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Journal of Chromatography A, 1057 (2004) 115–124
JOURNAL OF CHROMATOGRAPHY A
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Integrated process for purification of plasmid DNA using aqueous two-phase systems combined with membrane filtration and lid bead chromatography☆

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Received 20 July 2004; received in revised form 2 September 2004; accepted 16 September 2004

Abstract

An integrated process for purifying a 6.1 kilo base pair (kbp) plasmid from a clarified *Escherichia coli* cell lysate based on an ultra/diafiltration step combined with polymer/polymer aqueous two-phase system and a new type of chromatography is described. The process starts with a volume reduction (ultrafiltration) and buffer exchange (diafiltration) of the clarified lysate using a hollow fibre membrane system. The concentrated and desalted plasmid solution is then extracted in a thermoseparating aqueous two-phase system, where the contaminants (RNA and proteins) to a large extent are removed. While the buffer exchange (diafiltration) is necessary in order to extract the plasmid DNA exclusively to the top phase, experiments showed that the ultrafiltration step increased the productivity of the aqueous two-phase system by a factor of more than 10. The thermoseparated water phase was then subjected to a polishing step using lid bead chromatography. Lid beads are a new type of restricted access chromatography beads, here with a positively charged inner core that adsorbed the remaining RNA while its inert surface layer prevented adsorption of the plasmid DNA thus passing in the flow-through of the column. Differently-sized plasmid DNA in the range of 2.7–20.5 kbp were also partitioned in the aqueous two-phase system. Within this size range, all plasmid DNA was exclusively extracted to the top phase. The complete process is free of additives and easy scalable for use in large scale production of plasmid DNA. The overall process yield for plasmid DNA was 69%.

Keywords: Hollow fibre membrane; Restricted access material; Lid beads; Thermoseparation; Aqueous two-phase system; Plasmid; DNA; RNA; Purification

1. Introduction

Over the last couple of years there has been an increasing demand for plasmid DNA to be used as gene therapy vectors [1]. The plasmid vector offers a promising delivery system that is considered to be safer for gene therapy than viral vectors [1]. The low content of plasmid DNA (0–3%) in *Escherichia coli* cells and its similarity in size and charge properties to major contaminants like RNA and genomic DNA makes the purification process for plasmid DNA a challenging task. The purification of plasmid DNA is thus usually accomplished by a sequence of three to four different purification steps depending on the level of purity required [2–5]. Another issue to be considered is the low transfection efficiency of plasmid-based vectors compared to viral vectors, probably requiring milligram quantities of plasmid DNA for each treatment [1]. Thus, there is a demand for a scaleable and cost-effective process that can handle the purity requirements and quantity needed for plasmid-based vectors.

Plasmid DNA production usually begins with cultivation of transformed *E. coli* cells followed by cell lysis using the well-established alkaline lysis method [1–5]. This lysis however, leaves the plasmid mixture in a dilute form and in the

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doi:10.1016/j.chroma.2004.09.060

presence of high salt concentration, a complicating factor for further plasmid DNA purification. A volume reduction step accompanied with a buffer exchange is therefore commonly used. This can be achieved with a number of different techniques, e.g. precipitation methods using isopropanol [2] and poly(ethylene glycol) [1]. The following purification step typically focuses on the removal of contaminants such as RNA, genomic DNA, endotoxins and proteins from the plasmid DNA solution. In this area, many techniques, e.g. chromatography [1–6], precipitation with polycations [7] or spermidine [8], tangential flow filtration [9–11] and aqueous two-phase system [12,13] have proven to be successful.

Previously, Kepka et al. showed that an $EO_{50}PO_{50}$/Dextran T-500 aqueous two-phase system could to a high extent reduce contaminants such as RNA and proteins in a desalted clarified lysate without any loss of plasmid DNA [13]. The EO–PO copolymer (Breox PAG 50 A 1000) consists of 50% (w/w) ethylene oxide groups and 50% (w/w) propylene oxide groups that are randomly distributed within the polymer chain. The EO–PO copolymer has thermoseparating properties, i.e. after formation of the two aqueous phases, the top phase rich in the EO–PO copolymer, is recovered and when heated over its cloud point (CP) temperature the top phase separates into one water phase and one polymer phase [14–16]. The advantage of this system is that the plasmid DNA can be isolated in a polymer free water phase that is suitable for subsequent purification steps. The aqueous two-phase system was able to concentrate the target plasmid four times. The present thermoseparating polymer/polymer aqueous two-phase system has also proven to be suitable for scale-up in the purification of a recombinant protein [17].

Before partitioning the plasmid DNA solution in the $EO_{50}PO_{50}$/Dextran T-500 aqueous two-phase system, the excess of salts from the alkaline lysis has to be removed by a desalting step. Previously, this was accomplished by size exclusion chromatography [13]. In this work we have used hollow fibre membranes to accomplish both a concentration (ultrafiltration) and a buffer exchange (diafiltration) of the plasmid containing lysate, prior to the partitioning in the aqueous two-phase system. It was envisaged that by using a concentration step prior to the partitioning, the aqueous two-phase system should be able to accommodate a higher throughput in terms of processed plasmid DNA quantity.

Previously, the optimized $EO_{50}PO_{50}$/Dextran T-500 aqueous two-phase system removed 80% of total RNA and 60% of total protein in the desalted lysate [13]. However, remaining contaminants were still left in the plasmid DNA solution, a problem we have addressed in this work. Therefore, lid bead chromatography was chosen as a polishing step after the two-phase partitioning. Lid beads is a new type of restricted access chromatography beads, in this study with an anion-exchange ligand, that can effectively discriminate between the plasmid DNA and its contaminants, mainly smaller molecules like RNA and proteins [6]. The beads have an inert surface layer, with a pore size too small to allow the plasmid DNA to enter the positively charged inner core that will adsorb RNA and proteins. This results in a negative chromatography step where the target molecule passes the column while the contaminants are specifically retained by the chromatography material.

In this work, we propose a three-step process that is suitable for the large scale purification of plasmid DNA from a clarified alkaline lysate. The process starts with a volume reduction (ultrafiltration) and a buffer exchange (diafiltration) of the alkaline lysate with the hollow fibre membrane system. Contaminants (RNA and proteins) in the desalted and concentrated lysate are then significantly reduced in the following aqueous two-phase system. The thermoseparated water phase is then purified by lid bead chromatography to further remove the contaminants, resulting in a purified plasmid DNA fraction. Differently-sized plasmid DNA in the range of 2.7–20.5 kbp were also partitioned in the aqueous two-phase system.

2. Experimental

2.1. Instruments, columns and chromatography media

All chromatography experiments were performed using ÄKTAexplorer 10 controlled by UNICORN 4.11 software (GE Healthcare, Uppsala, Sweden). Agarose gel electrophoresis was performed on a Hoefer HE 33 Mini horizontal submarine unit powered by an EPS 301 supply (GE Healthcare). MiniQ column (PE 4.6/50), MonoS column (HR 5/5), XK 16/20, HR 5/5 and HR 5/10 columns were obtained from GE Healthcare. Sepharose HP was kindly provided by GE Healthcare. A Quixstand benchtop system and all hollow fibre membrane cartridges for ultra/diafiltration were obtained from GE Healthcare.

2.2. Chemicals

The polymer Breox PAG 50 A 1000 ($EO_{50}PO_{50}$, M_r = 3900) was obtained from International Specialty Chemicals (Southampton, UK). Dextran T-500 (weight-average molecular mass, M_w = 500,000) was purchased from GE Healthcare. Na_2HPO_4 (pro analysis) was obtained from Merck Eurolab (Stockholm, Sweden). The bicinchoninic acid (BCA) protein assay kit was acquired from Pierce (Rockford, IL, USA). Ampicillin and RNase were purchased from Sigma (St. Louis, MO, USA). Peptone and yeast extract were from BD Microbiology Systems (Sparks, MD, USA). The 1 kbp DNA ladder was obtained from MBI Fermentas (Vilnius, Lithuania) and the supercoiled DNA ladder (2–16 kbp) from Invitrogen (Groningen, The Netherlands).

2.3. Cultivation

E. coli TG1 cells harboring the plasmid pUC 19 (2.686 kbp) having an insert of the *dmg*A and *dem*A genes from a *Streptococcus dysgalactiae* strain (AJ243529,

3.433 kbp) [18] was provided by GE Healthcare. The plasmid is referred to as pJV4 later on in the text. *E. coli* TG1 cells harboring the plasmid pUC 18 (2.686 kbp) were obtained from MBI Fermentas and *E. coli* MM 294 cells harboring the plasmid pLUP 212 (20.5 kbp), were a kind gift from Dr. Mats Hansson (Department of Biochemistry, Lund University) [19].

The *E. coli* cells were precultivated overnight (37 °C, 175 rpm) in 10 ml of 2× YT medium (16 g/l peptone, 5 g/l NaCl, 10 g/l yeast extract and 100 mg/l ampicillin). One-ml volumes of the preculture were inoculated in 2 l baffled shake flasks each containing 500 ml 2× YT medium and 100 mg/l ampicillin. The bacterial cells were grown overnight (37 °C, 175 rpm).

2.4. Alkaline lysis

A modified alkaline lysis method was used [2]. The overnight cell cultures were harvested by centrifugation at 9000 rpm in a Sorvall SLA 3000 rotor for 10 min (4 °C) and the supernatants were carefully removed. The cultivations gave approximately 9 g cell pellet/l medium. Eighteen grams of the bacterial pellet was resuspended by gentle vortexing in 130 ml suspension buffer (61 mM glucose, 50 mM EDTA, 10 mM Tris–HCl, pH 8). After the cells were completely resuspended, 281 ml of lysis buffer (0.2 M NaOH, 1% (w/v) SDS) was added while stirring gently with a magnetic stirrer. To assure a complete mixing, the gentle stirring was continued for 10 min at room temperature. A volume of 211 ml cold (4 °C) neutralization buffer (3 M potassium acetate, pH 5.5) was added to the lysate. The solution was kept on an ice-bath under magnetic stirring for 20 min. A white precipitate was formed containing SDS, genomic DNA, proteins and cell debris. The precipitate was then removed by centrifugation in a Sorvall SLA 1500 rotor at 4 °C (30 min at 10,000 rpm). The supernatant was then carefully transferred to a fresh tube and stored at 4 °C for short time until further processing. Two alkaline lysis procedures were done in parallel each time (two times 18 g) thus giving a total volume of 1.1 l cleared lysate.

2.5. Membrane filtration

A Quixstand benchtop system with polysulphone hollow fibre cartridges with an M_r cut-off of 100,000, 300,000 and 500,000, all with an inner lumen diameter of 0.5 mm and a total membrane area of 650 cm^2 were used for ultra/diafiltration of the clarified alkaline lysate. The number of fibres in each cartridge was 140. Washing and equilibrating of the hollow fibre membrane cartridge was performed as recommended by the supplier. Clean water flux was determined to 750 ml/min, which corresponds to a shear rate of approximately 5000 s^{-1}. The permeate flow rate when recirculating water was 200 ml/min. After each ultra/diafiltration cycle, the membrane was cleaned in place by circulation of 500 ml distilled water (50 °C), followed by 500 ml 0.5 M NaOH (50 °C). The membrane was incubated overnight in 0.5 M NaOH. The NaOH solution was then removed by washing the membrane with distilled water (50 °C). Finally, 500 ml NaOCl solution (100 ppm) was circulated for 1 h through the membrane and then extensively washed with distilled water (50 °C) and room temperatured water.

2.6. Aqueous two-phase systems

Two-phase systems containing 4.5% (w/w) $EO_{50}PO_{50}$, 4.5% (w/w) Dextran T-500 and 50 mM Na_2HPO_4 or 2.5% (w/w) $EO_{50}PO_{50}$, 9% (w/w) Dextran T-500 and 50 mM Na_2HPO_4 were made up by weighing appropriate amounts from a 25% stock solution of Dextran and a 100% $EO_{50}PO_{50}$ stock solution in 10 ml graded test tubes. Fifty millimolar Na_2HPO_4 was used as the buffer salt, and added to the system from a 1 M stock solution. Finally, the concentrated and desalted alkaline lysate was added to obtain a final weight of 10 g. The system was mixed carefully until all polymers were dissolved and phase separation was accomplished by centrifugation (1600 × *g*, 10 min) at room temperature (primary system). The volumes of the top and bottom phase were determined and the phases were isolated in separate containers. The top phase was placed in a water bath at 55 °C for 3 min, followed by 2 min centrifugation (1600 × *g*) to obtain a water phase (thermoseparated water phase) and a concentrated polymer phase (thermoseparated $EO_{50}PO_{50}$ phase).

2.7. Synthesis of anion-exchange beads having a non-charged surface (lid beads)

The synthesis of the restricted access beads is based on a method described by Bergström et al. [20] and Berg et al. [21].

The synthesis of Q-Sephacryl S-500 HR beads having a non-charged surface was performed as described by Gustavsson et al. [6]. The synthesis briefly involved the following steps:

(1) Allylation of Sephacryl S-500 HR beads.
(2) Bromination and hydrolysis of an outer layer of the allylated beads.
(3) Bromination and trimethylamine reactions of the inner allyl groups of the beads.

The ion capacity of the produced lid beads was determined to be 59 μmol Cl^-/ml gel by titration as previously described [6].

The synthesis of EDA–Sephacryl S-500 HR beads having a non-charged surface was performed in the same way, except that the ion-exchange ligand trimethyl ammoniumchloride (Q) here was replaced by the ligand ethylene diamine (EDA). The ion capacity of the produced lid beads was determined to be 73 μmol Cl^-/ml gel.

2.8. Polishing of thermoseparated water phase by lid bead chromatography

Anion-exchange beads having a non-charged surface (lid beads) were packed into a HR 5/5 column to a final bed volume of 1 ml. The column was integrated with an ÄKTAexplorer 10 system and equilibrated with 1 mM EDTA, 25 mM Tris–HCl, pH 8. Thermoseparated water phase from the aqueous two-phase system was applied to the column at a flow rate of 0.25 ml/min. After washing all unbound material of the column, the adsorbed contaminants were eluted with 2 M NaCl, 1 mM EDTA, 25 mM Tris–HCl, pH 8 at a flow rate of 0.25 ml/min. All of the eluate was collected in 0.5 ml fractions. The plasmid containing fractions (visualized by agarose gel electrophoresis) were pooled and subjected to further analysis by analytical anion-exchange chromatography. After the purification process the column was cleaned in place with 0.5 M NaOH containing 20% ethanol followed by distilled water and elution buffer at a flow rate of 0.25 ml/min.

2.9. Agarose gel electrophoresis

Gel electrophoresis was performed on 0.7% (w/v) agarose gels (Duchefa, Haarlem, The Netherlands) in TBE buffer (0.089 M Tris–borate, pH 8, 2 mM EDTA) containing 15 μg/ml ethidium bromide (Quantum Biotechnologies, Carlsbad, USA) and was run at 60 V for 60 min. The agarose gel was analyzed and photographed using the gel documentation software AlphaImager 2200 v5.5 from Alpha Innotech (San Leandro, CA, USA).

2.10. Protein analysis

Protein concentration was measured by the BCA protein assay kit according to the manufacturer's instructions. Each sample was compensated for the presence of interfering substances by using samples from reference systems devoid of proteins. These reference systems were prepared by replacing the desalted alkaline lysate with 5 mM sodium phosphate buffer, pH 7 in the aqueous two-phase system process. In the case of protein measurements on eluates from the lid bead chromatography step, adsorption buffer (1 mM EDTA, 25 mM Tris–HCl, pH 8) and elution buffer (2 M NaCl, 1 mM EDTA, 25 mM Tris–HCl, pH 8) were used as blanks.

2.11. Chromatography analysis

The recovery and purity of plasmid DNA after each purification step was determined by ion-exchange chromatography analysis on a MiniQ column (PE 4.6/50) [13] or by size exclusion chromatography on a 5 ml Sepharose HP column (group separation [13]). The MiniQ analysis gave information about RNA content and different plasmid isoforms. Group separation analysis was used as a complementary method to quantify the total plasmid DNA content during the process. Moreover, the group separation method allowed faster analysis compared to the MiniQ analysis due to its non-interactive mode.

For the ion-exchange chromatography analysis, the MiniQ column was integrated to an ÄKTAexplorer 10 system and equilibrated at 0.4 ml/min with 0.5 M NaCl, 25 mM Tris–HCl, pH 8. Samples (100 μl), incubated with RNase (≈100 μg/ml) for 15 min prior to the chromatography run, were injected and elution carried out by applying a gradient from 0.5 M NaCl to 0.8 M NaCl in 18 column volumes. The chromatography runs were monitored at 260 nm. After the run the area under the plasmid peak was calculated. The plasmid content was then calculated by using the relationship 50 μg plasmid/ml = 1 AU [22], taking into account the volume of the phase and dilutions.

The MiniQ analysis was also used to quantitate the RNA in the samples by performing a second run of the sample without addition of RNase. In this case the relationship 40 μg RNA/ml = 1 AU was used [22]. The column was periodically cleaned in place with subsequent 2 ml injections of 1 M HCl, 2 M NaCl, 2 M NaOH and 2 M NaCl at a flow rate of 0.1 ml/min.

As previously described, size exclusion chromatography is able to separate DNA from RNA by compaction of RNA in the presence of 2 M ammonium sulphate [5,13]. In this study, a 5 ml Sepharose HP column was integrated to the ÄKTAexplorer 10 system and equilibrated at 2 ml/min with 2 M ammonium sulphate, 10 mM EDTA, 100 mM Tris–HCl, pH 7. A 1.5-ml sample was injected on the column and the eluted nucleic acids were monitored by UV absorbance at 260 nm. The column was periodically cleaned in place with distilled water, 0.5 M NaOH containing 20% ethanol, distilled water and re-equilibrated with 2 M ammonium sulphate, 10 mM EDTA, 100 mM Tris–HCl, pH 7 at a flow rate of 0.5 ml/min.

3. Results and discussion

3.1. Integrated process

In earlier work, it has been shown that plasmids can be completely recovered in a thermoseparating $EO_{50}PO_{50}$/Dextran T-500 aqueous two-phase systems [13]. In this work we propose an integrated process for purification of plasmid DNA from *E. coli* cultivations based on this system (Fig. 1). The process starts with a desalting and concentrating step of the alkaline lysate using cross-flow membrane filtration. The desalted and concentrated sample is then purified in a thermoseparating aqueous two-phase system where the major contaminants (RNA and proteins) to a large extent are removed. The thermoseparated water phase from the aqueous two-phase system is then finally subjected to a polishing step using lid bead chromatography. The results from the development of each step in this process are presented below.

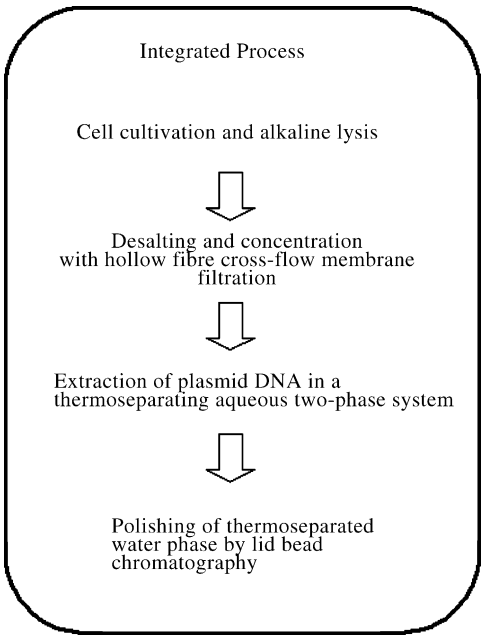

Fig. 1. Flow scheme for the integrated process of plasmid purification from *E. coli* cells.

3.2. Cross-flow membrane filtration

Before extraction of the plasmids from an alkaline lysate in the $EO_{50}PO_{50}$/Dextran T-500 aqueous two-phase system, a desalting step is required. The reason being that the high salt concentration in the alkaline lysate (≈1 M KAc) disturbs the extraction of the nucleic acid species to the top-phase in the aqueous two-phase system. Previously, the desalting step was accomplished by desalting chromatography [13]. From a large scale point of view, desalting is a tedious and time-consuming step. A viable alternative is to use cross-flow membrane filtration [9–11,23]. Cross-flow membrane filtration is a fast and convenient method for desalting a sample, while at the same time allowing concentration of the sample.

In this study, a polysulphone hollow fibre cross-flow membrane was used. The hollow fibre has a unique structure, lacking the macrovoids (large pores) normally found in the membrane substructure of other commercially available hollow fibres. The lack of macrovoids in its substructure makes the membrane capable of withstanding pressures over 4 bar and increases the long term stability and reliability of the membrane [24]. At the same time, these membranes have very sharp M_r cut-off-curves, allowing a very precise separation between different molecules. Cross-flow membranes with M_r cut-off between 100,000 and 500,000 were tested for their capability to concentrate the alkaline lysate. For evaluation purposes, two alkaline lysis batches, one containing a 2.6 kbp plasmid and the other one containing a 11.5 kbp plasmid, were concentrated using cross-flow membranes with M_r cut-off -values of 100,000, 300,000 and 500,000. Recovery and plasmid topology were studied after every experiment, but no differences could be observed between the different plasmids and cross-flow membranes (results not shown). Recovery was constant around 95% with the final 5% eluting in a single wash step. Plasmid topology was measured both by analytical chromatography and laser induced capillary gel electrophoresis, performed by PlasmidFactory (Bielefeld, Germany). No effect on the amount and topology of the plasmid DNA could be noticed. Interesting to note is that the amount of supercoiled plasmid DNA remained unchanged during the complete process. This is confirmed by another experiment where purified plasmid DNA was concentrated to 4.5 mg/ml without affecting the plasmid DNA quality.

In the proposed purification process of the 6.1 kbp plasmid, a membrane with a M_r cut-off of 100,000 was selected because of the slightly shorter processing time. Fig. 2 shows the experimental set-up for the membrane filtration process. To demonstrate the plasmid purification process, a volume of 1 l clarified lysate was first concentrated approximately five times (ultrafiltration mode, Fig. 2A). The purpose of choosing the ultrafiltration mode as the first step was to reduce the volume of the low salt buffer needed in the diafiltration step (step 2 below), which concomitantly means a reduction in process

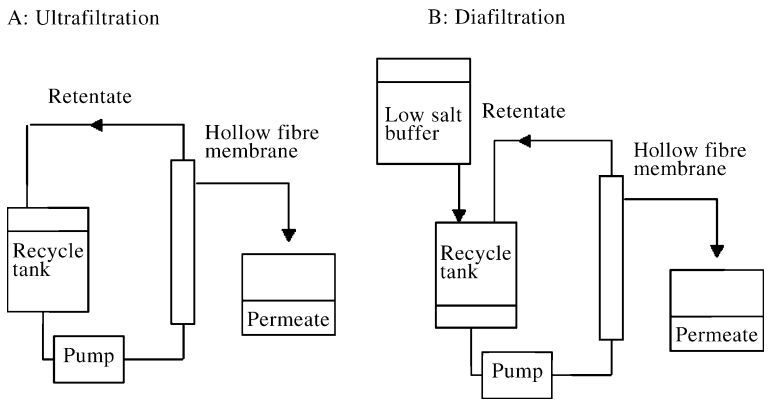

Fig. 2. Experimental set-up for the ultrafiltration (A) and the diafiltration mode (B) of the cross-flow membrane filtration step for desalting and concentration of a clarified alkaline lysate.

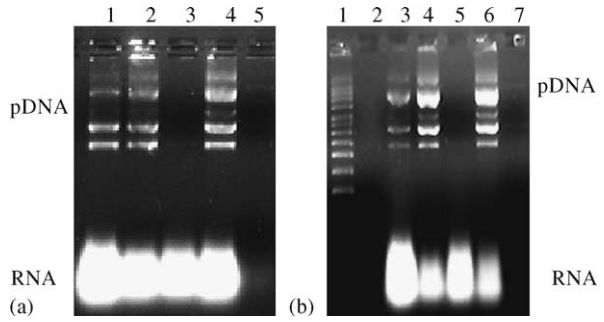

Fig. 3. The extraction of plasmid DNA (6.1 kbp) and RNA in aqueous two-phase systems as visualized by agarose gel electrophoresis. System composition: (a) 4.5% $EO_{50}PO_{50}$/4.5% Dextran T-500, 50 mM Na_2HPO_4 and (b) 2.5% $EO_{50}PO_{50}$/9% Dextran T-500, 50 mM Na_2HPO_4. All samples are diluted 10 times. (a) Lane 1: UF/DF lysate; lane 2: $EO_{50}PO_{50}$ phase; lane 3: Dextran phase; lane 4: thermoseparated water phase; lane 5; thermoseparated $EO_{50}PO_{50}$ phase. (b) Lane 1: Sc DNA ladder; lane 2: empty; lane 3: UF/DF lysate; lane 4: $EO_{50}PO_{50}$ phase; lane 5: Dextran phase; lane 6: thermoseparated water phase; lane 7: thermoseparated $EO_{50}PO_{50}$ phase. UF/DF lysate: ultrafiltrated and diafiltrated lysate. The plasmid band corresponds to linear, open circular, supercoiled and denatured plasmid DNA (from top to bottom).

time. In the second step, a buffer exchange was performed on the concentrated lysate (diafiltration mode, Fig. 2B). Since the aqueous two-phase system has proven to be sensitive to a high salt concentration, it was important to achieve a sample with conductivity similar to the 5 mM sodium phosphate buffer (≈700 μS/cm). In a first attempt, a diafiltration step with 4 lysate volumes of buffer was tested (800 ml). The diafiltrated sample with a conductivity of 5 mS/cm was then partitioned in a 4.5% $EO_{50}PO_{50}$/4.5% Dextran T-500 aqueous two-phase system, and it was found that some plasmid DNA partitioned to the bottom phase (data not shown). However, by using 8 lysate volumes of buffer (1.6 l), the lysate obtained a conductivity low enough (≈1 mS/cm) to not disturb the partitioning of the plasmid DNA to the $EO_{50}PO_{50}$ phase in the aqueous two-phase system (see lane 2 in Fig. 3a). This was followed by a further concentration of the desalted lysate (200 ml) to a volume of 90 ml (ultrafiltration). In conclusion, the volume of the lysate was reduced approximately 11 times compared to the start volume (Table 1[b]). A sample containing 580 μg plasmid DNA per ml in the desired buffer could thus be obtained in one straightforward process step (Table 1). It should however be noted that this concentrated alkaline lysate sample not only contains concentrated amounts of plasmid DNA, but also contains RNA and other contaminants, resulting in a viscous solution because of the high concentration of nucleic acids, of which the target molecule, plasmid DNA, only constitutes a minor part. However, a significant reduction of low molecular mass contaminants was observed. It was calculated that approximately 80% of total proteins were removed by the membrane filtration process. Also, a reduction of low molecular mass (LMM)-RNA was observed in the chromatography analysis. The operational transmembrane pressure (TMP) controls the ratio of permeate flow over retentate flow and was here kept constant during all experiments (12 psi or 0.9 bar) giving a permeate flow of on average 35 ml/min (initial permeate flow was 43 ml/min). The whole membrane step for processing of 1 l lysate was thus completed in approximately 1 h. After the cross-flow filtration process, the membrane was rinsed by re-circulating 100 ml of 5 mM sodium phosphate buffer pH 7 through the membrane (permeate outlet closed) for 10 min. Plasmid analysis on the washing solution (Table 1) showed that some plasmid (≈11%) could be recovered in this solution. As can be seen from Table 1 the mass balance is only 89% in this step. The reason for this is most likely that some plasmid DNA is still attached to the membrane, probably by hydrophobic interaction, and washed out in the cleaning step. Also, the somewhat lower yield (78%) for the 6.1 kbp plasmid compared to the yield for the 2.6 and 11.5 kbp plasmids (95%, see above) is puzzling. This however might be due to that the 6.1 kbp plasmid is produced in more different isoforms compared to the 2.6 and 11.5 kbp plasmids. One or more of these isoforms might then interact with the membrane thus leading to a reduction in plasmid yield. The membrane filtration method used is a well known technique and commercially standard systems capable of handling more than 100 l of lysate are now available [24] making it a good choice to complement the aqueous two-phase system.

Table 1
Overview of cross-flow membrane filtration step

Sample	Volume (ml)	Plasmid concentration (mg/ml)	Plasmid mass (mg)	Plasmid yield (%)
Alkaline lysate	1000	0.067	67	100
UF/DF lysate	90	0.58	52	78
Washing solution	100	0.073	7	11

UF/DF: ultrafiltrated and diafiltrated.

3.3. Thermoseparating aqueous two-phase systems

After desalting and concentration of the plasmid DNA sample by the cross-flow hollow fibre membrane filtration step, partial removal of major contaminants (i.e. RNA and proteins) was accomplished by the aqueous two-phase system. Two-phase systems are known to be able to have a high capacity, handle large volumes and to reduce the process volume of the sample [25], i.e. a further concentration of the plasmid DNA sample was accomplished in this step.

Two systems composed of 4.5% (w/w) $EO_{50}PO_{50}$, 4.5% (w/w) Dextran T-500, 50 mM Na_2HPO_4 (4.5/4.5) and 2.5% (w/w) $EO_{50}PO_{50}$, 9% (w/w) Dextran T-500, 50 mM Na_2HPO_4 (2.5/9), respectively were used for this study [13]. The difference between these systems is that the latter has a higher removal efficiency of contaminants and is also able to concentrate the plasmid DNA sample to a higher degree compared to the first system [13]. The systems were prepared (4.5/4.5 and 2.5/9) and after phase separation (primary systems), the $EO_{50}PO_{50}$ phase was isolated in a separate container. The $EO_{50}PO_{50}$ phase was then heated over its cloud

Table 2
Overview over the extraction of 6.1 kbp plasmid DNA in the aqueous two-phase system step

Sample	Plasmid mass (mg)	Yield (%) plasmid DNA	RNA mass (mg)	Protein mass (mg)	Concentrating effect (V_0/V_{TT})
UF/DF lysate (4.5/4.5)	4.2		47	4.4	
Thermoseparated water phase (4.5/4.5)	4.2	100	19	2.5	1.6
UF/DF lysate (2.5/9.0)	3.2		36	3.4	
Thermoseparated water phase (2.5/9.0)	3.2	100	2	1.0	3.9

V_0: volume of UF/DF lysate; V_{TT}: volume of thermoseparated water phase; UF/DF: ultrafiltrated and diafiltrated.

point (55 °C), causing the polymer phase to separate into two new phases (thermoseparated system), one water phase and one dense polymer phase. The phases were analyzed by electrophoresis on ethidium bromide stained agarose gels (Fig. 3a and b). From the agarose gel electrophoresis it can be seen that a full recovery of plasmid DNA to the thermoseparated water phase can be achieved in both systems. This was confirmed by chromatography analysis (Table 2), also showing complete recovery of the plasmid DNA to the thermoseparated water phase in both systems (4.5/4.5 and 2.5/9).

By moving a long a tie-line, a decrease of the top phase volume can be achieved. Thus, a further concentration of the plasmid DNA and a reduction of sample volume were achieved in the 2.5/9 system compared to the 4.5/4.5 system. It can be visualized from the agarose gel electrophoresis (Fig. 3b) and from the chromatography results (Table 2) that even at very high concentrations of plasmid DNA (2.1 mg/ml) the plasmid DNA still partitions exclusively to the thermoseparated water phase in the 2.5/9 system. From this it can be concluded that the aqueous two-phase system comprising of $EO_{50}PO_{50}$ and Dextran T-500 can be used for extraction of plasmid DNA at high DNA concentrations. By applying an ultrafiltration step prior to the partitioning in the aqueous two-phase system, the lysate volume was reduced 11 times which gave an increase of the productivity of the aqueous two-phase system by 11 times (see above). This shows that the productivity of plasmid purification by the aqueous two-phase system is to a large extent dependent on volume capacity rather than plasmid quantity in the lysate. The concentrating effect in the 2.5/9 system was 3.9 (Table 2). Thus, the total volume reduction of the plasmid containing lysate by the combination of membrane filtration and aqueous two-phase system was 43 times.

After extraction of the plasmid DNA in the aqueous two-phase systems, the concentration of contaminants was analyzed. As can be seen from Table 2, the 4.5/4.5 system removed 43% of the proteins and 60% of RNA from the plasmid containing lysate. However, by moving along the tie-line in the aqueous two-phase diagram, the phase ratio in the primary system can be changed and a more efficient removal rate can be obtained. Thus, the 2.5/9 system was able to remove 71% of the proteins and 94% of RNA from the plasmid DNA sample (Table 2). The high value for RNA removal (94%) can seem inconsistent with earlier presented results (80%) [13]. This can be explained by the use of the cross-flow hollow fibre membrane (M_r cut-off 100,000) in this study. This leads to a decrease in amount of LMM-RNA and therefore a relative higher presence of high molecular mass (HMM)-RNA. The aqueous two-phase system has proven to be efficient in removing HMM-RNA to the bottom phase [13]. In the current work, this leads to a high efficiency removal of RNA as illustrated by comparing Fig. 6a and b.

3.4. Extraction of different sizes of plasmid DNA in aqueous two-phase systems

The plasmid vectors that today are under gene therapy clinical trials can vary between 2 and 200 kbp in size depending on what target it is developed for [26]. This requires a robust purification process that can handle plasmid DNA of varying sizes. Two additional plasmids (2.7 and 20.5 kbp) were therefore partitioned in an aqueous two-phase system comprising of 2.5% (w/w) $EO_{50}PO_{50}$, 9.0% (w/w) Dextran T-500 and 50 mM Na_2HPO_4 to investigate the size impact on partitioning. The plasmid containing lysates were desalted on PD10 columns (GE Healthcare) prior to the partitioning in the two-phase systems. As can be seen from Fig. 4a and b, both plasmids are completely recovered in the thermoseparated water phase. By protein measurements (BCA analysis) it was determined that the total protein content could be reduced by approximately 80% for both the 2.7 and 20.5 kbp plasmid DNA. This flexible behaviour of the

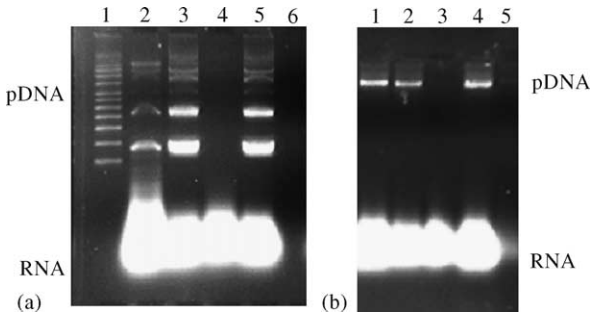

Fig. 4. The extraction of plasmid DNA and RNA in a system composed of 2.5% $EO_{50}PO_{50}$/9% Dextran T-500 and 50 mM Na_2HPO_4. Extraction of: (a) the 2.7 kbp plasmid DNA, and (b) the 20.5 kbp plasmid DNA. (a) Lane 1: Sc DNA ladder; lane 2: UF/DF lysate; lane 3: $EO_{50}PO_{50}$ phase; lane 4: Dextran phase; lane 5: thermoseparated water phase; lane 6: thermoseparated $EO_{50}PO_{50}$ phase. The plasmid band corresponds to multimeric forms, linear, open circular and supercoiled plasmid DNA (from top to bottom). (b) Lane 1: UF/DF lysate; lane 2: $EO_{50}PO_{50}$ phase; lane 3: Dextran phase; lane 4: thermoseparated water phase; lane 5: thermoseparated $EO_{50}PO_{50}$ phase.

two-phase system makes it a suitable system for purification and concentration of plasmid DNA in the range at least from 2.7 to 20.5 kbp.

3.5. Polishing of plasmid DNA sample by lid bead chromatography

After the aqueous two-phase system process a polishing step is necessary to remove residual contaminants. The 6.1 kbp plasmid DNA in the thermoseparated water phase is highly concentrated (≈2 mg plasmid/ml) and has a significantly reduced contaminant level. It was therefore considered suitable to use a negative chromatography step, i.e. adsorption of contaminants while plasmid DNA pass in the flow-through of the column. Previously, negative chromatography has been used for plasmid purification in hydrophobic interaction chromatography mode [4] and in anion-exchange chromatography mode [6]. Due to the low salt concentration of the thermoseparated water phase (≈5 mM) it was considered more suitable to use the anion-exchange chromatography mode. Recently, a new matrix suitable for RNA removal has been developed [6]. The chromatography matrix is produced to combine both anion-exchange and size exclusion principles. These beads have a positively charged core that enables RNA and negatively charged proteins to adsorb. The inert surface layer around the positively charged bead will however restrict the access of the plasmid DNA. The plasmid DNA will therefore elute unretarded in the flow-through while the contaminants though very similar to the target molecule in charge properties, will be adsorbed to the beads. Previously, a second column packed with regular anion-exchange beads was introduced downstream to the first column in order to capture the plasmid DNA to obtain a concentrating effect [6]. In this work, we have chosen to only use the first, restricted access column, since the concentrating effect has been accomplished by the previous two steps (membrane filtration and aqueous two-phase system). If only the first column is used, the lid beads in this column need to have a high contaminant capture efficiency to avoid contamination of the final product, the plasmid DNA. In the present study, the high capture efficiency of the lid beads was ascertained by two actions. First, a low salt concentration of the plasmid DNA solution avoids shielding of ionic interactions between the contaminants and the positively charged ligands on the lid beads. Secondly, the capture efficiency for contaminants by the anion-exchange ligand was increased by replacing the formerly used Q-ligand [6] with a different ligand, ethylene diamine, which has two positive charges per ligand molecule compared to the Q-ligand that has only one. This increases the local charge density on the matrix allowing a stronger binding of the contaminants, even in the presence of salts, enabling the capture of smaller RNA molecules.

The thermoseparated water phase containing the plasmid DNA was applied to the restricted access column packed with lid beads (EDA–Sephacryl S-500 HR) having an ion capacity of 73 μmol Cl^-/ml gel (Fig. 5). The eluate was collected in fractions (0.5 ml) and analyzed with agarose gel electrophoresis (Fig. 5, insert). Fig. 5 and Table 3 show that most of the plasmid DNA is recovered in the flow-through (fraction 4-14) while the RNA-peak (fraction 31-46) hardly contains any plasmid DNA. The plasmid containing volume (5.5 ml) thus gives a small dilution effect compared to the start volume of 3 ml. The plasmid DNA fraction was then analyzed on a MiniQ column for RNA content. The chromatogram

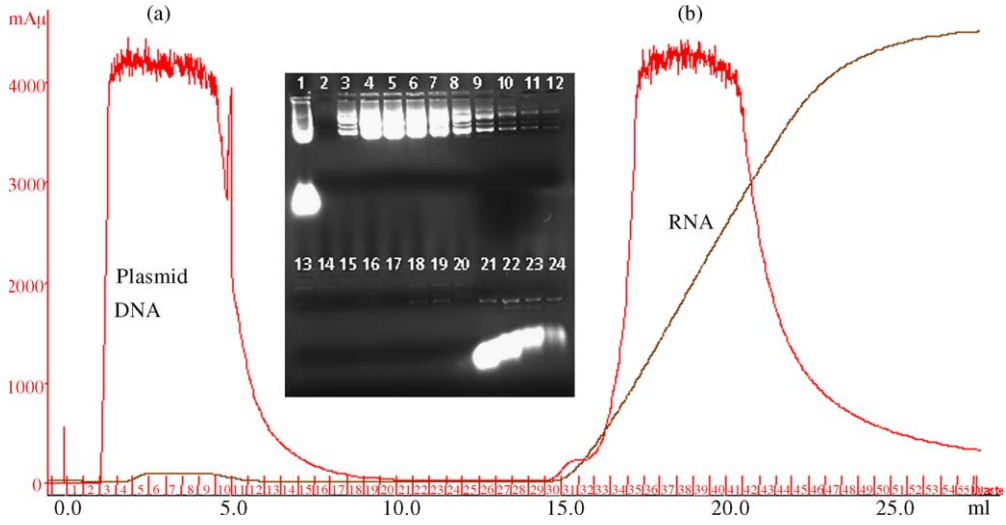

Fig. 5. Chromatogram showing the polishing step of the thermoseparated water phase using lid bead chromatography. The polishing step is divided into two parts indicated in the chromatogram: (a) loading of 3 ml thermoseparated water phase from the 2.5% $EO_{50}PO_{50}$/9% Dextran T-500, 50 mM Na_2HPO_4 system. The flow-through contains purified plasmid DNA. (b) Elution of adsorbed contaminants (RNA and proteins) from the lid bead column. The eluate was collected in 0.5-ml fractions. Conductivity is indicated with the hatched line. The chromatography run was monitored by UV absorbance at 260 nm. The agarose gel electrophoresis shows the eluted fractions obtained from the lid bead chromatography. The numbers indicated in the figure corresponds to different fractions in the chromatogram as follows. Lane 1: thermoseparated water phase; lane 2–9: fraction 3-14; lane 10–17: fraction 15-30; lane 18–24: fraction 31-46.

Table 3
Overview of the lid bead chromatography step shown in Fig. 6

Sample	Plasmid mass (MiniQ, mg)	Plasmid yield (%)	Protein mass (BCA, mg)	RNA mass (MiniQ, mg)
Thermoseparated water phase (2.5/9)	3.2		1	2
Flow-through (fraction 4-14)	2.8	88	0.8	0
Eluate (fraction 15-36)	0.08	3	0.2	n.d.
Eluate (fraction 37-46)	0.04	1	0.2	n.d.

n.d.: not determined.

(Fig. 6c) shows that the plasmid DNA peak is completely free of RNA compared to the starting material (thermoseparated water phase, Fig. 6b). This can also be compared with the membrane filtrated lysate depicted in Fig. 6a. Thus, the lid bead chromatography can be used for removal of residual RNA in a thermoseparated water phase containing plasmid DNA.

The higher capture efficiency of the EDA-ligand for LMM-RNA compared to the previously used Q-ligand was also confirmed by a separate experiment. In this experiment, thermoseparated water phase was applied to a Q-Sephacryl S-500 lid bead column (1 ml packed bed) and run under identical conditions as above. MiniQ analysis on the plasmid DNA containing fractions showed that the lid bead column removed 86% of the RNA in the thermoseparated water phase. Thus, the EDA–Sephacryl S-500 lid beads are more efficient for the removal of residual RNA in a thermoseparated water phase than the Q-Sephacryl S-500 lid beads.

However, the plasmid fraction from the EDA–Sephacryl S-500 column still contained some proteins (BCA-analysis, Table 3). The possibility that these contaminating proteins had a positive net-charge was investigated by re-chromatographing the plasmid DNA fraction using a column containing cation-exchange beads (MonoS). However, BCA-analysis on the plasmid DNA fraction showed that the proteins could still not be removed. Also, the possibility that the capacity of the lid bead column was too low for an efficient removal of proteins was also investigated. For this, the

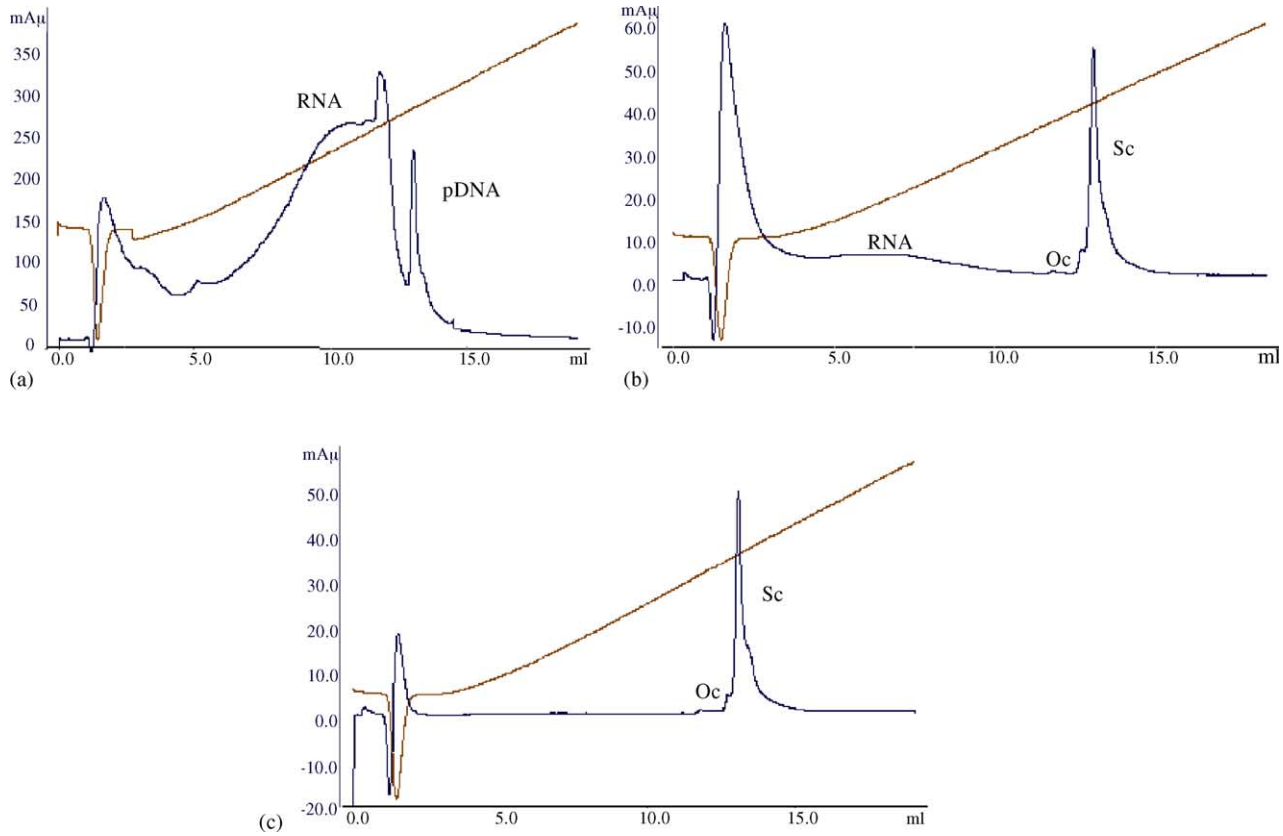

Fig. 6. Analytical anion-exchange chromatography of (a) 100 μl lysate after the membrane filtration step (ultrafiltration + diafiltration). (b) Thermoseparated water phase (100 μl) from a system composed of 2.5% $EO_{50}PO_{50}$/9% Dextran T-500, 50 mM Na_2HPO_4. (c) Plasmid fraction (100 μl) after the lid bead chromatography step. Conductivity is indicated with the hatched line. The chromatography run was monitored by UV absorbance at 260 nm. Sc: supercoiled plasmid DNA; Oc: open circular plasmid DNA; pDNA: plasmid DNA.

plasmid fraction from the EDA–Sephacryl S-500 lid bead column was re-chromatographed on a larger lid bead column (EDA–Sephacryl S-500, HR 5/10). However, analysis for proteins in the new plasmid DNA fraction showed that only 90% of the remaining proteins could be removed by this procedure. Thus, using a larger lid bead column to increase the capacity can only improve the removal efficiency of proteins and not solve the problem entirely. The possibility to use a size exclusion chromatography step after the lid bead column was also tested. For this experiment, the plasmid fraction from the first lid bead chromatography step was re-chromatographed on an XK 16/20 column packed with Sephacryl S-500 HR beads. After the group separation chromatography run the plasmid DNA fraction was analyzed for protein content using the BCA-method. No proteins could be detected with this method in the plasmid DNA fraction. It can thus be concluded that the residual proteins in the plasmid DNA fraction can be removed by group separation chromatography on a Sephacryl S-500 column. Furthermore, with this final polishing step, the plasmid DNA can be attained in a suitable buffer for formulation purposes.

4. Conclusions

This work demonstrates an integrated process for purifying plasmid DNA using a thermoseparating aqueous two-phase system to remove the main bulk of contaminants from a clarified alkaline lysate. By using a cross-flow membrane filtration step prior to the extraction, the productivity of the aqueous two-phase system process could be increased by more than 10 times. The volume of the membrane filtrated lysate containing the plasmid DNA was further reduced (four times) in the aqueous two-phase system, at the same time establishing efficient removal of contaminants such as protein and RNA. Finally, the thermoseparated water phase was subjected to a restricted access chromatography column yielding a RNA-free sample of plasmid DNA. The overall plasmid yield of the complete process was 69%. The results from this work show that three scalable individual steps can be integrated to obtain a concentrated plasmid DNA sample completely free from RNA, without the need of plasmid DNA interaction with a solid phase like a chromatography column.

Acknowledgements

Mr. Sherwin Mizani at GE Healthcare, Uppsala is gratefully acknowledged for the loan of the Quixstand benchtop system. Mrs. Truke Smoor, Delft Technical University, Delft, Holland is gratefully acknowledged for excellent technical assistance. Professor Folke Tjerneld at the Department of Biochemistry, Lund University is gratefully acknowledged for scientific discussions. The financial support from the Swedish Center for Bioseparation is gratefully acknowledged.

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