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Short communication

Two-step process for initial capture of plasmid DNA and partial removal of RNA using aqueous two-phase systems

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ABSTRACT

In this paper, a two-step process for initial capture of plasmid DNA (pDNA) and partial removal of RNA using polyethylene glycol (PEG) and di-potassium hydrogen phosphate aqueous two-phase systems (ATPS) has been investigated. A Kühni-type ATPS extraction column was prepared with 50 ml (12% (w/w) PEG 1450, 12% (w/w) phosphate) of stationary phase and loaded with crude mobile phase (26% (w/w) PEG 1450, 4% (w/w) phosphate and 70% (w/w) lysate) at a flow rate of 6 ml min^{-1} at an impeller speed of 200 rpm. The experiment was terminated after 100 min, and after complete resettling of the phases, 45 ml of stationary phase was harvested. During a subsequent second extraction step contained 18% (w/w) PEG 300 and 14% (w/w) phosphate, a proportion of RNA, which was also concentrated during the column process, was removed. It was demonstrated that the recovery of pDNA in the second bottom phase was 89.4%, which was similar to the initial recovery after column extraction (92.1%).

1. Introduction

Production of pDNA usually comprises cultivation of an *Escherichia coli* (*E. coli*) host, alkaline lysis of cells, clarification, concentration and purification [1–3]. It has been reported that [4,5] the pDNA concentration following alkaline lysis of *E. coli* cells is low (between 4 and 220 mg l^{-1}). It has been deemed useful to consider a concentration step prior to chromatographic adsorption processes [6]. From this follows that the product volume and hence the manufacturing costs can be reduced and the driving forces for chromatographic adsorption can be enhanced [7]. ATPS have been used in bioseparation operations as an excellent means of combining solid removal, concentration and primary capture of desired proteins directly from particulate containing feedstock [8–11]. The advantages of polymer–salt ATPS include low phase viscosities, low cost of phase-forming chemicals, low toxicity and easy scale-up [12,13].

The advantages of concentrating pDNA by ATPS are that phase mixing and separation can be accomplished rapidly and at low centrifugal or gravitational forces. This eliminates the use of high speed centrifugation, which is necessary for conventional PEG or organic solvent precipitation. Since partition equilibrium in ATPS is rapidly obtained [14,15], the extraction of pDNA may not be mass-transfer limited in the manner usually observed during chromatographic adsorption of pDNA [16,17]. The purification of pDNA by ATPS and hydrophobic interaction chromatography has been demonstrated successfully by Trindade et al. [3]. However, the study was carried out in test tubes with small volumes of lysate (0.5–2 ml). In this paper, a two-step process for initial capture of pDNA and partial removal of RNA using polymer–salt ATPS in a Kühni-type extraction column has been investigated. The application of columns in the ATPS has its origins in the study by Blomquist and Albertsson [18]. The application of different types of columns employing ATPS has been reviewed by Raghavarao et al. [19]. The current process might be applied as a new tool to semi-continuously processing of a relatively high lysate volume (420 ml).

2. Materials and methods

2.1. Materials

E. coli cell paste containing the pDNA vector (pTX0161) was kindly provided by Saethawat Chamsart, The University of Birmingham, UK [20]. PicoGreen™ double-stranded (ds) DNA quantification reagent was purchased from Molecular Probes, Inc. (Eugene, OR). Ribonuclease A (Type I-AS, from bovine pancreas), Lambda DNA BSTE II Digest, and all of the chemicals (analytical grade) were purchased from Sigma–Aldrich Ltd. (Dorset, UK). Bovine serum albumin (BSA), BCA protein assay reagents and Slide-A-Lyzer® mini dialysis units (10,000 MWCO) were purchased from Perbio Science Ltd. (Cheshire, UK).

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2.2. Two-step ATPS purification of pDNA

All of the ATPS carried out in this study were construct and described on a % (w/w) basis. A Kühni-type extraction column (inner diameter: 35.6 mm) consists of one mixing chamber and one separation chamber will used here [18,20]. The height of the column was 200 mm. The phase mixing was achieved by an impeller fitted in the column [20]. The operating and physical parameters (e.g. dispersed phase hold-up, mass transfer and residence time distribution studies) on ATPS extraction have been optimised and described in a previous study [20]. The column was prepared with 50 ml (12% (w/w) PEG 1450, 12% (w/w) phosphate) of stationary phase and loaded with crude mobile phase at a flow rate of 6 ml min^{-1} at an impeller speed of 200 rpm. The ATPS system was chosen based on the batch separation result (>95% recovery yield) [20]. The crude mobile phase composition contained 26% (w/w) PEG 1450, 4% (w/w) phosphate and 69.2% (w/w) lysate (420 ml). Crude *E. coli* cell lysis was prepared as described previously [22]. The measured pDNA concentration of the top phase was 85.6 (μg pDNA equivalent) ml^{-1}. The experiment was terminated after 75 min, and after complete resettling of the phases, 45 ml of stationary phase was harvested. During a subsequent second extraction step contained 18% (w/w) PEG 300 and 14% (w/w) phosphate, a proportion of RNA, which was also concentrated during the column process, was removed. The flowchart of the extraction process is shown in Fig. 2. Initially, the impeller was switched off and the selected volume of stationary (bottom) phase (S) was pumped into the column with a previously calibrated peristaltic pump (101 U Watson–Marlow Ltd., Falmouth, Cornwall, UK). Subsequently, the mobile phase (M) was pumped into the column until some initial drops had passed through the stationary phase. The column inlet was closed and the initial height H was measured. The experiment was initiated by opening the column inlet and setting the impeller speed and the mobile phase flow rate to their defined values. Mobile phase that had settled out of dispersion (M′) was removed from the column via a peristaltic pump. After a certain period of time, the process was terminated and the two phases were allowed to re-settle. Stationary phase (R) was then recovered by back-pumping.

The dispersed phase hold-up is related to the dispersed phase volume to the total volume of dispersion [20]:

$$\varepsilon_D = \frac{H_D - H}{H_D} = \frac{V_D - V_C}{V_D} \quad (1)$$

where H = stationary phase (bottom) height in column before run (mm); H_D = dispersion height in column during run (mm); V_C = bottom stationary phase volume (ml); and V_D = dispersion volume during run (ml).

Centrifugation was performed in centrifuge tubes (15 or 50 ml) in a Bench Jouan Centrifuge C422 (Jouan, France) [20].

2.3. PicoGreen analysis

The pDNA concentration was determined with an intercalating dye called PicoGreen™ [21].

2.4. BCA assay and gel electrophoresis

The protein concentration was analysed with the bicinchoninic acid (BCA) assay and the pDNA purity was assessed by 1% agarose gel electrophoresis [22].

3. Results and discussion

Fresh *E. coli* lysate without treatment with ribonuclease A was prepared for this experiment. Due to the interfering influence of RNA, the pDNA concentration of the mobile phase was 85.6 (μg pDNA equivalent) ml^{-1} (Table 1). In order to reduce the influence of RNA on the assay, samples were also treated with ribonuclease A before additional PicoGreen™ analysis. It has been demonstrated that 1 mg ml^{-1} of low molecular weight RNA caused a response equivalent to 17–20 μg ml^{-1} of pDNA [17]. As a result, the estimated pDNA concentration halved and the value for the mobile phase was 42.7 (μg pDNA equivalent) ml^{-1}. The process steps are outlined in Figs. 1 and 2. The parameters monitored during the process are shown in Fig. 3. The dispersed phase hold-up ε_D stabilised at 0.2. The pDNA concentration of the outlet mobile phase was about 3 (μg pDNA equivalent) ml^{-1} over the whole process time period. Only 6% of pDNA was lost in the outlet mobile phase with a concentration ratio C_T/C_{To} of 0.06. Also shown in Fig. 3 is the density and pH value of the outlet mobile phase. The density of the mobile phase on entry to the column was 1.11 g ml^{-1}, and this value did not change after it had passed the stationary phase. The original pH of the mobile phase was 6.9, but that of the outlet mobile phase decreased from 8.3 to 6.9 over a period of

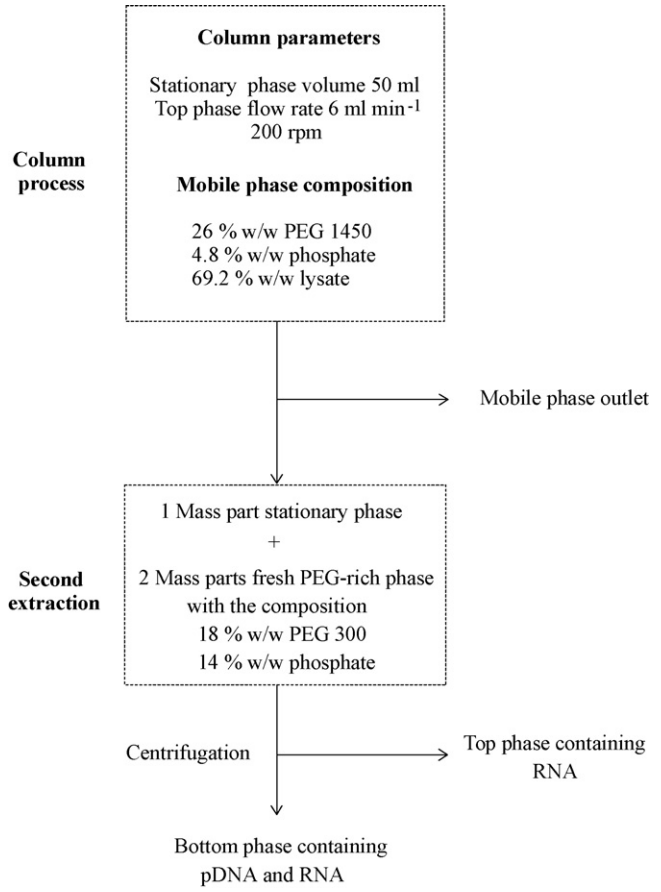

Fig. 1. Two-step process for initial capture of plasmid DNA and partial removal of RNA. In a column process, pDNA was semi-continuously concentrated. During a subsequent second extraction step, a proportion of RNA, which was also concentrated during the column process, was removed.

Table 1
Extraction results for the two-step concentration and purification of plasmid DNA using ATPS.

	Crude mobile phase	Harvested stationary phase	Bottom phase of second extraction
Volume (ml)	420	45	84
Concentration (μg plasmid equivalent) ml^{-1}			
Nucleic acids (directly measured)	85.6	736	308
Nucleic acids (after sample treatment with ribonuclease A)[a]	42.7	367	192
Recoveries of plasmid DNA			
Directly measured	–	92.1	71.5
After sample treatment	–	92.1	89.4

The two-step extraction process is outlined in Fig. 1. Due to the interfering influence of RNA in the lysate on PicoGreen™ analysis of pDNA, concentration measurements were carried out directly and after sample treatment with ribonuclease A. Results reported the mean of two independent experiments with an estimated error of ±5%.
[a] Samples were treated with 100 μg ml^{-1} ribonuclease A and incubated at laboratory temperature for 30 min.