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Adsorptive purification of pDNA on superporous rigid cross-linked cellulose matrix

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Abstract

Use of plasmid DNA (pDNA) in the emerging gene therapy requires pure DNA in large quantities requiring production of safe DNA on large scale. While a number of kit-based DNA purification techniques have become popular, large scale cost effective purification of DNA remains a technological challenge. Most traditional, as well as newly developed methods for DNA purification are expensive, tedious, use toxic reagents, and/or generally not amenable for scaled up production. Our attempts to develop a scalable adsorptive separation technology resulted in successful use of indigenously developed rigid cross-linked cellulose beads for single step purification of pDNA from alkaline cell lysates. This mode of purification employs a combination of intra-particle interactions that could give a product plasmid DNA free from chromosomal DNA, RNA and host proteins in a single scalable chromatographic step. The technology can be employed as a batch adsorption step on small scale, or on a large scale column chromatography. A high copy number 9.8 kb plasmid (from an *Escherichia coli* strain) was purified in yields of 77 and 52%, respectively in batch and column modes. The product obtained was homogeneous supercoiled plasmid with no RNA and protein contamination confirmed by quantitative analysis, agarose gel electrophoresis and SDS–PAGE.

Keywords: CELBEADS; DNA; Hydrophobic interaction chromatography; Plasmid

1. Introduction

Advances in molecular biology have led to improvements in our understanding and ability to diagnose and treat human diseases. Some of the newest and significant advances have been in the field of gene therapy and DNA vaccines. Gene therapy involves introduction of a gene to specific cells in a patient to produce therapeutic biomolecules, usually proteins that correct or modulate a disease. Different approaches to gene delivery systems are being studied based on different methods of gene administration and nature of the delivery vectors used (viral versus non-viral). Each of these approaches has certain advantages and disadvantages.

Plasmid (non-viral vector) borne genes are attracting increasing attention as a biotherapeutic in gene therapy and DNA vaccination. As regulatory approval is granted to increasing number of non-viral human gene therapy clinical trials, there is a corresponding rise in demand for pharmaceutical grade plasmid DNA (pDNA) since only one in every 1000 plasmid DNA molecules presented to the cells reaches the nucleus and is expressed [1]. Full treatments thus require milligram quantities of plasmid DNA, and one of the challenges associated with such a technology is the development of cost effective large-scale processes for the production of plasmid DNA [2]. Further, such a process should be capable of providing the product plasmid at required levels of product purity, potency, efficacy and safety.

Traditionally, DNA is purified by saccharose or cesium chloride–ethidium bromide density gradient ultracentrifugation. However, these methods are time consuming and difficult to scale up and use toxic and mutagenic reagents. Adsorptive separation or chromatography has emerged as the method of choice because it is scalable and reproducible, and uses chemicals that are generally regarded as safe. The technology has also met process approvals and validations

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[3]. Of the many chromatography modes, gel filtration and anion exchange have been scaled up to obtain final pure preparations of plasmid DNA usually using a combination of more than one chromatographic steps [4,5]. Commercial matrices like Q-SEPHAROSE, DEAE-STREAMLINE have been reportedly used for preparative and production scale purification of DNA [5,1]. Affinity chromatography and reversed phase chromatography have also been reported for purification of plasmid DNA [6,7]. A number of reports have recently appeared that use the relative hydrophobicities of the components in a cell extract for their separation from plasmid DNA using thiophilic interaction chromatography [8]. Components like proteins and nucleic acids (gDNA, pDNA and RNA) display different hydrophobic affinities to hydrophobic interaction matrices.

Plasmid DNA extractions routinely employ alkaline detergent cell lysis, followed by precipitation upon neutralization, for the primary recovery of the plasmid. This step, although key to the success of any purification strategy, is seldom sufficient to yield pure pDNA and gives substantial contamination from gDNA, RNA and host proteins. For therapeutic purposes, one requires high purity plasmid. FDA of USA has mentioned the following as requirements for the nucleic acids employed for therapeutic use [4]:

Endotoxins	<0.1 EU/μg of pDNA
Genomic DNA	<10 ng gDNA/μg pDNA
Host protein	Undetected by BCA assay or silver staining
RNA	Not seen in 0.8% agarose gel

Adsorptive interactions based on hydrophobic or ionic interactions have been reported and used for nucleic acid purification. Diogo et al. [9] described hydrophobic interaction chromatography (HIC) for purification of supercoiled plasmid DNA. They used sepharose gel derivatized with 1,4-butanedioldiglycidylether. The use of HIC takes advantage of the more hydrophobic character of single stranded nucleic acid impurities as compared to the double stranded plasmid DNA. Denatured genomic, plasmid DNA and RNA with large stretches of single strands, and lipopolysaccharides (LPS) are reported to display more hydrophobicity than supercoiled plasmid. Thus, all contaminants were retained and separated from non-binding plasmid DNA on HIC matrices. However, the reported work makes use of a pre-treatment step of ammonium sulfate precipitation of proteins from the alkaline cell lysate.

Most chromatographic matrices reported for plasmid DNA purification are traditional protein adsorbent matrices. Nucleic acids being much larger in molecular size have shown low binding capacities on these matrices [3]. Our laboratory has been able to prepare a highly porous new rigid cross-linked cellulose matrix CELBEADS that exhibits weak hydrophobicity [10]. CELBEADS are prepared as hard beads by cross-linking cellulose or cellulose derivatives and in a mean bead size from 150 to 500 μm, and a mean pore radius in the range of 1–3 μm, i.e. 10,000–30,000 Å. CELBEADS has been used for applications involving macromolecules like starch, and nano-particles like cell wall fragments, and semi-soluble polysaccharides [11]. It has been shown that CELBEADS offers low pore diffusional resistance to macromolecules entering the pores [12]. It was thought that possibly a combination of molecular exclusion of super-coiled DNA, and preferential adsorption of smaller proteins, RNA, and linear fragments of DNA may prove to be effective in separating components of cell lysate. Thus, it was decided to exploit the size and nature of the nucleic acid molecules (namely hydrophobicity), and the accessibility of the large nucleotide bases to hydrophobic locations in the super-pores of CELBEADS combined with the topological constraints imposed by supercoiling of the desired plasmid product. Thus, in this paper, we present a single step technology that makes use of a combination of factors like hydrophobicity and relative molecular size, to obtain purified super-coiled plasmid DNA. The indigenously developed and patented CELBEADS was used for purification of a pDNA (pSWEET plasmid) from the alkaline cell lysate of an *Escherichia coli* strain. Purification of the plasmid was attempted in both batch adsorption and column adsorption modes.

2. Experimental

2.1. Materials

E. coli harboring the plasmid pSWEET (9.8 kb) was obtained from Department of Biochemistry, McMaster University, Canada [13]. CELBEADS was prepared in the laboratory according to a procedure for which patent has been filed [10]. The procedure employs cross-linking of cellulose derivative/s in a porogen solvent. The cross-linked cellulose mass is then suspended repeatedly in aqueous buffer that drains off the porogen leaving pores of desired size depending on the concentration of cellulose used in the cross-linking step. Properties of the CELBEADS thus prepared and used are given in Table 1. Calf-thymus DNA was procured from SRL, India. All the chemicals used were of molecular biology grade and obtained from local suppliers.

Table 1
Characteristics of CELBEADS

Particle size range (μm)	150–500
Sphericity	0.8–0.9
Nature	Rigid porous matrix
Bulk density (kg/m^3)	1400–1800
Pore volume (%)	57–59
Average pore radius (μm)	∼3

2.2. Methods

2.2.1. Preparation of bacterial culture and cell lysis

E. coli harboring the plasmid pSWEET was grown for 12–16 h at 37 °C, in a 100 ml conical shake flask containing 20 ml LB broth medium with 50 μg/ml ampicillin. Larger culture volumes (1000 ml) were inoculated with the appropriate amount of log phase culture and incubated under the same conditions.

The bacterial culture was harvested by centrifugation (Sigma 6K10) at 4 °C for 15 min at 5000 × *g*. The harvested cells were lysed using the standard alkaline lysis method [14]. The lysate was centrifuged at 12,000 × *g* at 4 °C for 30 min to remove cellular debris. The supernatant (crude lysate) so obtained was used as such for adsorption experiments.

2.2.2. Purification of plasmid DNA

2.2.2.1. Batch purification. *X* ml of matrix was degassed and equilibrated with 2 M ammonium sulfate in 100 mM Tris–HCl, pH 7 buffer containing 10 mM EDTA, in a stoppered glass flask. Cell lysate, in volumes ranging from *X* to 3.5*X* ml, was added to the matrix. The mixture was incubated at room temperature (∼35 °C) for 4 h with gentle shaking. Thereafter, the supernatant was carefully decanted out and the nucleic acids in the supernatant solution were precipitated by 0.6 volume of 2-propanol in a centrifuge tube, and the mixture incubated at 4 °C for 30 min. The suspension was centrifuged at 12,000 × *g* for 30 min and the pellet separated and re-dissolved in a minimum volume of 25 mM Tris–HCl, pH 8 buffer containing 10 mM EDTA. This solution was analyzed for plasmid content and quality by agarose gel electrophoresis. Lipopolysaccharide content was tested by the limulus amoebocyte lysate (LAL) assay using the Bio Whittaker LAL kinetic assay kit. The quality of the plasmid was also checked by restriction enzyme *Bam*HI digestion at 37 °C for 1 h.

2.2.2.2. Column purification. A 10 mm diameter glass Bio-Rad ECONO column equipped with a top ECONO adaptor was packed with 10 ml of degassed CELBEADS. The resin was equilibrated with three bed volumes of equilibration buffer mentioned above. One column volume of the crude cell lysate was passed in to the column in up-flow mode at the rate of 0.2 ml/min. This was followed by the equilibration buffer at the same flow rate. The flow through DNA containing fractions, each of 2.5 ml, as indicated by an online 254 nm spectrophotometer flow cell, were collected and analyzed for nucleic acids by precipitation with 0.6 volume of 2-propanol in a centrifuge tube, and incubation at 4 °C for 30 min. The suspensions were centrifuged as mentioned above, and analyzed for plasmid content and quality, and protein content in each fraction.

2.2.3. Measurement of DNA

DNA concentration was determined by measuring the sample absorbance at 260 nm with one unit O.D.$_{260\,nm}$ corresponding to 50 μg/ml of double stranded DNA in the sample in a 10 mm path length cuvette. Relative pDNA and gDNA concentrations in a sample was determined by reading agarose gel on a Bio-Rad gel documentation system with the total deoxyribose content in the sample determined by the diphenylamine (DPA) test using calf thymus DNA as standard [15]. The $A_{260\,nm}/A_{280\,nm}$ was also measured as an indicator to sample DNA purity.

2.2.4. Agarose gel electrophoresis

Horizontal gel electrophoresis was performed using 0.8% agarose gel stained with ethidium bromide in 50 mM Tris–Borate EDTA buffer pH 8. The gel was visualized on the Bio-Rad Gel Doc 2000 system.

2.2.5. Protein determination

Total protein was analyzed on sodium dodecylsulfate 12% polyacrylamide gel on a vertical gel unit (Mini Protean II from Bio-Rad). Samples were run at 200 V and visualized using silver staining. Quantitative protein assay was done using standard Bradford reagent kit from Bio-Rad.

3. Results and discussion

In the batch adsorption method, the centrifuged crude lysate obtained from alkaline cell lysis method was directly applied to CELBEADS and equilibrated for 4 h. Such crude lysate contains large amounts of RNA, fragments of gDNA, linear and super-coiled pDNA, proteins and lipopolysaccharides. It was found that CELBEADS showed preferential adsorption of proteins, RNA and other fragments of DNA, over adsorption of the super-coiled pDNA. A time course batch adsorption showed at first adsorption of all other impurities and then followed by pDNA adsorption (data not shown). A contact time of 4 h was seen to give equilibrium results. It was necessary to arrive at optimum lysate loading to allow maximum amount of 'impurities' to be preferentially adsorbed while retaining all the super-coiled plasmid in the solution. Thus, different volumes of lysate (1–3.5 times the adsorbent volume) were loaded on the matrix and equilibrated. The results are shown in Fig. 1. A crude lysate volume of twice the adsorbent volume was found to be optimum as higher loading of crude lysate resulted in insufficient 'mopping up' of the contaminants including RNA. The supernatant of the optimized batch adsorption step when analyzed showed a sharp band of the plasmid with undetectable amount of impurities such as gDNA and RNA on 0.8% agarose gel (Fig. 2). When tested for proteins the supernatant showed no bands on SDS–PAGE, and the Bradford assay also indicated absence of protein. Details of a typical run are given in Table 2. Thus, from a centrifuged crude lysate with a pDNA concentration of 871 μg/ml in a DPA determined total DNA content of 1137 μg/ml, containing 111.4 μg/ml of protein, a purified supernatant product containing DPA determined 2707 μg DNA was obtained that by agarose gel was 100% pDNA giving an

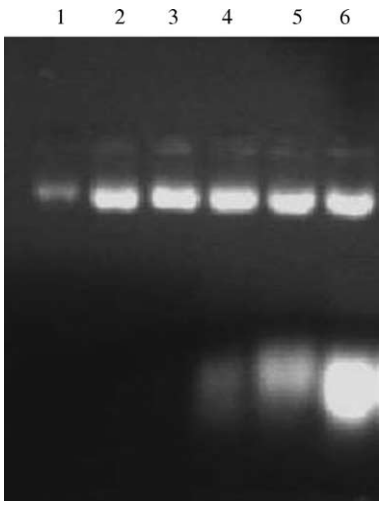

Fig. 1. Agarose gel (0.8%) electrophoresis performed on supernatants after equilibration of different volumes of crude lysate on to 2 ml of 2 M ammonium sulfate–Tris–HCl buffer equilibrated CELBEADS. Lane 1: 2 ml crude lysate; Lane 2: 3 ml crude lysate; Lane 3: 4 ml crude lysate; Lane 4: 5 ml crude lysate; Lane 5: 6 ml crude lysate; Lane 6: 7 ml crude lysate.

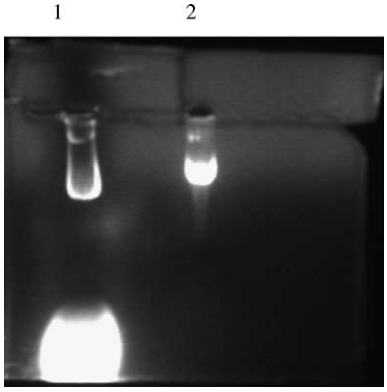

Fig. 2. Agarose gel (0.8%) electrophoresis of batch purified pDNA using optimized protocol on 2 ml of CELBEADS using 4 ml of crude lysate. Equilibration buffer was 2 M ammonium sulfate in 100 mM Tris–HCl, pH 7 buffer containing 10 mM EDTA. Lane 1: crude lysate; Lane 2: supernatant from batch adsorption.

Table 2
Purification of pSWEET plasmid in batch adsorption mode: 2 ml of CELBEADS equilibrated with 2 M ammonium sulfate in 100 mM Tris–HCl, pH 7 buffer containing 10 mM EDTA for 4 h

	Crude lysate load (μg)	Purified supernatant (μg)	Yield (%)
pDNA	3484	2707	77.6
gDNA	1065	Nil	–
RNA	Present	Nil	–
Total nucleic acids as DNA[a]	5760	2969	
Protein	444.6	Nil	–
A_{260}/A_{280}	1.7	2.1	

[a] By $A_{260\,nm}$.

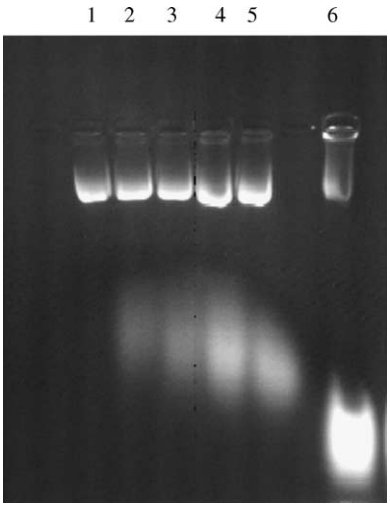

Fig. 3. Agarose gel (0.8%) electrophoresis of batch purified pDNA at different equilibrating ammonium sulfate concentrations. Lane 1: 2 M ammonium sulfate; Lane 2: 1.5 M ammonium sulfate; Lane 3: 1 M ammonium sulfate; Lane 4: 0.5 M ammonium sulfate; Lane 5: 0.25 M ammonium sulfate, Lane 6: crude lysate.

overall process yield of 77.6%. The $A_{260\,nm}/A_{280\,nm}$ ratio of the product was measured as 2.1. A DNA sample substantially devoid of proteins is known to give the ratio in the range 1.8–2.3, the value of 2.3 signifying highest purity. Further, the LAL test on the supernatant product showed undetectable amount of lipopolysaccharides.

The above results were obtained with 2 M ammonium sulphate equilibrated adsorbent, whereas a non-salt equilibrated adsorbent did not give same results. This necessitated study of the effect of ammonium sulfate concentration on the purification protocol. The results (Fig. 3) indicate that as salt concentration in equilibration buffer increased, binding of RNA to the adsorbent increased, with 2 M salt giving the best purification, higher concentration giving essentially same results as 2 M. It may be noted here that ammonium sulfate was used only in the buffer equilibration step, and no additional salt was added to the lysate that contains approximately 1 M potassium acetate from the lysis step.

In order to increase the throughput capacity of the pDNA purification step, purification of pDNA from the cell lysate was performed on a column packed with CELBEADS. Column adsorption is essentially a non-equilibrium process that generally shows a dynamic binding capacity less than that obtained with batch equilibrium experiments. Moreover, the dynamic capacity is a significant function of mobile phase linear velocity, the function being stronger with larger molecules. Breakthrough experiments with the batch optimized loading of two times the adsorbent volume resulted in incomplete adsorption of RNA, gDNA and proteins and the flow through gave low yield of purified plasmid. Fig. 4a shows the results from loading of one column volume of cell lysate at a linear mobile phase velocity of 15 cm/h (0.2 ml/min). The figure shows the quality of the DNA in four 2.5 ml size fractions. The first four fractions provided DNA free from RNA while

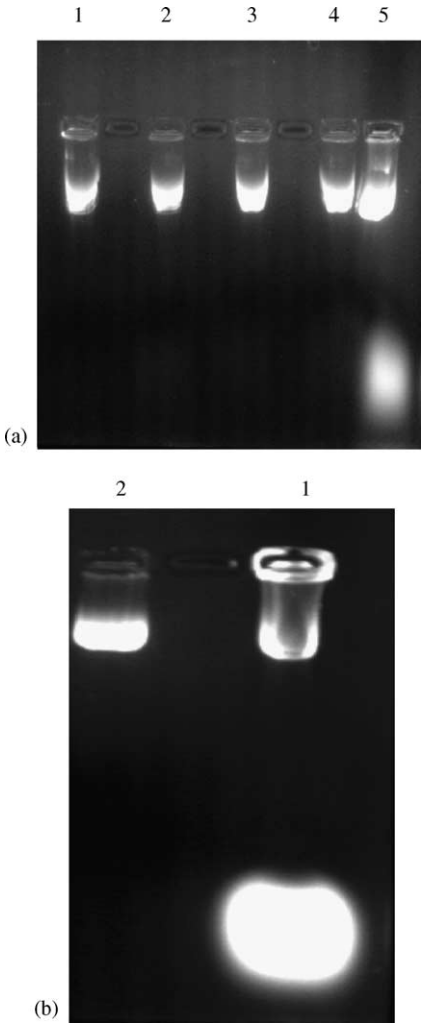

Fig. 4. Agarose gel electrophoresis of column purified pDNA. Crude lysate (10 ml) applied at 0.2 ml/min on 10 mm diameter column containing 10 ml CELBEADS pre-equilibrated in 2 M ammonium sulfate in Tris–HCl buffer. (a) Lanes 1–4: four flow through fractions (2.5 ml each) from column; Lane 5: crude lysate. (b) Lane 1: crude lysate; Lane 2: pooled fractions.

the fifth fraction indicated breakthrough of RNA (sample not loaded on agarose gel). Thus, on the column, the adsorbent capacity at 0.2 ml/min reduced by half compared to the batch capacity. Fig. 4b shows the quality of pDNA in the pooled four fractions against the crude lysate quality loaded. Table 3 presents the overall purification result from a typical column run. A yield of 51.5% was obtained on the column. However, initial results on scale-up have indicated that the plasmid yield drops significantly with increasing processing volumes on batch adsorption protocols due to damage to DNA caused by mixing conditions. Studies on scale-up and optimization of column adsorption are underway.

Performance of CELBEADS may be compared with performance of 1,4-butanediol diglycidyl ether derivatized Sepharose reported by Diogo et al. [9]. The said adsorbent was reported as hydrophobic matrix and resulted in purification of a pDNA (~4 kb) in much the same way as CELBEADS in this work. However, the work used two additional

Table 3
Purification of pSWEET plasmid in column mode: 10 ml of CELBEADS in 10 mm diameter glass column

	Crude lysate load (μg)	Purified supernatant (μg)	Yield (%)
pDNA	8325	4292	51.5
gDNA	2716	Nil	–
RNA	Present	Nil	–
Total nucleic acids as DNA[a]	12,180	5400	
Protein	1114	Nil	–
A_{260}/A_{280}	1.7	1.9	

Resin was equilibrated with 2 M ammonium sulfate in 100 mM Tris–HCl, pH 7 buffer containing 10 mM EDTA at a flow rate of 0.2 ml/min.
[a] By $A_{260\,nm}$.

steps of iso-propanol and ammonium sulphate precipitation to achieve an overall yield of 33%. In a patent report Butyl-650S resin from TosoHaas was used as HIC adsorbent for purification of plasmid free from endotoxins [16]. The patent presents data that indicates binding of a 4.6 kb plasmid from 2-propanol precipitated lysate preparation. The plasmid was reported to partially bind to the adsorbent and eluted in flow through, buffer wash and lower ionic strength buffers. Only the flow through fraction was found to be free from RNA and gave a 13% overall yield of purified plasmid.

One important desired characteristic of any adsorbent being its reusability, the reuse of CELBEADS was tested using different cleaning-in-place (CIP) methods. It was found that CELBEADS could be regenerated with 1 M NaOH, washed with de-ionized water, equilibrated and re-used again. Up to six purification cycles were tested without any loss of performance.

4. Conclusions

pSWEET (9.8 kb) was purified on CELBEADS using a combination of hydrophobicity and macroporosity of indigenous adsorbent matrix CELBEADS. It is shown that CELBEADS is able to bind substantial amounts of broken strands of gDNA and RNA along with proteins in the cell lysate. The adsorption capacity of CELBEADS for non-circular DNA was determined as 1400 and 680 μg/ml on batch and column protocols, respectively (Tables 3 and 4). These capacities are five to seven times higher than those reported for traditional protein binding matrices [3]. Significantly, the plasmid was purified through removal of all lysate contaminants and not through its specific adsorption. This result is similar to results reported by Diogo et al. [9] and Ramasubramanyam [16]. A plasmid is normally double stranded so that its hydrophobic bases are shielded inside the helix and are not available for interaction with hydrophobic sites. Genomic DNA though double stranded is rendered mostly single-stranded during the alkaline cell lysis and consequently is adsorbed on to hydrophobic sites in the macropores of CELBEADS. Lipopolysaccharides also interact with the CELBEADS via

lipid moieties. It is shown that the plasmid pSWEET was purified on CELBEADS by both batch and column protocols and yielded a product that seems suitable for therapeutical purposes. The yields obtained on the two protocols were 77 and 52%, respectively. These compare favorably against reported work on hydrophobic matrices. Further, CELBEADS gave a yield of 0.43 mg plasmid per ml adsorbent in column mode which is higher than reported yields of 0.05 and 0.19 mg plasmid per ml adsorbent reported by Diogo et al. [9] and Ramasubramanyam [16] for derivatized sepharose and Butyl650S, respectively. While the obtained plasmid was found to be free from RNA, gDNA, protein and endotoxins, the economy of the process is further improved through reuse of CELBEADS which could be used over many cycles in reproducible manner using an alkali based CIP procedure.

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