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Purification of Nanoparticle Bioproduct in Integrated Processes: Plasmid DNA Separation and Recovery

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ABSTRACT

The demand of high-purity plasmid DNA (pDNA) for gene-therapy and genetic vaccination is still increasing. Pharmaceutical-grade plasmid DNA for use in vaccines requires the development of reproducible and scaleable down stream processes. The aim of this study is investigation and comparison of pDNA separation by aqueous two phase system and anion-exchange chromatography as popular techniques in plasmid DNA purification. In this work anion exchange chromatography carried out in column with 1.3 cm diameter filled with 8 ml streamline DEAE and polymer-salt system (ATPS) consisted of polyethylene glycol (PEG300)-K_2HPO_4 was used for the purification of plasmid DNA vectors. Results show that 88% of pDNA purified by expanded bed chromatography in contrast with 84% recovery of pDNA in top phase of aqueous two phase system.

Keywords: expanded bed adsorption, aqueous two-phase system, plasmid DNA, purification

INTRODUCTION

One of the fundamental challenges in studying bioproducts (*e.g.* plasmid DNA nanoparticles) and their complexes in a biological system is isolating them in their structurally and functionally intact forms (Kim *et al*. 2010). Recent advances in gene therapy have increased the need for large-scale production of effective vectors (Teeters *et al*. 2003). Gene therapy and DNA vaccination have attracted a lot of interest as new ways of preventing or treating disease through gene transfer. Non-viral techniques including the intra-muscular injection of naked plasmid DNA or gene gun delivery of plasmid DNA coated onto gold particles into the epidermis appear very attractive as they offer several advantages over viral vectors, especially low immunogenicity, better safety profile and easier manufacture (Eon-Duval *et al*. 2004). There is therefore a need for recovery of plasmid DNA as nanoparticulate (nanoparticle) bioproducts (second generation of biotechnological products) as putative gene therapy vectors (Jahanshahi 2004). Many methods are available for the purification of small molecular, i.e. proteins (5-10 nm in size). However, these methods are often poorly suited for larger molecules such as plasmid DNA (>100 nm in size). Thus, there is a need for development of better-suited purification methods for plasmid DNA (Kepka *et al*. 2004). Plasmid purification strategies usually involve at least one chromatography step either to capture the plasmid or more often, for polishing. Techniques include: size-exclusion, reversed-phase, hydrophobic interaction, silica and triple-helix affinity (Eon-Duval *et al*. 2004). A plasmid purification method based on a unique anion exchange is the most popular chromatography technique in pDNA purification (Yang *et al*. 2008). This method takes advantage of the interaction between the negatively charged phosphate groups in the DNA back bone and the positively charged ligands on the matrix. The shape and size of the molecules may play an important role, particularly in the separation of plasmid variants. In fact in some anion exchangers, the more compact supercoiled form, which have a higher charge density, elute later than the open circular forms which have a lower, overall, charge density (Prazeres *et al*. 1998). Anion-exchange remains the chromatography technique that offers the advantages of rapid separation, no solvent requirement, sanitisation with sodium hydroxide and a wide selection of industrial media (Chandra *et al.* 1992). The aqueous two-phase system is a gentle approach that has strong potential as a primary recovery step for plasmid purification (Gomes *et al*. 2008). It is also one of the non-conventional methods which have recently received attention, since several features of early processing steps can be combined in only one operation and phase environment is non-toxic for biomolecules. Polyethylene glycol (PEG) is a linear polymer of ethylene oxide groups. The polymer is soluble in water and at a certain salt concentration a two-phase system consisting of PEG and salt is formed. By changing the molecular weight of the PEG polymer, the plasmid DNA can be partitioned in either phase (Trindade *et al*. 2005). in the present work pDNA in column on optimal condition with 1.3 cm diameter column, 25 cm height, 6 cm settled bed height was purified in expanded bed mode, also in this study experiments carried out with different PEG/salt ratio and recovery in three phases (top, bottom, interface) were measured. pDNA in optimized aqueous two-phase system (26% w/w PEG, 17% w/w K_2HPO_4 and molecular weight of PEG 300) was purified. The results of pDNA purification in two optimized system (ATPS and EBA) were compared.

MATERIALS AND METHODS

Materials

The *Escherichia coli* host strain DH5-α containing a 5.9 kb plasmid was developed in-house. Streamline DEAE adsorbent was provided by Amersham Bioscience. A glass column (13 mm ID, 25 cm height) was used for expanded experiments. All chemicals were bought from Merck and were of analytical grade. PEG 300 was obtained from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade.

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Short Communication

Column and setup used for expanded bed chromatography

The glass column (13 mm ID, 25 cm height) was used for expanded bed experiments. A sintered glass was fitted on to the lower end of the columns to act as a flow distributor and a support for the bed. Sintered was 2 mm in thickness, with a nominal pore size of 70 µm. The outlet of the column contained an adaptor at the top end of the expanded bed, which could be moved to any position. The top adaptor was positioned 0.5 ± 0.2 cm above the bed surface. The Liquid from the outlet of the column was transferred through the UV detector (WellChrom fast scanning spectrophotometer K-2600, Knauer, Berlin, Germany) and recorded with recorder.

Bacterial cell culture and lysis of plasmid DNA nanoparticles

E. coli cells were cultivated overnight in 1000 ml shake flasks containing 250 ml of Luria-Bertani medium supplemented with 50 µg/ml of kanamycin (Sigma), at 37°C and 250 rpm. Alkaline lysis, in combination with the detergent SDS, has been used to isolate plasmid DNA from *E. coli*. At first in the alkaline lysis method, 500 ml overnight cell culture was harvested by centrifugation at 5000 rpm for 10 min (4°C). The supernatant was carefully removed and 5 g of the bacterial pellet was resuspended in 10 ml of 10 mM Tris, 10 mM EDTA at pH = 8. Lysis was performed by adding 300 ml of NaOH 5N and 100 ml of 1% (w/v) SDS. The lysate was neutralized with 500 ml of 3M potassium acetate for 30 min at 4°C. Cell debris, chromosomal DNA, high molecular weight RNA and proteins were removed by centrifugation at 5000 rpm for 15 min. High-molecular-mass RNA was eluted using 5 µg/ml RNase for 30 min at 37°C. Total native plasmid (sc and oc) was quantified by anion exchange chromatography5 and also this prepared lysate was used for further processing with ATPS as described below. The average size of nanoparticles achieved was 70 nm.

Preparation of EBA column

The purification experiments were carried out at 25°C with a settle bed height of 6.0 ± 0.2 cm. The operating procedure was as follows:

(i) Expansion of the bed with the equilibration buffer.
(ii) Loading the feedstock until the plasmid DNA nanoparticle concentration of the outlet was equal to about 15% of the feed plasmid DNA nanoparticle concentration.
(iii) Washing the bed with the equilibration buffer to remove unbound nanoparticle.
(iv) Elution of the bound nanoparticles with the buffer containing 0.5-2 M salt gradient.

Preparation of aqueous two-phase system

All of the ATPS were made up and described on a % w/w basis. An ATPS composed of, for example, 15% w/w PEG 300 and 22% w/w potassium hydrogen orthophosphate was designated 15/22. Clean (blank) systems contained only PEG, salt and distilled water. Crude systems contained lysate. The pH of ATPS containing cell lysate was adjusted to 8 with concentrated HCl. Binodal curves were determined by titration according to Albertsson (1985). Water or lysate solution was added drop-wise to several biphasic systems with different compositions, until one phase systems were formed, after vortex mixing. The final composition of the system was then calculated and taken as a binodal point. Tie-lines were determined by calculating the composition of salt and PEG in both top and bottom phase of selected systems. Salt concentration in both phases was determined by conductivity measurements using a conductivimeter at 25°C after adequate dilution. PEG concentration was determined by refractive index measurements. Tie lines length (TLL) was determined by Eq. (1):

$$TLL(\%,W/W) = \sqrt{\Delta P^2 + \Delta C^2} \quad (1)$$

where ΔC is the difference between salt concentration in the two phases and ΔP is the difference between PEG concentrations in the two phases. After mixing in the experiment temperature until equilibrium and phase separation with centrifugation (1000 g, 3 min), the volume ratio was measured. The system volume ratio (VR) was determined by Eq. (2):

$$V_R = \frac{V_{TP}}{V_{BP}} \quad (2)$$

where V_{TP} is the volume of the top phase and V_{BP} is the volume of the bottom phase.

Then top, bottom and interface phases were analyzed for pDNA with spectrophotometer in 260 nm.

RESULTS AND DISCUSSION

Aqueous two-phase system

Different PEG/salt ratio was made and binodial curve was obtained for each ratio. The binodial curve is a curve, which separated the mono-phase from the bi-phase area, i.e. any mixture of PEG, salt and water to the right of the curve results in two phases. Many experiments carried out with different PEG/salt ratio and recovery in three phases (top, bottom, and interface) was measured. To test the effect of phase volume ratio in the current process, several PEG 300 systems with increasing phase volume ratio and with compositions along the optimal (shortest) tie-line were prepared. The partition and purification results were evaluated for a 30% (w/w) lysate load. The concentration of pDNA in the top phase increased with the phase volume ratio. A set of experiments was then carried out by varying the lysate load. An increase on this lysate load would be advantageous since larger feed volumes could be processed by the ATPS. However the concomitant increase in the amounts of both pDNA and contaminants in the systems could result in a decrease of performance of the ATPS, if limits of solubility are exceeded. Although the concentration of pDNA on the bottom phases increased with loading, an increase in protein contamination was also observed. In addition, the pDNA yield decreased and precipitated material could be observed in the top phases of systems with 40 and 50% (w/w) lysate load. This material is most certainly protein that has exceeded its limit of solubility, but some pDNA could also co-precipitate. The results show that for PEG/salt ratio of 26/17 with lysate of 30% about 84% of pDNA was recovered in top phase. Also maximum recovery in bottom phase was 50% that achieved for a PEG/salt ratio of 10/30. Results are shown in **Fig. 1**.

Expanded bed chromatography

Chromatographic purification in an expanded bed adsorption (EBA) format enables integrated capture and purification of biomolecules directly from unclarified feed-stock with the removal of suspended biomass (Graslund *et al*. 2002). When using AEC (anion exchange chromatography) in expanded mode to separate pDNA nanoparticle, the clarified lysate feed should always be loaded at a sufficiently high salt concentration (typically >0.5M NaCl) to avoid an unnecessary adsorption of low charge density impurities, such as low Mr RNA, oligonucleotides and proteins (Diogo *et al*. 2005). After the optimal condition was achieved the purification experiments were carried out in column with settle bed height of 6.0 ± 0.2 cm. The glass column was used to establish the separation. Stream line DEAE was used for the purification of plasmid DNA from a crude cell lysate by AEC in expanded mode. Total plasmid DNA nanoparticles concentration in feed stock was 56.2 µg (30% w/w). The flow rate was 9.2 ml/min. A Streamline 13 column filled with 8 ml of Streamline DEAE media, was equilibrated with 0.5 M NaCl in TE (10 mM Tris, 1 mM EDTA, pH = 8.0) buffer at an upward flow of 402.6 cm h^{-1}, *E. coli* lysates were injected in the column. After washing with a 0.5 M NaCl buffer out the unbound material, 0.5M-2M salt gradient used to displace the different nucleic acids which in principle should elute in order of increasing overall net

Table 1 Mass balance of pDNA recovery in expanded bed adsorption in column with 1.3 cm in diameter.

ID (cm)	Load (µg/ml)	oc fraction		sc fraction		Yield (%)		
		Volume (ml)	pDNA (µg)	Volume (ml)	pDNA (µg)	oc	sc	Total
1.3	56.2	66.2	8.66	66.2	41.3	15.4	73.4	88.8

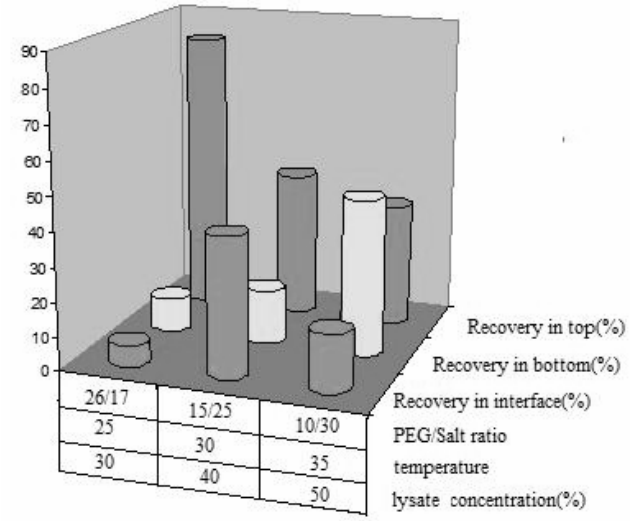

Fig. 1 The effect of three factors on plasmid DNA recovery in aqueous two-phase system.

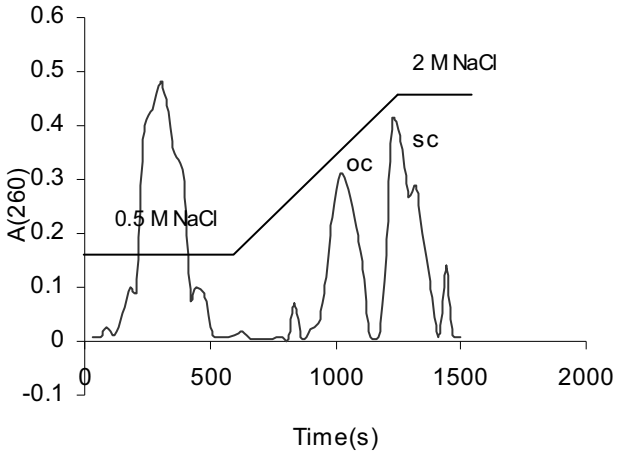

Fig. 2 Plasmid DNA purification by anion - exchange chromatography in a streamline DEAE column with 1.3cm in diameter.

charge. **Fig. 2** presents typical chromatogram obtained after loading clarified lysate for column with 1.3cm diameter. The characteristic flow through peak harbouring low Mr RNA and proteins is followed by DNA isoforms (oc and sc) that are selectively eluted with a shallow salt gradient. The purification result of PDNA nanoparticle in column listed in (**Table 1**).

CONCLUSIONS

Aqueous two-phase systems (ATPS) show an interesting alternative and an integration process since several features of early processing steps can be combined in only one operation. Anion-exchange chromatography is also fundamental in the downstream processing of plasmids both as a process and analytical technique. This work reported the use of a streamline DEAE column for the preparative purification of plasmid DNA nanoparticles. The experimental results also show that the value of plasmid DNA nanoparticles recovered with this procedure was 88% in 1.3cm diameter column. 84% of pDNA was also recovered in PEG/salt ratio of 26/17 with lysate of 30% in aqueous two-phase systems. It is concluded that expanded bed adsorption technology in contrast with aqueous two-phase systems is a more useful technique that can be employed for the nanoparticle purification.

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