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Rapid quantification of supercoiled plasmid deoxyribonucleic acid using a monolithic ion exchanger☆

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ABSTRACT

The demand for high-purity supercoiled plasmid DNA to be applied as a vector for new therapeutic strategies, such as gene therapy or DNA vaccination has increased in the last years. Thus, it is necessary to implement an analytical technique suitable to control the quality of the supercoiled plasmid as a pharmaceutical product during the manufacturing process. The present study describes a new methodology to quantify and monitor the purity of supercoiled plasmid DNA by using a monolithic column based on anion-exchange chromatography. This analytical method with UV detection allows the separation of the plasmid isoforms by combining a NaCl stepwise gradient. The specificity, linearity, accuracy, reproducibility and repeatability of the method have been evaluated, and the lower quantification and detection limits were also established. The validation was performed according to the guidelines, being demonstrated that the method is precise and accurate for a supercoiled plasmid concentration up to 200 μg/mL. The main advantage achieved by using this monolithic column is the possibility to quantify the supercoiled plasmid in a sample containing other plasmid topologies, in a 4 min experiment. This column also permits the assessment of the supercoiled plasmid DNA present in more complex samples, allowing to control its quality throughout the bioprocess. Therefore, these findings strengthen the possibility of using this monolithic column associated with a powerful analytical method to control the process development of supercoiled plasmid DNA production and purification for therapeutic applications.

1. Introduction

In a near future the use of plasmid DNA (pDNA) as non-viral vector for DNA vaccination and gene therapy purposes may be a reality. Several clinical trials are presently in progress using plasmid DNA as a biopharmaceutical product to treat or to prevent a wide range of diseases with complex etiologies [1,2]. The consequent use of pDNA vectors in these therapeutic approaches has increased the need for large amounts of highly pure pDNA with pharmaceutical-grade, as well as the development of large-scale and efficient pDNA purification processes [3,4].

Process development for pDNA manufacturing usually includes the upstream and downstream processing [5]. During these stages, pDNA can undergo several types of stress that may disrupt its structural stability [6]. Therefore, plasmid molecules that are mainly recovered in the supercoiled (sc) isoform, can also exist in a non-sc or relaxed form, such as the open circular (oc) conformation resulting from the damage of the sc form [7]. Supercoiled pDNA is the desired topological form since it induces the most efficient access to the nucleus of the cell and consequently improves gene expression in eukaryotic cells [8].

The challenges in pDNA downstream processing are essentially related to elimination of host impurities such as RNA, genomic DNA (gDNA), and endotoxins [2], as well as to isolate the sc pDNA from other plasmid isoforms. According to regulatory agencies, such as FDA, the product-quality is defined as a percentage of the sc isoform compared to the total pDNA. Thus, the purity of the sc pDNA must be 100% with the homogeneity degree higher than 97% of sc isoform [2]. However, the quantification and purity determination of active plasmid products for clinical applications depends on the availability of suitable analytical techniques [9]. Thus, it is crucial that quick, reliable, accurate, and inexpensive analytical methods can be established in order to ensure the required sc isoform purity. Liquid chromatography is one of the most useful analytical tools to monitor and control pDNA quality during the processing and in the final formulations [4,10].

The analytical methods already implemented to quantify total pDNA [11] or plasmid isoforms [12] from different samples were based in hydrophobic interaction chromatography (HIC) allied to a

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high-performance liquid chromatography (HPLC) system. Furthermore, Sousa and Queiroz have recently reported the assessment of the sc plasmid isoform using arginine-affinity chromatography [7]. Anion-exchange (AEX) chromatography remains one of the most used techniques for capture, purification and quantitation of pDNA, both at preparative [13–18] and analytical [9] scale. However, the majority of these methods are time-consuming or quantify only total pDNA. Fast pDNA separations can be obtained by reducing the mass transfer resistance within the pores/channels of super-porous matrices [19]. Monolithic columns have been described as a good option to achieve this purpose [20]. In fact, monolithic supports exhibit high binding capacity for pDNA [21,22] and excellent mass transfer properties, due to a high number of accessible binding sites for large biomolecules such as pDNA [23]. Moreover, these supports allow a fast and high-resolution separation of double stranded DNA (dsDNA) molecules, making them attractive for pDNA chromatography [4].

The purification of pDNA from an alkaline lysate using a CIM™ DEAE (Convective Interaction Media – diethylaminoethyl) monolith (BIA Separations) has already been described [20], being also demonstrated that the monolithic discs can be applied for fast analytical in-process control of the pDNA purity [20]. Moreover, the monolithic technology has also been applied to purify sc pDNA, by exploiting affinity interactions [3,22]. Knowing the advantages associated with these columns, it remains interesting to validate an analytical methodology to assess the sc pDNA quality by using this monolithic technology.

The present work describes a rapid analytical method, efficient for sc pDNA quantification and purity evaluation, employing a CIM™ pDNA analytical column from BIA Separations. The validation of the analytical method is based on the international guidelines [24,25]. Indeed, the establishment of an analytical method to control the sc pDNA quality, purity and yield during a biotechnological process requires the assurance of simplicity, linearity, accuracy and precision of the results [7].

2. Materials and methods

2.1. Materials

All solutions were freshly prepared using deionized water ultrapure grade, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and analytical grade reagents. The buffers were filtered through a 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. The samples to inject in the column were filtered through 0.22 μm pore size filter (Millipore, Bedford, MA, USA). CIMac™ pDNA analytical column, bearing weak anion exchanger (diethylamino), was kindly provided by BIA Separations (Ajdovščina, Slovenia). CIM™ monolithic column consists of a disk-shaped poly(glycidyl methacrylate-co-ethylene dimethacrylate) highly porous polymer matrix, with 0.32 mL of volume and a column of 15.0 mm length and 5.2 mm diameter.

2.2. Methods

2.2.1. Bacterial growth conditions and plasmid production

The 6.05-kbp pVAX1-*LacZ* (Invitrogen, Carlsband, CA, USA) amplification was obtained by fermentation of *Escherichia coli* (*E. coli*) DH5α (Invitrogen, Carlsband, CA, USA) after transformation, as described by Sousa and co-workers [7]. The cell growth was suspended at exponential phase ($OD_{600} \sim 5$). *E. coli* DH5α cells without plasmid were also grown under the same conditions, but with no antibiotic present.

2.2.2. Alkaline cell lysis and isolation of pVAX1-LacZ isoforms

The sc pDNA isoform was purified using the Qiagen (Hilden, Germany) plasmid maxi kit, according to the manufacturer's instructions. The protocol is based on a modified alkaline lysis procedure. The final pDNA pellet was resuspended in approximately 3 mL of 10 mM Tris-HCl with 700 mM NaCl, pH 8.0. Finally, 260 and 280 nm absorbance of the samples was determined using spectrophotometer to assess sc pDNA quantity and purity. Open circular pDNA isoform was prepared by incubating a sc pDNA sample without NaCl at room temperature (25 °C). The sample was monitored over the time by electrophoretic analysis, until the total conversion of sc plasmid to oc isoform was observed (about 3 days). All pDNA samples were stored at −80 °C until use.

2.2.3. Preparation of process samples

The evaluation of the applicability of the analytical method to quantify the sc pDNA isoform present in more complex samples required the preparation of *E. coli* lysates. Thus, *E. coli* cells were lysed using a modification of the alkaline method [26], as described by Diogo and co-workers [27]. After cell lysis and elimination of cellular debris, the concentration of the lysate was performed according to a previously published method [27]. Briefly, the pDNA present in the supernatant was precipitated with 0.7 volumes of ice-cold isopropanol and then redissolved in 6 mL of 10 mM Tris-HCl buffer, pH 8.0. Samples of this concentrated lysate were then kept and stored at −80 °C for further analysis.

2.2.4. Analytical chromatography

Chromatography experiments were performed using ÄKTA™ purifier system (GE Healthcare Biosciences, Uppsala, Sweden) controlled by UNICORN software, Version 5.11. All DNA concentrations were measured spectrophotometrically with Ultrospec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech, Cambridge, England). One unit of Optical Density (OD) 260 nm in a 10 mm cuvette is assumed to correspond to 50 μg/mL dsDNA. The chromatographic system was prepared with a 500 mM NaCl in 10 mM Tris-HCl buffer pH 8.0 in the pump A (mobile phase A), and 1 M NaCl in 10 mM Tris-HCl buffer pH 8.0 in the pump B (mobile phase B). The monolithic column was equilibrated with 52% of buffer B. Afterwards, 20 μL of a plasmid sample suitably diluted in the equilibration buffer were injected in the monolith and eluted at a flow rate of 1 mL/min. After elution of unbound species, the ionic strength of the buffer was instantaneously changed to 100% of buffer B to elute bound species. All experiments were performed at room temperature (25 °C). The control of the temperature is an important parameter for the selectivity to guarantee plasmid isoforms separation. The experiments developed with the *E. coli* lysate or with the isolated impurities were performed using the same elution gradient described above. Regeneration of CIM™ column was performed after approximately 50 chromatographic runs by washing with water followed by 10 column volumes of 0.5 M NaOH at a flow rate of 0.2 mL/min and finally washing with water.

2.2.5. Agarose gel electrophoresis

The fractions recovered from the experiments were analyzed by horizontal electrophoresis using 15 cm 1% agarose gels (Hoefer, San Francisco, CA, USA) stained with green safe (1 μg/mL) and visualized under UV light in a Vilber Lourmat system (ILC Lda, Lisbon, Portugal). Electrophoresis was carried out in TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) and run at 100 V for 40 min. Hyper Ladder I (Bioline, London, UK) was used as a DNA molecular weight marker.