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An innovative monolithic column preparation for the isolation of 25 kilo base pairs DNA

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ABSTRACT

The use of large DNAs in preparing multivalent vaccines that will eventually give protective immunity against multiple pathogenic microbes is becoming a major debate nowadays. One of the important issues in ensuring the successful implementation of the new vaccine technology is the development of a chromatographic technique that can handle larger DNAs. This paper reports the development of a novel conical monolithic column format with pore and surface characteristics engineered for the isolation of 25 kbp DNA in a single step fashion. An effective method of eliminating wall channelling, a defect of most conventional monolithic chromatography systems which has caused significant loss of product, was applied to maximise DNA recovery. This method was based on a systematic reduction of wall channel size based on a predetermined correlation between column's back pressure and wall channel size of a particular monolith pore size.

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1. Introduction

1.1. Fosmid DNA: characteristics and significance

Apart from the conventionally used plasmids (<10 kbp), DNA fragments which are considerably larger in size (25 kbp or more) have been frequently used for scientific investigations. Very large DNA fragments have been employed in genome sequencing [1] and their monodispersity and ability to get stained (and imaged by fluorescence microscopy) has made them an ideal candidate for polymer physics research [2–4]. It is important to see that whether the physiology of these larger DNA fragments is similar to their smaller counterparts or they vary significantly. The use of large DNA is an effective strategy in preparing multivalent vaccines that give protective immunity against multiple microbes [5]. In this study we employed an F-factor based cosmid (or fosmid), which was like a plasmid (circular DNA), but could contain much larger pieces of DNA.

The DNA used in this work is of medium size range ≈25,000 base pairs (bp), which generally has a very low copy number of 1–2 per cell and is quite stable in the stock cultures [3,6]. However, considering the fact that this may considerably lower the DNA yield during extraction and purification, a high copy number origin of replication (ori V) has been artificially incorporated into this 25 kbp DNA [3] and addition of L-arabinose as an inducer during DNA synthesis is advised [7].

1.2. Monolithic adsorbent: solving the 'wall channel' effect

Conventional isolation of high-quality large DNA molecules has routinely relied on size exclusion or some form of membrane separation processes [8,9]. The approach separates large DNA molecules from small size contaminating molecules accompanying the lysate phase from intracellular release of cytoplasmic and nucleic molecules after cell membrane disruption. The method utilises specific cut-off membrane pore size tailored for the screening of large DNA molecules under an applied pressure; the degree of which is a function of the membrane pore size and surface area, flow rate of feed, membrane clogging rate, and the flow configuration (dead-end or tangential). The selectivity of membrane separation could be improved using activated membrane filters harbouring a functional probe with active moieties to specifically target biomolecule of interest whilst enable unwanted biomolecules to flow through [10]. However, the application of size exclusion or general membrane purification processes for the isolation of large and viscous DNA molecules are confronted with serious challenges including scalability, low binding capacity and low productivity [11,12].

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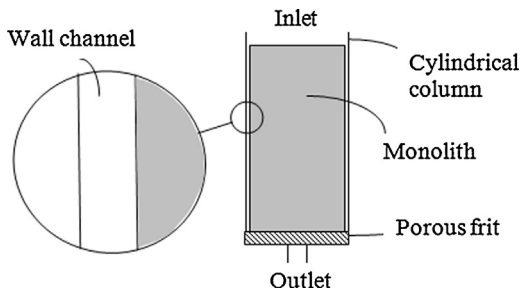

Fig. 1. Schematic representation of a 'wall channel' in a monolithic chromatography column. Wall channel is a gap between the column wall and the outer surface of the monolith.

Continuous monolithic supports have been widely explored for the chromatographic purification of different biomolecules including proteins, DNA and peptides [13]. However, work on the utilisation of monolithic columns for isolation and purification of large size DNA molecules is minimal. Large size DNA molecules particularly >5 kbp are densely electronegative, more susceptible to mechanical shearing, and with slow hydrodynamics and fluidity in solution, making their optimal isolation quite problematic [14]. One of the techniques used to overcome these flow-related drawbacks in large-size DNA isolation is through the incorporation of specific ligands on the adsorbent matrix. As a matter of fact, affinity chromatography has been preferred over other methods to isolate the supercoiled (sc) form of plasmid DNA from other plasmid DNA isoforms and host impurities [15]. Monolithic purification offers the platform for continuous isolation *via* convective hydrodynamics with virtually no resistance to the mass transfer. Monolithic columns can possess large pore sizes that allow easy adsorptive binding of large size DNA molecules with moderate back pressure. This results in rapid separation, high productivity, scalable and continuous operation, low operating pressure, and minimal loss of DNA supercoilty [13].

These monolithic columns are generally made of polymeric materials which are susceptible to shrinkage, leading to the development of a gap or 'wall channel' between the monolithic resin and the column wall as depicted in Fig. 1. The larger the pore size, the higher the degree of shrinkage. This in turn results in a portion of the mobile phase containing sample molecules effectively bypassing the stationary phase and remaining unseparated, thereby reducing the resolution of chromatographic peaks and contaminating the product fraction [16–18]. Attempts have been made to address the above described 'wall channelling problem' by providing covalent bonds between the column wall and the monolith by introducing a coupling agent, for example a silane-based methacrylate [19,20]. However, these methods are rather tedious to implement and may not provide a long-term solution to wall channelling. There is also a risk of leaching out the coupling agent during a column run and this can interfere with the product fraction. The interference may be toxic, and even if biocompatible, would necessitate further downstream purification, especially if the product fraction is to be used in biopharmaceutical applications. There is therefore a need for a method and a monolithic column which can address the wall channelling problem.

2. Materials and methods

2.1. Materials

Ethylene glycol dimethacrylate (EDMA) (M_w 198.22, 98%), glycidyl methacrylate (GMA) (M_w 142.15, 97%), cyclohexanol (M_w 100.16, 99%), 1-dodecanol (M_w 186.33, 98%), azobisisobutyronitrile (AIBN) (M_w 164.21, 98%), methanol (HPLC grade, M_w 32.04, 99.93%), diethylamine (DEA) (M_w 73.14, 99%), Na_2CO_3 (M_w 105.99, 99.5%) and EDTA (M_w 292.3, analytical grade) were purchased from Sigma–Aldrich, Australia. NaCl (Amresco, Australia, M_w 58.44, 99.5%), agarose (Promega, Australia), SDS (M_w 288.38, 99.0%) and Tris (M_w 121.14, 99.8%) from Amresco, Australia, ethidium bromide (Sigma, Australia, M_w 394.31, 10 mg/mL) and 1 kbp DNA marker (BioLabs, New England).

2.2. Cell line and DNA

The details of the 25 kbp fosmid DNA and its host [3] are given in Table 1.

2.3. Cell line propagation and cell harvesting

The attainment of high DNA mass yield is essential to meet the high *in vivo* dosage requirement of therapeutic DNA administration especially for large mammals. After procuring the sample (as of agar stab cultures of *Escherichia coli*), glycerol freeze stocks were made using 50% glycerol (15% glycerol in solution) and stored at −73 °C. In this way, the cultures are stable for several years and can be used to prepare DNA samples at any time [3]. A special variant of the LB medium was used for culturing *E. coli*. 1 L of the medium was composed of 5 g yeast, 10 g tryptone, 5 g glucose, 15 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NH_4Cl, 0.25 g $MgSO_4$ and 2.5 mL glycerol. The medium was sterilised in a steaming autoclave at 121 °C for 20 min. Standard molecular biology protocols [21] were followed for growing/culturing the cell line. 100 μL bacterial culture (from frozen glycerol stock) carrying the desired DNA construct was scrapped off and transferred to 250 mL of medium with chloramphenicol (12.5 μg/mL working concentration) and 0.01% L-arabinose and incubated for a prolonged period (22–24 h) at 37 °C with vigorous shaking (200 rpm). The grown cultures were always noted to possess an opaque or turbid appearance. The cells were harvested in 16 tubes (60 mL per tube) through double centrifugation and were preserved at −20 °C for a maximum of 1 week. Within this time period, DNA extraction and purification was done as per requirement.

2.4. Clarified cell lysate preparation

4 mL of suspension buffer (50 mM glucose, 25 mM Tris–HCl, 10 mM EDTA, pH 8.0) was added into each tube to resuspend the bacterial pellet and then incubated for 10 min at room temperature. The tubes were tapped against a hard rubber surface to break the pellets. 8 mL of freshly prepared lysis buffer (0.2 N NaOH, 1% SDS) was then added into each tube and mixed gently by inverting six times. This broke open the bacterial cell wall and released intracellular components including the DNA which would be denatured into single-stranded form [3]. Tubes were incubated for 5 min at room temperature. 6 mL of pre-chilled neutralisation buffer (5 M potassium acetate) was then added into each tube and gently mixed by inverting the tubes six times and incubated at 4 °C for 30 min. Tubes were centrifuged for 45 min at 4 °C and 4550 rpm to pellet out the unwanted precipitate. Following the centrifugation, the supernatant was transferred to fresh tubes.

2.5. Preparation of 25 kbp DNA sample by non-chromatographic method

Pre-existing approaches [3,21] involving alkaline lysis were employed for extraction and purification of the 25 kbp DNA from the *E. coli* cultures. RNase A (10 μg/mL) was added into each tube (to digest the contaminating RNA in the lysate) and gently inverted six times. The tubes were incubated in a water bath for 45 min at 60 °C.

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