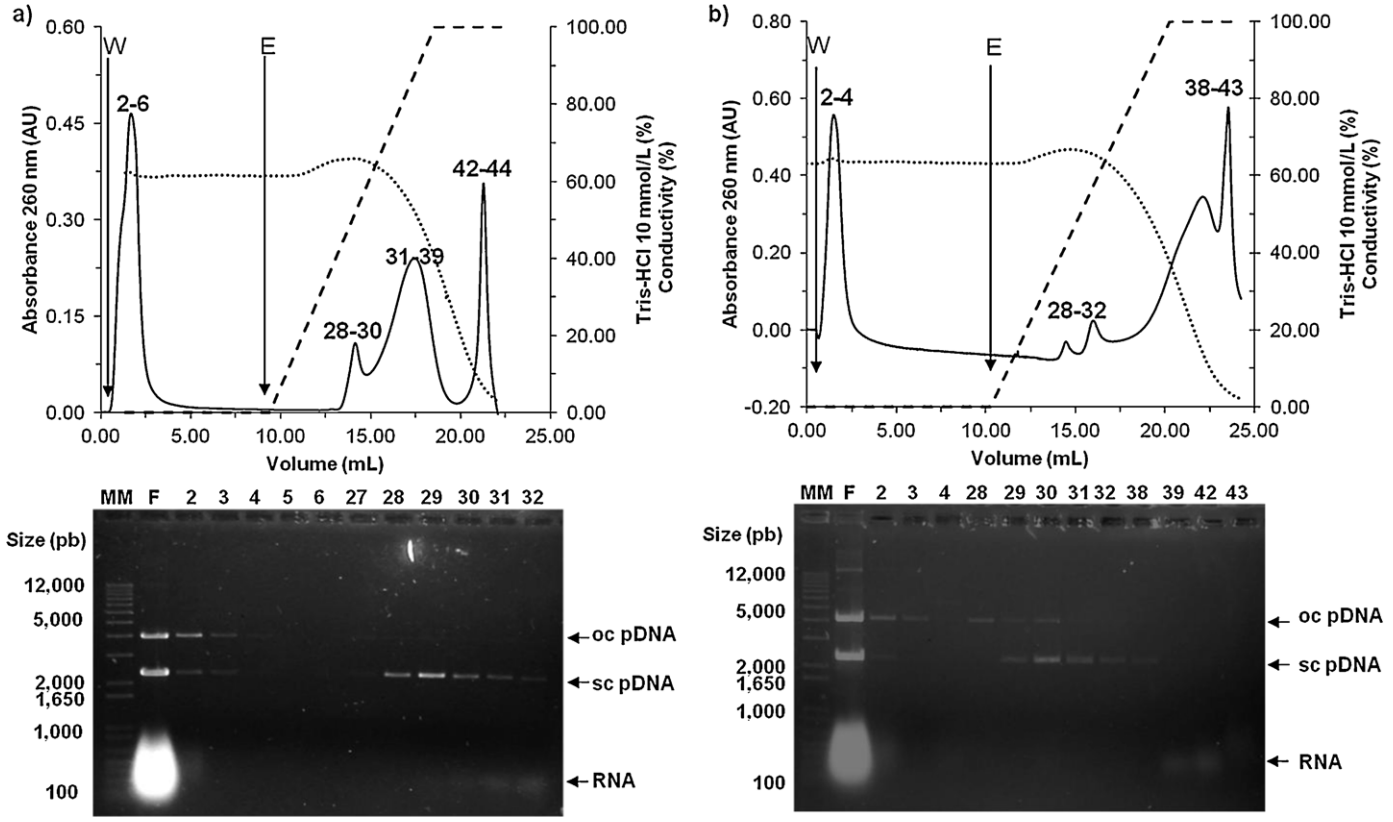

Fig. 3. Chromatography with 1.5 mol/L sodium citrate as adsorption buffer: (a) Chromatogram and electrophoretic analysis of neutralized lysate chromatography with phenyl-agarose adsorbent and (b) chromatogram and electrophoretic analysis of neutralized lysate chromatography with mercaptopyrimidine-agarose adsorbent. Numerical range implies the fractions of the respective absorbance peaks; W: washing step; E: elution step; MM: molecular mass; F: neutralized lysate. Solid line: absorbance at 260 nm (AU); dashed line: concentration of 10 mmol/L Tris–HCl in elution buffer (%); dotted line: conductivity (%).

Table 1
Comparison between data obtained using HIC with phenyl and TAC with mercaptopyrimidine ligands in pDNA chromatographic purification with 1.5 mol/L sodium citrate, 2.0 mol/L potassium phosphate, and 1.5 mol/L ammonium sulfate as adsorption buffers.

Adsorbent	Plasmid DNA			Impurity removal (%)		
	Yield (%)	Purity (%)	Purification factor	Proteins	gDNA	Endotoxins
1.5 mol/L sodium citrate						
Phenyl agarose [a]	59.1 ± 4.8	41.6 ± 5.6	6.8	>97.0	>99.9	>99.2
Mercaptopyrimidine agarose [b]	91.1 ± 2.7	13.4 ± 1.9	4.8	94.0 ± 3.0	99.5 ± 0.4	>99.9
2.0 mol/L potassium phosphate						
Phenyl agarose [c]	38.1 ± 0.0	75.5 ± 2.7	4.4	>98.0	>99.9	>99.7
Mercaptopyrimidine agarose [d]	68.5 ± 2.1	98.8 ± 0.3	12.2	91.7 ± 6.8	98.3 ± 0.9	>99.8
1.5 mol/L ammonium sulfate						
Phenyl agarose [e]	56.3 ± 1.3	82.6 ± 1.3	4.8	90.6 ± 5.2	>99.9	96.9 ± 0.7
Mercaptopyrimidine agarose [f]	58.0 ± 3.5	44.7 ± 2.6	9.9	>96.9	99.2 ± 0.2	>99.9

[a] Data shown corresponds to the peak composed of fractions 28, 29, and 30. Neutralized lysate composition: 16.2 μg pDNA, purity = 6.1%, 68.0 ± 6.7 μg/mL proteins, 1.8×10^5 ρg/μL gDNA, and 132.0 EU/mL.
[b] Data shown corresponds to the peak composed of fractions 29, 30, 31, and 32. Neutralized lysate composition: 11.0 μg pDNA, purity = 2.8%, 120.3 ± 6.6 μg/mL proteins, 1.1×10^5 ρg/μL gDNA, and 1051.8 EU/mL.
[c] Data shown corresponds to the peak composed of fractions 30, 31, and 32. Neutralized lysate composition: 32.4 μg pDNA, purity = 17.2%, 112.6 ± 45.2 μg/mL proteins, 2.1×10^5 ρg/μL gDNA, and 320.0 EU/mL.
[d] Data shown corresponds to the peak composed of fractions 32, 33, and 34. Neutralized lysate composition: 16.2 μg pDNA, purity = 8.1%, 131.1 ± 10.3 μg/mL proteins, 2.4×10^4 ρg/μL gDNA, and 447.0 EU/mL.
[e] Data shown corresponds to fraction 2. Neutralized lysate composition: 18.8 μg pDNA, purity = 17.2%, 112.6 ± 45.2 μg/mL proteins, 1.9×10^5 ρg/μL gDNA, and 329.1 EU/mL.
[f] Data shown corresponds to fraction 2. Neutralized lysate composition: 14.1 μg pDNA, purity = 4.5%, 130.0 ± 0.7 μg/mL proteins, 6.1×10^4 ρg/μL gDNA, and 1285.8 EU/mL.

This adsorbent/buffer combination permitted the recovery of 91.1% of the pDNA and fractions 28–32 had no measurable concentration of endotoxins, indicating a removal of above 99.9% of this impurity from the neutralized lysate (Table 1). The analysis indicates removals of 94.0 and 99.5% for protein and gDNA, respectively. Despite this removal of high levels of impurity from a crude lysate with a single-step purification, pDNA purity was low (13.4%), probably also due to RNA contamination below the electrophoresis detection limit, but detectable by HPLC.

Comparing the data obtained by HIC with phenyl-agarose adsorbent and by TAC with mercaptopyrimidine adsorbent (Table 1), the former was found to be superior in terms of sc pDNA selectivity, purity, and protein removal. However, TAC with mercaptopyrimidine agarose allowed better pDNA recovery, 91.1% as compared

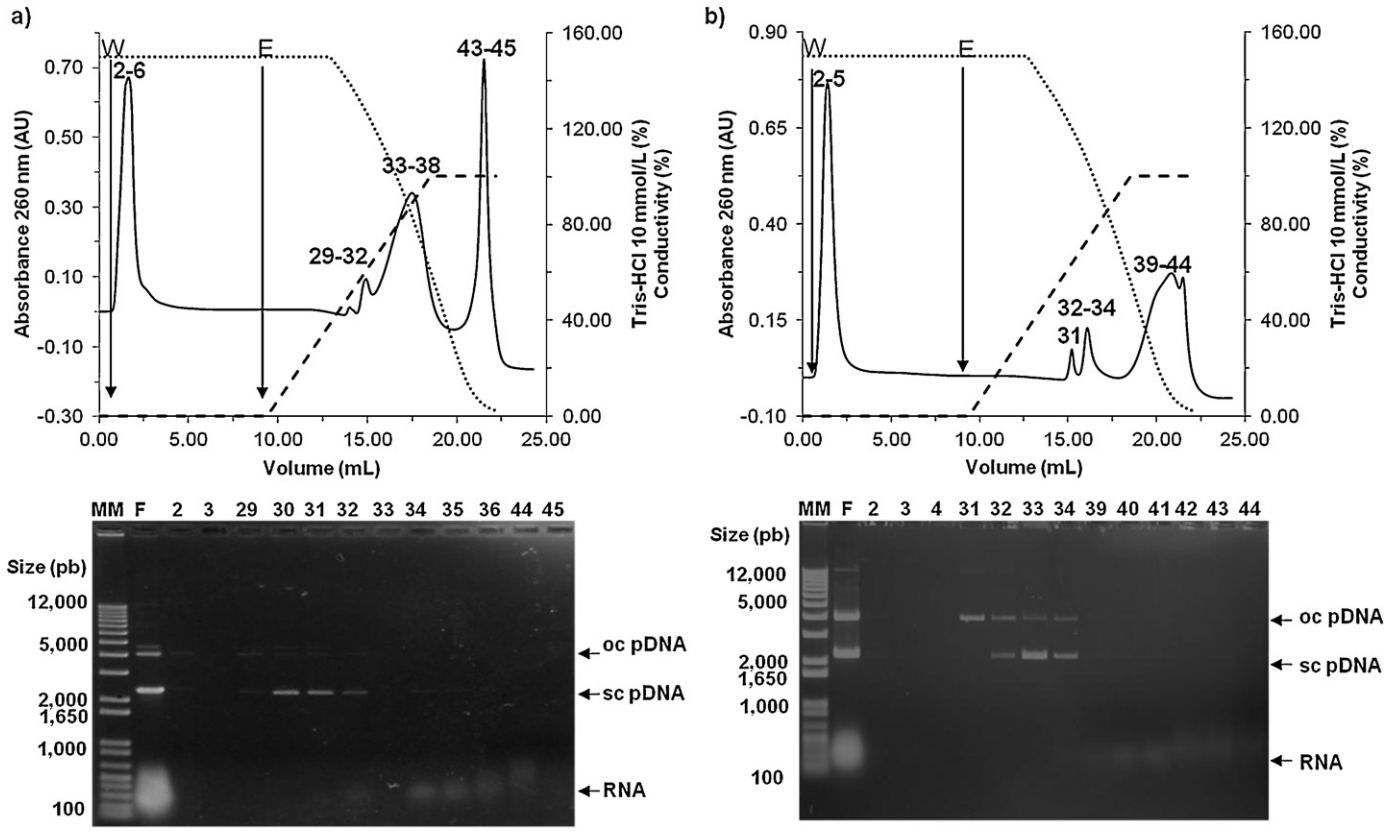

Fig. 4. Chromatography with 2.0 mol/L potassium phosphate as adsorption buffer: (a) chromatogram and electrophoretic analysis of neutralized lysate chromatography with phenyl-agarose adsorbent and (b) chromatogram and electrophoretic analysis of neutralized lysate chromatography with mercaptopyrimidine-agarose adsorbent. Numerical range implies the fractions of the respective absorbance peaks; W: washing step; E: elution step; MM: molecular mass; F: neutralized lysate. Solid line: absorbance at 260 nm (AU); dashed line: concentration of 10 mmol/L Tris–HCl in elution buffer (%); dotted line: conductivity (%).

to 59.1%. This higher pDNA recovery corroborates the work of Sandberg et al. [12], who showed that a higher electronegativity of the aromatic ring of ligands results in a stronger interaction with polynucleotides (mercaptopyrimidine contains two nitrogen atoms). Even though the neutralized lysate fed in the TAC experiments had the lowest purity (2.8%) and the highest level of endotoxins of all lysates, this adsorbent/buffer system provided remarkable endotoxin removal and a purification factor close to the one obtained with phenyl-agarose adsorbent. The high endotoxin level probably influenced selectivity of pDNA and its purification by occupation of the adsorption sites.

3.2. Potassium phosphate (2.0 mol/L) as adsorption buffer

3.2.1. HIC study

The use of 2.0 mol/L potassium phosphate as the adsorption buffer for the phenyl-agarose HIC produced a partial resolution between the oc and the sc isoforms of pDNA (Fig. 4a, fractions 30–32). These fractions were enriched with the supercoiled isoform. RNA molecules were eluted as ionic strength decreased along the elution gradient, showing a behavior similar to that observed with 1.5 mol/L sodium citrate mobile phase.

This HIC resulted in pDNA fractions with no proteins, gDNA, or endotoxins with impurity removals of over 98.0%, 99.9%, and 99.7%, respectively. pDNA recovery was 38.1% and purity was 75.5% (Table 1).

3.2.2. TAC study

The use of 2 mol/L potassium phosphate as the adsorption buffer in TAC with mercaptopyrimidine agarose as the adsorbent resulted in a chromatogram with four absorbance peaks (Fig. 4b). Electrophoretic analysis showed that the first elution peak (fraction 31) was composed of oc pDNA. The second elution peak (fractions 32–34) contained the oc and sc pDNA isoforms, but was enriched with the latter. The last elution peak (fractions 39–44) consisted mainly of RNA. This chromatography recovered up to 68.5% of the pDNA with an average removal of 91.7% of the proteins, 98.3% of the gDNA, and more than 99.8% of the endotoxins initially present (Table 1). Although it was possible to detect proteins and gDNA in the elution peak (fractions 32–34), its purity was close to 100% (98.8%). This inconsistency can also be explained by the fact that different methods with different sensitivities were applied to quantify purity, proteins, and gDNA. gDNA was quantified of the order of picograms (real-time PCR), a quantity probably below the detection limit of the method of pDNA purity determination (HPLC). Furthermore, using the method for determination of pDNA purity, peak absorbance was recorded at 260 nm, not the ideal wavelength for the detection of proteins (280 nm).

Potassium phosphate and mercaptopyrimidne agarose also gave good resolution between the pDNA and RNA. From these data on resolution and impurity removal it is possible to infer that the method of pDNA purity measurement was affected more by the resolution of these pDNA and RNA peaks than by the presence of other impurities (proteins and gDNA) in pDNA-containing fractions.

Three general conclusions can be drawn: (1) sodium citrate allowed more ligand selectivity toward sc pDNA, (2) potassium phosphate buffer produced higher purity, and (3) potassium phosphate required lower ionic strength in the elution buffer for pDNA desorption than sodium citrate. Sodium citrate allowed higher selectivity toward sc pDNA, probably due to its ability to compress more flexible molecules such as RNA and sc pDNA. Potassium phosphate at 2.0 mol/L promoted a stronger interaction between

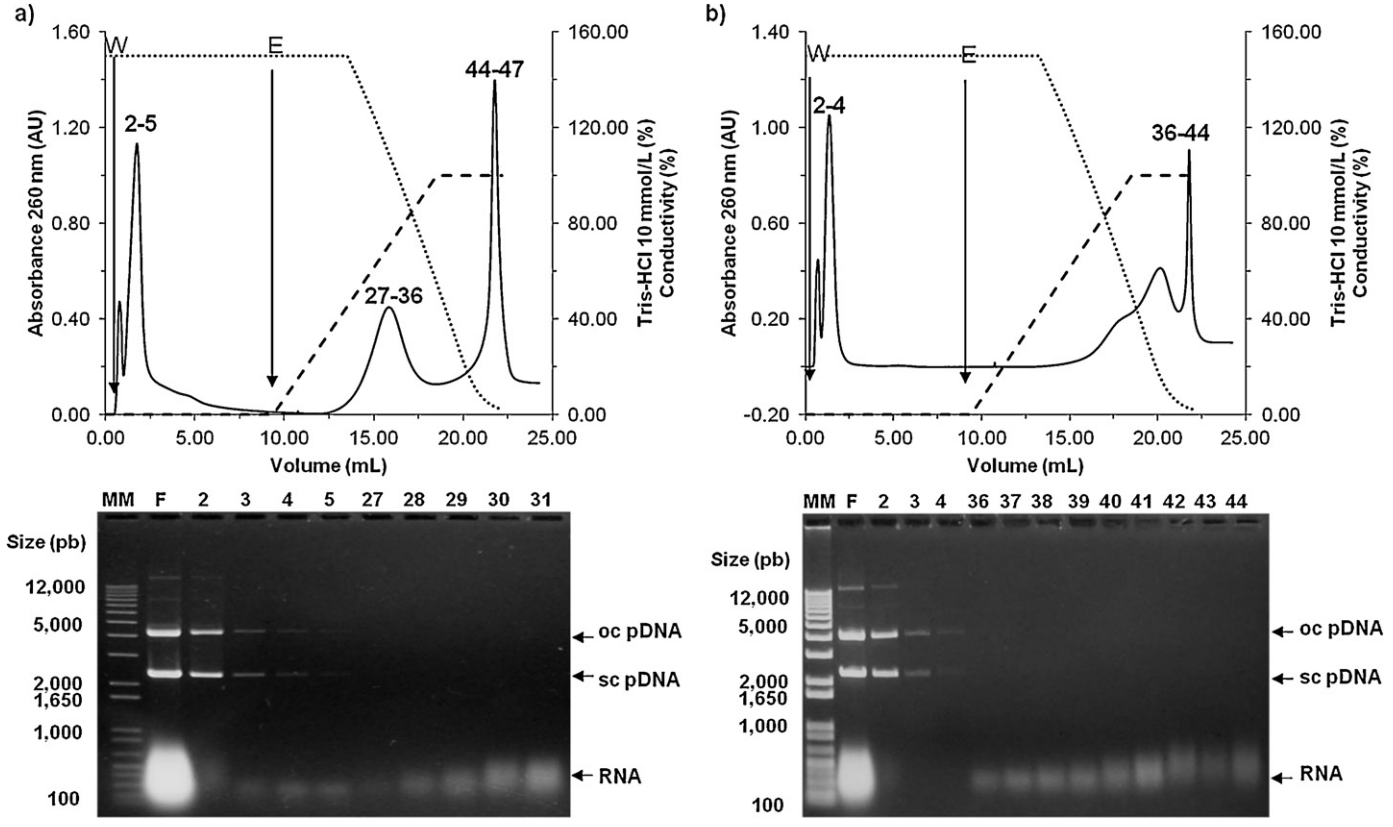

Fig. 5. Chromatography with 1.5 mol/L ammonium sulfate as adsorption buffer: (a) chromatogram and electrophoretic analysis of neutralized lysate chromatography with phenyl-agarose adsorbent and (b) chromatogram and electrophoretic analysis of neutralized lysate chromatography with mercaptopyrimidine-agarose adsorbent. Numerical range implies the fractions of the respective absorbance peaks; W: washing step; E: elution step; MM: molecular mass; F: neutralized lysate. Solid line: absorbance at 260 nm (AU); dashed line: concentration of 10 mmol/L Tris–HCl in elution buffer (%); dotted line: conductivity (%).

Table 2
Comparison of yields, HPLC purities and impurity removals of preliminary recovery steps and pDNA purification processes.

Application	Processes	Feed	Number of post-lyses stages	Yield (%)	Purity (%)	Impurity removal (%)			Reference
						Proteins	gDNA	Endotoxins	
Preliminary recovery	TAC with sodium citrate buffer	NL[a]	1	91.1	13.4	94.0	99.5	>99.9	This work
	Aqueous two phase system PEG 600/ammonium sulfate			96.0	58.5	90.0	99.5	98.3	[48]
	Tangential flow filtration			90.0	18.0	93.8	15.6	85.0	
	Precipitation with isopropanol + precipitation with ammonium sulfate		2	90.0	48.0	98.7	94.8	96.5	
	Tangential flow filtration + precipitation with ammonium sulfate			87.4	28.0	97.0	72.4	98.6	
	Tangential flow filtration + aqueous two phase system			58.0	94.0	96.7	85.0	99.8	
	Aqueous two phase system PEG 600/ammonium sulfate and sodium citrate			91.1	17.2	nr[c]	nr[c]	nr[c]	[30]
Final purification	TAC with potassium phosphate buffer	NL[a]	1	68.5	98.8	91.7	98.3	>99.8	This work
	Boronate adsorption			64.0	64.8	94.6	96.7	nr[c]	[20]
	Membrane HIC		2	73	60	nr[c]	nr[c]	nr[c]	[10]
	HIC with sodium citrate	CL[b]	3	73.5	~100	87.6	99.3	98.5	[9]
	Affinity chromatography with histidine ligand and ammonium sulfate as buffer			40	100	nr[c]	~100[d]	~100[d]	[46]

[a] NL: neutralized lysate.
[b] CL: clarified lysate.
[c] nr: non reported.
[d] This value was calculated based on the information given in the text.

pDNA and the adsorbent despite having a lower ionic strength than the 1.5 mol/L sodium citrate (5 mol/L and 7.5 mol/L, respectively), probably by promoting more compaction in pDNA molecules in general.

TAC was better than HIC (Table 1) in terms of pDNA recovery (TAC, 68.5%; HIC, 38.1%), purity (TAC, 98.8%; HIC, 75.5%), and resolution between pDNA and RNA. HIC was better than TAC at eliminating proteins and gDNA. The purity of the neutralized lysate fed into the TAC system was half that of the lysate employed in the HIC experiments, thus showing the efficiency of the mercaptopyrimidine-agarose adsorbent in pDNA purification. Using an electronegative ligand (mercaptopyrimidine), mixed interactions (electron donor/acceptor and hydrophobic interactions) may have resulted in stronger interactions between the adsorbent and polynucleotides [11]. In addition, these mixed interactions probably increased the interactions between adsorbent and proteins, resulting in the removal of a smaller amount of protein.

3.3. Ammonium sulfate (1.5 mol/L) as adsorption buffer

Ammonium sulfate is the most frequently employed adsorption buffer for pDNA purification by chromatography through affinity [42–46], hydrophobic [9,10,23,47], and thiophilic [11,12] interactions. Therefore, for comparison purposes chromatographies similar to those performed using sodium citrate and potassium phosphate were carried out using this salt.

The chromatographies using 1.5 mol/L ammonium sulfate in the adsorption buffer resulted in similar chromatograms for both adsorbents (Fig. 5). Electrophoretic analysis showed that this adsorption buffer did not promote adsorption of either one of the pDNA isoforms, with those recovered being contaminated with RNA in the washing fractions.

Both adsorbents were capable of adsorbing the RNA that had been desorbed in the elution step. However, the mercaptopyrimidine ligand was responsible for promoting a stronger interaction between the RNA and the adsorbent, since a much lower ionic strength was required to promote desorption of the RNA than in the case of the phenyl ligand. Stronger interactions were expected in TAC with mercaptopyrimidine ligand than in HIC, since in the former technique, besides the hydrophobic interaction, an electron donor-acceptor interaction with RNA also takes place.

Mercaptopyrimidine-agarose chromatography with 1.5 mol/L ammonium sulfate was not as efficient as phenyl-agarose chromatography in terms of pDNA purity (TAC, 44.7%; HIC, 82.6%) and removal of gDNA contamination (Table 1), which was too low for the method of detection (real-time PCR). Both chromatographic methods gave similar pDNA recovery values (TAC, 58.0%; HIC, 56.3%). TAC was better in terms of protein and endotoxin removal, reducing the concentrations to levels below the method's detection limit.

Table 2 contains a summary of the data obtained in this work and allows a comparison with related published work. Among the best results is the purity obtained with TAC/potassium phosphate and the high recovery obtained with TAC/sodium citrate.

4. Conclusions

This work showed that both buffer systems tested, sodium citrate and potassium phosphate, can be used as substitutes for ammonium sulfate in HIC and TAC aiming at the purification of pDNA. In addition, this work showed that this chromatographic system can be applied directly to neutralized lysate without the need for preliminary recovery steps. Sodium citrate at 1.5 mol/L can be used with mercaptopyrimidine agarose as an intermediate stage of recovery in the pDNA purification process due to its

ability to recover large amounts of pDNA and promote extensive removal of proteins, endotoxins, and RNA in a single chromatographic step. Potassium phosphate at 2.0 mol/L can be used as the adsorption buffer in a single chromatographic purification step with mercaptopyrimidine-agarose adsorbent, as this combination of matrix-ligand-buffer resulted in pDNA with a purity of 98.8%. This last finding is very relevant, since there is no report in the literature of a chromatographic step for purifying pDNA from neutralized lysate resulting in such a high purity. The presence of the gDNA and protein impurities in the pDNA purified with TAC/potassium phosphate is of no concern, since pDNA is a product for therapeutic use: it is mandatory to perform a buffer exchange step after the chromatographic purification. If this is performed with size exclusion chromatography, for example, gDNA and protein can be removed, since they are different in size from plasmid.

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