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Impact of lysine-affinity chromatography on supercoiled plasmid DNA purification

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ABSTRACT

Gene therapy and DNA vaccination cover a variety of applications using viral and non-viral vectors as vehicles of choice for treatment of genetic or acquired diseases. Recently, most therapeutic applications have been performed with non-viral biological agents preparations highly enriched in supercoiled plasmid molecules and it has been concluded that this isoform is more efficient at gene transfection than open circular isoform. This work describes for the first time a new strategy that uses lysine-chromatography to efficiently eliminate *Escherichia coli* impurities as well as other ineffective plasmid isoforms present in a complex clarified lysate to purify and obtain pharmaceutical-grade supercoiled plasmid DNA. The quality control tests indicated that the levels of impurities in the final plasmid product were below the generally accepted specifications. Furthermore, the delivery of the purified product to eukaryotic cells, the cell uptake and transfection efficiency were also analyzed. The results showed that the transfection efficiency reached with the application of the supercoiled plasmid conformation, purified with lysine-agarose, was higher than the values achieved for other plasmid topologies. Therefore, this study presents a new enabling technology to obtain the completely purified non-viral vector, able to act with good efficiency as gene therapy delivery vehicle in several diseases like cancer.

1. Introduction

In the last decades, a technological advance in medical and pharmaceutical areas allowed the accomplishment of several human clinical trials, testing the ability to inject plasmids providing therapeutic benefits in the untreatable pathologies [1–4]. Effective DNA vaccination and gene therapy require the control of both the location and function of therapeutic genes at specific target sites within the patient's body [5]. The main challenge in the development of molecular medicine is to introduce naked DNA into diseased cells overcoming the extra- and intracellular cell barriers. Most gene administration methods are based on viral vectors [6] but this delivery system has raised safety and regulatory concerns because of their toxicity and immunogenicity [7]. Thus, the non-viral vectors arise as a good alternative, overcoming the problems associated to the viral vector-mediated therapy [6], becoming the most attractive gene-transfer system to be used as biopharmaceutical product [5,8,9]. Therefore, it is important to continue the development of non-viral vectors, as plasmid DNA (pDNA), for efficient transfection and gene expression.

Almost all processes, for the manufacture of pDNA in sufficient quantities to clinical applications, needed to improve the productivity [10], mainly in the following operations: fermentation, lysis, isolation, purification and transfection (Fig. 1). Plasmids are mostly biosynthesized by *Escherichia coli* (*E. coli*) fermentation, intending to maintain high plasmid copy number per bacterium [1]. After the bacterial cell harvest, normally by centrifugation or microfiltration techniques, it is performed the cell lysis, which is considered the critical operation of the pDNA manufacturing process [11]. This step can affect the ratio of supercoiled (sc) plasmid to other forms (Fig. 1) and the plasmid amount, as well as the physicochemical characteristics of cellular impurities that must be removed during the downstream process. The isolation of crude plasmid can be done by precipitation with salt in order to reduce the presence of RNA and other host impurities [12], like proteins and endotoxins. The final step to obtain pDNA as a highly pure product for therapeutic applications is the plasmid purification, through of liquid chromatography [13].

Several common approaches exploit one or more of the following purification processes: size exclusion, anion exchange, hydrophobic interaction and affinity chromatography [14]. Nevertheless, the affinity concept is gaining impact with the development of new supports with specific ligands that improve the binding capacity for pDNA [15] and combine different interactions promoting a biorecognition of sc conformation [13].

Supercoiled pDNA, due to its structure extremely compact and functional, is considered the most efficient isoform at inducing gene expression comparing with other conformational variants

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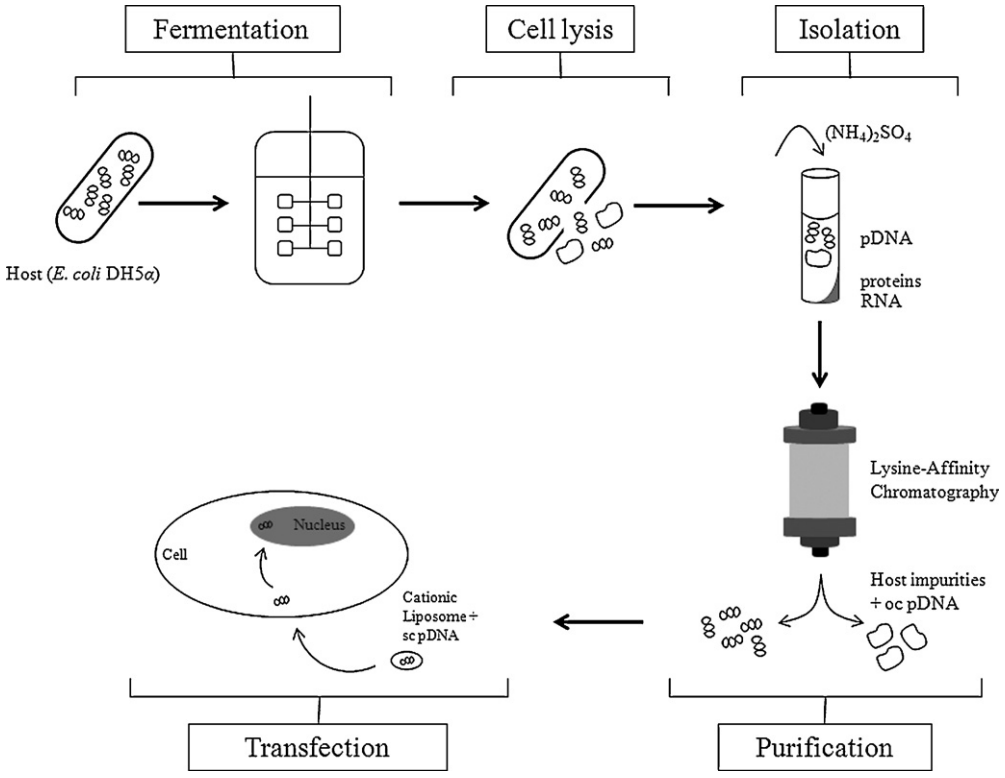

Fig. 1. Schematic representation of downstream processing of sc pDNA preparation, according to quantity and purity degree required for clinical applications, using lysine-affinity chromatography as a major purification step. *E. coli*, *Escherichia coli*; $(NH_4)_2SO_4$, ammonium sulfate; oc, open circular; sc pDNA, supercoiled plasmid DNA.

[16] (open circular (oc) and linear isoforms, obtained from the damage of the sc form) [1,17]. According to regulatory agencies, such as Food and Drug Administration (FDA), a content of sc form higher than 97% is required to apply in gene therapy and DNA vaccination [10]. This sc plasmid isoform should be relatively free from impurities, such as bacterial genomic DNA (gDNA) (<2 ng/μg of pDNA), endotoxins (<0.1 EU/μg of pDNA), RNA and host proteins (undetectable) [11]. Knowing that the total amount of pDNA present in the *E. coli* extract represents less than 3% of the global content, it becomes essential developing adequate purification processes to isolate the sc isoform, as it is represented in Fig. 1.

The selection of affinity matrices with amino acids ligands was mainly supported by the natural occurrence of protein–DNA complexes in biological systems [18] and because some atomic evidences suggested the existence of favored interactions between particular amino acids and nucleic acids bases [19–21]. Recently, several amino acids, such as histidine [22,23] and arginine [24,25] have been tested as affinity ligands in agarose chromatographic supports to specifically purify sc pDNA from a clarified *E. coli* lysate. A new agarose support with lysine ligand was first experimented with a pre-purified native (oc + sc) pDNA sample and the separation of both plasmid isoforms was achieved [26]. With this study, it was also possible to understand the interaction mechanism underlying the specificity of the support that allowed a biorecognition of the sc isoform. The applicability of this matrix to efficiently purify and isolate this isoform from the other plasmid topologies and *E. coli* host impurities in a single chromatographic step, considering the requirements of the regulatory agencies, is evaluated, for the first time, in the present study. These findings will strengthen the possibility of using lysine-affinity chromatography as a potential enabling technology in the downstream process of sc pDNA for therapeutic applications.

2. Materials and methods

2.1. Materials

Lysine-Sepharose 4B gel was obtained from GE Healthcare Biosciences (Uppsala, Sweden). Sodium chloride and ammonium sulfate were purchased from Panreac (Barcelona, Spain) and tris(hydroxymethyl) aminomethane (Tris) from Merck (Darmstadt, Germany). All solutions used in chromatographic experiments were freshly prepared using deionized water ultra-pure grade, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and analytical grade reagents. The elution buffers were filtered through a 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. The iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used for genomic DNA quantification. Unless otherwise stated, the reagents used for Hela and COS-7 culture were obtained from Sigma (St. Louis, MO, USA) and the reagents for the transfection experiments were obtained from Invitrogen (Carlsbad, CA, USA). The 6.05-kbp plasmid pVAX1-*LacZ* (Invitrogen, Carlsband, CA, USA), designed for the development of DNA vaccines, was used as a model plasmid. This vector contains the Human CytoMegalovirus (CMV) immediately early promoter/enhancer, the Bovine Growth Hormone (BGH) polyadenylation signal, a T7 promoter/printing site, a multiple cloning site, a kanamycin resistance gene, a pUC origin and a reporter (β-galactosidase) gene used as a positive control for transfection and expression in the cell line of choice. The host strain used to obtain the several nucleic acids was *E. coli* DH5α.

2.2. Plasmid production

The pVAX1-*LacZ* amplification was obtained by *E. coli* DH5α fermentation as described by Sousa *et al.* 2010 [27], carried out at 37 °C using the 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 kanamycin/mL. The cells were harvested at the late log phase by centrifugation and the pellets were stored at −20 °C. Plasmid-free *E. coli* DH5α cells were also grown under the same conditions as described before, but with no antibiotic present.

2.3. Alkaline lysis and clarification of E. coli lysate

Cells were lysed through a modification on the alkaline method [28] as described by Diogo et al. [29]. Bacterial pellets (obtained from centrifugation of 250 mL cell broth at 5445 × *g* (30 min, 4 °C)) were thawed and dissolved in 20 mL resuspension buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM ethylene-diamine tetraacetic acid (EDTA), pH 8.0). Alkaline lysis was performed by adding 20 mL of a 200 mM NaOH, 1% (w/v) sodium dodecylsulfate (SDS) solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated by adding and mixing 16 mL of prechilled 3 M potassium acetate, pH 5.0 (20 min on ice). The precipitate was removed by centrifuging twice at 20,000 × *g* (30 min, 4 °C) with a Sigma 3-18K centrifuge. The concentration and reduction of impurity content was performed according to a previously published method [29], since the main objective of this work is to explore the purification strategy. In a short description, the pDNA in the supernatant was precipitated by adding 0.7 volumes of isopropanol and incubating on ice for 30 min. The pDNA was recovered by centrifugation at 16,000 × *g* (30 min, 4 °C). The pellets were then redissolved in 2 mL of 10 mM Tris-HCl buffer, pH 8.0. Next, ammonium sulfate was dissolved in the pDNA solution up to a final concentration of 2.5 M, followed by a 15 min incubation on ice. Precipitated proteins and RNA were removed by centrifugation at 16,000 × *g* (20 min, 4 °C). The supernatant was desalted by passing through PD-10 desalting columns (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions, using 10 mM Tris-HCl buffer (pH 8.0) as the elution buffer.

2.4. Isolation of E. coli nucleic acids

Nucleic acids (gDNA and RNA) were isolated from pDNA-free DH5α *E. coli* cells. gDNA was isolated using the Wizard genomic DNA purification kit from Promega (Madison, WI, USA), according to the manufacturer's instructions. For RNA isolation, the cells were lysed by a modification of the alkaline method [28], the resultant lysate was clarified by ammonium acetate precipitation and nucleic acids were concentrated by polyethylene glycol 6000 precipitation as previously described [12].

2.5. Affinity chromatography

Chromatography was performed in a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden). A 16 mm diameter × 100 mm long (≈20 mL) column was individually packed with the commercial lysine-agarose gel. The chromatographic runs were performed at 5 °C, by using a specific column containing a water jacket tube connected to a circulating water bath to maintain the suitable temperature. The column was first equilibrated with 29% of buffer B (250 mM NaCl in 10 mM Tris-HCl buffer, pH 8.0) and the remaining 71% corresponds to buffer A (10 mM Tris-HCl buffer, pH 8.0) at a flow rate of 1 mL/min. After elution of unbound species the ionic strength of the buffer was increased stepwise to 37% of buffer B and then to 100% of the same buffer. The clarified lysate obtained after desalinization was loaded onto the column using a 200 μL loop at the same flow rate. In all separations and throughout the entire chromatographic run the optical density was monitored at 280 nm, while 2 mL fractions were pooled according to the chromatograms obtained, and used for further electrophoresis analysis. For the evaluation of the matrix efficiency on multiple isolations of the sc pDNA isoform and to assess the final content of impurities (proteins, gDNA and endotoxins) present in the sc pDNA purified product, they were used 5 different chromatographic runs. After chromatographic runs, it was necessary to wash the lysine matrix with at least 5 bed volumes of 2 M NaCl in 10 mM Tris-HCl buffer, pH 8.0.

2.6. Agarose gel electrophoresis

Pooled fractions were analyzed by horizontal electrophoresis using 15 cm-long 1% agarose gels (Hoefer, San Francisco, CA, USA), stained with ethidium bromide (0.5 μg/mL). Electrophoresis was carried out at 100 V, for 40 min, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). The gel was visualized in a Vilber Lourmat system (ILC Lda, Lisbon, Portugal). In order to confirm the homogeneity of isoforms recovered from the first and second chromatographic peaks, the band density of each sample pooled and analyzed by electrophoresis was evaluated through of Bio-Rad Quantity One® software (Hercules, CA, USA) using the band analysis toolbox.

2.7. Plasmid quantification

The pDNA concentration and purity was evaluated after each separation process, in the fractions pooled from lysine-chromatography, according to an adaptation of the analytical method previously described [30]. Briefly, the HPLC method based on hydrophobic interaction chromatography was developed for the assessment of pDNA concentration and purity in crude *E. coli* lysate or other extracts. The Phenyl Sepharose Source column (Amersham Biosciences) was used to separate the double-stranded pDNA molecules that elute in the flow through, from the more hydrophobic impurities that are delayed (gDNA and proteins) or retained (RNA and proteins) in the analytical column [30]. Therefore, the 4.6/100 mm HIC Source 15 PHE PE column (Amersham Biosciences) was connected to an ÄKTA Purifier system (GE Healthcare Biosciences). Initially, the column was equilibrated with 1.5 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer, pH 8.0. Afterward, 20 μL of a sample suitably diluted in the equilibration buffer were injected and eluted at a flow rate of 1 mL/min. After the injection of the sample, all unbound material was eluted with 1.5 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer, pH 8.0, for 4 min, and then the elution buffer was instantaneously changed to 10 mM Tris-HCl buffer pH 8.0 without ammonium sulfate. The last elution condition was maintained for 6 min to elute bound species. Next, the column was re-equilibrated for 10 min with the equilibration buffer to prepare the column for the next run. The absorbance of the eluate was recorded at 260 nm. The concentration of pDNA in each sample was calculated using a calibration curve constructed with pDNA standards (1–400 μg/mL) purified with a commercial Qiagen kit (Hilden, Germany). The purity degree was defined as the percentage of the pDNA peak area related with the total area (area of all peaks on the chromatogram). Isolated gDNA and RNA samples were also injected, in order to observe the retention pattern of these nucleic acids in the analytical column.

2.8. Protein quantification

Total protein was quantified using the micro-BCA (bicinchoninic acid) protein assay kit form Pierce (Rockford, USA), according to the manufacturer's instructions. A calibration curve was constructed with Bovine Serum Albumin (BSA) as the standard protein (0.01–0.1 mg/mL), using 10 mM Tris-HCl pH 8.0 to dilute the samples. Samples with high salt concentration were desalted against 10 mM Tris-HCl pH 8.0 before analysis.

2.9. Genomic DNA quantification

Genomic DNA was quantified through of real-time polymerase chain reaction (PCR) in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad), as described previously [31]. Sample collected after lysis was diluted 100 fold beforehand. Sense (5′-ACACGGTCCAGAACTCCTACG-3′) and antisense (5′-CCGGTGCTTCTTCTGCGGGTAACGTCA-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 I. The gDNA concentration was determined using a calibration curve generated by serial dilution of *E. coli* DH5α gDNA (purified with the Wizard gDNA purification kit; Promega) in the 5 pg to 50 ng/μL range. Negative controls (no template) were run at the same time of each analysis.

2.10. Endotoxin quantification

Endotoxin contamination was assessed using the ToxinSensor™ Chromogenic *Limulus* Amoebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript, USA, Inc.) according to the manufacturer's instructions. The calibration curve (from 0.005 to 0.1 EU/mL) was performed from a 10 EU/mL stock solution provided with the kit. To avoid the external endotoxins interference, samples to analyze and samples from the kit were diluted, or dissolved respectively, with non-pyrogenic water, which was also used as the blank. All tubes and tips used to perform this quantification were endotoxins-free.

2.11. Cell culture

Hela and COS-7 cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) at pH 7.4 containing 100 units/mL antibiotic-antimycotic (penicillin/streptomycin), supplemented with 10% Fetal Bovine Serum (FBS) (Biochrom AG, Berlin, Germany), at 37 °C in a 5% CO_2 atmosphere incubator.

2.12. FITC fluorescent labeling of pDNA

The preparation of fluorescently labeled pDNA was performed according to the method previously described [32] with some modifications. Briefly FITC (10 mg) was activated overnight with 2-(4-aminophenyl)-ethylamine in 150 μL of dimethyl-formamide (DMF) under stirring, at room temperature (≈24 °C). Afterwards, FITC-aniline conjugate was reacted with $NaNO_2$ (110 μmol) in 0.5 M HCl at 0 °C under stirring, for 5 min. The reaction was stopped by adding 100 μL of 1 M NaOH solution. FITC-diazonium salt was further mixed with the pVAX1-*LacZ* plasmid samples in Tris-HCl buffer (pH 8.0) at room temperature (≈24 °C), under stirring, for 25 min. The FITC-labeled plasmid was recovered by centrifugation at 16,000 × *g* after precipitation using 0.7 volumes of ice cold isopropanol.

2.13. In vitro transfection

To perform *in vitro* transfection experiments for fluorescence assay, Hela cells were seeded on a 24-well plate at a density of 2.5×10^4 cells/well (with a lamella per well coated with poly-D-lysine) and incubated during 20 h. Immediately before transfection, cells were rinsed and supplemented with 500 μL of fresh culture medium without antibiotic at pH 7.4, whereas FITC-pDNA/polycation complexes were prepared as briefly described below. FITC-labeled pDNA solutions and appropriate volumes of Lipofectamine™ 2000 were separately diluted in 50 μL (for each transfection assay) of Opti-MEM® I medium, according to the manufacturer's instructions. After a 5-min incubation of lipofectamine at room temperature (≈24 °C), the respective FITC-labeled pDNA solution was added and the mixture was incubated for 20 min at room temperature (≈24 °C) to allow the formulation of FITC-pDNA/lipofectamine complexes. A fraction of the mixture (100 μL) was then added to each well containing cells and medium. The cells were incubated at 37 °C under a 5% CO_2 atmosphere. After 6 h of incubation, the medium and complexes were removed, and cells were rinsed once with PBS and cultured with antibiotic and 10% FBS supplemented medium (DMEM).

2.14. Fluorescence

The fluorescence experiments were performed after Hela cells *in vitro* transfection. Following the change of the medium, transfected cells were fixed with 4% paraformaldehyde in PBS and incubated for 20 min. The cells were then permeabilized with 1% Triton X-100 and blocked with 10% FBS and 0.1% Tween 20 in PBS for 3 h. Subsequently the cells were washed 10 times with PBST. Hoechst 33342 dye (2 μM) (catalogue number: H1399; Molecular Probes, Invitrogen, Carlsbad, CA, USA) was then added and incubation proceeded for 15 min at room temperature (≈24 °C), followed by 10 washing steps with PBST. Afterwards the samples were then mounted with DAKO mounting medium and the fluorescence was visualized by confocal microscopy in a Zeiss LSM 510 Meta system (Zeiss Imaging/Systems), using a ×63 objective with an image zoom scan of 1.0.

2.15. Transfection efficiency quantification

The transfection efficiency was determined after 24 h of transfection by using the β-Gal Staining Kit (Invitrogen). In this case, the transfection experiments were performed as previously described, but with COS-7 cell line and without conjugation of FITC with pDNA/lipofectamine complexes. This method enables the determination of the efficiency of cell transfection with pVAX1-*LacZ*, because the *lacZ* gene product, β-galactosidase, catalyses the hydrolysis of β-galactosides such as X-gal, producing a blue color that can be visualized under the microscope using a ×10 objective with a image zoom scan of 1.0. The transfection efficiency is determined as the percentage of blue cells compared to the total cell number (5 randomly acquired fields in each well).

3. Results

3.1. Lysine affinity chromatography

In the present work, it is exploited that the applicability of lysine-affinity chromatography in the pDNA downstream process, to isolate sc plasmid conformation from a complex *E. coli* lysate. Therefore, several retention/elution experiments were performed in order to obtain different elution patterns for gDNA, pDNA isoforms and RNA present in the clarified *E. coli* lysate. In a first step, lysine-agarose column was equilibrated with 29% of buffer B (constituted by 250 mM NaCl in 10 mM Tris-HCl buffer, pH 8.0), while the remaining 71% corresponds to buffer A (10 mM Tris-HCl buffer, pH 8.0) at 5 °C. After injection of the pDNA-containing clarified lysate (200 μL) using an isocratic elution mode of 1 mL/min, it was obtained a first peak resultant from the elution of unbound material (Fig. 2A). The ionic strength of buffer B was then increased to 37% for 20 min in order to elute specific molecules in a second peak. The chromatographic run was concluded with a final elution step, using 100% of buffer B for 30 min, to recover the strongly bound species.

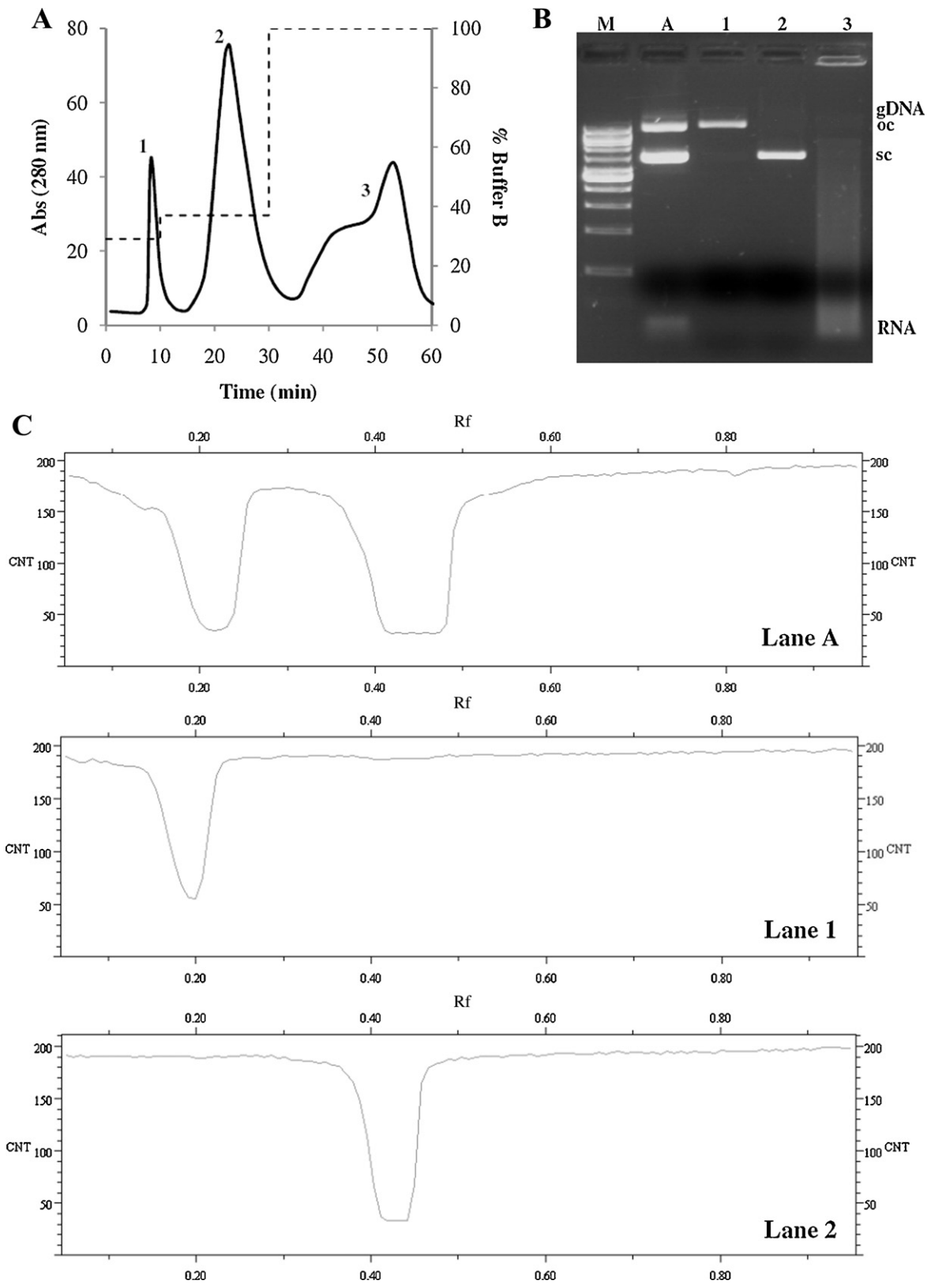

Fig. 2. (A) Chromatographic purification of sc plasmid isoform from a clarified *E. coli* lysate using lysine-affinity chromatography. Mobile phases – buffer A: 10 mM Tris-HCl buffer pH 8.0; buffer B: 250 mM NaCl in 10 mM Tris-HCl buffer pH 8.0. Elution was performed at 1 mL/min by stepwise gradient increasing the buffer B percentage in the eluent from 29% to 37% of buffer B, and finally to 100% of buffer B, as represented by the dashed line. (B) Agarose gel electrophoresis analysis of the samples recovered from each peak of the chromatogram. Lane M: molecular weight marker; Lane A: feed sample injected onto the column. (C) Lane densities of particular samples analyzed by electrophoresis.

With this study it was also verified that the chromatographic performance was dependent of a suitable conductivity control. Therefore, the salt concentration and environmental temperature should be monitored to attain the ideal plasmid separation conditions.

The presence of several peaks in the chromatogram (Fig. 2A) suggests a differential recognition of the plasmid isoforms and host biomolecules by the lysine matrix. The fractions pooled on each peak were analyzed by agarose electrophoresis. Fig. 2B confirms that the first peak mostly corresponds to the oc isoform that did not interact with the support (lane 1, peak 1), while sc isoform remained bound under the same conditions, being identified in the second peak (lane 2). The electrophoretic evaluation of the fractions recovered from the last peak revealed the presence of RNA (lane 3), by comparing with the bands of lane A (feed sample injected onto the column). The oc and sc isoforms homogeneity, analyzed on lanes 1 and 2 of electrophoresis, was also confirmed by the determination of the lanes density presented in Fig. 2C.

3.2. Analytic parameters quantification

The electrophoresis of the fractions pooled from the chromatographic experiment indicated that the sc pDNA eluted in the second peak is 100% pure, with no detection of RNA. However, to certify if the lysine-agarose chromatography can be applied in a single step to isolate sc pDNA from host biomolecules and other plasmid isoforms for therapeutic applications, further characterization of quality parameters is required.

3.2.1. Plasmid concentration and purity degree

The determination of pDNA concentration and the evaluation of residual impurities present in sc plasmid sample isolated by lysine preparative chromatography aforementioned, was performed according to an adaptation of the analytical method previously published [30], as briefly described in Section 2.7. Fig. 3 presents the analytical chromatographic profiles of several samples recovered throughout the manufacturing process of sc pDNA, as well as of the isolated gDNA and RNA samples to facilitate the identification of the impurities peaks. The analysis of the *E. coli* lysate sample showed a large quantity and diversity of biomolecules, as revealed by the multi-peak chromatogram (Fig. 3A). Knowing that the first peak eluting at 0.7 min corresponds to pDNA molecule [30], and by comparison with retention time of isolated nucleic acids (Fig. 3B and C), it was determined that the second and fourth peaks are due to the presence of gDNA and RNA respectively, while the remaining peaks (third and fifth) are other impurities. The isopropanol and ammonium sulfate precipitations, performed to clarify the lysate sample, contribute significantly to reduce the impurity content (Fig. 3D). When sc pDNA sample from second peak (Fig. 2A) of lysine-agarose chromatography was analyzed (Fig. 3E), only a single plasmid peak appeared in the chromatogram. The interference present after the elution of sc pDNA is due to the change of elution buffer as it is shown in Fig. 3F representing the negative control with the injection of binding buffer.

Table 1 presents the values of concentration and purity of pDNA, as well as the purification factor and yield achieved throughout the several steps of the process. The sc pDNA concentration obtained in the second peak of lysine chromatography was 7.34 ± 0.17 μg/mL, whereas the purity estimated from the relative peak area was 100%, what is in agreement with previous electrophoretic analysis and support the theory that the lysine matrix can be used as a final purification step. The total recovery of native plasmid (oc + sc) accomplished in the purification step corresponds to 55% yield, where 46% corresponds to the sc isoform recovery (Table 1). The relatively low yield can be related with the composition of the

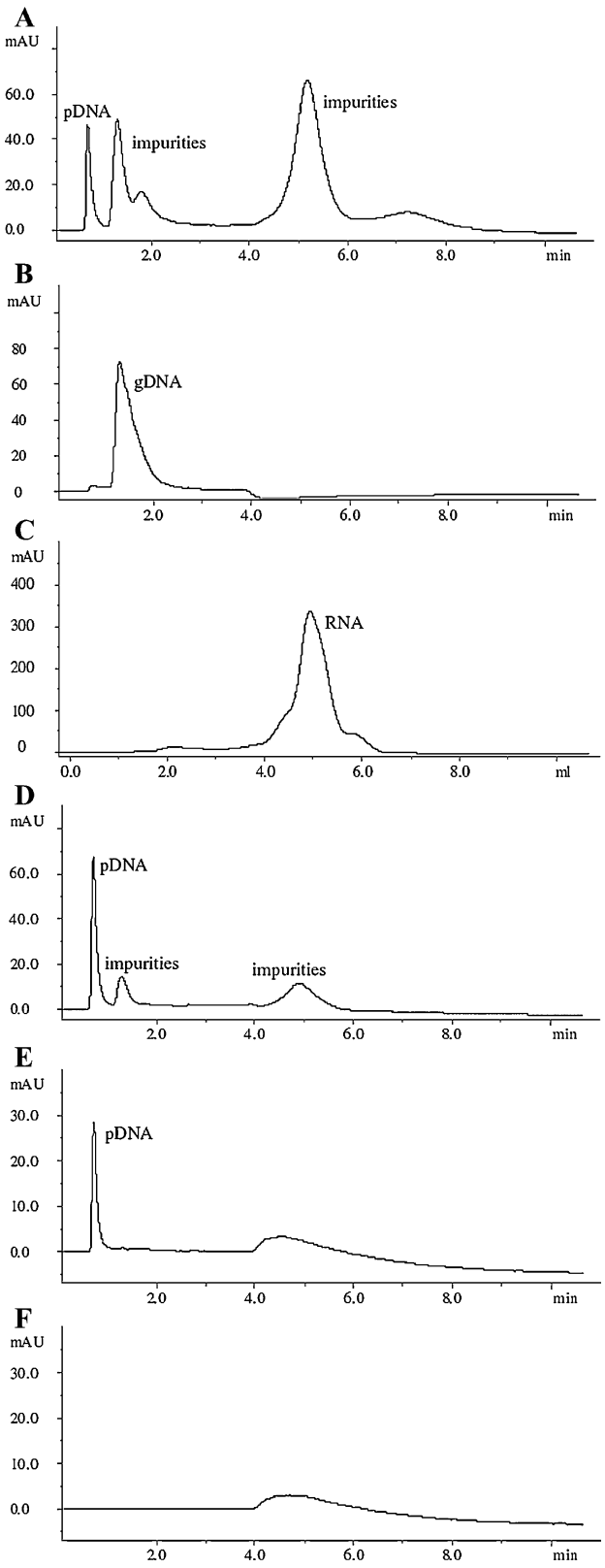

Fig. 3. Analytical chromatographic profiles of several nucleic acids and samples recovered throughout the purification process. (A) *E. coli* lysate; (B) genomic DNA; (C) RNA; (D) feed sample injected onto the column; (E) sc isoform fraction (peak 2); and (F) zero (injection of binding buffer).

Table 1
Analysis of purity and recovery yield of the sc pDNA isolated from *E. coli lysate* through of lysine-affinity chromatography.

Process step	Volume (mL)	pDNA		Purity (%)	Purification factor	Process yield (%)
		(μg/mL ± SD, $n = 5$)	(μg)			
Primary isolation						
Alkaline lysis	15	12.91 ± 0.43	193.58	6.76	–	–
$(NH_4)_2SO_4$ precipitation	10	18.07 ± 0.65	180.69	37.50	5.55	93.30
Lysine chromatography						
Peak 1 (oc)	4	4.49 ± 0.15	18.00	80.30	11.88	9.30
Peak 2 (sc)	12	7.34 ± 0.17	88.10	100	14.79	45.50

impure extract. Most of critical impurities share common characteristics of negative charge (gDNA, RNA and endotoxins), molecular mass (gDNA and endotoxins) and hydrophobicity (endotoxins) with pDNA [10]. Therefore, the clarification and purification are the two main steps that can affect the yield of sc pDNA manufacturing. The clarification step involves high ammonium sulfate concentration to promote the salting out phenomenon, favoring the precipitation of molecules containing hydrophobic areas. Thus, proteins, endotoxins, RNA and denatured gDNA are eliminated in this step, but a small amount of sc pDNA can also be precipitated. Furthermore, the characteristics of the contaminants significantly constrain the purification of target molecule, since the affinity ligands can also bind other biomolecules [33], leading to a decrease on the purification process yield. As it is shown in Fig. 2A, lysine matrix mainly interacts with the nucleic acids presenting higher bases exposition, such as RNA and sc pDNA. By this reason, RNA can be considered a key competitor for sc pDNA purification from *E. coli* lysate with lysine chromatography, decreasing the binding capacity of this support, or even the global yield of the sc plasmid recovery.

Nevertheless, the plasmid yield of 55% resultant in this work can be considered a reasonable value when compared with similar purification strategies. Previous studies of plasmids purification based on affinity chromatography strategies, such as triple-helix affinity chromatography [34] or histidine-affinity chromatography [23] achieved 42% and 45% of yield, respectively.

3.2.2. Proteins, gDNA and endotoxins contamination

The proteins were not detected on the purified oc and sc plasmid pools, by using the micro-BCA assay, while the RNA fraction had a residual concentration of 10.8 ± 0.54 μg/mL and the sample resulting from ammonium sulfate precipitation had, 12 ± 0.32 μg/mL. The analysis of gDNA contamination was performed by a suitable technique of real-time PCR. The results suggest a significant reduction on gDNA content throughout the clarification process and also in the lysine purification step, because the initial sample had 272 ± 1.35 ng gDNA/μg pDNA and the sc plasmid fraction had 2 ± 0.02 ng gDNA/μg pDNA (136-fold reduction). The methodology used to purify the sc pDNA was also efficient in removing endotoxins, which decreased from 9.5 ± 0.55 EU/μg of pDNA to 0.1 ± 0.01 EU/μg of pDNA (a 99% reduction).

These results were satisfactory considering the specifications recommended by the regulatory agencies [10,11]. However, it is known that the presence of these impurities in sc pDNA sample are undesirable due to their toxicity for the cells transfected with this sample [14]. Bearing this in mind, the transfection efficiency evaluation is required to investigate the biological effects of residual impurities, such as RNA, gDNA, proteins and endotoxins, in sc plasmid purified by this new lysine-affinity chromatographic strategy. In addition, the transfection level obtained with the plasmid sample purified with this matrix should be compared with the transfection level obtained in previous works using other chromatographic techniques, to analyze the efficiency of this new affinity strategy in the removal of host impurities.

3.3. Transfection experiments

3.3.1. Cellular localization of plasmid/lipofectamine complexes

The mechanism of cell uptake and localization of plasmid/lipofectamine complexes was observed with a confocal laser scanning microscope using FITC-pDNA and Hela cell line as it was established and optimized in previous work [32]. After the *in vitro* transfection experiments and fluorescence procedure, it was ascertained that the FITC-pDNA/lipofectamine complexes were condensed to form aggregates, which cross the cell membrane and can internalize into the cell. As it is observed in Fig. 4A, the FITC-pDNA/lipofectamine complexes correspond to the green areas that are near the nucleus blue-stained with Hoechst. Fig. 4B confirms that the green aggregates of stained plasmid are located inside the cell, particularly in the cytoplasm.

3.3.2. Transfection efficiency

To improve the characterization of sc pDNA sample resultant from *E. coli* lysate purification with lysine-affinity chromatography, the transfection efficiency experiments of isolated plasmid isoforms were performed and compared with the same plasmid purified with a commercial kit based on anion-exchange chromatography (Fig. 5). The COS-7 cell line is normally used for this procedure. Earlier studies have reported that some factors can lead to lower transfection efficiency, such as stoichiometry of pDNA/lipofectamine complex and serum concentration/pH of the transfection medium [35], as well as the type of cell line used [36]. However, in our study, the transfection efficiency (reflected in the expression level of β-galactosidase) obtained with sc plasmid fraction (43.96% ± 1.29) was practically three times higher than the obtained with the oc isoform fraction (17.06% ± 2.25), and it was also significantly higher when compared with the efficiency (22.12% ± 2.88) achieved with pDNA control sample (kit), as it is summarized in Table 2.

4. Discussion

The applicability of lysine-affinity matrix to purify the biological active sc pDNA from an impure *E. coli* lysate extract was evaluated. The characterization techniques allow the confirmation of the purity and activity of this plasmid. In the results of Fig. 2A and B, the separation of nucleic acids constituents from *E. coli* clarified lysate and the differential recognition by the lysine ligands is a consequence of several interactions. Particularly, electrostatic interactions can occur between the nucleic acids phosphate groups

Table 2
Summary of transfection efficiency achieved through of *in vitro* experiments using COS-7 cell line and applying different plasmid samples, with the same concentration (143 μg/mL).

Plasmid DNA	Transfection efficiency (% ± SD, $n = 3$)
Control (kit)	22.12 ± 2.88
oc	17.06 ± 2.25
sc	43.96 ± 1.29

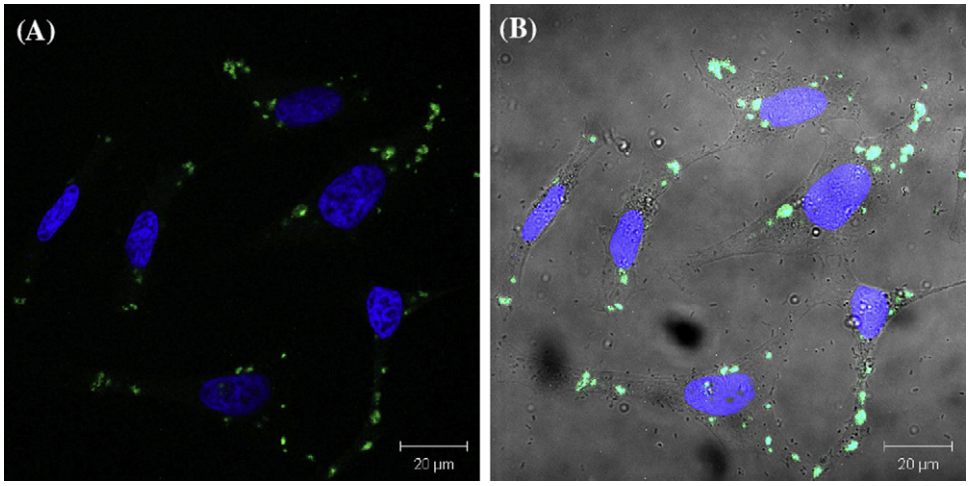

Fig. 4. Confocal microscopy of cellular localization of FITC-pDNA/lipofectamine complexes inside Hela cells (630×). (A) Co-localization of FITC-pDNA corresponds to the green areas, and the nuclei of cells were stained with Hoechst 33342 dye (blue) (image zoom scan, 1.0×). (B) The same image with phase contrast to observe the cellular delimitation.

and the lysine amino acid positively charged. Likewise, the lysine amino acid has some characteristics, such as the lateral side chain with significant length and capacity to interact in different conformations producing good hydrogen bond geometries, which reinforce the specific recognition mechanism. Molecular modeling studies evidenced that in protein–DNA interactions lysine promotes complex hydrogen-bonds preferentially with guanine base edges [21]. This positive amino acid performs an analogous interaction to the end-on conformation by placing N atom between more than one acceptor atom, being possible the same ligand to be acceptor and donor hydrogen atoms [20]. These multiple interactions can be responsible for the different retention of nucleic acids and lysine matrix, depending of the bases exposure degree. In this way, the oc plasmid isoform was first eluted because the bases are less exposed than in the sc isoform, which remain bound to lysine matrix. Indeed, the sc pDNA conformation results from the supercoiling phenomenon, which is a consequence of deformations induced by the torsional strain, leading to a more compact molecule with higher bases density than oc isoform [37]. The single-strand RNA molecule has the bases more exposed, being more retained than the other nucleic acids, in a similar way as obtained in our previous work [27].

Moreover, the fact that lysine amino acid can promote multiple interactions with the nucleic acids, also suggests that this support can be applied in the purification of different plasmids, even presenting different sizes, bases composition or exposure degree. In accordance to previous results [38], a slight adjustment of the binding and elution conditions will allow the target plasmid purification. In fact, this previous work shows that the retention on the histidine-agarose matrix is not directly affected by the plasmid size, because the smaller pUC19 (2.7 kbp) and the larger pVAX1-*LacZ*gag (7.4 kbp) plasmids were more retained than pVAX1-*LacZ* (6.05 kbp) [38]. The preferential retention of the pVAX1-*LacZ*gag to the histidine matrix was related with the 1.3 kbp adenine-enriched insert [38]. Taking into account that the lysine amino acid recognizes preferentially the guanine base [21], it will be expected an increased retention of plasmids with inserts mainly constituted by guanine.

In general, the differential interaction that was verified with both plasmid isoforms is in accordance with a previous study [26] that reported the biorecognition of pDNA isoforms in lysine-affinity chromatography. Fig. 2C confirms that the plasmid pool recovered in the peak 2 contains 100% of the sc isoform, without other nucleic acid impurities, in agreement with the recommendation of the regulatory agencies [10]. The temperature used on the chromatographic runs was 5 °C, because in a previous work [27], we have verified an increased RNA retention time using low temperature conditions what led to an improvement on separation between this impurity and sc pDNA. The analytical chromatograms in Fig. 3 clearly show a gradual reduction of impurities throughout the process until to obtain the sc plasmid isoform totally purified, confirming the result of agarose electrophoresis. Likewise, the content of residual proteins, gDNA and endotoxins present in sc plasmid sample is below to the level recommended by the regulatory agencies [10,11]. The global quantification of proteins

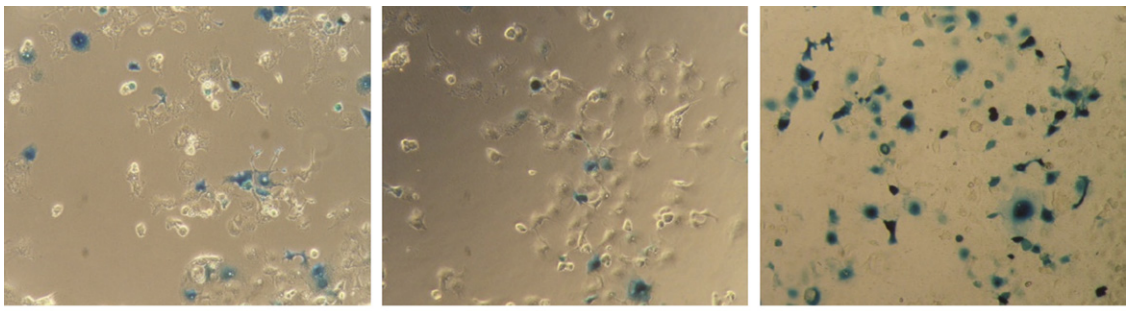

Fig. 5. Typical results of *in vitro* transfection experiments with COS-7 cells (100×) using different plasmid samples, with the same concentration (143 μg/mL). (A) Native pDNA (oc + sc) control sample obtained with a Qiagen kit; (B) oc plasmid and (C) sc plasmid obtained from chromatography with lysine-agarose support.

and endotoxins revealed that the ammonium sulfate precipitation step was very efficient [39], and the residual amount of these impurities was further eliminated during the purification procedure. With relation to gDNA contaminant, a recent study reports that the presence of bacterial gDNA in a purified plasmid sample for therapeutic benefits causes significant necrosis of skeletal muscle cells [40]. By this way, the gDNA must be reduced to the lowest value as possible, in plasmid preparation for vaccines because of the risk of insertion [1]. In fact, the gDNA molecule was mostly eluted in the first peak together with oc isoform (Fig. 2A) because the bases exposure degree is lower than in sc pDNA or RNA molecules.

Similar to the study of Ishii and collaborators, about the mechanism of cell transfection with plasmid/chitosan complexes [35], in our work it was also verified that the preparation of fluorescently labeled pDNA with FITC was efficient, as well as the cell uptake and the beginning of accumulation near the nucleus (Fig. 4). As illustrated in Fig. 5 and confirmed by the values present in Table 2, the transfection efficiency obtained with sc plasmid topology was almost three times higher than the transfection rate achieved with oc isoform (both purified with lysine-affinity chromatography) and the double comparing with the native pDNA purified with a commercial kit (based on ionic chromatography). Although different cell lines have been used, the transfection efficiency of COS-7 cells (44%) obtained with sc pDNA conformation purified with the process developed in the present work was identical to the transfection efficiency of CHO cells (50%) achieved with sc pDNA purified with histidine-agarose matrix [23]. Likewise, other processes developed to achieve a clinical pDNA product revealed that the transfection efficiency is enhanced when using the sc pDNA conformation [17,41]. A particular study using arginine-agarose to purify a therapeutic plasmid encoding the tumor suppressor gene p53 showed that sc pDNA isoform is also responsible for a significant enhancement in the expression of the target protein [41]. Thus, the results obtained confirmed that the process developed with lysine-agarose for purification of clinical grade pDNA present a good performance. Overall, the novelty presented in this study is the application of the lysine-affinity chromatography, for the first time, to specifically and efficiently purify the most biologically active sc plasmid conformation from a much more complex and impure extract, resultant from the recombinant production of the plasmid in *E. coli*. Considering the relevance of obtaining pharmaceutical-grade pDNA, the quality and purity of the plasmid product obtained was evaluated by using the methodologies required by FDA. Also the biological activity of the supercoiled plasmid was verified by measuring the transfection efficiency, in *in vitro* tests. The levels of impurities present in isolated sc pDNA sample are in accordance with the specifications of regulatory agencies in terms of RNA and proteins (undetectable), gDNA (2 ± 0.02 ng/μg of pDNA), endotoxins (0.1 ± 0.01 EU/μg of pDNA) and sc pDNA homogeneity (100% sc). Thus, the lysine ligands provided high specificity and ability to purify the sc pDNA under a pharmaceutical-grade. Furthermore, the knowledge of the ideal conditions for sc pDNA purification and the selectivity provided by the lysine matrix for the different nucleic acids can be useful in a future association of the lysine ligands with a specific chromatographic support of higher capacity than traditional agarose supports. With this future approach it is also expected to improve the scalability and the performance of this downstream operation integrated in a global process.

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