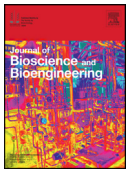

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Large-scale purification of pharmaceutical-grade plasmid DNA using tangential flow filtration and multi-step chromatography

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The demand for pharmaceutical-grade plasmid DNA in vaccine applications and gene therapy has been increasing in recent years. In the present study, a process consisting of alkaline lysis, tangential flow filtration, purification by anion exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography was developed. The final product met the requirements for pharmaceutical-grade plasmid DNA. The chromosomal DNA content was <1 μg/mg plasmid DNA, and RNA was not detectable by agarose gel electrophoresis. Moreover, the protein content was <2 μg/mg plasmid DNA, and the endotoxin content was <10 EU/mg plasmid DNA. The process was scaled up to yield 800 mg of pharmaceutical-grade plasmid DNA from approximately 2 kg of bacterial cell paste. The overall yield of the final plasmid DNA reached 48%. Therefore, we have established a rapid and efficient production process for pharmaceutical-grade plasmid DNA.

[**Key words:** DNA vaccine; Purification; Gene therapy; Chromatography; Alkaline lysis]

DNA vaccines allow foreign genes to be transiently expressed in transfected cells, mimicking intracellular pathogenic infection and triggering both humoral and cellular immune responses (1–3). While considerable attention has been paid to enhancing the immunogenicity of DNA vaccines (4–6), substantially less consideration has been given to the practical challenges of larger scale production of plasmid DNA for both pre-clinical studies and therapeutic usage.

Large-scale production of plasmid DNA has to meet several criteria. First, safety of the final product is a key point in the clinical use of plasmid DNA. The product should be free of animal-derived enzymes (e.g., RNase, lysozyme, proteinase K), organic solvents (e.g., phenol, ethanol, isopropanol) and toxic reagents (e.g., cesium chloride, ethidium bromide). Second, the entire process should be economical using easily-obtained materials and have a short production period, which can be achieved using fast flow and large adsorption chromatography gels. In addition, the production process must meet good manufacturing practice (GMP) requirements and criteria of regulatory authorities such as the World Health Organization (WHO), Food and Drug Administration (FDA) and European Medicines Agency (EMEA) (7–13).

There have been intensive investigations into optimizing the production of heterogeneous proteins over the past three decades. The processes for producing plasmid DNA and proteins have much in common, requiring fermentation cell harvesting, product recovery, chromatography purification, dilution/concentration and sterilization. However, different characteristics of DNA and proteins require different processes at each step. For example, high-pressure homogenization and ultrasonication are not suitable for purification of plasmid DNA due to its sensitivity to shear forces (14).

In principle, the process of plasmid DNA vaccine production involves the following steps: fermentation, alkaline lysis, purification and concentration. Alkaline lysis, first reported by Birnboim and Doly in 1979 (15), is usually the best choice in plasmid DNA production for disruption of bacterial cells. The release of plasmid DNA, RNA, chromosomal DNA and proteins from bacterial cells after lysis results in a highly viscous solution (16). Thus, it is necessary to reduce the amount of impurities before the chromatography step, which can be achieved using a number of protocols (17–23). Such large-scale and lab-scale protocols for the isolation of plasmid DNA in the lysate fall into three categories. First, isopropanol, polyethylene glycol (PEG), compaction agents and chaotropic salts have been used in DNA precipitation (24–26). The second category involves tangential flow filtration (TFF) for concentration and removal of contaminating RNA and proteins to purify plasmid DNA after the clarification step (27). Filtration technology is considered a separation tool at the last step of plasmid purification. In the third category, chromatography methods, such as size exclusion

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chromatography (SEC) (28,29), ion exchange chromatography (IEC) (30), hydrophobic interaction chromatography (HIC) (31,32), reversed-phase HPLC (RP-HPLC) (33) and affinity chromatography (AC) (34,35), are often used for refinement after primary purification of plasmid DNA. However, no single type of chromatography media can remove all residual proteins, chromosomal DNA, endotoxins and RNA. Therefore, an optimal multi-step chromatography process is required. In the last few years, much effort has been placed on development of downstream processes for the manufacture of pharmaceutical-grade plasmid. Urthaler et al. (36) developed a process consisting of a cell disruption step, followed by three different chromatography steps. In that protocol, cell lysis is performed by an automated continuous reactor. The clarified lysate is further purified by HIC, ion exchange chromatography (IEC), SEC and finally by an ultrafiltration step. Guerrero-German et al. conducted the purification of plasmid DNA from bacterial cell lysate using hollow-fiber tangential filtration, frontal IEC and HIC (37,38).

In the present work, we developed a large-scale purification process for pharmaceutical-grade plasmid DNA encoding HIV core antigen. The process begins with RNA precipitation of the alkaline lysate by high salt. Low molecular weight RNA and the clarified alkaline lysate were then essentially removed using a tangential flow filtration system. The raw product is further purified by chromatography steps based on three different principles, resulting in high-purity plasmid free of RNA, chromosomal DNA, proteins and endotoxin. The final plasmid DNA is concentrated by ultrafiltration and sterile filtered in the last step. The integrated system for the purification of plasmid DNA from *Escherichia coli* is outlined in Fig. 1.

MATERIALS AND METHODS

Instruments, columns and chromatography media Mini Q (PE 4.6/50), Q Sepharose XL, Phenyl Sepharose 6 FF (low sub) and Sepharose 6 FF were purchased from GE Healthcare Life Sciences (USA). Filters and the Centramate TFF system were obtained from Pall (USA).

Cultivation The *E. coli* DH5α host strain, harboring a 6.4 kb plasmid encoding the HIV core antigen, was grown in a 500 ml shake flask containing 100 ml of a complex growth medium supplemented with 50 μg/ml kanamycin (37°C, 230 rpm, 12 h) to an absorbance of 2 (A_{600} nm). Ninety-five milliliters of this culture was used to inoculate two shake flasks (2000 ml), each containing 1000 ml fermentation medium, and the cells were grown for 5 h (37°C, 230 rpm) to an average absorbance of 5 (A_{600} nm). All of this culture (2000 ml) was used to inoculate a 40 L BioFlo 5000 fermenter (New Brunswick Scientific, USA) containing 25 L fermentation medium. The agitation speed was controlled by the oxygen demand. The culture was centrifuged at 5020 ×*g* for 20 min in a Beckman J-6M1, and the supernatant was discarded. The bacterial paste was stored at −20°C.

Alkaline lysis Two thousand grams of bacterial cell paste was suspended in 16 L of suspension buffer (50 mM Tris−HCl, 10 mM EDTA, pH 8.0) by stirring the mixture at 4°C until a homogenous suspension was obtained. The cell suspension was transferred to a 100-L vessel, and the lysis was carried out by stirring with an overhead low-shear impeller in order to mix the cells with lysis solution (0.2 M NaOH, 1.0% SDS) for 10 min. The lysate was neutralized by addition of 16 L of neutralization buffer (3 M KAc, pH 5.5) for 30 min, and then 16 L of 2 M $CaCl_2$ was added directly to the unclarified lysate under gentle stirring and incubated for 1 h until a tight floating layer of solids formed. The cleared lysate beneath was drained and passed through a 1.0-μm filter membrane.

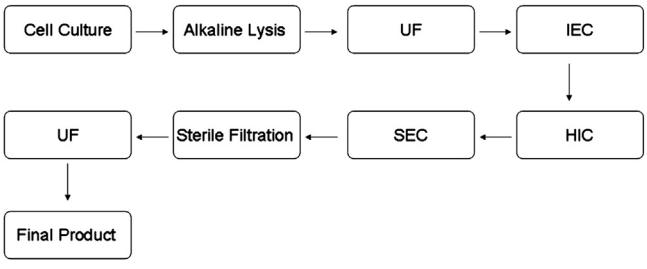

FIG. 1. Process flow sheet for purification of plasmid DNA. IEC: ion exchange chromatography; SEC: size exclusion chromatography; HIC: hydrophobic interaction chromatography; UF: ultrafiltration.

Tangential flow filtration (TFF) The clarified lysate was processed by TFF to remove the remaining low molecular weight RNA and concentrated, and the buffer was exchanged. The lysate was processed with a Centrasette TFF cassette with a polyethersulfone (PES) membrane (1 ft^2, 300 kDa) on a Centramate LV holder (Pall) at a pressure of 10−15 psi. The solution was concentrated ten times and then dialyzed against 10 volumes of 0.5 M KAc, pH 5.5.

Ion exchange chromatography (IEC) Plasmid DNA purification by IEC was performed using Q Sepharose XL resin packed on a BPG100/500 column (GE Healthcare Life Sciences) to a final bed volume of 2.5 L and equilibrated with 0.5 M KAc, pH 5.5. Plasmid samples were then loaded at a flow rate of 100 ml/min. RNA was eluted with 0.6 M NaCl, 50 mM Tris−HCl, 10 mM EDTA, pH 8.0. The plasmid DNA was eluted with 1.0 M NaCl, 50 mM Tris−HCl, 10 mM EDTA, pH 8.0. After each run, columns were cleaned with five volumes of 2.0 M NaCl, 0.5 M NaOH. All chromatography experiments were carried out on the ÄKTA purifier 100 system (GE Healthcare Life Sciences) installed with the Unicorn 5.1 software for data acquisition and processing. The outlet stream was continuously detected with a UV monitor at 280 nm, and appropriate fractions were collected for further analysis.

Hydrophobic interaction chromatography (HIC) Solid ammonium sulfate was added to the plasmid solution to a final concentration of 2.0 M and incubated at 4°C for 20 min. The supernatant was then loaded directly onto a HIC column. The XK 50/30 column (GE Healthcare Life Sciences) packed with 500 ml Phenyl Sepharose 6 FF (low sub) was equilibrated with 2.0 M ammonium sulfate, 50 mM Tris−HCl, 10 mM EDTA, pH 8.0, at a flow rate of 30 ml/min. Plasmid DNA samples were then loaded at the same flow rate. The flow-through peak was collected, and impurities were eluted with 50 mM Tris−HCl, 10 mM EDTA, pH 8.0. The column was cleaned with two column volumes of 2.0 M NaCl, 0.5 M NaOH.

Size exclusion chromatography (SEC) Peak fractions were injected into a SEC column. The BPG100/950 column packed with 5.5 L Sepharose 6 FF resin (GE Healthcare Life Sciences) was equilibrated with 20 mM PBS, pH 7.2, at a flow rate of 60 ml/min. The main peak was collected and saved as the plasmid DNA fraction.

Final filtration and concentration of plasmid DNA Peak fractions from SEC was passed through a 0.22-μm filter membrane for sterilization and concentrated using the QuixStand hollow fiber ultrafiltration system (300 kD MWCO, GE Healthcare Life Sciences) to a level above 2.0 mg/ml.

Purity Plasmid purity, or percentage of covalently closed circular plasmid, was estimated after all plasmid forms present in the sample (supercoiled, denatured and open circular) were separated by 0.8% agarose gel electrophoresis, photographed and analyzed by Labworks version 4.5 software from UVP (39).

Plasmid DNA starting at 1 μg was 2-fold serially diluted, and each dilution was loaded onto the gel. Estimation of plasmid purity was made by comparison of the series dilution number (n_p) at which the supercoiled DNA band was no longer visible with that of an observed contaminant (n_c). The reciprocal of 2 to the power of the difference ($n_p - n_c$) multiplied by 100% is a measure of the relative abundance of that contaminant:

$$\text{Observed contaminant}(\%) = 100{*}2(n_p - n_c)^{-1} \quad (1)$$

The supercoiled DNA content was determined as 100% minus the sum of the percentage values for each observed contaminant.

Analytical chromatography The recovery and purity of plasmid DNA were analyzed by a Mini Q column (GE Healthcare Life Sciences), which was coupled to the ÄKTA purifier 10 and equilibrated with buffer (0.5 M NaCl, 25 mM Tris−HCl, pH 8.0). Samples (100 μl) were eluted by applying a gradient from 0.5 M NaCl to 0.8 M NaCl in 20 column volumes at a rate of 1 ml/min. The chromatography runs were monitored at 260 nm. The Mini Q analysis was used to quantitate the RNA using the relationship 40 μg plasmid/ml = 1 AU and the plasmid with 50 μg plasmid/ml = 1 AU (40).

Protein concentration determination Protein concentrations, including that of the final product of purified plasmid sample, were determined by using a MicroBCA Kit (Pierce), according to the manufacturer's protocol. Briefly, the reaction mixture (100 μl protein sample plus 100 μl microBCA reagent) was incubated at 60°C for 30 min, and adsorption was measured at 562 nm.

Chromosomal DNA analysis Chromosomal DNA contamination in the purified plasmid was assessed using Southern blot analysis. A 361-bp sequence of the 16S ribosomal RNA gene from *E. coli* DH5α was amplified by PCR (forward primer, 5′-ACACGGTCCAGACTCCTACG-3′, reverse primer, 5′-TACACCTGGAATTCTACCCC-3′) and labeled by random priming with digoxigenin-11-dUTP (Boehringer Mannheim, Germany). The hybridized probes were immunodetected with anti-digoxigenin conjugated to alkaline phosphatase and then visualized with the colorimetric substrate NBT/BCIP (Boehringer Mannheim).

Endotoxin analysis Endotoxin contamination was assessed by an LAL-gel clotting assay kit (Zhanjiang Biological Ltd., China) according to manufacturer's recommendation.