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Effect of salt on purification of plasmid DNA using size-exclusion chromatography

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Abstract

In the present study, we compared the performances of size-exclusion chromatography for the purification of plasmid DNA when different concentrations (0.5 M, 1 M, 2 M, respectively) of two types of salt (NaCl and $(NH_4)_2SO_4$) are present in running buffers. Our experiment results displayed that it is not only the resolution of RNA but also those of supercoiled plasmid DNA and host's genomic DNA were increased greatly in the presence of high concentration of water-structure salt. We deduce that two separation modes may be involved in the process: The supercoiled plasmid DNA is influenced mainly by compaction effect and eluted in the size-exclusion mode; whereas, RNA and genomic DNA are influenced mainly by hydrophobic effect due to their stretched and loose structures and eluted in the interaction mode. This method led to an improved efficiency of size-exclusion chromatography.

Keywords: Size-exclusion chromatography; High salt; Genomic DNA; Supercoiled plasmid DNA; RNA; Size-exclusion mode; Interaction mode

1. Introduction

In recent years, developments in molecular therapies such as non-viral gene therapy and DNA vaccination have fostered the development of efficient plasmid DNA (pDNA) purification processes [1]. At the same time, the requirements of regulatory agencies regarding purity, potency, safety and efficacy must be met [2,3]. Plasmid DNA production normally begins with a cultivation, followed by an alkaline lysis method to release the plasmid, and then, most of the cell walls, organelles, proteins, genomic DNA (gDNA) and RNA should be removed. Therefore, at least one chromatography step either to capture the plasmid or to polish usually be involved. Techniques include: hydrophobic interaction [4], anion-exchange [5], thiophilic interaction [6] and so on. Among various methods that have been developed, size-exclusion chromatography (SEC) is widely used in the large-scale purification of plasmids and generally as a step for group separation [7–9]. Its' chromatograms are characterized by two peaks. The first peak harbours all forms of plasmid DNA and genomic DNA; second peak contains RNA and other small contaminants. The resolution attainable by size-exclusion is usually inferior to that of other methods [10]. Theoretically, gDNA can be distinguished as a leading shoulder, but in fact, the separation is usually poor and not reproducible. This is attributed to shearing and breakage of gDNA during alkaling lysis [1]. On the other hand, the elution of plasmid isoforms presents a superimposition of chromatograms, and the resolution becomes worse in case of feedstock with higher pDNA concentrations. Therefore, more steps usually need to be taken together with SEC to obtain purer supercoiled (sc) pDNA. And thus, the strategies of purification become more complicated and often lead to low yield. Therefore, in order to simplify the procedure of purification, people must find ways to improve the efficiency of SEC.

Recently, researchers have noticed that selectivity of RNA versus pDNA was improved when high concentration of salts were present in SEC buffers [9]. Nevertheless, the performances of the "high-salts SEC" for separation of genomic DNA and supercoiled plasmid DNA received less attention. The objective of our work is comparing the effect of different concentrations of two types of salts on SEC, evaluating the chromatographic behavior of gDNA and sc pDNA as well as RNA, and thus improving the efficiency of SEC mode. Besides, the potential mechanism was also discussed in this paper and two separation

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modes (SEC mode and interaction mode) were considered to be involved in the "high-salt" SEC process.

2. Experimental

2.1. Materials

The *E. coli* host strain is DH5α.

7074 bp (base pairs) plasmid pGP140, constructed by the Department of Virus and Immunology (China CDC) for the HIV DNA vaccine, was used as a model plasmid. This plasmid contains the human cytomegalovirus (CMV) immediate early promoter, the BGH polyadenylation sequence, a kanamycin resistant gene and an antigen gene of HIV gp140.

Mono Q FPLC (100 mm × 4.6 mm), sepharose 6 Fast Flow, Sephacryl S-1000 SF, a XK 16 (1000 mm × 16 mm) column, AKTA Purifier system and Frac-920 fraction collector were all purchased from Amersham Bioscience (Uppsala, Sweden).

Horizontal gel electrophoresis unit, PowerPac Basic power supply unit, and Geldoc image system were all purchased from Bio-Rad Laboratories Inc. (Hercules, USA).

SYBR Premix Ex Taq was from TaKaRa (TaKaRa biotechnology (Dalian) Co., Ltd., Dalia, China).

DNeasy tissue kit was from Qiagen (QIAGEN, Hilden, Germany).

Other reagents used were of analytical-reagent grade or with equivalent purity.

2.2. Cell growth and lysis

DH5α *E. coli* harbouring the 7 kbp gp140 (HIV antigen)-encoding plasmid (pGP140) were grown in a 5 l Bioreactor (East Biotech Co. Zhenjiang, China) at 37 °C. Dissolved oxygen was set to 15% of the saturation value and controlled by changing air flow and agitation speed. Cells were harvested at late log phase (6 h; A600 nm = 10) by centrifugation at 4000 × *g* for 10 min at 4 °C, resuspended in 50 mM Tris–HCl (pH 8.0), 5 mM EDTA and 50 mM glucose (50 ml of buffer per one liter cultivation broth), then lysed by the alkaline lysis method (0.1 M NaOH, 1% SDS) without using enzymes, at room temperature for 5 min. The lysate was neutralized with 3 M potassium acetate for 30 min, at 4 °C. The precipitate formed was removed by centrifugation at 20,000 × *g* for 15 min, and the supernatant was clarified by isopropanol precipitation (0.6 volume per 1 volume of the supernatant). The precipitated nucleic acids were recovered by centrifugation (20,000 × *g* for 10 min) and resuspended in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). Then the same volume of 5 M lithium chloride was added, the precipitate was removed by centrifugation at 6000 × *g* for 10 min, and the supernatant was clarified by isopropanol precipitation again followed by resuspension in TE buffer.

2.3. Size-exclusion chromatography

One hundred eighty milliliters of Sephacryl S-1000 SF media was packed into a XK 16 column (bed height 90 cm), the column was connected to an AKTA Purifier system, and the system UV monitor was set to 254 nm. 0.1 Column volume (CV) of sample was loaded in each run and the maximum flow is 17 cm h^{-1}. Seven projects were designed to perform the chromatography. Different types and concentrations of salts were present in the running buffer of these projects: **1**. (TE); **2**. (2 M $(NH_4)_2SO_4$ in TE); **3**. (1 M $(NH_4)_2SO_4$ in TE); **4**. (0.5 M $(NH_4)_2SO_4$ in TE); **5**. (2 M NaCl in TE); **6**. (1 M NaCl in TE); **7**. (0.5 M NaCl in TE). In each process, 21 fractions were collected at the exactly same retention volume by Frac-920 fraction collector.

2.4. Desalting

Samples containing high salts were desalted prior to all of the analysis steps to prevent the interference with the assay. 0.5 ml sample was loaded on the Sepharose 6 Fast Flow column (packed in the XK16, bed height is 5 cm) and was first eluted in TE buffer (pH 8.0) at 0.2 ml min^{-1}, followed by salt's peak.

2.5. Agarose gel electrophoresis

A 0.8% agarose gel containing 0.5 μg ml^{-1} ethidium bromide ran in a horizontal gel electrophoresis unit with TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) used as running buffer. Electrophoresis was carried out at 120 V for 30 min on a PowerPac Basic power supply unit. Gels were scanned by the Geldoc image system and analyzed by Quantity One software (Bio-Rad Laboratories, Inc. Hercules, USA).

2.6. Real-time PCR

The approach presented here uses the change in fluorescence of SYBR Green I upon binding with double-stranded DNA [11–13]. Change in fluorescence during the binding-extension step allows for real-time monitoring of the PCR reaction.

PCR conditions: Oligonucleotide primers 23S-sense (5′ GAA AGG CGC GCG ATA CAG 3′) and 23S-antisense (5′ GTC CCG CCC TAC TCA TCG A 3′) were used to amplify a 76 bp fragment of the 23S ribosomal RNA gene, present in seven copies in the *E. coli* genome. Smith et al. originally reported the use of this primer pair [14]. DNA polymerase, reaction buffer, dNTP, and SYBR Green were premixed in SYBR Premix Ex Taq.

DH5α *E. coli* genomic DNA prepared with the DNeasy tissue kit was used to generate standard curves from 100 ng to 10 fg (100 ng μl^{-1}, 10 ng μl^{-1}, 1 ng μl^{-1}, 100 pg μl^{-1}, 10 pg μl^{-1}, 1 pg μl^{-1}, 100 fg μl^{-1}, 10 fg μl^{-1}).

PCR reactions were carried out and monitored in a LightCycler (Roche Diagnostics Corporation, Basel, Switzerland). Activation of Taq polymerase was achieved by incubation at 95 °C for 10 s, followed by 44 cycles of incubation at 95 °C for 5 s, and incubation at 70 °C for 20 s. Fluorescence sensors were active during the annealing-extension step (70 °C).

2.7. Southern-blotting analysis

DIG DNA Labeling and Detection Kit (Roche Diagnostics Corporation, Basel, Switzerland) was used. Probe was produced by the following method: digest 10–15 μg DH5α *E. coli* genomic DNA with restriction enzyme BspT107 I (TaKaRa biotechnology (Dalian) Co., Ltd., Dalia, China) overnight at