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Journal of Chromatography B, 804 (2004) 327–335
JOURNAL OF CHROMATOGRAPHY B
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Purification of pharmaceutical-grade plasmid DNA by anion-exchange chromatography in an RNase-free process

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Received 17 October 2003; received in revised form 15 January 2004; accepted 19 January 2004

Abstract

Anion-exchange is the most popular chromatography technique in plasmid DNA purification. However, poor resolution of plasmid DNA from RNA often results in the addition of bovine-derived ribonuclease (RNase) A to degrade RNA impurities which raises regulatory concerns for the production of pharmaceutical-grade plasmid DNA. Low capacity for plasmid of most commercial media is another issue affecting the suitability of anion-exchange chromatography for large-scale processing. This study reports the use of anion-exchange chromatography to remove RNA in an RNase-free plasmid purification process. Resolution was achieved through careful selection of adsorbent and operating conditions as well as RNA reduction steps before chromatography. Dynamic capacity for plasmid was significantly increased (to 3.0 mg/ml) so that it is now possible to envisage the large-scale manufacturing of therapeutic-grade plasmid DNA in the absence of added RNase using anion-exchange chromatography as a polishing step.

Keywords: Purification; Plasmid DNA; RNase

1. Introduction

In recent years, gene therapy and DNA vaccination [1,2] have attracted a lot of interest as new ways of preventing or treating disease through gene transfer. Non-viral techniques including the intra-muscular injection of naked plasmid DNA or gene gun delivery of plasmid DNA coated onto gold particles into the epidermis appear very attractive as they offer several advantages over viral vectors, especially low immunogenicity, better safety profile and easier manufacture. There is therefore a need for large-scale processes to manufacture plasmid DNA of a high level of purity for use as therapeutic agent. At the same time, the requirements of regulatory agencies regarding purity, potency, safety and efficacy must be met [3–5].

Plasmid purification strategies usually involve at least one chromatography step either to capture the plasmid or more often, for polishing. Techniques include: size-exclusion [6–8], reversed-phase [9,10], hydrophobic interaction [11–13], hydroxyapatite [14–16], silica [17–19] and triple-helix affinity [20,21]. Anion-exchange remains the most popular chromatography technique as it offers the advantages of rapid separation, no solvent requirement, sanitisation with sodium hydroxide and a wide selection of industrial media. DNA is a polyanionic molecule due to the presence of phosphate groups on the nucleic acid backbone and is therefore conveniently captured on a resin derivatised with positively charged functional groups. Protocols for both packed [22–24] and expanded bed [25–27] operation have been developed.

Current plasmid purification methods suffer from several drawbacks that make them unsuitable for the manufacture of pharmaceutical-grade plasmid DNA. They often involve the use of solvents (ethanol, isopropanol), toxic chemicals (cesium chloride, ethidium bromide, phenol, chloroform) or animal-derived enzymes (ribonuclease A, lysozyme, proteinase K) that are either not approved or not recommended by regulatory agencies. Removal of key impurities such as chromosomal DNA, RNA, proteins and endotoxins is often insufficient. Finally, many techniques were designed to produce small quantities of plasmid DNA for laboratory use and are not suitable for the production of therapeutic

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doi:10.1016/j.jchromb.2004.01.033

material at large scale. A common limitation of commercial chromatography media is the low capacity for plasmid DNA due to the inability of large molecules to penetrate porous beads [22,28].

Resolution of plasmid DNA from RNA, especially high molecular weight molecules by anion-exchange chromatography is insufficient [24,26]. As a result, ribonuclease A (RNase A) is commonly added to degrade RNA impurities [22–25]. Apart from the obvious costs involved, there are concerns regarding RNase because it is purified from bovine pancreas and regulatory authorities recommend that bovine derived materials should be avoided in the production of bio-therapeutics following the outbreak of new-variant Creutzfeld–Jakob disease in the UK [29].

In this paper, we investigate the suitability of anion-exchange chromatography to purify plasmid DNA in the absence of exogenous RNase. RNA reduction steps were necessary prior to chromatography to allow complete resolution from plasmid DNA. We evaluated a range of anion-exchangers for their ability to resolve plasmid DNA from RNA, high capacity and robustness. This allowed us to identify one adsorbent with suitable characteristics for the large-scale production of pharmaceutical-grade plasmid DNA.

2. Experimental

2.1. Materials

The *E. coli* host strain DH5α containing a 5.9 kb plasmid encoding hepatitis B surface and core antigen was developed in-house. Anion-exchange chromatography media were purchased as follows: Q Sepharose Fast Flow, DEAE Sepharose Fast Flow and SOURCE 30Q from Amersham Biosciences (Little Chalfont, UK), Q Ceramic HyperD F and DEAE Ceramic HyperD F from BioSepra (Cergy-Saint-Christophe, France), Fractogel EMD TMAE (M), Fractogel EMD DMAE (M) and Fractogel EMD DEAE (M) from Merck KGaA (Darmstadt, Germany), Macro-Prep DEAE, Macro-Prep High Q and UNOSphere Q from BioRad (Hercules, CA, USA), POROS 50HQ, POROS 50PI and POROS 50D from Applied Biosystems (Warrington, UK), Toyopearl DEAE 650 M and Toyopearl SuperQ M from TosoHaas (Stuttgart, Germany) and UFC DEAE and UFC PEI from UpFront Chromatography (Copenhagen, Denmark). Analytical HPLC columns were obtained as follows: DNAPac PA-100, 4 mm × 250 mm from Dionex (Sunnyvale, CA, USA) and TSKgel G-DNA PW, 7.8 mm × 300 mm and TSKgel G6000 PWXL, 7.8 mm × 300 mm from Phenomenex (Macclesfield, UK). Preparative chromatography studies were conducted on an Äkta-FPLC system with UFC900 control unit, P920 pumps and Frac950 fraction collector from Amersham Biosciences (Little Chalfont, UK). Analytical HPLC equipment consisted of a Waters 2690 Separations module and Waters 996 Photodiode Array Detector from Waters (Watford, UK). The tangential flow filtration Centramate rig was purchased from PallFiltron (Portsmouth, UK) together with the Omega 100 K Centramate 0.1 ft^2 open channel membrane and 500 ml ultrareservoir. The centrifuge used was a Sorvall RC5C Plus from Kendro (Newtown, CT, USA). Sartobran P 0.45/0.2 μm sterile capsules were purchased from Sartorius (Göttingen, Germany). The pump used for tangential flow filtration was a WatsonMarlow (Falmouth, UK) 505S. The 10 l bioreactor was purchased from New Brunswick Discovery. Slide-A-Lyzer 3–15 ml, 10,000 MWCO dialysis cassettes were bought from Pierce (Rockford, IL, USA). All chemicals were bought from Merck (Poole, UK) and were analytical grade.

2.2. Methods

2.2.1. Bacterial cell culture

A 10 l bioreactor (5 l working volume) was inoculated with 300 ml of an overnight shake-flask culture (230 rpm, 37 °C). The medium was a complex growth medium (in-house formulation) supplemented with 50 μg/ml kanamycin. The fermenter culture was grown at 37 °C, pH 7. Dissolved oxygen was set to 30% of the saturation value and controlled by changing air flow and agitation speed. The culture was harvested when a drop in oxygen consumption was observed. Cells were centrifuged at 3200 × *g* for 20 min on a MSE Mistral 6000 and the supernatant was discarded.

2.2.2. Lysis and plasmid purification

Bacteria were lysed using a modification of the alkaline method described by Birnboim and Doly [30]. Bacteria cell paste (typically 100 g) was re-suspended in 500 ml of 25 mM Tris, 10 mM EDTA, 55 mM dextrose, pH 8.0. Lysis was performed by adding 834 ml of 0.96% (w/v) NaOH and 166 ml of 6% (w/v) SDS for 30 min at 4 °C. The lysate was neutralised with 500 ml of 3 M potassium acetate for 30 min at 4 °C. RNA levels were reduced by addition of 1 vol. of 5 M calcium chloride to 4 vol. of neutralised lysate. Precipitated material including cell debris, chromosomal DNA, high molecular weight RNA and proteins was removed by centrifugation at 8000 × *g* for 10 min followed by clarification through a 0.45/0.2 μm Sartobran P filter. The clarified lysate was processed by tangential flow filtration (TFF) to remove remaining proteins and low molecular weight RNA, concentrate and buffer exchange. Clarified lysate was concentrated to 100 ml on a Pall Filtron Centramate rig fitted with a 1 ft^2, 100 K Centramate membrane. Cross-flow rate was maintained at 1 l/(min ft^2) using a WatsonMarlow 505S pump. Trans-membrane pressure was set at 35 kPa. Diafiltration with 50 vol. of 50 mM Tris, 0.54 M NaCl, pH 8.5 was then carried out under the same conditions. The retentate from TFF was used as the load material for anion-exchange chromatography after dialysis into a suitable buffer. Dialysis was performed against two changes of loading buffer in 10,000 MWCO Slide-A-Lyzer cassettes.

2.2.3. Analytical chromatography

Plasmid concentration was determined by anion-exchange HPLC (IE-HPLC) on a Dionex DNAPac PA-100 column. Injection of 10 μl of sample was followed by a linear gradient of 0.7–0.9 M KCl in 20 mM Tris, pH 7.5 for 9.5 min. The column was re-equilibrated with 0.7 M KCl in 20 mM Tris, pH 7.5 for 4.5 min. The flow rate was 1 ml/min. Absorbance was monitored at 260 nm. Plasmid standards of concentrations ranging from 5 to 275 μg/ml were injected on the column and the surface area under the peak of absorbance at 260 nm was plotted against the plasmid concentration. Samples of unknown plasmid concentration were injected and from the resulting peak area, concentration was determined from the standard curve.

RNA levels were measured as a percentage of RNA to plasmid by size-exclusion HPLC (SEC-HPLC). After the tangential flow filtration step samples were free of contaminant chromosomal DNA and proteins so there was no interference with the assay [31]. The only substances absorbing at 260 nm present at this stage were plasmid and RNA. Injection of 100 μl of sample was followed by isocratic elution in 0.1 M Tris, 0.3 M NaCl, 1 mM EDTA, pH 7.5 for 70 min at 0.5 ml/min on TSKgel G-DNA PW followed by TSKgel G6000 PWXL in series. Absorbance was monitored at 260 nm. The surface area under the first peak (plasmid) and the second peak (RNA) was integrated and the percentage of each peak calculated.

2.2.4. Preparative anion-exchange chromatography

HR5/5 Amersham Biosciences columns were packed with 1 ml of anion-exchange adsorbent according to manufacturer's instructions. All columns were equilibrated with 5 ml of loading buffer (typically 50 mM sodium phosphate, pH 7.0) at a linear flow rate of 150 cm/h. Dialysed retentate from TFF was injected on each column (typically 0.5 ml except for breakthrough experiments). The columns were washed with 5 ml of loading buffer.

For the screening experiments, a 40 ml gradient of 0 to 1 M NaCl in loading buffer was applied to resolve RNA and plasmid. For the step elution experiments, RNA was eluted in one 5 ml step of loading buffer containing sodium chloride at various concentrations, followed by plasmid elution in a 10 ml gradient to 1 M NaCl.

For the breakthrough experiments, the TFF retentate was dialysed into loading buffer containing sodium chloride at the concentration determined in the step experiment to prevent binding of RNA to the column without affecting plasmid recovery. The column was washed with 5 ml of dialysis buffer followed by plasmid elution in a gradient to 1 M NaCl.

All columns were then regenerated with 4 ml of 1 M NaCl in loading buffer, sanitised with 5 ml of 0.5 M NaOH followed by 5 ml re-equilibration in loading buffer. Absorbance was monitored at 254 nm.

2.2.5. Agarose gel electrophoresis

A 0.8% agarose gel containing 0.5 μg/ml ethidium bromide was run in a horizontal gel electrophoresis unit (Mini-Sub DNA cell, BioRad). The running buffer was TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). Electrophoresis was carried out at 100 V for 1 h on an Amersham Pharmacia Biotech power supply unit ECPS3000/150.

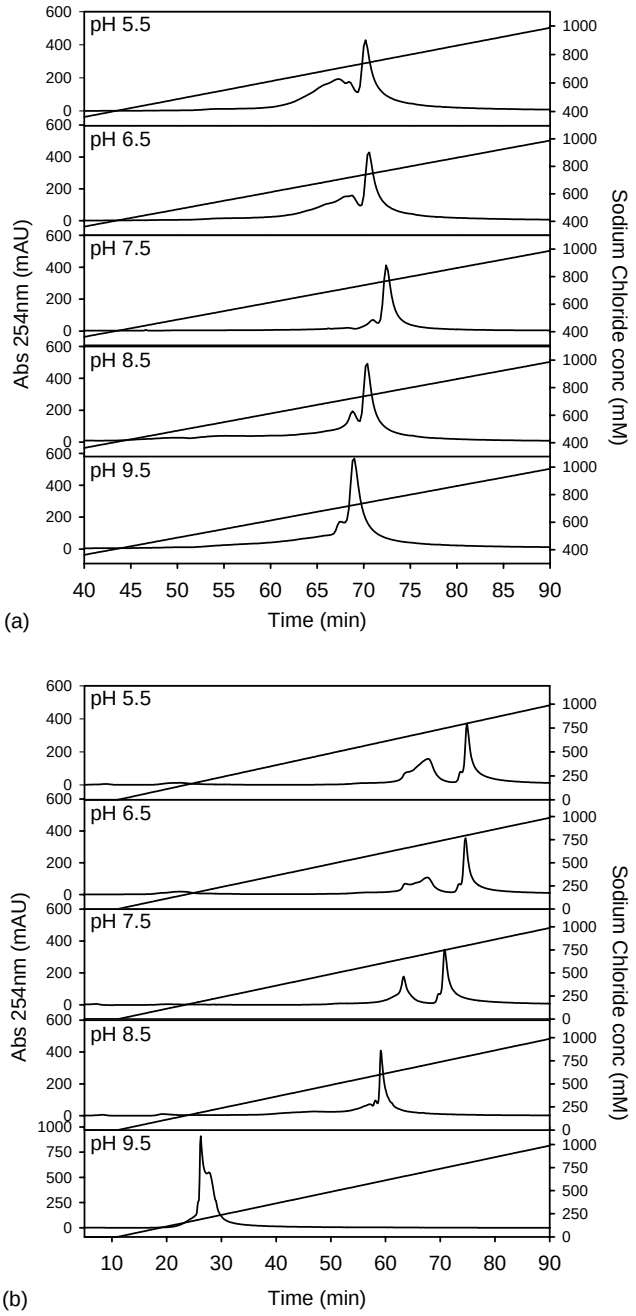

Fig. 1. Elution profile of plasmid and RNA on a 1 ml Fractogel EMD TMAE (a) and Fractogel EMD DEAE (b) column using a linear NaCl gradient at five different buffer pH values: 5.5, 6.5, 7.5, 8.5 and 9.5. The first peak represents the elution of RNA while plasmid elutes in a second peak.

3. Results

3.1. Anion-exchange chromatography: effect of pH

To determine the effect of pH on the performance of a selected strong (Fractogel TMAE) and weak (Fractogel DEAE) anion-exchanger, both columns were run in a universal buffer of 20 mM Tris, 20 mM MES, 20 mM glycine at pH 5.5, 6.5, 7.5, 8.5 and 9.5. Results are shown in Fig. 1a (Fractogel TMAE) and Fig. 1b (Fractogel DEAE). As expected, pH had a very limited effect on plasmid retention on the strong anion-exchanger within the range investigated. Resolution of RNA (first peak) from plasmid (second peak) was insufficient. The weak anion-exchanger (Fractogel DEAE) gave baseline separation of RNA from plasmid in the range of pH between 5.5 and 7.5. As pH was increased above 7.5, not only was plasmid retention significantly reduced but resolution was also lost.

Although results obtained from an experiment with just one weak and one strong anion-exchanger cannot be directly generalised to all other adsorbents, a phosphate buffer at pH 7 was selected for all subsequent experiments so that separation of RNA from plasmid was maximised.

3.2. Anion-exchange chromatography: screening of adsorbents

Table 1 shows the characteristics of 18 anion-exchange chromatography media screened, all of which are suitable for large-scale manufacturing. Pore sizes of 50–100 μm were preferred for better comparability and potential scale-up. A broad range of weak (DEAE, DEAP, DMAE, PEI non-Q) and strong (Q, TMAE, PEI Q) functional groups was included. Matrices were diverse (methacrylate, agarose, polystyrene divinylbenzene, acrylamide) but always sanitisable with sodium hydroxide.

Only four anion-exchangers gave baseline resolution of RNA from plasmid: Fractogel DEAE (Fig. 2a), Q Sepharose FF (Fig. 2b), POROS 50HQ (Fig. 2c) and Q Ceramic HyperD F (Fig. 2d).

3.3. Anion-exchange chromatography: step elution of RNA

A step elution was devised to remove RNA adsorbed to the anion-exchanger followed by plasmid elution in a gradient. The conditions required for complete RNA elution in the step were determined by varying the salt concentration in the step. Under optimised conditions, only RNA eluted in the step while plasmid essentially free of RNA was recovered in the gradient. The levels of plasmid and RNA in the step and gradient fractions were measured by size-exclusion HPLC and expressed as a percentage of each species present. Results in Table 2 show that all four selected anion-exchangers gave complete RNA removal and recovery of almost pure plasmid under optimised conditions.

3.4. Anion-exchange chromatography: breakthrough experiments

Sequential elution of RNA and plasmid has now been achieved by selecting the anion-exchanger together with a suitable pH and salt concentration for RNA elution. However, the fact that RNA binds to the anion-exchanger means that fewer binding sites are available for plasmid capture and as a result dynamic capacity for plasmid is reduced. By selecting loading conditions that prevent binding of RNA, plasmid capture can be maximised and dynamic capacity significantly increased. This was achieved by optimising loading

Table 1
Characteristics of the commercial anion-exchange chromatography media evaluated

Support	Functional group	Matrix	Particle size (μm)	Pore size (Å)	Ligand density (μeq./ml)
Q Sepharose FF	Q	Agarose	90	1900	180–250
DEAE Sepharose FF	DEAE	Agarose	90	1900	110–160
SOURCE 30Q	Q	PSDB[a]	30	20–1000	NA
Q Ceramic HyperD F	Q	Hydrogel/ceramic	50	3000	>200
DEAE Ceramic HyperD F	DEAE	Hydrogel/ceramic	50	3000	>180
Fractogel EMD TMAE	TMAE	Methacrylate	40–90	800	NA
Fractogel EMD DMAE	DMAE	Methacrylate	40–90	800	NA
Fractogel EMD DEAE	DEAE	Methacrylate	40–90	800	NA
Macro-Prep DEAE	DEAE	Methacrylate	50	1000	175
Macro-Prep High Q	Q	Methacrylate	50	1000	400
UNO Sphere Q	Q	Acrylamide	120	10000	NA
POROS 50HQ	PEI Q	PSDB[a]	50	<8000	NA
POROS 50PI	PEI non-Q	PSDB[a]	50	<8000	NA
POROS 50D	DEAP	PSDB[a]	50	<8000	NA
Toyopearl DEAE 650	DEAE	Methacrylate	40–90	1000	80–120
Toyopearl SuperQ 650	Q	Methacrylate	40–90	1000	200–300
UFC DEAE	DEAE	Agarose	100–300	NA	50–100
UFC PEI	PEI	Agarose	100–300	NA	180–200

NA: not available.
[a] Polystyrene divinylbenzene.

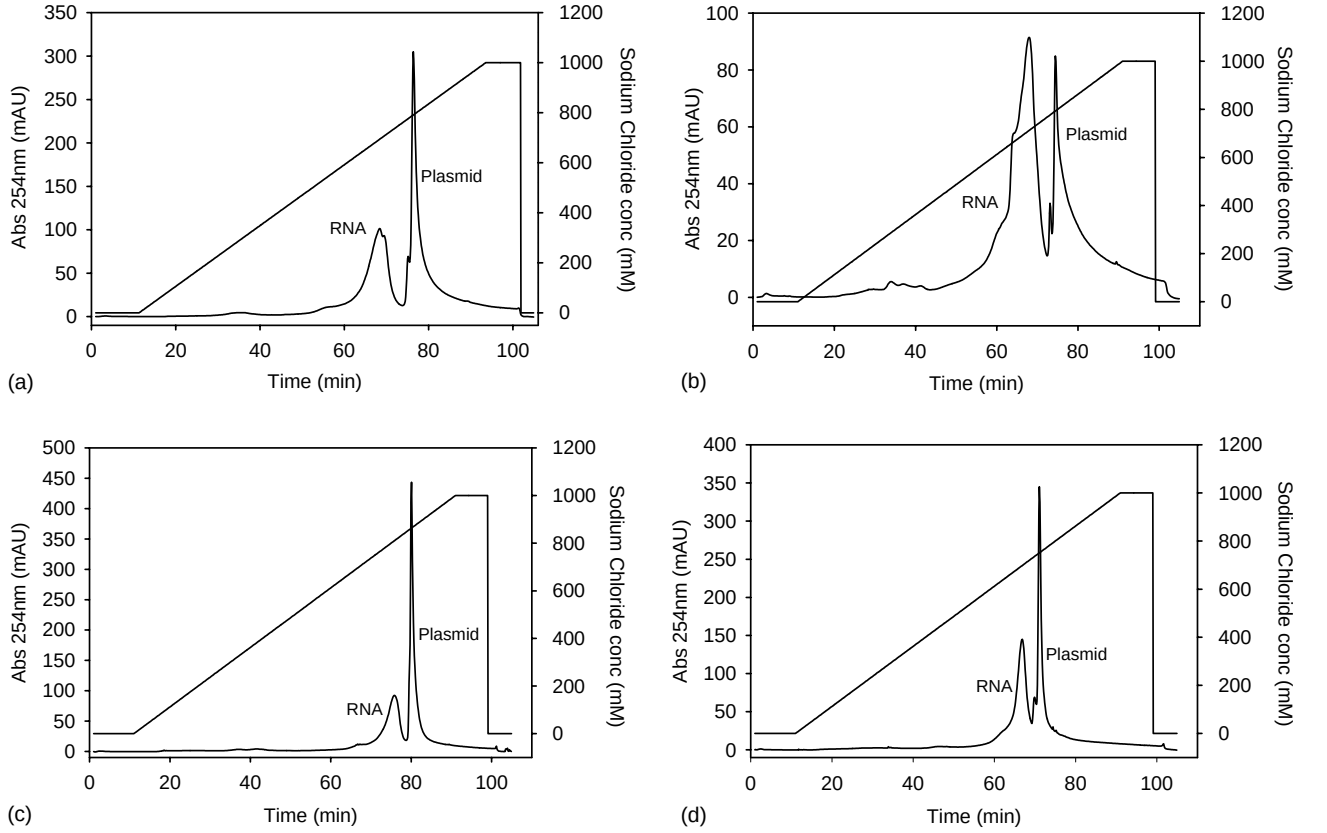

Fig. 2. Elution profile of plasmid and RNA on a 1ml Fractogel EMD DEAE (a), Q Sepharose FF (b), POROS 50HQ (c) and Q Ceramic HyperD F (d) column using a linear NaCl gradient in 50 mM sodium phosphate buffer at pH 7.0.

Table 2
Sequential elution of RNA in a step and plasmid in a gradient by anion-exchange chromatography

Anion-exchange column	Step NaCl concentration (M)	SEC-HPLC	
		RNA content in step fraction (%)	Plasmid content in gradient fraction (%)
Q Sepharose FF	0.67	100.0	94.5
Q Ceramic HyperD F	0.67	100.0	92.8
Fractogel DEAE	0.70	100.0	99.5
POROS 50HQ	0.81	100.0	97.7

0.5 ml of TFF retentate containing plasmid and RNA was loaded on a 1 ml anion-exchange column. RNA was recovered by step elution with NaCl in 50 mM sodium phosphate buffer at pH 7.0. Plasmid was then eluted with a NaCl gradient to 1 M in 50 mM sodium phosphate at pH 7.0. Step and gradient elution fractions were analysed by size-exclusion HPLC (SEC-HPLC) and the percentage of plasmid and RNA determined in each fraction.

salt concentration in a breakthrough experiment where RNA and plasmid levels were measured in the column flowthrough during loading by size-exclusion HPLC. Dynamic capacity was determined as the amount of plasmid loaded when plasmid starts to appear in the column breakthrough. Table 3 shows that loading salt concentrations determined in the previous section were too high and salt concentration in the load had to be reduced to prevent early plasmid breakthrough. Only three of the four selected anion-exchangers had sufficient capacity: Q Ceramic HyperD F, Fractogel DEAE and POROS 50HQ. Q Ceramic HyperD F had a dynamic capacity of >5.3 mg/ml when loading in 50 mM phosphate, 0.63 M NaCl, pH 7 at 150 cm/h. Fractogel DEAE had a dynamic capacity of 2.4 mg/ml under the same conditions except for

Table 3
Dynamic capacity for plasmid of several anion-exchangers when loading in high salt

Anion-exchange column	NaCl concentration in the load (M)	Dynamic capacity (mg plasmid/ml gel)
Q Sepharose FF	0.61	0.72
Q Ceramic HyperD F	0.63	>5.3
Fractogel DEAE	0.69	2.45
POROS 50HQ	0.72	2.12

Each 1 ml anion-exchange column was loaded with a mixture of plasmid and RNA in high NaCl in 50 mM sodium phosphate buffer at pH 7.0. Dynamic capacity was determined as the amount of plasmid loaded when plasmid begins to appear in the column breakthrough as detected by size-exclusion HPLC (SEC-HPLC).

Table 4
Effect of NaCl concentration in the load on RNA removal and plasmid recovery when loading anion-exchange columns to capacity

Anion-exchange column	NaCl concentration in the load (M)	SEC-HPLC		IE-HPLC
		RNA content in breakthrough (%)	Plasmid content in eluate (%)	Plasmid recovery (%)
Q Ceramic HyperD F	0.63	100.0	100.0	72.0
Fractogel DEAE	0.69	100.0	100.0	97.6
	0.61	100.0	100.0	100.0
	0.59	100.0	99.2	100.0
POROS 50HQ	0.73	99.7	100.0	95.0
	0.72	100.0	100.0	96.0
	0.71	100.0	99.7	95.0

Each 1 ml anion-exchange column was loaded to capacity in high NaCl in 50 mM sodium phosphate buffer at pH 7.0. The breakthrough fraction was recovered and analysed by size-exclusion HPLC (SEC-HPLC) to determine the RNA content. The columns were then washed in high NaCl containing buffer and plasmid eluted with a linear gradient of NaCl to 1 M in 50 mM sodium phosphate at pH 7.0. The eluate was analysed by size-exclusion HPLC to determine the plasmid content and by anion-exchange HPLC (IE-HPLC) to measure plasmid recovery.

a loading salt concentration of 0.69 M. POROS 50HQ had the lowest dynamic capacity at 2.1 mg/ml under the same conditions except that the salt concentration in the load was 0.72 M.

3.5. Anion-exchange chromatography: loading to capacity

The robustness of the three anion-exchangers selected for their high capacity was tested by determining their loading range of operation at capacity. The highest loading salt concentration that resulted in complete recovery of plasmid was determined. Similarly, the lowest loading salt concentration that prevented binding of RNA to the column was also determined. Table 4 shows that Fractogel DEAE had a wider range of operation (0.61–0.69 M NaCl in the load) compared with POROS 50HQ (0.72–0.73 M NaCl) and Q Ceramic HyperD F. In fact, with Q Ceramic HyperD F consistency of column performance was a problem and loading conditions could not be reproducibly established. Loading the Fractogel DEAE column at the lower end of the operating range (0.63 M NaCl) also resulted in improved dynamic capacity (3 mg/ml). Within their range of operation, all three anion-exchangers gave complete separation of RNA in the breakthrough from plasmid in the eluate. Fig. 3a shows the size-exclusion HPLC analysis of the loading material. The first elution peak between 25 and 30 min is plasmid with 72% of the total peak area while RNA elutes as a second peak between 40 and 45 min and represents 28% of the total peak area. Fig. 3b shows the eluate material from anion-exchange chromatography on Fractogel DEAE: only the plasmid peak is present. Pure plasmid free of contaminant RNA was recovered at high yield (>95% for Fractogel DEAE and POROS 50HQ) except for Q Ceramic HyperD F where plasmid recovery was low (72.0%). Subsequent sanitisation cycles on Q Ceramic HyperD F revealed that some of the plasmid was strongly bound to the column and difficult to elute with a salt gradient.

Three repeat runs on Fractogel DEAE loading plasmid at 3 mg/ml in 50 mM phosphate, 0.63 M NaCl, pH7.0 at 150 cm/h showed good reproducibility in plasmid recovery and RNA clearance (Table 5).

Three repeats runs on POROS 50HQ loading plasmid at 1.9 mg/ml in 50 mM phosphate, 0.72 M NaCl, pH 7.0 at 150 cm/h also showed good reproducibility (Table 5).

Table 5
RNA clearance and plasmid recovery after three repeat runs on anion-exchange chromatography columns under optimised conditions

Anion-exchange column	NaCl concentration in the load (M)	IE-HPLC	SEC-HPLC	
		Plasmid recovery (%)	RNA content in breakthrough (%)	Plasmid content in eluate (%)
Fractogel DEAE	0.63	94.0	98.9	100.0
		94.0	99.4	100.0
		94.0	99.3	100.0
POROS 50HQ	0.72	95.0	99.6	100.0
		95.0	99.3	100.0
		90.0	100.0	100.0

Each 1 ml anion-exchange column was loaded to capacity in high NaCl in 50 mM sodium phosphate buffer at pH 7.0. The breakthrough fraction was recovered and analysed by size-exclusion HPLC (SEC-HPLC) to determine the RNA content. The columns were then washed in high NaCl containing buffer and plasmid eluted with a linear gradient of NaCl to 1 M in 50 mM sodium phosphate at pH 7.0. The eluate was analysed by size-exclusion HPLC to determine the plasmid content and by anion-exchange HPLC (IE-HPLC) to measure plasmid recovery. This process was repeated a further two times with sanitisation in 0.5 M NaOH between each run.

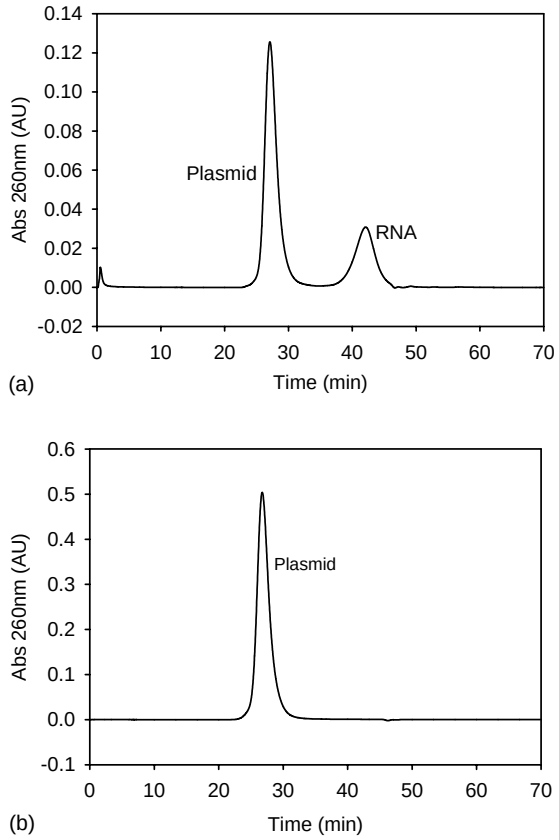

Fig. 3. Size-exclusion HPLC analysis of loading material (a) and eluate (b) from anion-exchange chromatography on Fractogel EMD DEAE under optimised conditions.

4. Discussion

Anion-exchange chromatography as a tool for the purification of pharmaceutical-grade plasmid DNA suffers from several drawbacks. Loading crude lysate containing large amounts of impurities such as chromosomal DNA, RNA, proteins and endotoxins directly on an anion-exchanger is not recommended and primary purification involving precipitation or filtration steps is essential. Resolution from impurities with a similar chemical composition and structure (chromosomal DNA, RNA) or charge (endotoxin) is limited. Low capacity for plasmid of most commercial chromatography media is also a concern for large-scale manufacture. Bovine-derived RNase is commonly added to degrade RNA but is not recommended by regulatory agencies. The absence of added RNase in the purification process results in very high RNA levels: about 25 times the amount of plasmid DNA by weight in the clarified lysate. We have developed a plasmid purification protocol incorporating an anion-exchange polishing step that addresses these issues.

First, RNA levels were reduced by precipitation with calcium chloride to remove high molecular weight molecules [31] followed by tangential flow filtration to clear low molecular weight RNA [32]. As a result the ratio of RNA to plasmid DNA in the load of the chromatography column was decreased to 28:72 wt.%. RNA removal steps prior to chromatography were necessary not only to reduce the RNA burden on the column but also to allow better control of loading buffer conditions which is not possible when loading lysate directly.

Careful selection of the anion-exchange chromatography adsorbent is essential. But before starting a screening experiment, it was important to have the right conditions for optimal resolution of plasmid from RNA. Since it would have been too time-consuming to scout each adsorbent for the effect of pH, only one weak and one strong anion-exchanger were investigated (Fractogel DEAE and TMAE, respectively). As expected, pH had no significant effect on plasmid retention on the strong anion-exchanger but was an important factor in the selectivity of the weak anion-exchanger. Only at pH 7.5 or less was baseline resolution of RNA from plasmid achieved. As pH was increased above 7.5, not only was plasmid retention affected but resolution was also lost. As a result, phosphate buffer at pH 7.0 was used to conduct all subsequent experiments.

A wide range of anion-exchangers (18 in total) was tested under the same conditions of elution with a linear salt gradient. Only four gave baseline separation of RNA from plasmid: Q Sepharose FF, Q Ceramic HyperD F, Fractogel DEAE and POROS 50HQ. We found no obvious explanation for the high selectivity of these adsorbents under the same operating conditions: the heterogeneity of functional groups (DEA and Q) and matrix (agarose, ceramic, methacrylate and polystyrene divinylbenzene) cannot account for their performance.

The screening experiments show that both plasmid and RNA bind to the chromatography support but that RNA has a lower affinity. Both plasmid and RNA are polyanionic molecules and interact with the positively charged functional groups on the chromatography support through the negatively charged phosphate residues on the nucleic acid backbone. With nucleic acids, overall charge is a function of size and RNA molecules are therefore expected to be displaced ahead of the plasmid. However, high molecular weight RNA molecules have been found to co-elute with plasmid [24,26]. It is thought that the supercoiled conformation of plasmids reduces the overall charge density and may be responsible for early elution. RNA reduction steps before the chromatography step are therefore essential (Fig. 4).

Anion-exchange chromatography may be considered for large-scale processing only if capacity for plasmid is sufficient. The typical anion-exchanger capacity of 40–200 μg/ml quoted by Prazeres et al. [28] is well below that for globular proteins (up to >100 mg/ml) and is a challenge for scale-up. We have found that dynamic capacity for plasmid can be increased significantly by using a suitable salt concentration in the load that prevents binding of RNA to the adsorbent, therefore maximising plasmid capture. These loading conditions were optimised for each of the four selected anion-exchangers and breakthrough experiments conducted to determine capacity. Q Sepharose

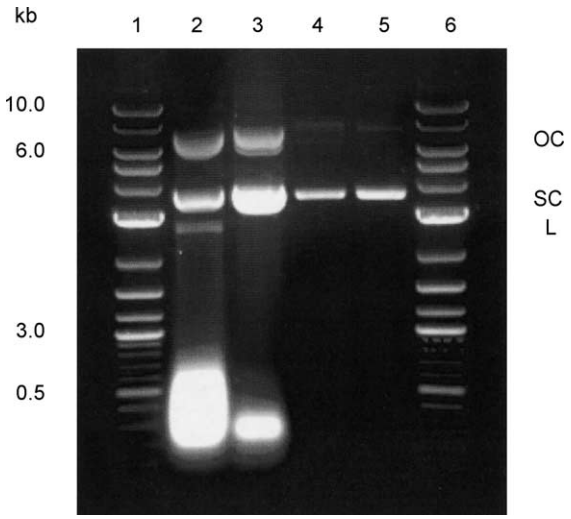

Fig. 4. Analysis of RNA removal by electrophoresis on a 0.8% agarose gel: molecular weight markers (lanes 1 and 6); clarified lysate (lane 2); lysate post calcium chloride precipitation (lane 3); TFF retentate (lane 4), and Fractogel DEAE eluate (lane 5). Plasmid isoforms include open circular (OC), supercoiled (SC), and linear (L).

FF had a low capacity (<1 mg/ml) compared with POROS 50HQ (2.1 mg/ml), Fractogel DEAE (3 mg/ml) and especially Q Ceramic HyperD F (>5.3 mg/ml). Most commercial anion-exchangers were designed for the purification of globular proteins and have a porous bead structure with pore sizes of up to 2000 Å. Supercoiled plasmids are several thousands of angstroms in length and are therefore essentially excluded from the pores [22–28]. Binding is achieved mostly through interaction with functional groups on the surface of the beads resulting in low capacity. This is the case for the Q Sepharose FF adsorbent with a pore size of about 1900 Å. POROS 50HQ exhibits large through-pores (6000–8000 Å) which may allow some penetration of plasmids [25]. Fractogel DEAE has a "tentacular" structure where functional groups are located at the end of long arms grafted to the bead surface that circumvent the steric hindrance caused by large plasmid molecules [25]. Q Ceramic HyperD F adsorbent is made of a polymeric cross-linked hydrogel homogeneously distributed within a porous ceramic material. It is unlikely that plasmid molecules penetrate the hydrogel contained within the ceramic shell. One explanation for high capacity is that plasmids not only bind to the hydrogel coating the surface of the particle but also to the hydrogel filling the pores although surface ruggedness has also been mentioned as a possible source of high capacity [33].

When the three anion-exchangers selected for their high capacity and selectivity were loaded to capacity under optimal conditions of high loading salt concentration, plasmid free of contaminating RNA (as determined by size-exclusion HPLC) was eluted from the column while RNA was cleared in the column breakthrough. Plasmid recovery was a problem with Q Ceramic HyperD F: only 72% could be eluted from the column. Significant peak tailing during gradient elution and removal of material absorbing at 254 nm during sanitisation with sodium hydroxide suggests diffusion limitations. In terms of robustness, Fractogel DEAE showed a wider range of operation: selectivity at high capacity was achieved within a wider range of loading salt concentration than with POROS 50HQ or Q Ceramic HyperD F. Plasmid recovery and reproducibility over three repeat runs was also excellent.

In conclusion, one anion-exchanger (Fractogel DEAE) used under optimal conditions met the criteria of purity, high capacity, high recovery, robustness and reproducibility required for the large-scale manufacture of pharmaceutical-grade plasmid DNA. Anion-exchange chromatography was the polishing step in a downstream process with no added RNase. Primary purification was achieved by precipitation and TFF to reduce RNA levels before chromatography.

Acknowledgements

We would like to thank Cora Henwood for her comments and Hella Bosteels for the fermentation.

References

[1] A. Mountain, Trends Biotechnol. 18 (2000) 119.
[2] H.L. Robinson, Int. J. Mol. Med. 4 (1999) 549.
[3] M. Schleef, Biotechnology, vol. 5a, Wiley, Weinheim, 1999, p. 443.
[4] US Food and Drug Administration, Points to consider on plasmid DNA vaccines for preventive infectious disease indications, US Food and Drug Administration, Rockville, MD, USA, 1996.
[5] US Food and Drug Administration, Guidance for industry: guidance for human somatic cell therapy and gene therapy, US Food and Drug Administration, Rockville, MD, USA, 1998.
[6] N.A. Horn, J.A. Meek, G. Budahazi, M. Marquet, Hum. Gene Ther. 6 (1995) 565.
[7] J.K. McClung, R.A. Gonzales, Anal. Biochem. 177 (1989) 378.
[8] G.J. Raymond, P.K. Bryant, A. Nelson, J.D. Johnson, Anal. Biochem. 173 (1988) 125.
[9] R.B. Sparks, J.H. Elder, Anal. Biochem. 135 (1983) 345.
[10] A.P. Green, G.M. Prior, N.M. Helveston, B.E. Taittinger, X. Lu, J.A. Thompson, BioPharm 10 (1997) 52.
[11] M.M. Diogo, J.A. Queiroz, G.A. Monteiro, D.M.F. Prazeres, Anal. Biochem. 275 (1999) 122.
[12] M.M. Diogo, J.A. Queiroz, G.A. Monteiro, S.A.M. Martins, G.N.M. Ferreira, D.M.F. Prazeres, Biotechnol. Bioeng. 68 (2000) 576.
[13] M.M. Diogo, S. Ribeiro, J.A. Queiroz, G.A. Monteiro, P. Perrin, N. Tordo, D.M.F. Prazeres, Biotechnol. Lett. 22 (2000) 1397.
[14] A. Colman, M.J. Byers, S.B. Primrose, A. Lyons, Eur. J. Biochem. 91 (1978) 303.
[15] T.R. Johnson, J. Ilan, Anal. Biochem. 132 (1983) 20.
[16] R. Giovannini, R. Freitag, Biotechnol. Bioeng. 77 (2002) 445.
[17] M.J. Carter, I.D. Milton, Nucleic Acids Res. 21 (1993) 1044.
[18] R. Boom, C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim-Van Dillen, J. Van der Noordaa, J. Clin. Microbiol. 28 (1990) 495.
[19] R. Lakshmi, V. Baskar, U. Ranga, Anal. Biochem. 271 (1999) 109.
[20] P. Wils, V. Escriou, A. Warnery, F. Lacroix, D. Lagneaux, M. Ollivier, J. Crouzet, J.-F. Mayaux, D. Scherman, Gene Ther. 4 (1997) 323.

[21] T. Schluep, C.L. Cooney, Nucleic Acids Res. 26 (1998) 4524.
[22] D.M.F. Prazeres, T. Schluep, C. Cooney, J. Chromatogr. A 806 (1998) 31.
[23] R.N. Hines, K.C. O'Connor, G. Vella, W. Warren, BioTechniques 12 (1992) 430.
[24] G. Chandra, P. Patel, T.A. Kost, J.G. Gray, Anal. Biochem. 203 (1992) 169.
[25] I. Theodossiou, M. Sondergaard, O.R.T. Thomas, Bioseparation 10 (2001) 31.
[26] G.N.M. Ferreira, J.M.S. Cabral, D.M.F. Prazeres, Bioseparation 9 (2000) 1.
[27] E. Thwaites, S.C. Burton, A. Lyddiatt, J. Chromatogr. A 943 (2002) 77.
[28] D.M.F. Prazeres, G.N.M. Ferreira, G.A. Monteiro, C.L. Cooney, J.M.S. Cabral, Trends Biotechnol. 17 (1999) 169.
[29] G.D. Cooke, R.M. Cranenburgh, J.A.J. Hanak, P. Dunnill, D.R. Thatcher, J.M. Ward, J. Biotechnol. 85 (2001) 297.
[30] H.C. Birnboim, J. Doly, Nucleic Acids Res. 7 (1979) 1513.
[31] A. Eon-Duval, K. Gumbs, C. Ellett, Biotechnol. Bioeng. 83 (2003) 544.
[32] A. Eon-Duval, R.H. MacDuff, C.A. Fisher, M.J. Harris, C. Brook, Anal. Biochem. 316 (2003) 66.
[33] J. Coffman, E. Boschetti, Bioseparation and Bioprocessing, vol. 1, Wiley, Weinheim, 1998, p. 157.