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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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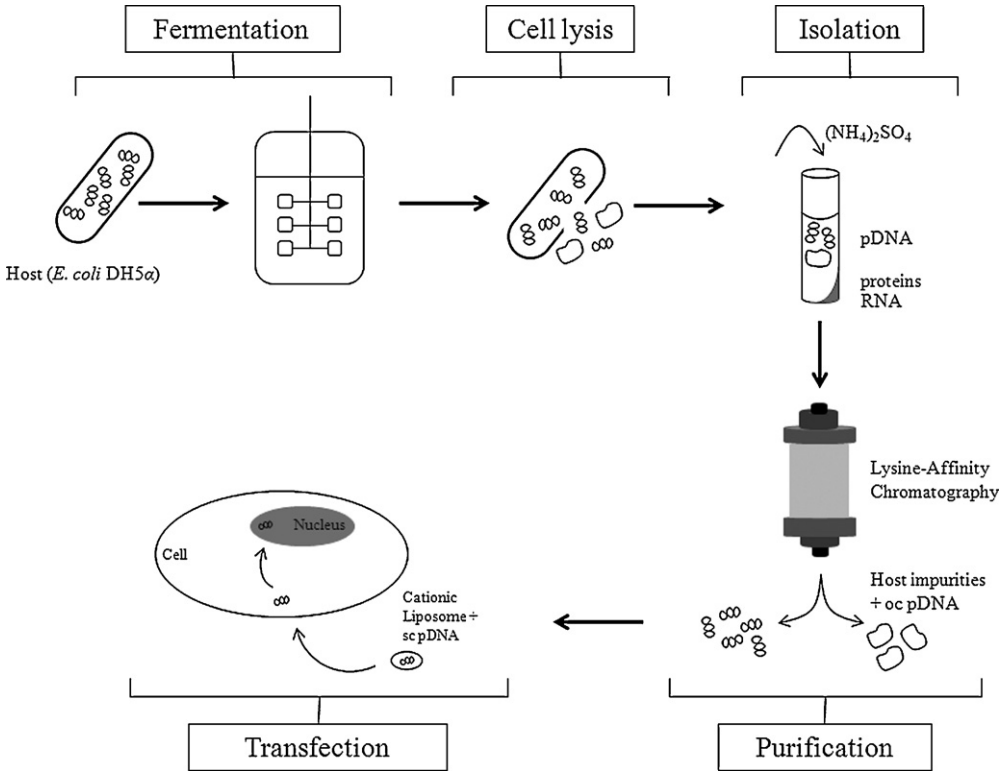

Fig. 1. Schematic representation of downstream processing of sc pDNA preparation, according to quantity and purity degree required for clinical applications, using lysine-affinity chromatography as a major purification step. *E. coli*, *Escherichia coli*; $(NH_4)_2SO_4$, ammonium sulfate; oc, open circular;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