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Purification of plasmid DNA with a new type of anion-exchange beads having a non-charged surface☆

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Abstract

We have prepared a new type of anion exchanger, which effectively discriminates between RNA and plasmid DNA. The material is based on a Sephacryl S-500 HR matrix provided with quartenary amine anion-exchange groups. A distinguishing feature of the beads is that a thin (2–3 μm) outer layer of the beads lacks ion-exchange groups. In the synthesis of these beads the vinyl groups in the outer layer of vinylalkyl substituted Sephacryl S-500 HR beads are reacted with bromine. The resulting layer of bromoalkyl groups are hydrolysed, creating an inert outer layer of hydroxyalkyl groups. Finally, bromination and trimethylamine reactions of the inner vinyl groups provide the beads with a core of cationic groups. Large plasmid molecules will not bind to such beads since they are too large to enter the pores and therefore cannot come into contact with the charged matrix in the inner parts of the beads. RNA and protein molecules present in a cleared lysate, on the other hand, readily enter the pores and become adsorbed. A two-column strategy was developed for plasmid purification (recombinant pBluescript, 5.9 kilo base pairs, kbp). The first column was packed with the restricted access anion-exchanger beads (lid beads) and the second column with normal ion-exchange material (same ligand density as the lid beads). Diluted (3×), cleared lysate was pumped through the tandem columns. The first column was subsequently disconnected from the system and the purified plasmid adsorbed on the second column was eluted in a concentrated form (6×) and with 89% recovery. The two-column procedure removed 99.5% of the RNA and 96% of the proteins.

Keywords: Restricted access material; Anion exchangers; Stationary phases, LC; Coupled columns; DNA; RNA

1. Introduction

The on-going gene therapy trials world-wide, raise the demand for large-scale purification of gene vectors, such as e.g. plasmid DNA [1]. In this process, high-resolution steps such as column chromatography are considered essential to achieve the high purity requirements for gene therapy products [2]. Among the different chromatography modes available for plasmid purification, anion-exchange chromatography [1,3,4] and size-exclusion chromatography [1,5,6] are probably the most utilised methods, although hydrophobic chromatography [7,8], hydroxyapatite chromatography [9], affinity chromatography [10,11] and thiophilic aromatic chromatography [12] have also been used.

When dealing with protein purification, anion-exchange chromatography is a high resolution and high capacity method. When plasmid purification is involved two major problems arise. One problem is the large size of plasmids, which results in a low capacity when standard anion-exchange chromatography matrices are used [1,2]. This problem could be tackled by chromatography support development [1,2] and has been addressed by increasing the available surface e.g. by adding surface-extenders or using large-pore supports made by polystyrene [2] and agarose i.e. superporous agarose beads [13]. The other problem is that RNA and plasmid DNA have very similar charge properties

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and consequently behave similarly on ion-exchangers. This problem can be solved by addition of RNase, as made in lab-scale plasmid purification kits [14]. However, the addition of extraneous enzymes from e.g. bovine sources imposes serious validation problems in large-scale purifications of gene vectors [1,2]. Therefore, other methods have been suggested including the use of endogenous RNase [15] and degradation of RNA by heat treatment [16].

Size-exclusion chromatography in plasmid purification is based on the difference in size of the plasmid DNA and RNA. Here, the separation between plasmid DNA and RNA can be improved by compaction of the RNA in the presence of high concentration of salts e.g. ammonium sulphate [12] that will suppress charge repulsion and enhancing hydrophobic interactions. A drawback of this method is the limited amount of material that can be processed e.g. 0.3–0.5 ml lysate/ml bed volume [12]. It is furthermore not possible to obtain a concentrating effect of the plasmid by this method as plasmid DNA appears in the flow-through.

However, by combining anion-exchange and size-exclusion chromatography, an improved plasmid purification process could be envisaged, one which has both a high capacity and a good discriminating ability between the high-molecular-mass (HMM) portion of RNA and the plasmid DNA. In this context, restricted access packing would be beneficial. Restricted access materials have been employed in chromatography to solve various problems, mostly involving hydrophobic matrices with an inert hydrophilic outer surface [17]. Recently, a synthesis method was developed to introduce different functional layers in a chromatography bead [18,19]. We have used this method to produce a new type of chromatography matrix that combines size exclusion and anion-exchange principles. This restricted access material has a positively charged core that will adsorb large amounts of RNA while its inert surface layer will exclude the target plasmids from coming into contact with the inner parts of the bead. A two-column procedure was subsequently developed to efficiently purify plasmids. The first column packed with the new restricted access material removed RNA and proteins while the second column packed with an ordinary type anion-exchanger adsorbed the plasmids. Separate elution of the second column produced purified plasmid DNA.

2. Experimental

2.1. Instruments, columns, and chromatography media

All column chromatography experiments were performed using ÄKTAexplorer 10 controlled by UNICORN 4.11 software (Amersham Biosciences, Uppsala, Sweden). Electrophoresis was performed on a Hoefer HE 33 Mini horizontal submarine unit powered by an EPS 301 supply (Amersham Biosciences). Titration was performed on a DL 50 Graphix (Mettler-Toledo, Schwerzenbach, Switzerland). MiniQ (PE 4.6/50), XK 16/20 columns, 150 ml superloop and Sephacryl S-500 HR media were obtained from Amersham Biosciences.

2.2. Chemicals

Agarose L (low electroendosmosis) was from Amersham Biosciences. Glycidyltrimetylammonium chloride and sodium borohydride ($NaBH_4$) were purchased from Fluka (Buchs, Switzerland). Bromine and trimetylammonium chloride were from Aldrich (Milwaukee, WI, USA). Allyl glycidyl ether was obtained from Acros Organics (Geel, Belgium). Congo red was purchased from Merck (Darmstadt, Germany). Bicinchoninic acid (BCA) and μ-BCA protein assay kits were obtained from Pierce (Rockford, IL, USA). Supercoiled DNA ladder ((2-16 kilo base pairs (kbp)) was from Invitrogen (Groningen, The Netherlands). DNA molecular mass marker *Hind*III digest of lambda phage DNA and restriction endonuclease *Bam*HI were from Fermentas (Vilnius, Lithuania).

2.2.1. Fermentation medium

Tryptone soya broth was obtained from Oxoid (Hampshire, UK). Yeast extract was purchased from DSM (Delft, The Netherlands). Dextrose was from AB R. Lundberg (Malmö, Sweden). Ampicillin was obtained from Sigma (St. Louis, MO, USA). The fermentation medium consisted of tryptone 30 g/l, yeast extract 10 g/l, dextrose 10 g/l and ampicillin 100 mg/l. *Plasmid DNA: Escherichia coli* XL1 Blue harbouring the plasmid pBluescript II KS (±) 2.9 kbp having an insert of a xylanase gene from *Rhodothermus marinus* (3 kbp) giving a total plasmid size of 5.9 kbp was chosen for this study [20].

Buffer A used for chromatography and the BCA protein assay was composed to mimic the solvent after the alkaline lysis step. Thus, buffer A was prepared by mixing 180 ml of 10 mM Tris–HCl, pH 8, 61 mM glucose, 50 mM EDTA, 390 ml of 0.2 M NaOH containing 1% (w/v) Sodium dodecyl sulfate (SDS) and 295 ml of cold (5 °C) 3 M KAc, pH 5.5. The resulting solution containing a precipitate was filtered through a 0.2 μm filter and then finally diluted three times with distilled water.

2.3. Cell cultivation

E. coli cells harbouring the plasmid were grown in a 500 ml shake flask containing 100 ml fermentation medium (37 °C, 160 rpm, 12 h) to an absorbance of 2 ($A_{600\,nm}$). Ten ml of this culture was used to inoculate four shake flasks (500 ml) each containing 100 ml fermentation medium and the cells were grown further for 9 h (37 °C, 160 rpm) to an absorbance of average 6.5 ($A_{600\,nm}$). All of this culture (400 ml) was used to inoculate a 15 l fermentor (Electrolux) containing 10 l of fermentation medium. The cells were grown for 8.5 h (37 °C, 600 rpm) to an absorbance of 12.5 ($A_{600\,nm}$). During fermentation the pH of the medium was

kept at 7 by addition of 1 M NaOH and foam was inhibited by occasional addition of Adeka NOL LG (Asahi Denka Kogyo, Japan). The 10 l cell culture was pumped through sterile tubing into a 784 l fermentor (Belach Bioteknik AB, Stockholm, Sweden) containing 400 l of fermentation medium. The cells were grown for 10 h (37 °C) to an absorbance of 13 ($A_{600\,nm}$). During fermentation the pH of the medium was kept at 7 by addition of 5 M NaOH and foam was inhibited by addition of Adeka NOL LG. The 3.5 kg of dextrose in 8 l of water was also pumped into the fermentor during the cultivation (fed-batch). The agitation speed was controlled by the oxygen demand. After cultivation the fermentor was cooled to 16 °C and the cells were harvested by centrifugation at 15,000 rpm in a Sharpless centrifuge operated with a feed rate of 1.2 l/min. The harvest step gave a cell loss of 16% at the used feed rate but was deemed necessary to cut down the processing time. The centrifuge was emptied three times during a period of 7 h. The yield of cell paste was 7.7 kg with a dry mass of 0.25 g/g cell paste. The paste was stored as 5, 25, and 300 g aliquots at −80 °C. The supercoiled plasmid content in the cell paste was determined by MiniQ chromatography analysis on clarified alkaline lysates. The plasmid content was 125 ± 28 μg/g cell paste (x ± S.D., n = 17). The large standard deviation indicate that the amount of plasmid which is extracted from the cell paste is very much dependent on the success of the lysis procedure.

2.4. Cell lysis

Cell lysis was performed by the alkaline lysis method according to Horn et al. [21] as follows: 5 g of cell paste prepared as described in Section 2.3, was thawed and completely resuspended by gentle vortexing in 36 ml of 10 mM Tris–HCl, pH 8, 61 mM glucose, 50 mM EDTA. The cell suspension was transferred to a plastic beaker equipped with magnetic stirring and 78 ml of 0.2 M NaOH containing 1% (w/v) SDS was added and the gentle stirring was continued for 10 min at room temperature. After this incubation period, 59 ml of cold (5 °C) 3 M KAc, pH 5.5 was added and the solution was gently mixed by magnetic stirring for 20 min in an ice bath. The white precipitate containing genomic DNA, proteins and cellular fragments was removed by 30 min centrifugation at 10,000 rpm in a Sorvall GSA rotor (4 °C). The supernatant was filtered through a 40 μm nylon net (Falcon Cell Strainer, BD Biosciences, San Jose, CA, USA), transferred to a fresh container and stored overnight at 4 °C.

2.5. Analytical anion-exchange chromatography

The recovery and purity of supercoiled plasmid preparations were determined by ion-exchange chromatography analysis on a MiniQ column at room temperature. The column was equilibrated with 0.5 M NaCl, 25 mM Tris–HCl, pH 8. Samples (100 μl) were injected and elution carried out by applying a gradient from 0.5 M NaCl to 0.8 M NaCl in 18 column volumes, all at 0.4 ml/min. 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 total volume of sample and dilution. The plasmid content in clarified lysate was determined in the same way with the exception that the lysate sample was diluted three times with distilled water and incubated with RNase (≈100 μg/ml) for 15 min prior to the chromatography run. The supercoiled plasmid form was identified as the peak eluting with a retention volume of 12.7 ml. The small open circular plasmid peak eluted well in advance of the supercoiled plasmid peak.

The MiniQ analysis was also used to quantitate the RNA in the samples. This was performed by 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.

2.6. Analysis by gel electrophoresis

Gel electrophoresis was performed on 0.7% agarose gels (10 × 6.5 cm) in TBE buffer (0.089 M Tris–borate, pH 8.0, 2 mM EDTA). 15 μl of sample (including 2.5 μl of a 50% solution of glycerol in water) was loaded in each well and the samples were run at 60 V for 60 min. When the electrophoresis was completed the agarose gel was stained with ethidium bromide by soaking the gel in 100 ml TBE buffer containing 1.5 μg ethidium bromide/ml for 30 min. The agarose gel was analysed and photographed using the gel documentation software AlphaImager 2200 v5.5 from Alpha Innotech. (San Leandro, CA, USA).

2.7. Protein analysis

Protein concentration was measured by the bicinchoninic acid assay. Samples of 100 μl were added to 2 ml of BCA reagent and incubated for 30 min at 37 °C. Absorbance was measured at 562 nm. Buffer A, prepared as described in Section 2.2.1 was used as blank. Protein concentrations were then calculated by comparing with a calibration curve of bovine serum albumin standards.

Final protein concentrations in the purified plasmid fractions were determined by the μ-BCA assay. One ml samples were added to 1 ml μ-BCA reagent and incubated for 60 min at 60 °C. Absorbance was measured at 562 nm. 0.5 M NaCl, 1 mM EDTA, 25 mM Tris–HCl, pH 8 was used as blank. Also in this case, bovine serum albumin standards were used to construct a calibration curve.

2.8. Synthesis of Q-Sephacryl S-500 HR anion exchanger (homogeneous beads)

To obtain an anion exchanger with quaternary amine (Q) groups, glycidyltrimethylammonium chloride (G-MAC) was

coupled to Sephacryl S-500 HR beads by an established method [23]. Briefly, 39 ml Sephacryl S-500 HR beads were added to a 250 ml round-bottom flask together with 10 ml distilled water and 45 ml G-MAC was added under stirring at room temperature. 2.7 ml 50% (w/w) NaOH solution and 0.19 g $NaBH_4$ were added and the reaction was allowed to proceed overnight. The beads were washed with distilled water, 1 M NaCl and again with distilled water.

The ion capacity was determined by washing the beads (1.0 ml gel) with 0.5 M HCl followed by 1 mM HCl. The chloride ion capacity of the beads was then determined by argentometric titration with 0.1 M $AgNO_3$ using a combined silver ring electrode (detection of Ag^+ after precipitation of AgCl). The ion capacity was 52 μmol Cl^-/ml gel.

2.9. Synthesis of Q-Sephacryl S-500 HR anion exchanger having a non-charged surface (lid beads)

The synthesis is based on a method described by Bergström et al. [18] and Berg et al. [19].

Step 1. Allylation of Sephacryl S-500 HR: 60 ml sedimented bed volume of Sephacryl S-500 HR beads was washed on a glass filter funnel with distilled water and the surplus of water was removed by vacuum suction. The beads were added to a 250 ml round-bottom flask together with 24 ml sodium hydroxide solution (50%, w/w). 0.25 g $NaBH_4$ and 6.7 g sodium sulphate were added to the flask under stirring. The flask was thermostated to 50 °C in a water bath and the stirring continued for 1 h. Subsequently 51 ml allyl glycidyl ether was added to the flask and the reaction was allowed to proceed overnight at a stirring speed high enough to obtain a homogeneous emulsion. The beads were then washed on a glass filter with distilled water followed by ethanol and finally distilled water.

The allyl group content was determined by reacting 1.0 ml beads with an excess of bromine water, then removing the surplus of volatile bromine by evaporation. To speed up this evaporation process, vacuum was applied. Finally, the allyl content of the beads was determined by argentometric titration with 0.1 M $AgNO_3$ using a combined silver ring electrode (detection of Ag^+ after precipitation of AgBr). The allyl group content was 172 μmol/ml beads.

Step 2. Partial bromination of allylated beads (bromination and hydrolysis of an outer layer of the beads): 25 ml allylated beads were added to a 500 ml round-bottom flask together with 20 ml of a sucrose solution (2 g/ml). 0.55 g sodium acetate and 40 g sucrose were added under stirring. After dissolving the sucrose, the stirring speed was increased to 800 rpm. Sixty microliter bromine was added to 10 ml sucrose solution (2 g/ml) in a tube which was sealed and the bromine was allowed to dissolve by occasional vigorous shaking. The bromine solution was added to the round-bottom flask and the stirring was continued until the bromine colour disappeared. The beads were washed on a glass filter funnel with distilled water and put back in the round-bottom flask together with 25 ml distilled water. The 2.3 g NaOH was dissolved in 2.3 g distilled water and was added to the flask together with 0.10 g $NaBH_4$. The round-bottom flask was thermostated to 40 °C and the deactivation (hydrolysis of the bromine groups) was allowed to proceed overnight under stirring. The beads were then washed with distilled water. To improve the inertness of the outer layer, the bromination/hydrolysis procedures were carried out twice. Finally, the allyl content of the beads were determined as described above and found to be 127 μmol/ml beads.

Step 3. Coupling of quaternary amines (Q): 23 ml of the above beads were added to a 500 ml round-bottom flask together with 10 ml distilled water. The 1.1 g sodium acetate was added under stirring (400 rpm). Bromine in water was added until a permanent yellow colour appeared. The beads were washed on a glass filter funnel with distilled water until the yellow colour disappeared. The beads were put back in the round-bottom flask together with 10 ml distilled water. Fifteen gram of a trimethylammonium chloride solution (65%, w/w) was added under stirring. 3.9 g NaOH was dissolved in 3.9 g distilled water and was added to the reaction flask together with 0.1 g $NaBH_4$. The reaction was allowed to proceed overnight at room temperature. The beads were washed with distilled water, 1 M NaCl solution and distilled water.

The ion capacity of the beads was determined according to Section 2.8 and was 59 μmol Cl^-/ml.

2.10. Microscopy analysis

Approximately 1 ml of each prototype was incubated with an aqueous solution of the negatively charged dye Congo red for 15 min and then washed with distilled water on a glass filter for 5 min (several filtering/resuspension cycles). The washed beads were studied under a Nikon Labophot-2 light microscope and photographed using a Nikon Coolpix 950 digital camera.

2.11. Purification of recombinant pBluescript by the two-column approach

Q-derivatized Sephacryl S-500 HR beads having a non-charged surface (lid beads) were packed into a XK 16/20 column to a final bed volume of 10 ml (Column 1). Homogeneous Q-derivatized Sephacryl S-500 HR beads were also packed into a XK 16/20 column to a final bed volume of 13 ml (Column 2). The two columns were connected in series, integrated with an ÄKTAexplorer 10 system and equilibrated with buffer A. Clarified lysate (70 ml) was diluted three times with distilled water (1:2) and applied to the columns via a 150 ml superloop (two consecutive injections) at a flow rate of 1 ml/min. After the loading step the columns were washed with buffer A until the 260 nm absorbance had returned to baseline level. Column 1 was disconnected from the system and the adsorbed nucleic acids (mainly plasmid) on Column 2 were eluted by 2 M

NaCl, 1 mM EDTA, 25 mM Tris–HCl, pH 8 at a flow rate of 0.25 ml/min. Adsorbed nucleic acids on Column 1 were then eluted by 2 M NaCl, 1 mM EDTA, 25 mM Tris–HCl, pH 8 at a flow rate of 1 ml/min. The chromatography run was monitored by UV absorbance at 260 nm. The eluate was collected in different fractions for subsequent analysis by analytical anion-exchange chromatography and agarose gel electrophoresis. The fractions containing plasmids were pooled and then subjected to a final polishing step, i.e. size exclusion chromatography separation on a Sephacryl S-500 HR column (XK 16/20, equilibrated with 0.5 M NaCl, 1 mM EDTA, 25 mM Tris–HCl, pH 8) at a flow rate of 0.5 ml/min. After the purification process the columns were cleaned in place with 0.5 M NaOH containing 20% ethanol followed by distilled water and elution buffer at a flow rate of 0.5 ml/min.

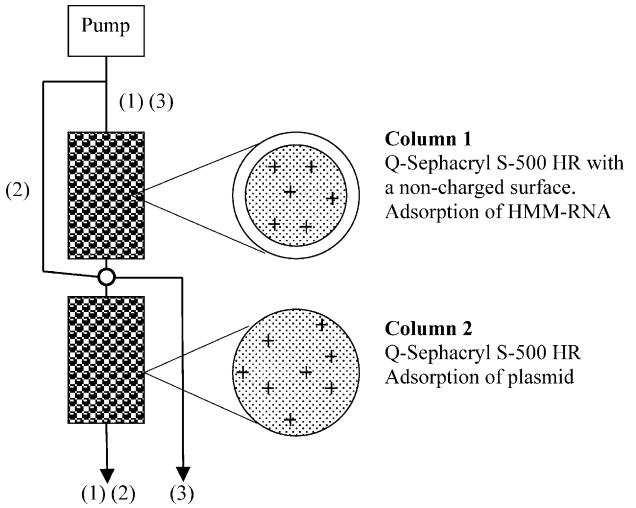

Fig. 2. Tandem columns for plasmid separation. The normal operating procedure for alkaline lysates consist of the following steps: (1) loading of lysate (both columns connected in series); (2) elution of plasmid DNA from Column 2 (Column 1 is off-line); (3) elution of RNA (mainly high-molecular-mass RNA) from Column 1 (Column 2 is off-line). The numbers in the figure indicate liquid flow in the different steps.

3. Results and discussion

3.1. Purification concept

A much-preferred initial procedure when purifying plasmids is the alkaline lysis step. It effectively removes most of the cell walls, organelles, proteins and genomic DNA leaving RNA as the main contaminant of the target plasmid [12]. A complicating factor for further plasmid DNA purification is that the lysis step leaves the plasmid mixture in a dilute form and in the presence of high salt concentration. In this report we have addressed this problem and devised an effective one-step procedure to isolate the plasmid from the contaminants and deliver it in a concentrated form for subsequent polishing steps.

Our approach is centred around a new type of restricted access material which consists of an anion-exchanger having a non-charged surface layer, so called lid beads (Fig. 1). Plasmids are too large to enter the layer and are therefore unaffected by the particle, while RNA and proteins readily diffuse through the inert layer and becomes adsorbed in the charged core. A column packed with this material will thus rapidly remove the main contaminants. If a second column packed with the same type of anion-exchange material but without the inert layer is placed downstream, the plasmid is effectively trapped on the second column. After detachment of the first column and separate elution of the second column the plasmid is obtained in a concentrated and pure form (Fig. 2).

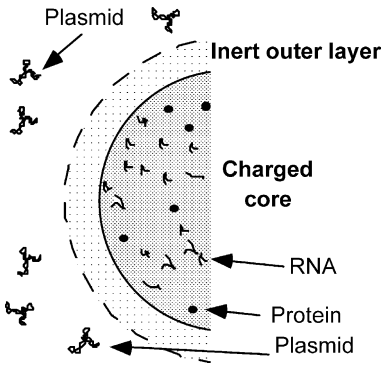

Fig. 1. Schematic view of restricted access material (lid beads) for plasmid purification.

For the concept to work properly it is important that the ligand density of the second column is approximately the same as that of the first column. The practical use of the two-column concept in this report involves alkaline lysates, which contains a high amount of salts. In such cases low-molecular-mass RNA (LMM-RNA) and most proteins are not enough charged to become adsorbed on the first column, even if they have full access to the interior of the beads. Instead these molecules will enter the second column, which they will also pass provided the beads here have a charge density equal or preferably slightly lower than that of the beads in the first column. On the other hand, it must be pointed out that the charge density of the second column must not be too low either, since this would prevent the proper adsorption of the plasmids.

3.2. Synthesis of restricted access beads (lid beads)

Fig. 3 gives a schematic overview of the synthesis of the new type of restricted access bead. The basic outline of the synthesis was obtained from a patent exemplifying other separation materials [18]. The methodology was modified to suit the separation task at hand. The size exclusion chromatography material, Sephacryl S-500 HR, was chosen as a base matrix for several reasons. It is readily available, biocompatible (allyldextran/bisacrylamide composite material), has a fairly small particle size (50 μm) and is readily modified

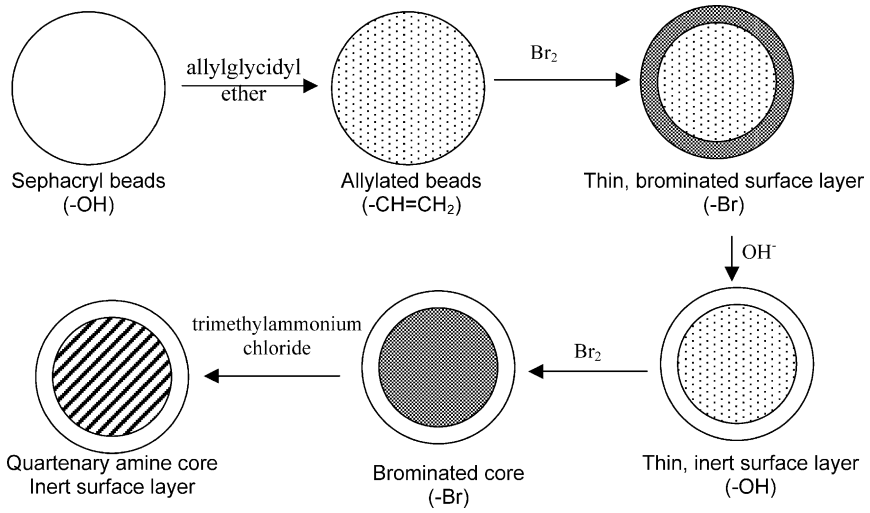

Fig. 3. Synthesis scheme for restricted access anion-exchange beads prepared from large-pore Sephacryl S-500 HR beads. Details of the synthesis are given in the text.

with various chemical reagents. A most important property is the pore size (200 nm). With these pores Sephacryl S-500 has a nucleic acid exclusion limit of about 1000 bp, which is large enough to allow diffusion of RNA molecules as well as proteins into the beads but small enough to exclude most of the plasmids used for biotechnological purposes.

Other aspects considered of the restricted access material were the thickness and the quality of the inert outer layer. Clearly the outer layer should be as thin as possible, since it will otherwise reduce the inner, charged volume of the bead and the RNA binding capacity. Thus, a layer thickness of only 10% of the bead diameter will reduce the binding capacity with 50%. The inertness of the outer layer was also considered important. Only limited amounts of charges could be allowed in the layer. Otherwise the plasmids would be adsorbed to the bead surface, compromising the very idea with a layered bead structure.

A number of bead syntheses were carried out, where several parameters affecting layer thickness/layer inertness were investigated. The beads were analysed at the various stages of synthesis by their allyl content, ion binding capacity and also microscopically and from these data the quality/thickness of the layer could be deduced. As expected, the use of a comparatively large amount of bromine in the bromination/hydrolysis step gave a layer with a desired low plasmid binding capacity, but unfortunately also made the layer too thick, thus reducing capacity and turning the bead practically ineffective. We settled for an experimental procedure in which the bromination was carried out in two steps and in the presence of sucrose to increase the viscosity of the reaction medium. A few of these prototype beads are described in Table 1, restricted access beads as well as homogeneous beads.

Fig. 4a shows a micrograph of Q-derivatized Sephacryl S-500 HR beads provided with an inert outer layer and prepared according to the standard protocol given in the experimental section. The beads were stained with the negatively charged dye Congo red. The non-charged outer layer of the beads appeared in the microscope completely uncoloured by the dye, whereas the centre part of the beads was strongly coloured. The demarcation between coloured and uncoloured was usually very distinct. The standard protocol resulted in beads with an average 2–3 μm thick inert layer, which is reasonably acceptable from a capacity point of view. Such a layer would reduce the capacity of the beads (compared to normal beads) with about 30%.

Table 1
Overview of anion-exchange prototypes

Prototype no.	Bead type[a] (RAM/Hom.)	Ion capacity (μmol Cl^-/ml gel)	Surface layer volume[b] (percent of total bead volume)	Surface layer thickness[b] (percent of bead diameter)
1	RAM	96	12	2
2	RAM	34	32	6
3	RAM	59	26	5
4	Hom.	72	–	–
5	Hom.	52	–	–

[a] RAM = restricted access material, i.e. beads with an inert surface layer; Hom. = homogeneous beads. Both beads based on a Sephacryl S-500 matrix.
[b] Surface layer volume and surface layer thickness is calculated from the decrease in allyl content of the bead after the introduction of the layer (see manufacturing protocol). Surface layer thickness were based on calculations using an average bead diameter of 50 μm.

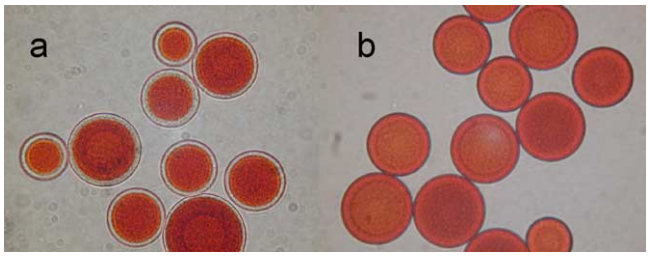

Fig. 4. Micrographs of anion-exchange beads stained with the negatively charged dye Congo red. (a) Q-Sephacryl S-500 HR beads having a non-charged surface (restricted access beads; lid beads). (b) Homogeneous Q-Sephacryl S-500 HR beads. The thin halo surrounding the beads in (a) and (b) is a diffraction effect.

In a reference experiment, homogeneous beads were also dyed with Congo red. As expected no colourless outer layer was observed here, i.e. the beads were homogeneously stained with the dye (Fig. 4b) indicating the homogeneous distribution of the ion-exchange ligands.

It must be noted that the presented method of analysing the beads (titration/standard light microscopy) does not possess the high sensitivity needed to detect trace amounts of charges in the inert layer, as for example confocal microscopy can detect [24,25]. If such charges would be present they would, on the other hand, not be a problem for the separation protocols we were using, since low charge densities would be effectively masked by the ion strength adopted in the separation protocols. Nevertheless, this type of information would be interesting to further optimise the quality of the layer.

3.3. Plasmid purification by the two-column approach

3.3.1. Initial studies of the two-column approach

Preliminary experiments showed that in order for the HMM-RNA to bind properly to Column 1 (lid bead column) a dilution of the clarified alkaline lysate was necessary. The lysate was therefore diluted with water (1:2) which gave a conductivity value of around 30 mS/cm, corresponding to a potassium acetate concentration of about 0.35 M. A higher dilution in order to capture a larger portion of the bulk RNA is certainly possible but was deemed counter-productive due to the increase in processing time. However, in certain applications, e.g. in polishing of plasmid preparations at later stages of a purification process, the salt concentration could be lower, resulting in a more complete capture of impurities. Under such conditions the lid bead column alone could be used to purify an already concentrated plasmid preparation from trace amounts of contaminants.

A somewhat lower degree of dilution is also possible, but would reduce the plasmid-binding capacity on the second column. Therefore, to have a safety window, a dilution factor of three was considered appropriate.

The volumetric capacity of the two-column process was determined by checking the RNA binding to Column 1, the lid bead column, the rationale being that Column 1 is the capacity-limiting column, due to the rather low plasmid content of the lysate preparation. The capacity of the lid beads was determined to be 42 ml diluted lysate/ml gel (14 ml lysate/ml gel) before HMM-RNA could be detected in the flow-through (based on beads with an ion capacity of 34 μmol/ml gel). The volumetric capacity of 42 ml diluted lysate/ml gel corresponds in this case to a RNA binding capacity of 4.6 mg/ml column volume.

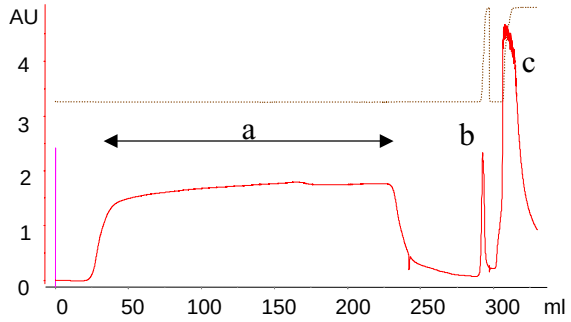

Fig. 5. Chromatogram showing the purification of recombinant pBluescript from a three times diluted clarified lysate using the two-column approach. The process is divided into three steps indicated in the chromatogram: (a) loading of 205 ml diluted lysate on the columns (two consecutive injections with the 150 ml superloop). The flow-through mainly contains LMM-RNA. (b) Elution of plasmid DNA from the second column. (c) Elution of HMM-RNA from the first column. Conductivity is indicated with the hatched line.

3.3.2. Purification of recombinant pBluescript from clarified lysate

Fig. 5 shows a chromatography run of the purification of recombinant pBluescript from a three times diluted clarified lysate. Diluted lysate was applied on the tandem columns (10 ml lid beads and 13 ml homogeneous beads) in an amount of 205 ml which corresponded to about 50% of the maximum capacity of the system. Approximately 40% of the 260 nm absorbance of the diluted lysate passed both columns (“a” in Fig. 5). Fig. 6a shows a MiniQ chromatography analysis of the diluted clarified lysate. The chromatogram illustrates the separation problem between the HMM-RNA and plasmid DNA normally encountered with anion-exchange columns. The plasmid DNA peak in Fig. 6a is not fully separated from the very broad HMM-RNA peak, which makes quantification difficult. This problem can be overcome by using a shallower salt gradient. However, a more convenient method used in this work, is the treatment of the diluted lysate with RNase prior to analysis.

Fig. 6b shows a MiniQ chromatography analysis of the flow-through fraction from the tandem columns. LMM-RNA is seen but neither HMM-RNA nor plasmid DNA. The HMM portion of RNA is defined in this context as the fraction of RNA that binds to the MiniQ column at a NaCl concentration of 0.5 M.

Protein analysis of the diluted clarified lysate and the flow-through showed that almost all proteins passed the tandem columns unadsorbed. This fact can be explained by the

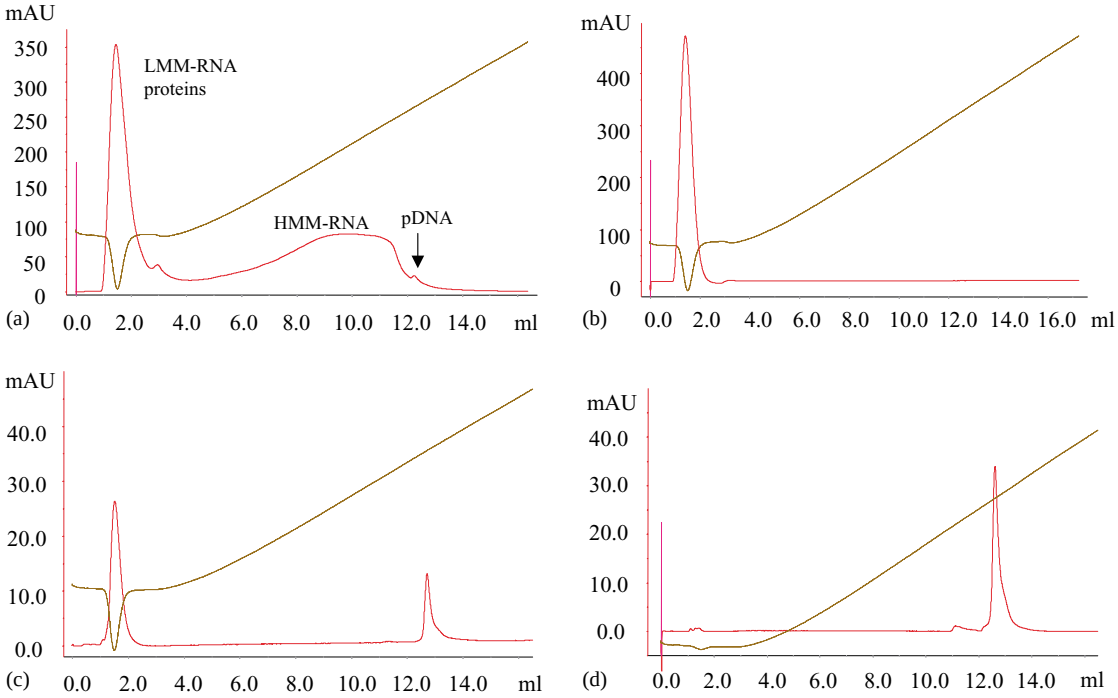

Fig. 6. Analytical anion-exchange chromatography of samples from the two-column process (Fig. 5). (a) 100 μl clarified lysate diluted three times with distilled water. (b) 100 μl flow-through from the columns in the loading step (pooled fractions). (c) 100 μl of the pooled plasmid fractions from the second column diluted seven times with distilled water. (d) 100 μl of the plasmid fractions after polishing step on Sephacryl S-500 HR column. Conductivity is indicated with the hatched line.

low charge density of the two types of beads in combination with the relatively high salt concentration of the applied sample. It was calculated that the protein only contributed with 1–2% of the 260 nm absorbance in the flow-through from the columns (calculated by comparing the molar absorptivity at 260 nm of the average protein bovine serum albumin and RNA). Thus, the major part of the 260 nm absorbance in the flow-through sprung from LMM-RNA not able to adsorb to the columns. Fig. 7 shows agarose gel electrophoresis analysis on diluted clarified lysate (lane 1) and the flow-through

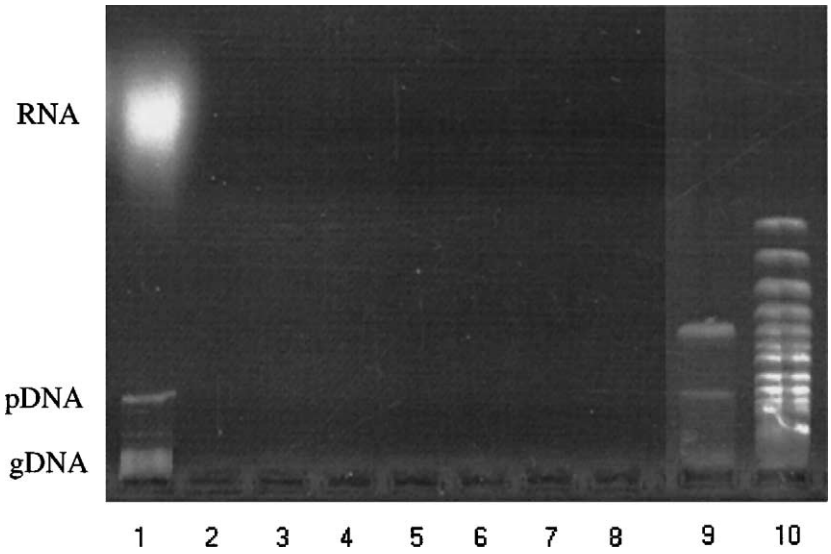

Fig. 7. Agarose gel electrophoresis analysis of samples obtained from the two-column plasmid process (Fig. 5). Lanes 1: clarified lysate diluted three times, 2: empty, 3: flow-through fraction 5 from the loading step, 4: flow-through fraction 10, 5: flow-through fraction 15, 6: flow-through fraction 20, 7: flow-through fraction 25, 8: pooled flow-through fractions, 9: pooled plasmid fractions after desalting on a Sephadex G-25 column, 10: supercoiled DNA ladder. The different migration rates for the plasmid DNA in lanes 1 and 9 are due to the difference in salt concentration of the samples.

from the columns (lanes 3–8). It is seen that no plasmid DNA passed the tandem columns during the loading step and that no HMM-RNA could be detected in the flow-through.

After the loading of the diluted lysate on the tandem columns, the first column was disconnected from the system and the adsorbed plasmid DNA was eluted from the second column as shown in Fig. 5 (“b”). Preliminary runs had shown slow elution kinetics on the second column. This was solved by incubating the column with elution buffer for 45 min after the first elution (Fig. 5 “b”). At this stage, Column 2 was already filled with elution buffer and the incubation proceeded while eluting the first column (Fig. 5 “c”) whereafter Column 2 was eluted a second time. This produced two plasmid fractions, which were pooled and gave a total volume of 11 ml. This incubation procedure improved the plasmid recovery by approximately 25% and produced a plasmid preparation 19 times as concentrated as in the sample originally applied to the column (diluted lysate). The overall duration of the two-column process for plasmid purification including the loading, washing, incubation and plasmid elution step was 400 min.

Fig. 6c shows a MiniQ chromatography analysis of the pooled plasmid fractions. By comparing Fig. 6a and Fig. 6c, the high degree of purification obtained with the tandem column procedure becomes obvious. Fig. 6c shows a contamination by presumably LMM-RNA and proteins not detectable by agarose gel electrophoresis analysis (lane 9, Fig. 7). This contamination might be due to unspecific binding of LMM-RNA and proteins to the beads in Column 2. μ-BCA analysis of the pooled plasmid fraction showed that 96% of the proteins had been removed by the process. The plasmid recovery after the two-column process was 89%. The loss in recovery was due to plasmid adsorption to the first column, which was evident after MiniQ chromatography analysis on the eluate from the first column (Fig. 5 “c”). Plasmid identity was confirmed by linearization with *Bam*HI followed by agarose gel electrophoresis. The main plasmid conformation present was the supercoiled form but also a minor band was visualised on agarose gel electrophoresis corresponding to the open circular form (lane 9, Fig. 7). The different migration rates for the plasmid DNA in lanes 1 and 9 are due to the difference in salt concentration of the samples.

Table 2 summarises the two-column process for the purification of recombinant pBluescript from diluted clarified lysate. It must be emphasised here that the MiniQ chromatography used to quantitate RNA in the samples slightly overestimates the RNA content, due to the contribution of proteins to the 260 nm absorbance. However, by doing similar calculations as for the flow-through above (comparing the molar absorptivity at 260 nm of the average protein bovine serum albumin and RNA), one can estimate this contribution. These calculations indicated that the contribution of proteins to the 260 nm absorbance in the plasmid fraction (Table 2) is approximately 5% and thus negligible. It could be calculated that the two-column process removed 99.5% of the RNA, the remaining being LMM-RNA. By comparing lane 1 with lane 9 in Fig. 7 it can also be noticed that the gDNA contamination has been significantly reduced. This can be explained by that gDNA remained bound to Column 2 after elution of the plasmid. By employing a stronger elution buffer (50 mM sodium phosphate, pH 10, 1 M NaCl) the gDNA could be eluted and identified by agarose gel electrophoresis (data not shown). A similar observation concerning the much stronger binding of gDNA to anion-exchange supports was made by Prazeres et al. [4].

Table 3
Comparison of different prototypes for plasmid purification from diluted clarified lysate

Run	Prototype pair[a]	Applied sample volume (ml)	Plasmid yield (%)
1	1 + 4	218	69
2	1 + 4	235	72
3	1 + 4	300	88
4[b]	3 + 5	205	89

[a] The ion capacity of the prototypes are given in Table 1.
[b] Run 4 is described in detail in Table 2.

Table 3 summarises purification runs using two other prototypes (prototypes 1 and 4 from Table 1) performed in the same way as the above described process (run 4). Prototype 1 had a smaller thickness of the inert layer (Table 1) compared to prototype 3 and gave less plasmid recovery. However, by increasing the applied volume of the lysate the recovery was increased, possibly suggesting the existence of a limited amount of adsorption sites in the inert layer. Run 4 was subjected to a polishing step described below.

Buffer A was used for equilibration and washing of the tandem columns (see Section 2.11) to avoid refractive index changes in the UV cell (thereby avoiding ghost peaks). However, another buffer having similar conductivity was also tried (i.e. 0.3 M NaCl, 1 mM EDTA, 25 mM Tris–HCl, pH 8) and gave similar results.

Table 2
Overview of plasmid purification process

	Volume (ml)	Protein mass (BCA, mg)	RNA mass (MiniQ, mg[a])	Plasmid mass (MiniQ, mg[b])
Diluted lysate	205	26.4	62.5	0.18
Two-column process	11	1.1	0.33	0.16

[a] Based on UV absorbance at 260 nm, 1 AU = 40 μg RNA/ml
[b] Based on UV absorbance at 260 nm, 1 AU = 50 μg DNA/ml

3.3.3. Polishing of plasmid preparation

The remaining contaminants (LMM-RNA and proteins) in the plasmid preparation after the tandem column process were removed by size exclusion chromatography on a Sephacryl S-500 HR column. Fig. 6d shows a MiniQ chromatography analysis of the plasmid preparation after the polishing step that shows no RNA contamination. μ-BCA analysis on the plasmid preparation showed also that no protein could be detected. It should be emphasised that size exclusion chromatography is only one of several candidates for a polishing step, hydrophobic interaction and thiophilic aromatic chromatography or even anion-exchange chromatography being other alternatives.

4. Conclusions

This work demonstrates that the new type of restricted access anion-exchange packing simplifies plasmid purification considerably. By distributing the nucleic acids species on two coupled columns, followed by separate elution of each column, the method avoids the co-elution of HMM-RNA and plasmid DNA normally experienced with preparative anion-exchange columns. Compared to size exclusion chromatography the present method has a much higher volumetric capacity and delivers the plasmids in a concentrated form. Thus, the present technique combines the high capacity of anion-exchange chromatography with the discriminating ability of size exclusion chromatography for RNA and plasmid DNA. The new technique shows great promise as a single-unit primary recovery step for the purification and concentration of plasmid DNA from clarified alkaline lysates.

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References

[1] D.M.F. Prazeres, G.N.M. Ferreira, G.A. Monteiro, C.L. Cooney, J.M.S. Cabral, TIBTECH 17 (1999) 169.
[2] M.S. Levy, R.D. O'Kennedy, P. Ayazi-Shamlou, P. Dunnill, TIBTECH. 18 (2000) 296.
[3] D.L. Varley, A.G. Hitchcock, A.M.E. Weiss, W.A. Horler, R. Cowell, L. Peddie, G.S. Sharpe, D.R. Thatcher, J.A.J. Hanak, Bioseparation 8 (1999) 209.
[4] D.M.F. Prazeres, T. Schluep, C. Cooney, J. Chromatogr. A 806 (1998) 31.
[5] M. Bywater, R. Bywater, L. Hellman, Anal. Biochem. 132 (1983) 219.
[6] P.A.D. Edwardson, T. Atkinson, C.R. Lowe, D.A.P. Small, Anal. Biochem. 152 (1986) 215.
[7] R.B. Sparks, J.H. Elder, Anal. Biochem. 135 (1983) 345.
[8] M.M. Diogo, S. Ribeiro, J.A. Queiroz, G.A. Monteiro, P. Perrin, N. Tordo, D.M.F. Prazeres, Biotechnol. Lett. 22 (2000) 1397.
[9] R. Giovannini, R. Freitag, Biotechnol. Bioeng. 77 (2002) 445.
[10] T. Schluep, C.L. Cooney, Nucl. Acids Res. 26 (1998) 4524.
[11] W.S. Vincent III, E.S. Goldstein, Anal. Biochem. 110 (1981) 123.
[12] R. Lemmens, U. Olsson, T. Nyhammar, J. Stadler, J. Chromatogr. B 784 (2003) 291.
[13] P.E. Gustavsson, et al., unpublished results.
[14] Qiagen Plasmid Purification Handbook Qiagen Inc., Hilden, Germany, 2000.
[15] G.A. Monteiro, G.N.M. Ferreira, J.M.S. Cabral, D.M.F. Prazeres, Biotechnol. Bioeng. 66 (1999) 189.
[16] G.N.M. Ferreira, J.M.S. Cabral, D.M.F. Prazeres, Biotechnol. Prog. 15 (1999) 725.
[17] T.C. Pinkerton, J. Chromatogr. 544 (1991) 13.
[18] J. Bergström, R. Berglund, L. Söderberg, Process for Introducing a Functionality, WO 98/39364.
[19] H. Berg, P. Busson, M. Carlsson, PCT/SE03/01284.
[20] E. Nordberg Karlsson, Ph.D. thesis, Department of Biotechnology, Lund University, Sweden, 1999.
[21] N.A. Horn, J.A. Meek, G. Budahazi, M. Marquet, Hum. Gene Ther. 6 (1995) 565.
[22] Sambrook, Fritsch, Maniatis, Molecular Cloning, a Laboratory Manual, CSH Press, New York, second ed., 1989.
[23] G.T. Hermanson, A.K. Mallia, P.K. Smith, Immobilized Affinity Ligand Techniques, Academic Press, San Diego, CA, 1992.
[24] A. Ljunglöf, P. Bergvall, R. Bhikhabhai, R. Hjorth, J. Chromatogr. A 844 (1999) 129.
[25] J. Hubbuch, T. Linden, E. Knieps, J. Thömmes, M.-R. Kula, Biotechnol. Bioeng. 80 (2002) 359.