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Supercoiled plasmid DNA: selective purification by thiophilic/aromatic adsorption

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Abstract

Separation of the different plasmid isoforms is a major challenge in purifying plasmid DNA. We describe a new type of biochemical interaction that occurs in the presence of high concentrations of lyotropic salt and results in the selective adsorption of supercoiled plasmid DNA to aromatic thioether ligands. Under well-defined conditions, these ligands are capable of separating supercoiled plasmid DNA (ccc) from its isoform, i.e. open circular (oc) form. Integrated in a process, preceded by group separation and followed by anion-exchange chromatography, this new purification method may facilitate the production of highly purified supercoiled plasmid DNA for use in gene therapy and DNA vaccine applications.

Keywords: Supercoiled plasmid; Gene therapy; Purification; DNA

1. Introduction

In the past decade, the administration of therapeutic genes to patients has become a reality in many clinical trials for preventing or treating various diseases [1]. Non-viral vectors would be preferred in clinical applications to minimize the risk of viral infection [2]. This increases the demand for highly purified plasmids for use in gene therapy and plasmid-based vaccines. The stringent guidelines and rules set forth by health authorities [3–8] further require highly purified and homogeneous preparations of supercoiled plasmid DNA for clinical applications.

The purification of plasmid DNA from cell or tissue extracts involves essentially the removal of RNA. This can be achieved using a variety of separation methods, e.g. heat treatment [9] or digestion with RNase followed by dialysis or gel filtration. Other techniques used include adsorption on hydroxyapatite [10], centrifugation in cesium chloride gradients [11], ion-exchange chromatography [12], electrophoresis [13], gel filtration [14], triple helix affinity chromatography [15,16], and hydrophobic interaction chromatography [17]. In practice, only a few of these techniques, viz. adsorption on hydroxyapatite, gel filtration, affinity chromatography and ion-exchange [15,17,18], are useful for small-scale preparative applications. Moreover, the purified plasmid DNA obtained was not homogeneous with

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respect to the shape of the plasmid, but was a mixture of the closed circular covalently linked (ccc) and the open circular (oc) forms of the plasmid DNA. The most desirable form of plasmid DNA is the native supercoiled (ccc) form because of its higher degree of infectivity [19,20]. In order to obtain homogeneous preparations of supercoiled plasmid DNA in sufficient quantities for non-analytical applications, efforts in the past were concentrated upon refinements of sample preparation techniques that would minimize the formation of open circular isoforms of plasmid DNA. However, this approach was difficult to reproduce and the quality of the plasmid DNA thus produced did not meet the stringent specifications set forth for the final product [21]. This situation clearly indicated the need for the development of a new and robust purification protocol for supercoiled plasmid DNA that would significantly reduce the tedious efforts for optimization of sample preparation techniques.

In our continued effort to devise suitable separation methods that could be adopted for the large-scale purification of the ccc form of plasmid DNA for applications in gene therapy, we re-examined the suitability of thiophilic interaction chromatography performed in the presence of varying concentrations of so-called structure-forming salts. This approach was motivated by the fact that high concentration of salts may result in the compaction of the nucleic acids leading to conformational changes that could be exploited for their purification. To this end, we tested a range of commercially available, and newly developed, thioether containing adsorbents that were operated in the presence of high concentrations of ammonium sulfate. We found that a thioether-type adsorbent [22,23] based on 2-mercaptopyridine coupled to Sepharose™ 6 Fast Flow (Amersham Biosciences, Uppsala, Sweden) gave the best results. In this report a three-step, efficient, robust, reproducible and scalable chromatographic procedure for the purification of supercoiled plasmid DNA from alkaline lysate of *E. coli* cells is described. The procedure leads not only to the efficient removal of RNA (by far the major contaminant in crude plasmid preparations) but also reduces other critical impurities such as proteins, chromosomal *E. coli* DNA and endotoxins to levels that correspond to the stringent recommendations set forth by health and regulatory authorities. Data to show the homogeneity of purified supercoiled plasmid DNA are presented.

2. Experimental

2.1. Plasmid DNA

A 6125 base pair recombinant pUC19 plasmid was used for this study. The plasmid was transfected and grown in *E. coli* TG1α by well-established protocol [24]. Clarified alkaline lysate was prepared according to Horn et al. [25].

2.2. Analytical methods

Peaks were judged to be non-nucleic acid, open circular plasmid DNA, supercoiled plasmid DNA and RNA, respectively, by their 260/280 nm absorption ratios, by laser induced fluorescence capillary gel electrophoresis [26] and by their electrophoretic mobility pattern on 0.8% agarose gel electrophoresis with ethidium bromide staining [24]. Ready to use 0.8% agarose gels with ethidium bromide included in the gel (E-gel, Invitrogen, Groningen, The Netherlands) and 1 kb molecular mass standards (Amersham Biosciences, Uppsala, Sweden) were used.

2.3. Endotoxin model

To estimate the elution profile of endotoxins in the chromatographic method samples were spiked with 2.5 μg/ml of a fluorescently labeled 10 000 Da lipopolysaccharide (LPS) isoform from *E. coli* (isotype 055:B5; Molecular Probes, Leiden, The Netherlands). During chromatography fractions were collected and fluorescence was subsequently recorded according to the manufacturer's instructions. In a different set of experiments, the distribution of endotoxins was tested with the *Limulus* amebocyte lysate (LAL) test (Coatech, Kungsbacka, Sweden) and found to confirm the elution profile as seen with fluorescently labeled LPS (not shown).

2.4. Chromatography system

All column chromatography experiments were performed using ÄKTA™ explorer (Amersham Biosciences, Uppsala, Sweden).

2.5. Chromatographic media

Sepharose 6 Fast Flow and SOURCE™ 30Q were from Amersham Biosciences (Uppsala, Sweden). All ligands (inserts in Figs. 2 and 3) were coupled to epoxy-activated Sepharose 6 Fast Flow by established methods [27]. The ligand concentration was chosen to be in excess over available binding sites and after washing, the amount of coupled ligand was checked by elemental analysis (sulfur or nitrogen).

2.6. Buffer solutions

The buffers used are described in the text or in the figure legends. The following buffers are referred to as buffer A and buffer B:

Buffer A: 2.0 *M* $(NH_4)_2SO_4$, 10 m*M* EDTA, 100 m*M* Tris–HCl, pH 7.0.

Buffer B: 2.0 *M* NaCl, 2.0 *M* $(NH_4)_2SO_4$, 10 m*M* EDTA, 100 m*M* Tris–HCl, pH 7.0.

2.7. Group separation

Sepharose 6 Fast Flow was used as a gelfiltration medium for group separation of plasmid DNA from RNA. Sepharose 6 Fast Flow was packed in columns and equilibrated in buffer A if nothing else is indicated. One liter of clarified alkaline lysate, prepared according to Ref. [25] containing plasmid DNA (here 6125 base pairs), was group separated on Sepharose 6 Fast Flow packed in an INdEX™ 100 column (Amersham Biosciences, Uppsala, Sweden) (10 cm diameter×25 cm bed height) at 30 cm/h in order to remove RNA and change the buffer to buffer A (not shown). Group separated material containing supercoiled and open circular DNA equilibrated in buffer A was subsequently used in Figs. 2–4 (and eventually in Fig. 5).

2.8. Thiophilic aromatic chromatography

Ligands were coupled to Sepharose 6 Fast Flow as described above. 4.6 mm Omegachrom™ columns (Upchurch Scientific, Oak Harbor, WA, USA) packed to 15 cm bed height were used in Figs. 2–4. Group separated samples containing supercoiled and open circular DNA were loaded in buffer A if nothing else is indicated and eluted as described in the figure legends. The flow-rate was 0.5 ml/min (180 cm/h) throughout Figs. 2–4.

2.9. Anion-exchange

Samples eluted from the previous step (thiophilic aromatic chromatography) containing only supercoiled plasmid DNA were concentrated and $(NH_4)_2SO_4$ was removed by binding the sample to and subsequent elution from a SOURCE 30Q column. Also for anion-exchange 4.6 mm Omegachrom columns packed to 15 cm bed height were used. The flow-rate was 0.5 ml/min (180 cm/h). Samples were first diluted in order to bind to the anion-exchange column. Immobilized sample was washed and subsequently eluted as described in the legend to Fig. 5.

2.10. Purification process

An XK 50/30 column (5 cm diameter) packed to 25 cm bed height was used for cumulative group separation of the clarified bacterial lysate at 10 ml/min (30 cm/h) in buffer A. Thiophilic aromatic chromatography was performed on an XK 16/20 column (1.6 cm diameter) packed to 12 cm bed height with Sepharose 6 Fast Flow with 2-thiopyridine as ligand. The sample was applied in buffer A and eluted with 5 column volumes of buffer B at 4 ml/min (120 cm/h). Anion-exchange was performed on an XK 16/20 column packed to 12 cm bed height, also at 4 ml/min (120 cm/h). The sample was diluted four times with water before loading on the column, washed with 10 column volumes of 0.4 *M* NaCl, 10 m*M* EDTA, 100 m*M* Tris–HCl, pH 7.0 and eluted in a gradient of 5 column volumes to 1.0 *M* NaCl, 10 m*M* EDTA, 100 m*M* Tris–HCl, pH 7.0. All analyses in Table 1 were performed by PlasmidFactory (Bielefeld, Germany,

Table 1
Analysis of purified plasmid DNA

	Test/sample							
	Capillary gel electrophoresis		TaqMan-PCR [plasmid] (μg/ml)	A_{260} [plasmid] (μg/ml)	LAL test [endotoxins] (EU/mg plasmid)	Ribogreen [RNA] (μg/mg plasmid)	BCA [proteins] (μg/mg plasmid)	TaqMan-PCR gDNA (μg/mg plasmid)
	ccc plasmid DNA (%)	oc plasmid DNA (%)						
Clarified lysate	54	46	13		150 000	5200	160 000	150
Sepharose 6 Fast Flow	71	29	14	24	4800	<200	2300	6
Thiophilic/aromatic chromatography	96	4	140	240	470	<2	58	6
SOURCE 30Q	97	3	284	350	9	<0.2	<3	2

http://www.plasmidfactory.com). The 5′nuclease (TaqMan) assay (real-time PCR; Applied Biosystems, Foster City, CA, USA) [28] was used to quantitate plasmid DNA and genomic DNA (gDNA). RNA was quantitated with Ribogreen (Molecular Probes, Eugene, OR, USA) [29].

3. Results

3.1. Group separation

Preliminary experiments indicated that addition of salt enabled the separation of plasmid DNA from RNA by gelfiltration on Sepharose 6 Fast Flow (used as a medium for size-exclusion chromatography). Plasmid DNA was found to elute in the void volume of the column. However, in the absence of enough salt, the elution peaks of plasmid DNA and RNA were congruent. In the presence of more than 1.5 *M* $(NH_4)_2SO_4$, plasmid DNA was completely separated from RNA (Fig. 1). Please note that in contrast to conventional gelfiltration as used for polishing where it is not recommended to load more than 2% of the column volume, the sample volume in Fig. 1 exceeds 30% of the column volume. This makes the group separation as applied in Fig. 1 a powerful method not only in terms of its ability to separate DNA from RNA but also in terms of mass (volume) throughput, in particular if it is combined with a concentrating step such as diafiltration.

3.2. Thiophilic aromatic chromatography

In a systematic search for a suitable column chromatography medium for the selective purification of supercoiled plasmid DNA several ligands were tested in the presence of a high concentration of $(NH_4)_2SO_4$. In Fig. 2a–c, the ligand structures of three media that selectively interacted with the supercoiled DNA and the elution patterns obtained using these media are shown. Three other structures that did not show specific adsorption properties and which did not retain plasmid DNA under the conditions used are also shown (Fig. 2d–f). The results indicated that ligands containing an aromatic ring as well as a thioether moiety retained supercoiled plasmid DNA in the presence of 2 *M* $(NH_4)_2SO_4$. Substituting the aromatic moiety with aliphatic structures led to a chromatography medium that did not bind supercoiled plasmid DNA under above described conditions (Fig. 2d). Moreover, exchanging the sulfur atom of the thioether bond for nitrogen (Fig. 2e) or oxygen (Fig. 2f) resulted in no binding of supercoiled plasmid DNA. Sepharose 6 Fast Flow derivatised with 2-mercaptopyridine (Fig. 2a) as well as with 2-pyridine ethanethiol (Fig. 2b), benzenethiol (Fig. 2c) and benzenemethanethiol (not shown) retained supercoiled plasmid DNA. The presence of only the aromatic ring does not appear to be sufficient for the interaction of the immobilized ligands with plasmid DNA (Fig. 2e and f).

If the minimal requirement, i.e. an aromatic ring and at least one thioether moiety, was maintained,

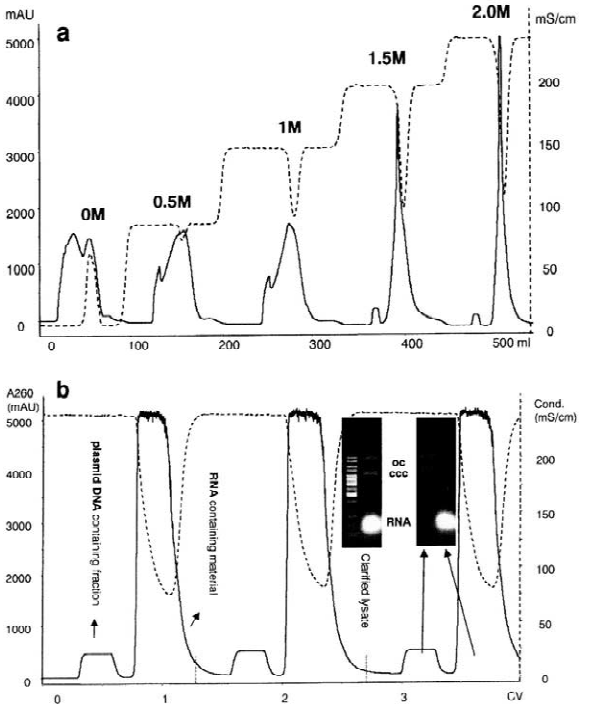

Fig. 1. Group separation of clarified bacterial cell lysate. In panel a, the sample was loaded in batches of 15 ml on a XK16/40 column (Amersham Biosciences, 1.6 cm diameter) packed to 30 cm (packed bed volume=60 ml) with Sepharose 6 Fast Flow. Between each batch the column was equilibrated with buffer containing 10 m*M* EDTA, 100 m*M* Tris–HCl, pH 7.0, and the indicated amount of $(NH_4)_2SO_4$. The flow-rate was 6 ml/min (180 cm/h). In panel b, the same sample was loaded in batches of 150 ml on a larger XK 50/30 column packed to 20 cm (packed bed volume=393 ml) with Sepharose 6 Fast Flow equilibrated with buffer A. The flow-rate was 10 ml/min (30 cm/h). The contents of the eluting peaks are indicated. An ethidium bromide stained 1% agarose gel electrophoresis photo is inserted indicating the polynucleotide composition of the crude sample and the group separated material. Elution profiles were recorded at 260 nm (solid line). Conductivity is indicated with hatched line.

substitutions on the aromatic ring structure with additional sulfur-, oxygen- or nitro-containing groups, or up to two halogens, did not adversely affect the binding of supercoiled plasmid DNA to such media (Fig. 3a–e). However, electron-donating substitutions on the aromatic ring appeared to reinforce interactions between plasmid DNA and the ligand. Thus supercoiled plasmid DNA eluted at a lower conductivity from media containing the structures shown in Fig. 3b–e compared to that in Fig. 3a. The structures, shown in Fig. 3b,d and e, gave elution profiles where open circular plasmid DNA was also retained in a buffer containing 2 *M* $(NH_4)_2SO_4$ and eluted before supercoiled plasmid DNA in the gradient. Multiple substitutions on the aromatic ring with halogens changed the binding characteristics and elution profile of supercoiled plasmid DNA, but the pentafluorobenzenethioether ligand still appeared to retain supercoiled plasmid DNA (Fig. 3f). It thus appears that changing the electronegativity of the substituted groups may affect the salt concentration needed for total plasmid DNA binding and also affect the selectivity for the supercoiled plasmid DNA. However, it is clear that both the aryl-group and the sulfur atom are required for selective interaction of supercoiled plasmid DNA with the immobilized ligand (Fig. 2).

A 6125 base pair recombinant plasmid (pUC19) was used for this study. The plasmid was transfected and grown as described in the experimental section. Thiophilic aromatic chromatography as in Figs. 2 and 3 has been tested and found to work well with several plasmids in the size range 3–14 kb pairs (not shown).

In large-scale purification processes, it is desirable to remove traces of contaminating proteins and RNA and not least endotoxins from the plasmid preparation. Our experiments indicated a potential risk of co-elution of traces of endotoxins with the elution of supercoiled plasmid DNA when applying a gradient to lower conductivity as in Figs. 2 and 3. Interestingly, we found that an alternative mode of elution completely eliminated the risk of co-elution. Plasmid DNA could be eluted from the aromatic thioether medium by a gradient to 2 *M* NaCl in the buffer containing high amounts of $(NH_4)_2SO_4$ (Fig. 4). By maintaining the concentration of $(NH_4)_2SO_4$ while increasing the NaCl-concentration, we were able to specifically elute plasmid DNA. Trace amounts of protein, RNA and endotoxins could not be eluted by the addition of this salt, even if the NaCl concentration was increased to saturation (≈3 *M*), and had to be removed from the chromatography medium by decreasing the $(NH_4)_2SO_4$ concentration. As mentioned above, the structures in Fig. 3b–f presented increased binding of plasmid DNA (compared

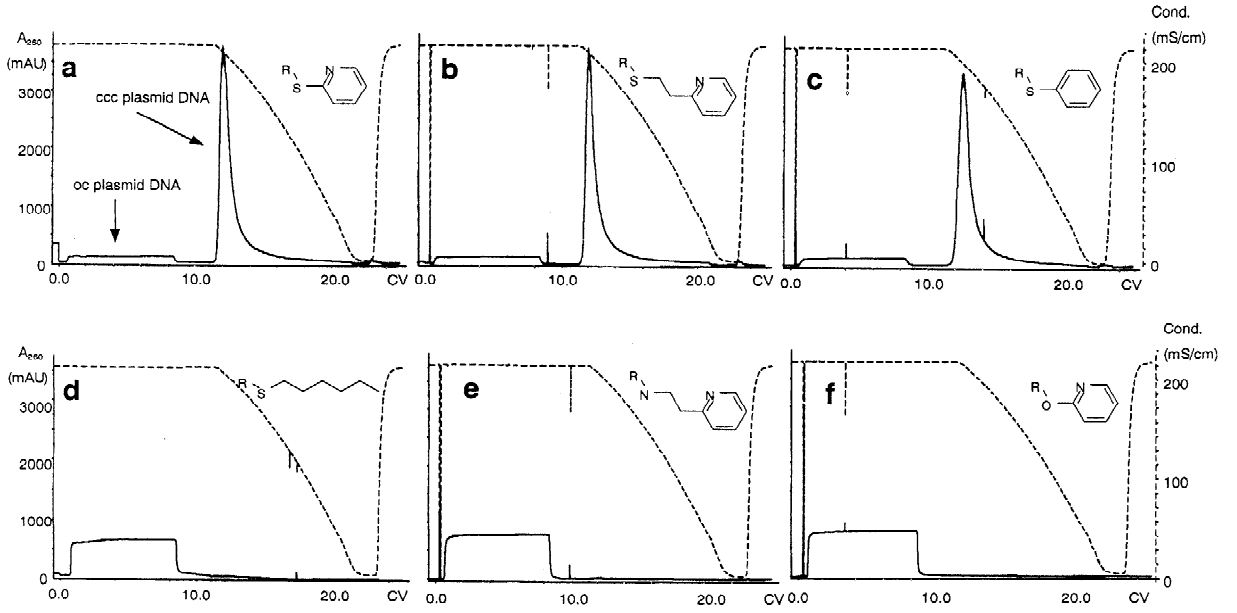

Fig. 2. Chromatograms obtained after binding and elution of plasmid DNA preparations on different media. Ten ml of the sample containing supercoiled and open circular plasmid DNA was loaded on columns containing Sepharose 6 Fast Flow (R) derivatised with the indicated ligands (insert) and equilibrated in buffer A. Elution profiles were recorded at 260 nm (solid line). Conductivity is indicated with hatched line. The columns did not retain open circular forms of plasmid at this $(NH_4)_2SO_4$ concentration (see text and Fig. 4). Bound supercoiled plasmid (6125 base pairs) could be eluted from the column with a linear gradient to H_2O (conductivity, hatched), as illustrated in panels a–c. The plasmid DNA was not retained on Sepharose 6 Fast Flow derivatised with the ligands shown in panels c–f.

to Fig. 3a). Consequently, attempts to elute plasmid DNA from those columns even with gradients up to saturated NaCl in 2 *M* $(NH_4)_2SO_4$ failed. This was also the case with the structure shown in Fig. 2c (benzenethioether) that in addition appeared to have a slightly higher affinity for plasmid DNA than the structure shown in Figs. 2a and 3a (2-mercaptopyridine).

The binding capacity was over one mg supercoiled plasmid DNA/ml resin. The step yield for the purification of supercoiled plasmid DNA by thiophilic aromatic chromatography was 70%, but could drastically be increased by incubating the column for 15 min with elution buffer, indicating the slow kinetics of the elution.

In summary, aromatic thiol structures (Figs. 2 and 3) were identified that, when coupled to Sepharose 6 Fast Flow as thioethers, show the ability to separate the supercoiled form of plasmid DNA from open circular forms and other contaminants. This was best accomplished by a combination of lyotropic salt-promoted adsorption [22,23] and subsequent elution of the bound materials with an increase in NaCl in the column equilibration buffer (Fig. 4).

3.3. Anion-exchange

The group separation described above removed the majority of host cell material including RNA and resulted in a sample containing essentially only plasmid DNA (open circular and supercoiled). Thiophilic aromatic chromatography further purified the sample to a homogenous preparation of supercoiled plasmid DNA with the removal of impurities. However, a final polishing step was still needed to concentrate the sample and to remove ammonium sulfate and potential traces of endotoxins. This may be particularly advantageous in clinical applications. Anion-exchange is known for concentrating the sample and it has been used for removal of endotoxins [30]. In Fig. 5 the elution profile of a homogenous supercoiled plasmid DNA sample prepared by thiophilic aromatic chromatography on a column packed with a SOURCE 30Q anion-exchanger is

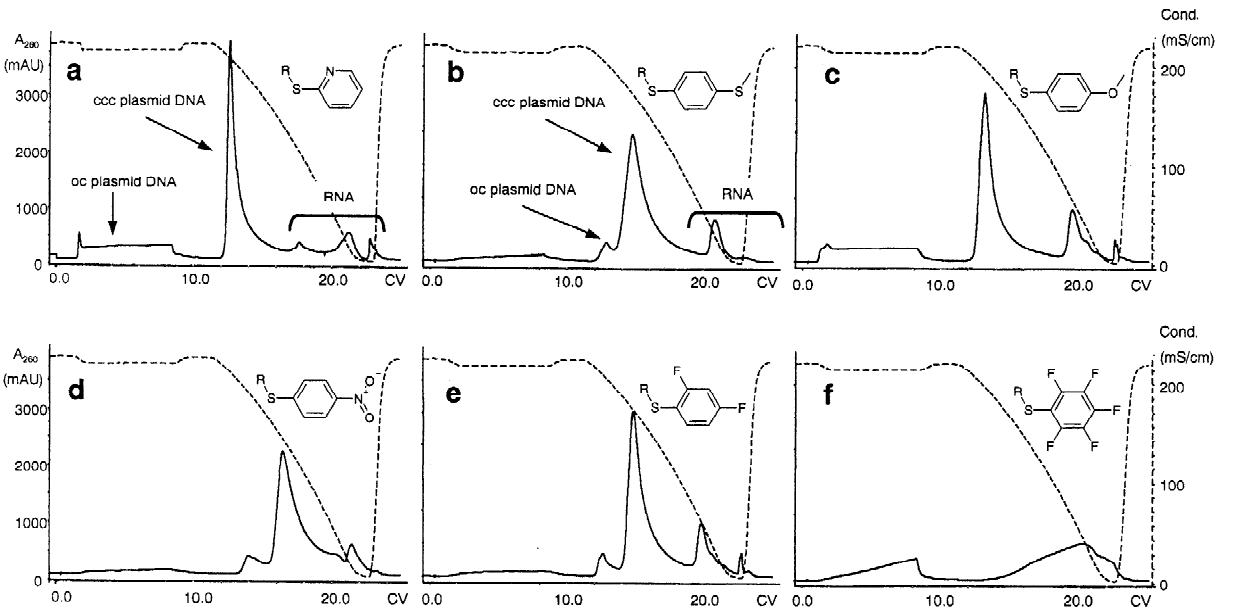

Fig. 3. Changing the electronegativity of the aromatic substituents. By changing the electronegativity of the aromatic substituents the binding and elution conditions for the different isoforms could be manipulated. For comparison of specificity the sample was spiked with RNA, which was bound and then eluted at very low conductivity values. Ligands (insert in each panel) were coupled to Sepharose 6 Fast Flow (R) as described in the experimental section. Columns, buffers, material, and experimental conditions were as described in the legend to Fig. 2 and in Section 2.

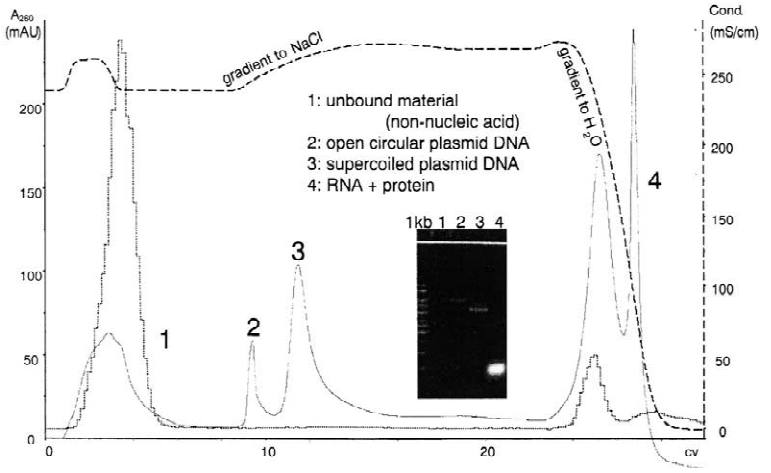

Fig. 4. Polynucleotide separation on Sepharose 6 Fast Flow with 2-thiopyridine as ligand. Five ml of sample was spiked with crude clarified alkaline lysate (containing RNA) and 2.5 μg/ml of a fluorescently labeled LPS and the $(NH_4)_2SO_4$ concentration was adjusted to 2.25 *M*. This sample was separated on a column packed with 2-mercaptopyridine derivatised Sepharose 6 Fast Flow (same as in Figs. 2a and 3a) at 2.25 *M* $(NH_4)_2SO_4$ (higher than in Figs. 2 and 3). Elution profile was recorded at 260 nm (solid line). Conductivity is indicated with hatched line. Open circular plasmid DNA eluted early in a NaCl gradient to 2 *M* NaCl, followed by supercoiled plasmid DNA. RNA (and contaminating protein) and LPS (dotted) eluted only after partial removal of lyotropic salts by a gradient to lower conductivity (water). A 0.8% agarose gel electrophoresis picture (insert) is showing the electrophoretic mobility of polynucleotides in fractions collected from each peak in a parallel run, where more sample was loaded to enable detection with Ethidium Bromide.

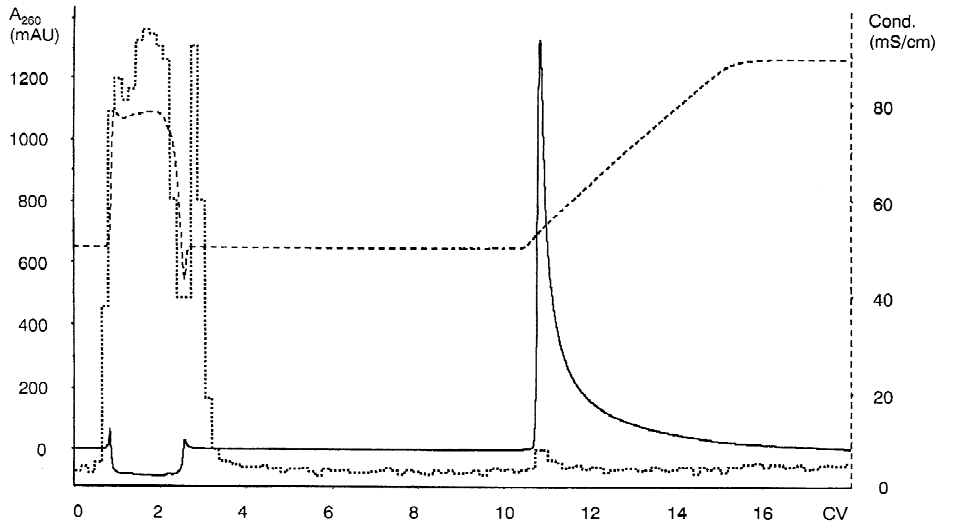

Fig. 5. Anion-exchange chromatography. Five ml of a sample containing only supercoiled plasmid DNA (purified by group separation and thiophilic aromatic chromatography) was spiked with 2.5 μg/ml of a fluorescently labeled LPS. The sample was separated on a column packed with SOURCE 30Q. The sample was diluted 4 times with water before loading on the column. The column was washed with 10 column volumes of 0.4 *M* NaCl, 10 m*M* EDTA, 100 m*M* Tris–HCl, pH 7.0 before gradient elution with 5 column volumes to 1.0 *M* NaCl, 10 m*M* EDTA, 100 m*M* Tris–HCl, pH 7.0. Elution profile was recorded at 260 nm (solid line). Conductivity is indicated with hatched line. Fractions were collected and fluorescence was recorded as described above.

shown. To illustrate the elution pattern of endotoxins on anion-exchange, the sample was spiked with fluorescently labeled endotoxins. Endotoxins do not bind the anion-exchange column at the used NaCl concentration (0.4 *M*) and is removed during washing of unbound material from the column (Fig. 5).

3.4. Purification process

The three purification steps described above, group separation, thiophilic aromatic chromatography and anion-exchange chromatography may be combined and as such encompasses a purification process. Thus this purification process was used to purify plasmid DNA from a larger volume of clarified bacterial lysate. The 275 g of *E. coli* cell paste containing a 6125 base pairs plasmid from a non-optimized fermentation resulted in 7 l of clarified bacterial lysate as starting material for the purification process. The purification process was performed under normal, non-GMP laboratory conditions. The prerequisites were that the resulting clarified bacterial lysate contained large amounts of endotoxins and only low amounts of plasmid DNA (approximately 10 μg/ml). Only 54% of the plasmid DNA in the starting material was of the desired supercoiled conformation. After the three purification steps 97% of the plasmid DNA was of supercoiled conformation and the level of endotoxins was reduced to 9 EU/mg (Table 1). These figures and the data for RNA and genomic DNA traces are better (with margin) than corresponding data for plasmid DNA preparations that have already been approved for clinical trials in gene therapy and vaccine applications [31]. Thus the content of genomic DNA after three purification steps in Table 1 was <2 μg/mg plasmid DNA, while <5 and <10 μg genomic DNA/mg plasmid DNA have been reported in approved preparations. The content of RNA in Table 1 was less than 0.2 μg/mg plasmid DNA (detection limit using Ribogreen assay). The current recommendation of regulatory authorities is that RNA should be invisible on ethidium bromide stained 0.8% agarose gel electrophoresis, Under these conditions, fairly high amounts of RNA remain undetected since RNA stains less intensively than DNA

(see Fig. 4). Protein content was below the detection limit in approved preparations and in Table 1.

4. Discussion

In contrast to protein separations, it appears difficult to separate polynucleotides on the basis of differences in their physical properties. Because of their polymeric composition of only four building blocks with similar characteristics, RNA, genomic DNA and isoforms of plasmid DNA appear to be similar by physical and chemical parameters. The group separation and the thiophilic aromatic chromatography approach described here overcome problems faced by traditional chromatography methods.

Because of their double-stranded circular shape, the influence of the structuring salts on the plasmid DNA will be less pronounced than on the RNA. This results in a different compacting effect of the RNA, leading to an efficient separation of both polynucleotides based on size-exclusion chromatography.

Thiophilic interactions between proteins and ligands are promoted by relatively high concentrations of water-structuring salts [23] and are probably a result of combined electron donor/acceptor type of interactions [32] and possibly a mixed mode, hydrophobic/thiophilic type of interactions [33]. There is evidence that the nature of the thiophilic aromatic interaction between polynucleotides and the ligands may be different. First, proteins interact also with aliphatic thioether ligands [23,32,33], while polynucleotides were not retained by such ligands (Fig. 2d). Second, an aromatic ring and a vicinal sulfur appeared to be the minimal requirements for the interaction, fairly independent of additional structural characteristics of the ligand (Figs. 2 and 3). This implies that the aromatic ring structure and the sulfur both contribute to the interaction between the ligand and the polynucleotides. Third, proteins could not be eluted from the column with a NaCl gradient (Fig. 4). The water structuring salts may have different compacting effects on RNA and DNA because of the inherent structural differences between these polynucleotides, thus facilitating the separation of plasmid DNA from RNA. It is tempting to speculate that the aromatic ring structure could participate in intercalating hydrophobic ($\pi-\pi$) type of interaction with the supercoiled, double helix form of plasmid DNA as proposed for topoisomerases [34,35]. The crucial role of the sulfur in the retention behavior remains elusive. It may participate with an electron donating (charge transfer) role in interaction with specific nucleotides. The fact that it is possible to elute associated supercoiled and open circular plasmid DNA from the column with a NaCl gradient (Fig. 4) per se may indicate ion-pair interactions.

The work presented here was all performed with one and the same recombinant plasmid of 6125 base pairs. However, as mentioned above, thiophilic aromatic chromatography has been tested and found to work well with several plasmids in the size range of 3–14 kb pairs. There is no reason to believe that the group separation and anion-exchange would behave differently with other sizes of plasmids. Larger plasmids are easily disrupted by shearing forces during culture and preparation of the bacterial lysate (for a recent review, see Ref. [36]). Therefore thiophilic aromatic chromatography is a useful technique for selective purification of those plasmids in their native supercoiled conformation. It may be easier to scale up to amounts used in clinical trials than high-pressure anion-exchange HPLC that have been reported to give good separation between open circular and supercoiled conformations of plasmid DNA in small scale (microgram) separations suitable for applications such as subcloning [37].

In conclusion, a systematic search for suitable ligands for purifying plasmid DNA led to the identification of a group of structures (aromatic thioethers) that require water structuring salts (e.g. $(NH_4)_2SO_4$) for their interaction with plasmid DNA. The interaction was structure-dependent, allowing topoisomer-selective purification of the desired supercoiled plasmid DNA. Furthermore, co-elution of contaminants (traces of protein and endotoxins) could be avoided by applying a linear gradient of NaCl for eluting the ccc form of plasmid DNA. This new column chromatography method is generic and can be integrated in a robust three-step purification process, highly suitable for the purification of supercoiled plasmid DNA for gene therapy and vaccine applications. Thiophilic aromatic chromatography may also be used in combination with other purification protocols whenever separation of oc and ccc forms of plasmid DNA is essential.

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