Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Chromatography A, 1069 (2005) 3–22

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

Chromatography of plasmid DNA

M.M. Diogo[a], J.A. Queiroz[b], D.M.F. Prazeres[a,*]

[a] Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal
[b] Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, 6200-001 Covilhã, Portugal

Abstract

Liquid chromatography plays a central role in process-scale manufacturing of therapeutic plasmid DNA (pDNA) for gene therapy and DNA vaccination. Apart from its use as a preparative purification step, it is also very useful as an analytical tool to monitor and control pDNA quality during processing and in final formulations. This paper gives an overview of the use of pDNA chromatography. The specificity of pDNA purification and the consequent limitations to the performance of chromatography are described. Strategies currently used to overcome those limitations, as well as other possible solutions are presented. Applications of the different types of chromatography to the purification of therapeutic pDNA are reviewed, and the main advantages and disadvantages behind each technique highlighted.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Plasmid DNA; Gene therapy; Purification; Chromatography; RNA; Genomic DNA; Endotoxins

Contents

1. Introduction 4
   1.1. Physical–chemical characteristics of pDNA 4
   1.2. Biosynthesis and primary isolation 4
2. The role of chromatography in pDNA purification 5
   2.1. Overview 5
   2.2. Limitations of pDNA chromatography and possible solutions 6
3. Applications of chromatography in pDNA purification 8
   3.1. Size-related chromatography 8
      3.1.1. Size-exclusion chromatography 8
      3.1.2. Slalom chromatography 10
   3.2. Charge-related chromatography 10
      3.2.1. Anion-exchange chromatography 10
      3.2.2. Hydroxyapatite chromatography 13
   3.3. Hydrophobicity-related chromatography 14
      3.3.1. Reversed-phase liquid chromatography 14
      3.3.2. Reversed-phase ion-pair chromatography 15
      3.3.3. Hydrophobic interaction chromatography 15
      3.3.4. Thiophilic adsorption chromatography 16

* Corresponding author. Tel.: +351 21 841 9133; fax: +351 21 841 9062.
E-mail address: prazeres@alfa.ist.utl.pt (D.M.F. Prazeres).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.09.050

3.4. Affinity-related chromatography . . . 17
3.4.1. Triple-helix affinity chromatography . . . 17
3.4.2. Protein–DNA affinity chromatography . . . 18
3.4.3. Immobilised metal affinity chromatography . . . 18
3.4.4. Boronate affinity chromatography . . . 18
3.4.5. Polymyxin B affinity chromatography . . . 18
4. Selection of chromatographic techniques . . . 18
5. Future trends . . . 20
Acknowledgements . . . 20
References . . . 20

1. Introduction

In the last two decades, the purification of plasmid DNA (pDNA) molecules became a routine procedure in laboratory and industrial environments. First, the introduction and expansion of recombinant DNA technology as a general molecular biology tool increased the demand for laboratory-scale protocols for the isolation of pDNA. Likewise, the more recent developments in therapeutic approaches, such as DNA vaccination and gene therapy have fostered the development of large-scale pDNA purification processes. Briefly, DNA vaccines based on genes can provide immunity against major-killers, such as malaria [1] while gene therapy relies on the introduction of one or more functional genes as a way of preventing, treating or curing genetic defects, such as cystic fibrosis, or acquired diseases like cancer and AIDS [2]. Although the therapeutic genes can be transported by several types of viral vectors [2], pDNA vectors have been considered to be safer, simpler to use and easier to produce on a large-scale [3,4]. The consequent use of pDNA vectors in pre-clinical and clinical trials of gene therapy and DNA vaccination has increased the need for large amounts of highly pure pDNA with a therapeutic or pharmaceutical-grade pDNA.

1.1. Physical–chemical characteristics of pDNA

Plasmids are covalently closed, double stranded, DNA molecules. Those intended for therapeutic applications contain human or non-human genes, and are very large (M_r >10^6, sizes in the μm range) when compared with proteins [5]. Each strand of a pDNA molecule is a linear polymer of deoxyribonucleotides linked by phosphodiester bonds. These phosphate groups are negatively charged for pH > 4 [6]. The winding of the two anti-parallel DNA strands around each other and around a common axis originates the classic right handed double helix structure, which is stabilised by Watson-Crick hydrogen bonds between AT and GC base pairs and by stacking forces [6]. The inside of the double helix is highly hydrophobic due to the close packing of the aromatic bases. The helix axis of pDNA can also be coiled in space, forming a higher order molecule named supercoiled (SC) pDNA. A fraction of a population of pDNA molecules can also exist in a non-supercoiled or open circular (OC) form. Other variants, such as linear, denatured or oligomeric pDNA can also be found in cell lysates. The linear form results from chemical/enzymatic cleavage of the phosphodiester bonds in opposite DNA strands, denatured forms exhibit a conformation where the hydrogen bonding between complementary strands at certain locations has been disrupted and oligomers are a consequence of homologous recombination [7].

1.2. Biosynthesis and primary isolation

Plasmids are biosynthesised by autonomous replication in *Escherichia coli*, a bacterium with a history of safe use in the bio-industry as a producer of many recombinant proteins. Although several techniques can be used to disrupt *E. coli* cells, and hence, release pDNA molecules (<1% dry cell mass), the most widely used method is alkaline lysis [8] or variations thereof [9]. Alkaline lysis relies on the disruption of cells at high pH with NaOH and in the presence of SDS, followed by the release and denaturation of genomic DNA (gDNA), cell wall material and most of the cellular proteins. Although SC pDNA isoforms also unwind as a consequence of the alkali-promoted hydrogen bond disruption, if the pH is maintained below 12.5, anchor base pairs remain which prevent the complete separation of complementary strands. These anchor bases serve as nuclei for the complete renaturation of pDNA molecules during a subsequent neutralisation step [10]. If cell lysis is carried out at a pH above 12.5, or if local pH extremes are present in solution, the plasmid anchor base pairs may be lost resulting in irreversibly denatured pDNA forms that contain large regions of single stranded material. After the lysis step, the solution is neutralised with potassium acetate, which precipitates SDS together with denatured gDNA and cellular debris. Different operations can remove this insoluble material, whereas the majority of pDNA remains in the supernatant. During these manipulations care should be taken to avoid shear-induced cleavage of genomic DNA (gDNA), which may result in small fragments that will not aggregate. The resulting clarified alkaline lysate typically contains proteins, RNA, lipopolysaccharides (LPS), gDNA fragments and less than 1% (w/w) pDNA.

The subsequent recovery and purification of pDNA molecules from the clarified cell lysate may involve several techniques. Molecular biologists developed a range of efficient lab-scale protocols, which use operations, such as ultracentrifugation or solvent extraction [11]. Although routinely

used for obtaining pDNA for research applications, many of these protocols cannot be scaled-up or use reagents, such as phenol, ethidium bromide, CsCl or animal derived enzymes that are not compatible with the recommendations of regulatory agencies for the manufacturing of therapeutics [12]. Further disadvantages include limited capacity, low recovery and high cost. It is in this context that liquid chromatography stands as a central purification technique, both at laboratory and process-scale.

2. The role of chromatography in pDNA purification

2.1. Overview

Liquid chromatography is central in process-scale manufacturing of therapeutic pDNA (Fig. 1). The overall process must deliver a pDNA product, which meets quality specifications set or recommended by regulatory agencies, such as the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medical Products (EMEA) [4]. Typically, a final bulk pDNA should be free from host gDNA (<0.05 μg/μg pDNA), host proteins (undetectable), RNA (undetectable) and endotoxins (<0.1 EU/μg pDNA) [4]. Additionally, more than 90% of the pDNA should be SC, since this isoform is thought to be more effective at transferring gene expression than other variants [4,5]. The removal of endotoxins is particularly important, since these LPSs components of the *E. coli* cell wall [13] can produce symptoms of toxic shock syndrome if present in large enough quantities in vivo [13]. Furthermore, endotoxins may dramatically reduce transfection efficiencies in various cell lines and display cytotoxic effects on mammalian cells [13].

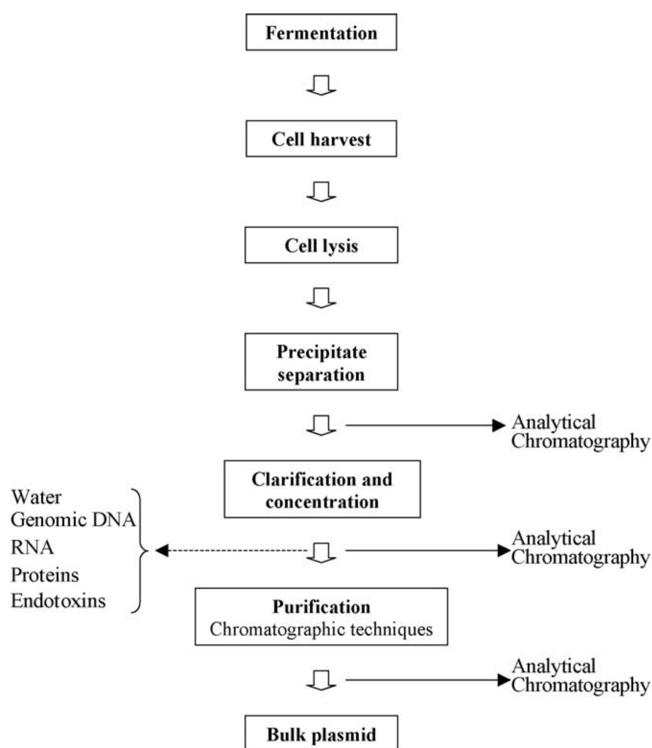

Fig. 1. The role of liquid chromatography in process-scale manufacturing of therapeutic pDNA.

Although attempts have been made to include chromatography after cell lysis, it is generally included after the impurity load and the process volume has been reduced by clarification and concentration operations, respectively (Fig. 1) [14]. The role of chromatography is then to remove those cellular host components (RNA, proteins, gDNA fragments, endotoxins) and non-SC pDNA variants, which are virtually impossible to remove by other unit operations (Fig. 1) [4]. Chromatographic modalities, such as size-exclusion (SEC), anion-exchange (AEC), hydrophobic interaction (HIC), reversed-phase (RPLC), ion-pair reversed-phase (RPIPC), thiophilic adsorption (TAC) and affinity (AC) have been integrated, singly or combined, into several

Table 1
The use of chromatography in processes for the manufacturing of therapeutic pDNA

Process	Description	References
I	Isopropanol pp Ammonium acetate pp Polyethylene glycol-8000 pp SEC Ethanol pp	[16,17]
II	RNase digestion Bag Filtration EB AEC	[18]
III	Isopropanol pp Ammonium acetate pp Isopropanol pp RPIPC	[19]
IV	Isopropanol pp Ammonium sulphate pp HIC Dialysis	[20]
V	RNase digestion Diafiltration AEC RPLC	[21]
VI	RNase digestion AEC 1 AEC 2 HIC	[22]
VII	$CaCl_2$ pp AEC 1 AEC 2	[23]
VIII	Conditioning with 2 M $(NH_4)_2SO_4$ SEC TC AEC	[24]

Abbreviations: pp, precipitation; SEC, size-exclusion chromatography; EB-AEC, expanded bed anion-exchange chromatography; RPLC, reversed-phase chromatography; RPIPC, ion-pair reversed-phase chromatography; HIC, hydrophobic interaction chromatography; TAC, thiophilic adsorption chromatography.

Table 2
Current limitations of pDNA chromatography and possible solutions

Specificities of pDNA separations and consequent limitations	Solutions
Physical and chemical similarities between impurities and pDNA	Reduce impurity load prior to chromatography
Poor selectivity due to competition	
Co-elution of impurities in pDNA pools	
High viscosity of feed solutions	Avoid small beads
Increase in pressure drop	Dilute feed streams
Low linear flow rates and throughput	Use expanded beds
Large pDNA size, large size of high M_r RNA, small pore size	Compaction of pDNA
Small diffusion coefficients and hence poor internal mass transfer	Decrease bead size
Broad peaks, low recovery	Use beads with “superpores” or “tentacles”
Small flow rates, long separation times	Use formats, such as membrane layers and monoliths
Poor capacity for pDNA due to lack of accessibility	Pre-digestion of high M_r RNA with RNase
	Use micropellicular stationary phases

processes for the manufacture of therapeutic pDNA (Table 1) [12]. Liquid chromatography is also very useful as an analytical tool to monitor manufacturing and control pDNA quality during processing and in the final formulations (Fig. 1). This type of analysis is fundamental to guarantee the production of a consistent product that meets the desired specification [15].

If the purpose is to purify pDNA at lab-scale for general molecular biology applications, it is possible to choose between a number of commercially available disposable gravity-flow or “spin” chromatographic columns [25]. High-throughput and automated protocols and procedures that use these column cartridges are also becoming available, enabling the processing of multiple samples while minimising hands-on preparation time and reducing the risk of sample mix-ups [26].

2.2. *Limitations of pDNA chromatography and possible solutions*

The separation and purification of pDNA by chromatography is faced with a number of limitations (Table 2), which are related to the structural nature of the available stationary phases, and of the molecules involved–pDNA and associated impurities.

Poor selectivity and co-elution are problems directly attributable to the physical and chemical similarities between impurities and pDNA (Table 3). This problem can be partially tackled by reducing the impurity load prior to chromatography, using a number of operations (Table 4). Chromatographic processes can then advantageously explore some differences in properties, such as charge, molecular size and hydrophobicity.

The high M_r of pDNA and nucleic acid impurities (Table 3) originates high viscosity in solutions with a high content of these solutes [39]. For instances, the viscosity of an alkaline lysate may reach 15–60 MPa, depending on the shear rate [40]. For chromatography carried out in a conventional packed bed mode, high viscosity may translate into high-pressure drops, and consequently, limit the range of linear flow rates available and throughput. This problem can be alleviated by diluting the process feed, by avoiding the use of chromatographic particles with small diameters or by carrying out chromatography in the expanded bed mode.

Diffusion coefficients of pDNA in solution are significantly lower when compared with those of protein [41]. This reflects not only the differences in mass between pDNA and most proteins, but also differences in size and structure.

Table 3
Characterisation of nucleic acids and other components in an *E. coli* cell (adapted from [27])

Species	Different species/cell	Average $M_r \times 10^{-3}$	Observations
Water	1	0.018	–
Nucleic acids			
Genomic DNA	1[a]	2.8×10^6	Polyanionic, highly fragmented, single/double stranded
Transfer RNA	40	28	Polyanionic, single stranded
Ribosomal RNA	3	500–1000	Polyanionic, single stranded
Messenger RNA	400–800	660–90	Polyanionic, single stranded
Plasmid DNA	1	3300[b]	Polyanionic, double stranded, isoforms compact
Proteins	1100	8–200	Anionic, cationic, neutral, hydrophobic, hydrophilic
Endotoxins		10	Anionic, lipidic part
Small molecules and ions	800–2000	<1	–

[a] Rapid growing *E. coli* cells have on average four molecules of gDNA.
[b] A pDNA molecule with an average 5 kb is considered.

Table 4
Unit operations used to reduce impurity load before chromatography

Operations	References
Filtration through	
Diatomaceous earth	[28]
Cellulose acetate	[29]
Nitrocellulose	[30,31]
Tangential flow filtration	[32–34]
RNase digestion step	[18,35]
High temperatures (≈70 °C)	[29]
Hot Mg^{2+} treatment	[36]
Precipitation with	
Isopropanol (0.6, v/v)	[14,35]
Polyethylene glycol (PEG)	[14,16,17]
Compaction agents (e.g. spermine, spermidine)	[37]
Chaotropic salts (e.g. LiCl, KAc and NH_4Ac)	[14]
Divalent alkaline earth metal ions	[23,38]

Effectively, most proteins are globular, measuring 2–10 nm across [42] while plasmids display a micron-sized (>0.2 μm) structure, which is intermediate between a flexible coil and a rigid interwound structure [41]. The typical diffusion coefficient of SC pDNA is of the order of 10^{-8} cm^2/s, while that of a protein with equivalent mass is one order of magnitude larger [42]. The larger nucleic acid impurities are also characterised by small diffusion coefficients. In chromatography, these low diffusion coefficients will result in low internal mass transfer, a situation that is clearly worsened by the fact that the pores of most stationary phases have not been engineered to handle such large macromolecules. The limitations in internal mass transfer result in broad peaks and low recovery [43]. Furthermore, the consequent need to use small flow rates and shallow gradients leads to long separation times [43].

Contamination of the pDNA peak or pool can also result from constraints in the diffusion of macromolecules in the particle pores. For example, the slow diffusion of RNA molecules through the pores may retard these molecules enough to contaminate pDNA fractions. The pDNA peak can also be contaminated with impurities that would normally elute earlier.

One of the most reported drawbacks of pDNA chromatography is poor capacity of stationary phases for pDNA binding, as illustrated by the anion-exchange capacity data collected from the literature and listed in Table 5. This limitation results from an inadequacy of the pores of most stationary phases (<0.2 μm) [53], originally designed to bind proteins, to accommodate macromolecules like pDNA [35]. A confocal microscopy visualisation of pDNA in Q-Sepharose XL [54] and DEAE Streamline [55] anion exchangers confirmed this by revealing that pDNA adsorbs mainly in an outer layer

Table 5
Static and dynamic (†) binding capacity of pDNA on commercial anion-exchange adsorbents

Stationary phase	Bead size (μm)	Pore size (μm)	Capacity (mg/ml)		pDNA (kb)	References
			pDNA	Protein		
Beads						
Q-Sepharose Big Beads	200	n.a.	0.7	70	4.8	[44]
Q-Sepharose Fast Flow	90	0.19	1.3	120	4.8	[44]
	90	0.19	0.72†	120	5.9	[33]
Q-Sepharose High Performance	34	n.a.	2.5	70	4.8	[44]
Streamline QXL	200	n.a.	3	>110	4.8	[44]
Q Hyper D	80	0.30	1.2	n.a.	3.5	[45]
	60	0.30	2	∼106	3.5	[45]
	60	0.30	3.3†	∼106	7.1	[46]
	50	0.30	>5.3†	∼90	5.9	[33]
	20	0.30	5.4	∼85	3.5	[45]
	10	0.30	10.0	∼90	3.5	[45]
Fractogel EMD DEAE	40–90	0.80	2.45†	100	5.9	[33]
	40–90	0.80	2.0†	100	7.1	[46]
Poros 50 HQ	50	<0.80	∼10.0	60	3.5	[47]
	50	<0.80	2.12†	60	5.9	[33]
Monoliths						
DEAE-CIM	–	0.01–4	10.0	n.a.	n.a.	[48]
Membranes						
Mustang-Q	–	0.8	10.0	56	6.1	[49]
	–	0.8	15.0†	56	6.3	[50]
	–	0.8	6.0†	56	4.5	[51]
Cell microcarrier						
Cytopore	230	30	∼31	n.a.	4.8	[52]

Capacity data for proteins is shown for comparison.

of the beads. Studies with anion-exchanger beads of different size further showed the existence of an inverse correlation between bead radius and pDNA static [44,45] and dynamic [46] binding capacity, thus confirming that adsorption is mainly superficial. This suggests that capacity can be improved simply by decreasing bead size [54]. The capacity of chromatographic beads for pDNA may also depend on surface topography or "roughness" [46,55,56].

The diffusion and capacity limitations can both be partially overcome either by compaction of pDNA or by increasing the size of pores in stationary phases. The first strategy relies on the use of multivalent cations or compaction agents that decrease repulsion between DNA phosphates, and thus, condense pDNA molecules from an elongated coiled state to a compacted globular state [37]. For instances, by using the cationic compaction agents spermidine and spermine, the capacity of the Q-Sepharose (Amersham Biosciences) anion exchanger for pDNA increased by as much as 40% [57]. This increase was attributed to an easier access of pDNA to pores and also to a closer packing of pDNA on the surface of the adsorbent, and it was found to work best at low ionic strength [57]. The condensation of DNA molecules may additionally account for a more homogeneous behaviour during chromatography, ultimately leading to sharper peaks, higher yields and increased selectivity. For instances, use of PEG prior to AEC with Q-Sepharose increased recovery from as little as 20–80% [58], while the use of a 2 M ammonium sulfate buffer during SEC resulted in high productivity [24,53].

The use of stationary phases engineered to accommodate "superpores" or convective through pores (>0.2 μm) is another strategy that may partially solve the pDNA capacity/diffusion problem [59]. "Superporosity" not only improves the access of pDNA to the internal voids but may also allow convective pore flow to take place, and consequently, improve internal mass transfer [60,61]. For instances, one of the highest figures reported for pDNA binding capacity in anion-exchange beads (∼10 mg/ml) [47] has been obtained with the support Poros (Applied BioSystems), characteristic of the so-called perfusion chromatography. This enhanced capacity was attributed to the penetration of the pDNA molecules into the large 0.6–0.8 μm "superpores" which are characteristic of the Poros architecture [46,61]. A further advantage of perfusion chromatography is the claimed acceleration of separations 10–100 times relative to conventional HPLC.

Experiments carried out with the anion-exchange cell microcarrier Cytopore (30 μm pore diameter) yielded a pDNA capacity of 1240 mg/g (∼31 mg/ml), approximately one order of magnitude higher when compared with the capacity of conventional chromatographic supports [52]. This extraordinary capacity is associated with a deep penetration of pDNA into the "superpores" of Cytopore beads as demonstrated by confocal microscopy [53]. Although the Cytopore media cannot be used in chromatography due to poor mechanical stability [53], similar capacity figures are likely to be obtained if superporous agarose beads (25–75 μm pore diameters), such as the ones described by Gustavsson and Larsson are used [60].

High accessibility to binding sites and reduction in internal mass transfer limitation are also characteristic of monolithic supports and adsorptive membranes, making them attractive for pDNA chromatography [48–51,62,63]. Monoliths (Bia Separations) are single pieces of porous material characterised by a highly interconnected network of channels (0.01–4 μm) [48], while the adsorptive membranes developed by Pall have characteristic 0.8 μm pores [49]. The improved static capacity for pDNA binding in anion-exchange monoliths and membranes (Table 5) clearly confirms the advantage of "superpores".

Surface topography can also be engineered with tentacle-like structures that protrude from the surface and enable pDNA to be captured more densely at the particle exterior [46]. This is the case for instances of Streamline QXL, which is characterised by long dextran chains coupled to an agarose matrix. In this case one of the best relationships between static capacity and bead diameter has been reported (3 mg/ml for 200 μm beads; Table 5).

The internal mass transport problem can also be eliminated by using non-porous, micropellicular beads [43]. These stationary phases are characterised by a core of fluid-impervious material covered by a thin adsorptive layer. Although total surface area is low, these supports present very favourable mass transfer properties and allow a fast and high-resolution separation of double stranded DNA molecules [43]. Volumetric capacity, however, remains low.

3. Applications of chromatography in pDNA purification

3.1. Size-related chromatography

3.1.1. Size-exclusion chromatography

Size-exclusion chromatography (SEC) has been extensively used to purify pDNA (Table 6). Earlier experiments did not yield significant results due to the lack of stationary phases adequate for the separation of nucleic acids with high molecular mass (M_r) and complex conformations [64,65]. This situation changed with the introduction of rigid and highly porous composite polyacrylamide/dextran stationary phases.

Sephacryl S-1000 (Amersham Biosciences) is one of the stationary phases most widely used in SEC of pDNA [14,16,17,64,66–70]. This stationary phase presents an exclusion limit of 20 kbp for linear DNA and 400 nm for spherical particles. SEC with Sephacryl S-1000 is reported to be simple, inexpensive and reproducible, yielding milligram amounts of highly pure SC pDNA from a partially purified *E. coli* lysate. Independently of the pDNA targeted, chromatograms obtained after feeding clarified lysates to Sephacryl S-1000 columns are always characterised by two

Table 6
Size-related chromatography of pDNA

Type	Stationary phases	Supplier	References
SEC	Sephacryl S-1000	Amersham Biosciences	[16,17,64,66–70]
	Sephacryl S-500	Amersham Biosciences	[18]
	Superose 6B	Amersham Biosciences	[67,69,71]
	Sepharose 6 FF	Amersham Biosciences	[24]
	Zorbax GF 250	Agilent Technologies	[72]
	Fractogel TSK	Merck	[73]
SLC	Asahipak GS-310	Asahi Chemical	[74]

well-separated peaks. The first peak is usually broad and non-Gaussian, and may contain a leading shoulder. This large peak harbours all forms of DNA present in the lysate: gDNA, linear, denatured, OC and SC pDNA. Fractions corresponding to the leading edge of the peak are enriched in high M_r gDNA, pDNA multimers and OC pDNA. Subsequent fractions contain essentially pDNA with the latest fractions particularly rich in the SC isoform. The ability of Sephacryl S-1000 to fractionate OC and SC pDNA is illustrated in Fig. 2, which presents a superimposition of chromatograms obtained after injection of OC and SC pDNA standards in a 30 cm column. The fractionation of the isoforms present in the first peak was found to be dependent on the pDNA M_r. With small pDNA (4.4 kbp) the isoforms partially overlap, whereas for sizes above 10 kbp the separation is complete [69]. It has been claimed that the OC form elutes earlier than the SC form because of steric hindrance [69]. Several authors reported that pDNA yields of 80–90% are obtained, when using a low pDNA concentration (0.5–1.0 μg/ml) in the feed [64,68,70]. In this case, gDNA can even be distinguished as a leading shoulder. In the case of lysates with higher pDNA concentrations (2–4 μg/ml) [64], this gDNA shoulder is no longer distinguishable. Whichever the case, it is always necessary to take appropriate cuts and discard the front part of the peak in order to obtain pDNA free from measurable amounts of gDNA. Yields close to 70% can be obtained in this case [67].

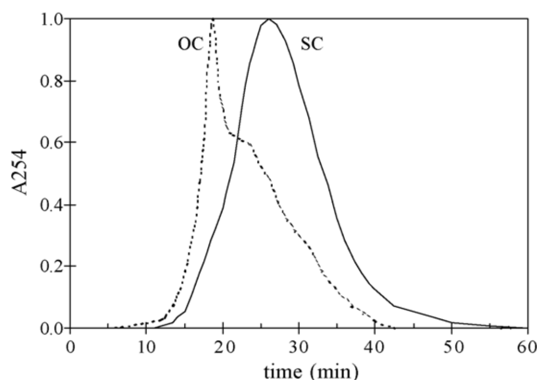

Fig. 2. Size exclusion chromatography of open circular (OC) and supercoiled (SC) standards (8 μg) of a 2.7 kb pDNA in a Sephacryl S-1000 column (30 cm × 1 cm). Flow rate, 0.5 ml/min; injection volume, 1 ml; elution buffer, 10 mM Tris, 1 mM EDTA, 100 mM NaCl; pH 8.0 (D.M.F. Prazeres, unpublished results).

This overlapping between gDNA and pDNA was also reported, when processing high M_r plasmids [75].

The second peak in Sephacryl S-1000 chromatograms corresponds to small M_r molecules, such as oligonucleotides, RNA and proteins. A third peak corresponding to low M_r RNA is sometimes obtained. In the case of lysates extensively contaminated with RNA, some overlapping can occur between the DNA and RNA peaks with the consequent decrease in yield [75]. Chromatography with Sephacryl S-1000 is lengthy and up to 200–300 min may be necessary to complete a run, when using a standard low-pressure column. This drawback can be partially overcome by operating columns at a medium pressure of 20 bar, the maximum tolerated by Sephacryl S-1000 [69]. Although, in this case, pDNA elution was complete in 45 min, isoform separation was poor.

Another widely used SEC media for pDNA purification is Superose 6B (Amersham Biosciences) [67,69,71]. This stationary phase has a much lower size-exclusion limit for DNA (450 bp) when compared with Sephacryl S-1000. However, since the Superose 6B beads are more resistant to pressure, elution of pDNA can be achieved in only 20 min at much higher concentrations [69]. Nevertheless, a preliminary treatment of the lysate with RNase is essential to avoid overloading of the column. Additionally, it is very difficult to remove gDNA and non-SC isoforms with this support. This procedure was later optimised in order to increase even more the resolution and the elution time [71]. In that case, the elution of plasmids with different sizes (4.12–150 kbp) was achieved in approximately 8 min. Furthermore, the pDNA retention volume was not affected significantly by size. Due to the improved resolution of the pDNA peak, the capacity of the column for pDNA was greatly improved. A comparative study of the performance of Sephacryl S-1000 and Superose 6 showed that both stationary phases were able to resolve a 4.8 kbp pDNA and gDNA, from lower M_r impurities [67]. From this point of view, the performance of Superose 6 was superior, since better process yields were obtained with higher flow rates. However, Sephacryl S-1000 was better at resolving pDNA isoforms. Although, the differences in pore diameter between Sephacryl S-1000 and Superose 6 (400 versus 40 nm) would also make Sephacryl S-1000 better at resolving intact gDNA from pDNA, the separation was poor and not reproducible. This was attributed to shearing and breakage of gDNA during alkaline lysis, an operation, which is usually difficult to control [67].

SEC with Sephacryl 500 (Amersham Biosciences) has also been included as the final polishing step in a process for the production of therapeutic pDNA. A clear separation between pDNA and RNA molecules previously digested with RNase was obtained [18]. Simultaneously, the pDNA was exchanged into a buffer more appropriate for gene therapy applications [18].

The selectivity for pDNA versus RNA has been drastically increased by combining the use of Sepharose 6 Fast Flow (Amersham Biosciences) with an elution buffer with high ammonium sulfate concentrations (>1.5 M) [24,53]. This has been attributed to a different compacting effect of ammonium sulphate on pDNA and RNA. More than one-third of the column volume could be loaded per run, resulting in an unusually high productivity for a SEC step.

SEC with the high performance Zorbax GF250 media is rapid (6 min for pDNA elution) and pDNA is obtained free from RNA and proteins but a pre-digestion with RNase is required to avoid the overlapping of the RNA and DNA peaks [72].

Fractogel TSK (Merck), a SEC matrix made of hydrophilic vinyl polymers which has very good mechanical properties [73], combines high capacity with high resolution for pDNA purification. As for the case of Sephacryl S-1000, a column of Fractogel TSK is claimed to separate SC pDNA from impurities and other pDNA isoforms. The method, however, is highly time-consuming. Interestingly, and contrary to the results obtained by Vo-Quang et al. [69], the overlapping between the SC and OC isoforms was found to be higher for a larger pDNA (26.5 kbp) when compared with a smaller one (4.4 kbp).

All systems described above enable the recovery of pDNA with a quality adequate for molecular biology applications. In most of the studies reviewed, however, pDNA purity was evaluated only by agarose gel electrophoresis and restriction analysis. The possibility of including SEC with Sephacryl S-1000 in a process for the purification of therapeutic pDNA was evaluated more thoroughly by rigorous monitorization of impurities, such as endotoxins and gDNA [16,17]. In the process described (process I in Table 1), pDNA was isolated, pre-purified and concentrated prior to SEC by sequentially performing alkaline lysis, isopropanol precipitation, ammonium acetate precipitation, PEG-8000 precipitation and filtration. The compacting effect of PEG resulted in a more homogeneous behaviour of pDNA during the subsequent SEC step [58]. A typical Sephacryl S-1000 elution profile, with a 51% yield, was obtained: a first peak with a leading shoulder of gDNA followed by pDNA fractionated to some extent in the SC and OC circular isoforms. Process reproducibility, a characteristic which reassures regulatory agencies that the process is well controlled, was proved by superimposing chromatograms from three separate runs [17]. The large differences in M_r between the pDNA used (3×10^6) and endotoxins (10^4 to 3×10^5), together with the fractionation range of Sephacryl S-1000 (10^5 to 10^8) resulted in a 3000–8000-fold reduction in the endotoxin load [17]. Impurities, such as protein and RNA were not detected in the final pDNA pool, but residual *E. coli* gDNA exceeded the level required for a recombinant pharmaceutical. Plasmid potency was found to be comparable to a reference standard.

3.1.2. Slalom chromatography

Slalom chromatography (SLC) is a non-typical, size-related chromatographic technique that can be used for pDNA purification [74]. The method was first developed with columns packed with HPLC microbeads (diameter <20 μm) and eluted at relatively fast flow rates (>0.3 ml/min) [74]. SEC [76] and reversed-phase [77] HPLC columns have both been used for SLC. The technique is only applicable to large DNA molecules (>5 kbp) that, unlike what is expected for SEC, elute in order of increasing molecular size. Although the precise separation mechanism is still not clearly understood, hydrodynamic effects probably play the most important role. What is known is that the use of a fast flow rate originates a steep flow rate gradient in the narrow open spaces between the microbeads, which causes DNA molecules to extend. Since larger DNA molecules have longer reorientation times, passage between particles takes longer. Results showed that the retardation occurred on the basis of DNA length and not mass. In fact, OC forms were retarded to only half of the extent in comparison with linear forms of the same size. Solvent viscosity, temperature, particle size and flow rate were also found to be important.

3.2. Charge-related chromatography

3.2.1. Anion-exchange chromatography

Anion-exchange chromatography (AEC) remains one of the most used techniques for pDNA capture, purification and quantitation (Table 7), as it offers the advantages of rapid separation, no solvent requirement, sanitisation with sodium hydroxide and a wide selection of process-grade stationary phases [33]. It relies on the interaction between negatively charged phosphate groups in the DNA backbone and positively charged ligands on the stationary phase [35]. A salt gradient is employed to displace the different nucleic acids that should elute in order of increasing charge density, a property, which in turn is a function of chain length and conformation [4]. In some cases, base sequence and composition affect the elution pattern of nucleic acids in anion exchangers [88]. Several inversions in retention time as a function of chain length were attributed to a higher AT content of the more retarded molecule [43]. This correlates with nucleic acid bending at polyA sequences that may induce alterations in the overall conformation of the molecule and hence in its charge density [6].

When using AEC to separate pDNA, the clarified lysate feed should always be loaded at a sufficiently high salt concentration (typically >0.5 M NaCl) to avoid an unnecessary adsorption of low charge density impurities, such as low M_r RNA, oligonucleotides and proteins. Under these conditions, a significant amount of impurities elute in the flow

Table 7
Charge-related chromatography of pDNA

Type	Stationary phases	Supplier	References
AEC (analytical)	Nucleogen-DMA-4000	Macherey-Nagel	[78]
	DEAE-NPR,	Tosoh	[79]
	DEAE-5PW	Tosoh	[79]
	DEAE-PS-DVB	Non-commercial	[80]
	DMAE-LiChrospher	Merck	[17]
	Poros HQ, QE, PI	PerSeptive Biosystems	[20,35,81,82]
AEC (preparative)	QIAGEN	QIAGEN	[83]
	Protein-Pak DEAE 8HR	Waters	[84]
	Protein-Pak DEAE 5PW	Waters	[85]
	Gen-Pak FAX	Waters	[85]
	Mono Q-Sepharose	Amersham Biosciences	[29]
	HI-Load Q-Sepharose	Amersham Biosciences	[29]
	Q-Sepharose Big Beads	Amersham Biosciences	[14]
	Q-Sepharose XL	Amersham Biosciences	[23]
	Q-Sepharose FF	Amersham Biosciences	[33,35]
	Q-Sepharose	Amersham Biosciences	[86]
	Source15Q, 30Q	Amersham Biosciences	[23,24]
	Poros HQ, PI	PerSeptive Biosystems	[21,33,47]
	Fractogel DEAE	Merck	[33]
	Q Ceramic HyperD F	BioSepra	[33]
	Q Sephacryl S500 HR	Non-commercial	[87]
	Streamline QXL	Amersham Biosciences	[44]
	DEAE Streamline	Amersham Biosciences	[18]
	CIM monoliths	Bia Separations	[48]
	Mustang-Q membranes	Pall	[49–51]

through and capacity can be fully exploited for pDNA adsorption [33,78]. Molecules with a high charge density, such as pDNA isoforms, high M_r RNA and gDNA are retained and subsequently eluted by increasing the ionic strength of the mobile phase. Many AEC stationary phases display poor separation selectivity towards pDNA and impurities (high M_r RNA, pDNA concatamers, gDNA, non-SC pDNA isoforms, endotoxins) due to their similar binding affinities. In the case of gDNA, separation will be even more difficult to accomplish if alkaline lysis generates fragments with sizes closer to pDNA size [5]. This lack of selectivity makes purification of pDNA very difficult to achieve in a single AEC step [89]. In most cases a second chromatographic step is needed to meet the required purity (check processes V to VIII in Table 1) or pre-purification steps are required (Table 4). For instances, a recent example describes the combined use of calcium chloride precipitation and tangential flow filtration to improve the ratio of pDNA to RNA from 0.042 (w/w) to 2.57 (w/w) prior to AEC [33].

The Protein-Pak DEAE 8HR polymer-based column (Waters) allowed the purification of up to 5 mg of total, biologically active pDNA in a single 50-min run, without exceeding the capacity of the column [84]. A preparative Protein-Pak DEAE 5PW column (Waters) was also able to deliver 7.5 mg of total pDNA in a single, 1-h run [85].

Q-Sepharose (Amersham Biosciences) has been extensively used for AEC of pDNA (Table 7). This strong anion exchanger is made of 6% highly cross-linked agarose particles derivatised with quaternary amino groups. A comparison of the performance of Mono Q- and Hi-load Q-Sepharose columns on the small and large-scale purification of pDNA showed that both columns were able to separate SC and OC isoforms [29]. With the Mono Q-Sepharose column however, pDNA larger than 8 kbp could not be eluted and backpressure developed. In opposition, the Hi-load Q-Sepharose column could be loaded with very large pDNAs and was able to handle very large volumes (equivalent to 0.5–2.0 l of bacterial culture) without causing development of backpressure. Other Q-Sepharose-based media, such as Q-Sepharose Big Beads [14,66], Q-Sepharose XL [23], Source 15Q [23,24] have been tested for the preparative purification of therapeutic SC pDNA. The use of Q-Sepharose Big Beads made it possible to proceed directly from cell lysis to AEC and then to SEC to obtain a high quality/purity pDNA [14]. A reduction in the dielectric constant of buffers by incorporation of alcohols, such as isopropanol or ethanol has been shown to improve binding selectivity for pDNA and RNA [86].

Process questions related to the preparative purification of SC pDNA, using Q-Sepharose Fast Flow (FF) have been addressed, using 1, 10 and 40 ml columns [35]. The smaller column was first used to establish and optimise the separation. Fig. 3 presents a typical chromatogram obtained after loading an RNase-treated clarified lysate. The characteristic flowthrough peak harbouring low M_r RNA and proteins is followed by pDNA isoforms (denatured, OC and SC) that are selectively eluted with a shallow salt gradient. The interaction of gDNA with the Q-Sepharose FF column was very strong and could only be removed after NaOH treatment [35]. Conformation and the consequent charge/shape relation clearly plays an important role in the separation of pDNA isoforms

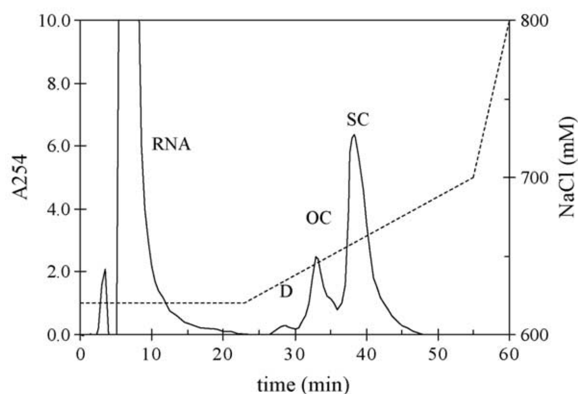

Fig. 3. Plasmid DNA purification by anion-exchange chromatography in a 1 ml Q-Sepharose column (5 cm × 0.5 cm). Flow rate: 1 ml/min; feed: 1 ml of a clarified lysate, containing 22 μg of a 2.7 kb pDNA; washing and elution buffers: 10 mM Tris, 1 mM EDTA, pH 8.0 with NaCl at the concentrations shown by the dashed line. Peaks corresponding to the supercoiled (SC), open circular (OC) and denatured (D) pDNA isoforms are identified (reprinted with permission from [35]).

[43,88]. The stronger binding of the more compact SC pDNA can be attributed to its higher overall, charge density relative to the other isoforms [29,78,84] or to a better fit and interaction with ligands within the stationary phase pore curvatures [78].

Scale-up (10 and 40 times) was straightforward, and consistent results were obtained in terms of yield, purity, quality and elution profiles. Independently of the scale, gradient used to elute pDNA and loading, an average yield of 62 ± 8% of SC pDNA was obtained [35]. A dynamic capacity of 40 μg SC pDNA/ml support was determined, whereas the capacity reported for human serum albumin is 120 mg/ml gel [35]. This low capacity was attributed to the fact that only a small fraction of the internal pore volume (exclusion limits ≈1900 Å) is accessible to the long (≈3700 Å) and thin (≈113 Å) SC pDNA molecules [35].

Anion-exchange perfusion chromatography with the "superporous" stationary phase Poros PI/M has been carried out at a process-scale [21]. A pDNA yield of 80% was obtained when loading a filtered lysate, containing 5 g of SC pDNA onto a 3.6 L column. Separation factors of 2, 5.3, 22.1 and 283 were obtained for gDNA, protein and RNA and endotoxins, respectively [21].

A recent report describes the evaluation of a range of commercial anion exchangers for their ability to resolve total pDNA from RNA, high capacity and robustness [33]. Fractogel DEAE operated under optimised conditions (loading at 0.63 M NaCl, elution with linear gradient to 1 M NaCl) was identified as the best anion exchanger in terms of pDNA purity (100%), dynamic capacity (2.45 mg/ml), recovery (94%), robustness and reproducibility required for the large-scale manufacture of therapeutic pDNA.

A new type of anion-exchange beads made of a Sephacryl S-500 HR matrix (Amersham Biosciences) derivatised with quaternary amine groups has been described which effectively discriminates between RNA and pDNA [87]. A distinguishable feature of the beads is a thin (2–3 μm) outer layer devoid of ion-exchange groups. The pores in this layer prevent the large pDNA molecules from coming into contact with the charged interior, while RNA and proteins readily pass through the pores and adsorb. A pDNA purification strategy was developed which uses a first column packed with these restricted access beads and a second, normal, AEC column. The procedure removed 99.5% of the RNA and 96% of proteins and yielded 89% of pDNA [87].

Several AEC columns in pDNA purification kits such as the one from Qiagen are based on DEAE-modified resins [25,83]. The Qiagen resin consists of defined silica beads with a particle size of 100 m, large pore size and a hydrophilic surface coating. The Qiagen resins allow a tight binding of pDNA molecules. Thus, while at lower ionic strength (<1.0 M NaCl) the proteins and RNA are washed away, pDNA elutes only at high salt conditions (1.6 M NaCl, pH 7.0) [83]. The selection of double stranded pDNA molecules and any single-stranded DNA is also claimed. AEC with Qiagen resins has also been used at pilot scale (7-l column packed with 2.8 kg of resin) as part of a process used for various clinical phase I/II manufacturing runs [83]. A disadvantage of the process is the need to use RNase A during alkaline lysis.

The use of AEC for the quantitation of pDNA has been described in numerous publications ([17,20,35,78-82]; Table 7). In earlier experiments, an HPLC macroporous Nucleogen-DMA-4000 column (Macherey-Nagel) was reported to separate RNA and gDNA from pDNA, which eluted as a peak consisting of 95% of the SC isoform [78]. Total pDNA analysis in the context of pDNA manufacturing has been described more recently with anion exchangers, such as Poros HQ [66], Poros QE [35], Poros PI [20,81,82] and DMAE-LiChrospher [17].

Although unable to resolve OC, SC and linearized pDNA, the Poros columns are suitable for the assessment of purity and total pDNA quantitation in process solutions [20,35,66,81,82]. Interestingly, the Poros QE column was able to separate native and denatured forms of pDNA—the denatured form eluted after the total pDNA peak, with some partial overlapping [35]. This extra retention of denatured pDNA was attributed to non-specific interactions of the exposed hydrophobic aromatic bases in denatured pDNA with the very hydrophobic polystyrene-divinylbenzene (PS-DVB) Poros matrix. This interaction is less intense in the case of native pDNA, where hydrophobic bases are shielded inside the double helix. Although a hydrophilic polymer (PEI) is used as a coating in the Poros material, this may not completely shield the hydrophobic nature of the matrix [35].

The separation and quantitation of OC and SC isoforms has been achieved with DEAE-NPR and DEAE-5PW (Tosoh) columns [79]. The presence of nanograms of OC pDNA could be determined within 30 min [79]. A much faster analysis of pDNA isoforms was obtained with a non-porous, micropelicular DEAE anion exchanger based on highly cross-linked PS-DVB particles [80]. The very

favourable mass transfer properties of this type of supports allowed the resolution of different pDNA isoforms within less than 3 min.

Anion-exchange expanded bed chromatography (AE-EBC) is an efficient alternative to packed bed AEC. In EBC a stationary phase made of dense particles of a defined size distribution is fluidised by a mobile phase directed upwards to form a stable bed [56]. The consequent increase in the extra-particle void volume makes it possible to process the high viscosity solutions characteristic of pDNA separations with high-throughput but without the build up of high-pressure drops [39]. Furthermore, under certain circumstances, an expanded bed can process a feed with small particulate material, and thus, it may be possible to by-pass some clarification and concentration operations [4].

Streamline QXL (Amersham Biosciences) was used for the primary purification of pDNA from a crude cell lysate by AEC in expanded bed mode [39]. The loading of the column with more than one sedimented volume of feed led to particle aggregation and to the collapse of the bed, resulting in a decrease in the yield and purity of the pDNA-containing fractions. To avoid this problem a precipitation with isopropanol was carried out which reduced significantly the amount of host impurities, the viscosity of the streams and the volume of the feed. In this case, no media aggregation or channelling was observed, and recovery yields increased significantly. However, the authors found no advantages in performing AEC in expanded mode. The large-scale purification of therapeutic pDNA by AEC in expanded bed mode was also tested, using DEAE Streamline (Amersham Biosciences) [18]. The process developed included only scaleable unit operations, such as a bag filtration step designed to deliver a clarified lysate feed for expanded bed AEC (process II in Table 1). Although the clarity of the bag filtration feedstock is insufficient for conventional packed bed AEC, it was ideally suited for an operation in expanded bed mode. After elution, the pDNA was further purified and buffer exchanged by SEC to yield a pharmaceutical-grade product.

The realisation that AE-EBC of pDNA is hampered by a lack of suitable adsorbents has prompted the design of new small (20–40 μm in diameter) high-density (>3.7 g/cm^3) prototype EBC beads for the capture of very large biological molecules [46]. These materials, obtained by functionalizing beads made of a stainless steel core encased in a thin layer of 6% agarose, with DEAE and PEI, possessed much higher dynamic binding capacities (25–70-fold higher) for pDNA when compared with other 200 μm commercial EBC supports. In opposition to the PEI-beads, the directly DEAE-linked beads that lead to high capture of pDNA with few operational difficulties were considered a significant improvement over the commercial AE-EBC supports. The PEI-linked beads, however, exhibited severe sensitivity to inter-particle bridging by nucleic acid polymers, gave low recoveries and proved to be difficult to regenerate. In fact, highly charged polymers, such as PEI, are well known for their ability to condense nucleic acids [56]. In this way, it was concluded that the use of weakly derivatised structures seems to be the best option for DNA purification as it enhances the capacity without increasing the sensitivity to inter-particle cross-linking [56]. Despite the initial success of the application of AE-EBC for pDNA purification, the authors claim that the technique is currently not sufficiently robust to encourage widespread industrial application [56].

Fast pDNA separations can be obtained by reducing the mass transfer resistance within the pores/channels of super-porous matrices [48]. High-performance membranes [49–51,62,63] and monolithic columns [48] have been described as a good option to achieve this purpose. A Mustang-Q chromatography capsule (Pall), containing a polyether sulfone membrane with quaternary amine groups and wound around a cylindrical core was used to capture pDNA from a dilute lysate (1.52 g of pDNA in 71 l) [51]. After washing with a 0.6 M NaCl buffer, pDNA was eluted with 1.2 M NaCl. Around 95% of pDNA were recovered in a 7.25 l pool, representing a 10-fold concentration in pDNA. The overall duration of the step was 35 min and purity of the pDNA was estimated to be between 85 and 90%.

An RNase-free process designed to purify pDNA from an alkaline lysate by performing AEC on a CIM (Convective Interaction Media)-DEAE monolith (Bia Separations) has been described [48]. RNA was found mostly in the flowthrough while pDNA eluted as a sharp peak. The pDNA quality, when assessed by agarose electrophoresis, restriction digestion and transformation experiments, was comparable to the quality of pDNA isolated with a commercial Qiagen kit. Successful regeneration was demonstrated and the characteristic shortness of the monolith resulted in a fast separation (approx. 7 min) [48].

3.2.2. Hydroxyapatite chromatography

The naturally occurring inorganic material $Ca_{10}(PO_4)_6(OH)_2$ known as hydroxyapatite (HA) is particularly effective for pDNA purification [90–93]. Hydroxyapatite chromatography (HAC) operates by mixed-mode ion exchange due to the inclusion of both positively and negatively charged moieties. Nucleic acids are assumed to bind to HA by means of coordination bonds between the phosphate moieties of the DNA backbone and the calcium sites on the surface of the stationary phase [93]. Most of the protocols for DNA purification using HA call for elution with a gradient of increasing phosphate concentration. In fact, the phosphate ions act like a competing agent for the stationary phase binding sites [93]. The method is able to differentiate rather well between the most important impurities that elute earlier and pDNA [90,93]. The presence of urea has a strong influence on the retention and elution behaviour of the nucleic acids in HAC. As urea concentration is raised, the difference between the phosphate elution molarities of pDNA and RNA becomes increasingly large [92]. This effect is probably due to the partial denaturation of nucleic acids by urea. HAC was also found to be useful on resolving different isoforms [93]. A significant disadvantage of HAC in the context of pDNA pu-

rification is that acetate ions from cell lysis interact with HA causing dissolution of the medium [90]. To avoid this problem pDNA-containing lysates, are previously treated by precipitation, desalting, diafiltration or dialysis. Alternatively, the cell lysate can be acidified by a mineral acid in the presence of an inorganic salt, rather than by acetate buffer [90]. In this way, the resulting clarified lysate can be directly loaded onto the HA column.

The possibility of purifying pDNA from a lysate by using HA in continuous annular chromatography (CAC) has been studied in comparison with a conventional HA batch column [93]. The quality of pDNA obtained by the two methods was similar, but scale-up of CAC is direct and straightforward.

3.3. Hydrophobicity-related chromatography

Chromatographic operations that explore hydrophobicity have been tested for the separation and purification of pDNA. A number of these applications is listed in Table 8.

3.3.1. Reversed-phase liquid chromatography

Retention in reversed-phase liquid chromatography (RPLC) of nucleic acids is attributed to hydrophobic interactions between the aromatic hydrophobic bases and hydrophobic ligands in the matrix. Bound molecules are eluted by increasing order of hydrophobicity, a property that is defined by characteristics, such as base composition, chain length, and secondary structure [95]. In this way, nucleic acids of increasing size will be retained longer in RPLC. On the other hand, the presence of AT-rich locations which usually form single stranded regions within DNA molecules, leads to exposure of the bases to the ligands, and thus, to an increase in the hydrophobic interaction strength [43]. This hypothesis is consistent with the fact that single-stranded oligonucleotides bind more tightly to RPLC matrices than double-stranded fragments of the same size [95]. The exposition of the hydrophobic bases also explains the higher retention times of partially denatured DNA molecules.

A RPLC-5 column (Ashland Chemical Co.) was successfully used for the purification of tRNAs, oligonucleotides, DNA fragments [78] and pDNA [94]. In the case of pDNA purification, a good separation of the three isoforms was obtained with an optimised gradient (0.05 M NaCl/ml, 28–30 °C). Improved separation of the SC isoform from the linear and OC forms was achieved at relatively low temperatures. However, since the column could not be operated at high pressure, 13–14 h were needed to complete a run and large peak volumes were thus unavoidable. When using these conditions, the SC isoform eluted at the lowest salt concentrations together with a small amount of the OC isoform. The OC and the linear form of the pDNA and the gDNA were mainly eluted in the last portion of the peak, at higher salt concentrations. The factors regulating the elution of tRNAs from RPLC columns have led to the conclusion that the order of elution is governed also by the secondary and tertiary structures [94]. This also seems to be the case for pDNA isoforms. However, the authors did not explain in what way the structural differences in pDNA isoforms affect the elution behaviour.

Other RPLC protocols for pDNA purification are based on the use of more hydrophobic C_{18} silica beads. In one of the examples reported, ammonium sulphate was used to precipitate the majority of RNA and proteins prior to RPLC [102]. This procedure, however, yielded a pDNA pool with about 25% RNA contamination because the column was not able to preferentially elute pDNA and RNA. A LiChrosorb RP18 HPLC-RPLC column (Merck) could separate SC pDNA from most impurities found in crude cell lysates (low-M_r RNA, gDNA fragments and linear pDNA) but not from ribosomal RNA [95] As expected for RPLC, single stranded DNA was retained more than double-stranded DNA due to the exposition of the hydrophobic bases. In opposition with the results obtained by Best and co-workers [94], the SC pDNA eluted after the OC and linear isoforms. The SC isoform fraction is expected to elute later in RPLC, since its negative torsional constraints, known to cause local denaturation of the DNA, render the bases more accessible to interaction with the stationary phase. The broadness obtained for the SC pDNA peak also suggests a separation of different SC pDNA species on the basis of their supercoiling number. In the case of the separation of RNA, the authors claimed that the difference between ribose and deoxyribose also influences the retention behaviour, thus differentiating RNA from DNA. The

Table 8
Hydrophobicity-related chromatography of pDNA

Type	Stationary phases	Supplier	References
RPLC	Adogen 464	Ashland Chemical Co.	[94]
	LiChrosorb RP18	Merck	[95]
	PRP-∞	Hamilton	[96]
	Poros R1	PerSeptive Biosystems	[97]
	Poros R2	PerSeptive Biosystems	[21]
RPIPC	PolyFlo	Puresyn	[19]
HIC	1,4-Butanediol diglycidyl ether-Sepharose	Non-commercial	[20,98]
	Butyl-6PW/Octyl-6PW	Pharmacia	[99]
	Source 15PHE	Amersham Biosciences	[100]
	TSKgel Butyl-NPR	Tosoh Biosep LLC	[101]
TAC	2-Mercaptopyridine-Sepharose 6 FF	Non-commercial	[24]

technique was used both for analytical and preparative purposes.

Another RPLC–HPLC technique for pDNA purification uses the PRP-∞ media (Hamilton) [96]. The column can be used to separate RNA from DNA present in a bacterial cell lysate, since the DNA is adsorbed onto the column whereas the RNA is eluted.

Reversed-phase perfusion chromatography with the "superporous" stationary phases Poros has been included in processes for the purification of therapeutic pDNA [21,97]. In one of the applications, a Poros 50 R1 (PerSeptive Biosystems) column was used [97]. A crucial step in the process is an ammonium sulphate precipitation prior to RPLC, which decreases gDNA contamination to less than 1%. After equilibrating the column with an acetate buffer saturated to 80% with ammonium sulfate, the pDNA solution is loaded. Washing and pDNA elution are carried out with the same buffer supplemented with 2.85 and 11.7% ethanol, respectively. After RPLC, AEC is used as a final polishing step.

In the second application (process V in Table 1), RPLC with Poros R2/M (PerSeptive Biosystems) was carried out at a process-scale [21]. The column was loaded with an anion-exchange pDNA pool (with pH adjusted to 8.5 and isopropanol to 1.2%), containing 3.2 g of SC pDNA onto a 7.0 l column. After extensive washing, a gradient of isopropanol from 1.2 to 11.2% was performed. A SC pDNA fraction eluted at 4% isopropanol with a yield of 77%. Separation factors of 14.1, >30, >10 and 194 were obtained for gDNA, protein and RNA and endotoxins respectively [21].

3.3.2. Reversed-phase ion-pair chromatography

Reversed-phase ion-pair chromatography (RPIPC) uses ion-pairing modifiers in the mobile phase to alter selectivity and allow separation of charged solutes. The stationary phase is hydrophobic but the mobile phase consists on a hydroorganic eluent, containing an amphiphilic ion and a small, hydrophilic counterion [43]. When charged solutes bind to the amphiphilic ion its hydrophobicity effectively increases. In some cases, this is an absolute requirement for the binding of the solute to the stationary phase. One of the most widely used ion pair agents in the case of nucleic acids is triethylamine. Therapeutic SC pDNA was purified from cell lysates at pilot scale by RPIPC, using a non-porous, inert, polymer matrix PolyFlo (Puresyn) [19]. Chromatograms showed the elution of low-M_r RNA in the void volume. After washing the column with a buffer containing acetonitrile, high-M_r RNA, high-M_r gDNA and non-monomeric forms of pDNA eluted as a single peak. A second gradient elution with high concentrations of acetonitrile yielded a peak containing a majority of monomeric SC pDNA. Similar chromatographic results were obtained regardless of whether pDNA was purified from 1, 10 or 100 g of cell paste. At each scale, more than 90% of the SC pDNA was recovered after chromatography. The process was considered to be reproducible in terms of chromatograms and yields. The final pDNA contained undetectable or acceptable trace levels of impurities, including RNA, protein, gDNA, endotoxins and other pDNA isoforms. When compared with AEC and SEC, the biological activity of pDNA purified by PolyFlo chromatography consistently demonstrated a significantly increased ability to transform competent *E. coli* cells. The high affinity of the column towards the highly hydrophobic lipid A component of endotoxins (50000 EU/ml packed resin) resulted in a 200,000-fold reduction down to 3.3–6.6 EU/mg pDNA. Furthermore, bound endotoxins did not co-elute with pDNA and were removed only during regeneration with 0.5 M NaOH. This is an important result, since the similar charge of pDNA and endotoxins prevents the use of AEC as a single-step procedure for efficient removal of this impurity. The authors claimed that whether used alone or in combination with other operations, RPIPC is sufficient to generate therapeutic pDNA consistent with cGMP manufacturing guidelines.

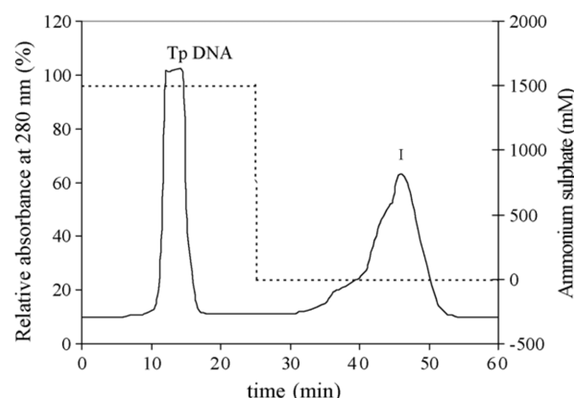

Fig. 4. Plasmid DNA purification by hydrophobic interaction chromatography in a column (20 cm × 1.6 cm) packed with 334 ml of Sepharose CL-6B derivatised with 1,4 butanediol diglycidyl ether. Flow rate, 10 ml/min; feed, 60 ml of a lysate previously purified by precipitation with 2.5 M ammonium sulphate, containing 4981 μg of a 6.7 kb pDNA; washing and elution buffers, 10 mM Tris/HCL, pH 8.0 with ammonium sulphate at the concentrations shown by the dashed line. Peaks corresponding to total pDNA (T pDNA) and impurities (I) are identified. (M.M. Diogo, unpublished results).

3.3.3. Hydrophobic interaction chromatography

The purification of pDNA by hydrophobic interaction chromatography (HIC) explores differences in hydrophobicity between pDNA, single-stranded nucleic acid impurities and endotoxins [101,103]. Experiments carried out with Sepharose CL-6B (Amersham Biosciences) derivatised with a mildly hydrophobic ligand (1,4-butanediol diglycidyl ether), proved that is possible to separate native SC pDNA from the more hydrophobic nucleic acid impurities (RNA, gDNA, oligonucleotides, denatured pDNA) under non-denaturing conditions [103]. In the presence of 1.5 M of ammonium sulphate, total pDNA eluted in the void volume whereas impurities eluted later, well separated from the pDNA peak (Fig. 4). This behaviour is explained by the fact that pDNA molecules have the hydrophobic bases packed and shielded inside the double helix, and thus, the hydrophobic interaction with the HIC media is minimal. On the other hand, single stranded nucleic acids impurities show a higher

exposure of the hydrophobic bases, and thus, interact with the hydrophobic ligands [103]. Other cellulose- and Sepharose-based HIC materials have also been found to promote the binding of poly A, denatured DNA or viral RNA [104]. The nature of the ligands and the dependency of salt suggest that these separations are based on hydrophobic interactions as well [104]. As described for RPIPC [19], the highly hydrophobic endotoxins are also retained longer in a HIC column when compared with pDNA [20,81,98].

The HIC support obtained by derivatization of Sepharose CL-6B with 1,4 butanediol diglycidyl ether was included in a process for the purification of a cystic fibrosis gene therapy vector (pCF1-CFTR) of clinical grade [98]. The method developed included alkaline cell lysis followed by a precipitation with ammonium sulphate and HIC. The HIC yield was 70% and the quality of the recovered pDNA met all regulatory standards. Further work with a pDNA vaccine against rabies demonstrated the feasibility of scaling-up the process 60 times with no loss in pDNA quality and gains in yield (95%) [20,81]. Subsequent biological activity studies and protection experiments in mice showed that the experimental vaccine displays immunogenic activity and potency similar to, or higher, than that of the vaccine prepared using a commercial purification kit [20]. Both small and large scale processes showed a run-to-run consistency of the HIC step even after sanitisation and cleaning cycles of caustic washing.

A pDNA purification process was developed on the basis of HIC performed with Butyl-6PW (Tosoh) and Octyl-6PW (Tosoh) hydrophobic columns used in tandem [99]. Columns equilibrated with a high salt concentration (2.0 M ammonium sulphate) were fed with a clarified lysate. The first column adsorbed protein and RNA but not pDNA. The pDNA, containing eluate of this column was fed to the second column at the same salt concentration in order to bind pDNA and gDNA. The columns were then disconnected and pDNA in the second column was eluted by decreasing the salt concentration. The pDNA-containing eluate was further purified by AEC. According to agarose gel electrophoresis analysis, a high purity SC pDNA fraction was obtained. In comparison, many impurities were present in a pDNA fraction obtained by means of AEC alone. The capacity of the Octyl-6 PW column for pDNA was around 1.1 mg/ml and a recovery yield of 90% was obtained.

HIC has also been used analytically for the monitoring and control of pDNA quality. Sepharose CL-6B derivatised with 1,4-butanediol diglycidyl ether was successfully used for the separation of pDNA isoforms in the presence of 1.5 M of ammonium sulphate [10]. Native pDNA (SC + OC) eluted in the flowthrough followed by denatured pDNA. The denatured form was retained longer in the column due to hydrophobic interactions of large stretches of single-stranded DNA with the support. Analytical HIC has been described recently for the assessment of purity and quantification of pDNA in process solutions [100]. A HPLC Phenyl Sepharose column (Amersham Biosciences) was used for the rapid separation (7 min) of total double-stranded pDNA from more hydrophobic nucleic acids impurities present in samples process streams. The column was capable of handling heavily contaminated samples (<5% pDNA), without the need of using any sample pre-treatment. Replicate analysis of pure and impure samples containing pDNA yielded standard deviation values lower than 2% and the detection limit of the method was determined to be near 1 μg/ml. The method stood as a good alternative to other less robust quantitative analytical techniques, such as capillary electrophoresis or HPLC based on AEC or RPLC principles.

SC and OC isoforms of pDNA ranging from 4 to 30 kbp were successfully resolved in a TSKgel Butyl-NPR analytical column (Tosoh) [101]. The hydrophobic ligand is a butyl group, attached to a non-porous methacrylate backbone. Due to the predominantly hydrophilic nature of pDNA, very high salt concentrations (3 M of ammonium sulphate) were required to bind the pDNA onto the resin surface. A reverse salt gradient was used to elute the OC and SC isoforms sequentially. The separation of the isoforms was attributed to the increased exposition of hydrophobic bases as a consequence of the underwinding of the SC form. Larger pDNA molecules required a lower salt concentration to bind to the column and eluted later, when using the same salt concentration. A third peak that eluted after the OC and SC forms was identified through light scattering as a concatameric form of pDNA. The resolution was found to increase with a decrease in the flow rate in a way that was dependent on the temperature but not on pDNA size. On the other hand, the resolution also increased with the temperature in a way that was dependent on the flow rate. The pDNA isoforms could be analysed in less than 7 min at 1 ml/min for plasmids up to 30 kbp.

3.3.4. Thiophilic adsorption chromatography

The name HIC was first introduced to describe salt mediated separations of proteins on weakly hydrophobic matrices [105]. Later, however, all types of interactions that take place in the presence of high concentrations of salt were regrouped as: thiophilic adsorption chromatography (TAC), electron donor-acceptor chromatography (EDAC) and HIC. A pDNA purification process with three chromatographic steps (SEC–TAC–AEC), which includes TAC was recently proposed [24] (process VIII in Table 1). First, the pDNA present in a clarified cell lysate is separated from RNA by SEC in group separation mode using Sepharose 6 Fast Flow (Amersham Biosciences) and in the presence of 1.5 M or more of ammonium sulphate. In contrast with typical SEC, columns could be loaded with more than 30% of the column volume and yields consistently higher than 90% were obtained with no significant dilution. Furthermore, linear flow rates can be as high as 80 cm/h and bed heights as low as 25 cm. The subsequent TAC step explores the selective interaction of molecules with aromatic thioether ligands, containing a vicinal sulfur in the presence of high concentrations of an anti-chaotropic salt. A column packed with a thioether-type adsorbent based on 2-mercaptopyridine coupled to Sepharose 6 FF and equilibrated with 2 M of

ammonium sulphate was able to retain SC pDNA but not OC pDNA. SC pDNA could be eluted both by decreasing the ammonium sulphate concentration on the mobile phase or by performing a gradient to 2 M NaCl in the buffer containing ammonium sulphate. Since some impurities co-eluted with SC pDNA when applying the gradient to lower conductivity, elution by a gradient to 2 M of NaCl was chosen. In fact, trace amounts of protein, RNA and endotoxins could not be eluted by the addition of this salt even if the concentration of NaCl was increased to saturation (∼3 M). These impurities had to be removed from the chromatography medium by decreasing the ammonium sulphate concentration. The authors speculate that the aromatic ring structure of the ligand could participate in intercalating hydrophobic (π–π) type of interaction with the SC, double helix form of pDNA, as proposed for topoisomerases [24]. They also proposed that the sulfur atom participates with an electron donating (charge transfer) role in interaction with specific nucleotides. The fact that it is possible to elute pDNA from the column with a NaCl gradient indicates the presence of ion-pair interactions. The binding capacity of the column was over 1 mg SC pDNA/ml resin and the step yield was 70% [24]. The last step of the process consists on the concentration and polishing of the sample using the AEC with the Source 30Q resin (Amersham Biosciences).

3.4. *Affinity-related chromatography*

Chromatographic operations based on affinity interactions between pDNA or impurities with specific ligands immobilised in stationary phases have not been used extensively for pDNA purification. Nevertheless, a few relevant applications have been described in the literature, which deserve mentioning (Table 9).

3.4.1. *Triple-helix affinity chromatography*

Triple-helix affinity chromatography (THAC) is based on the formation of a triplex between an oligonucleotide covalently linked to a chromatographic matrix, and a specific duplex sequence in the target pDNA [107,108]. The best characterised triplex forms when a homopyrimidine oligonucleotide strand binds to the major groove of a homopurine–homopyrimidine duplex DNA through the formation of Hoogsteen hydrogen bonds: thymine (T) recognises adenine (A) to form TA-T triplexes, and protonated cytosine (C^+) recognises guanine (G) to form CG-C^+ triplexes [106–108,113]. The formation of the triplex is generally slow. A repetitive target sequence is chosen in order to provide multiple hybridisation positions of the oligonucleotide with the pDNA [108]. Triple-helices are stable at acidic pH values close to cytosine's pK_a (4.8), since protonation of cytosines in the oligonucleotide are required. Thus, the frequency and the distribution of the CG-C^+ triplets within the target sequence have a strong influence on the pH stability of the triplex [106]. Acidic cell lysates provide an adequate environment for the formation of stable triple-helices. Triplexes are also stabilised at high salt concentrations, since cations shield coulombic repulsion between the sugar-phosphates.

Two reports describe the capture of pDNA directly from bacterial cell lysates by formation of a triple-helix with a 20-base long biotinilated oligonucleotide attached to streptavidin-coated magnetic beads [114,115]. The first paper describes the potential of the technique for the enrichment and screening of recombinant DNA libraries. However, the method was found to be time-consuming, since the two steps of oligo immobilisation and triple-helix formation were performed separately over a period of 3 h [114]. In the second paper, the authors proved that the two steps can be performed at the same time, requiring only 15 min. Several plasmids were isolated and found to be suitable for the digestion with specific restriction enzymes. Although, attractive for lab-scale applications, magnetic beads are not suited for process-scale operations.

A scalable THAC was developed for integration into a pilot scale DNA production process [108]. Polypyrimidine sequences [$(CTT)_7$, $(CT)_{11}$, $(CCT)_7$] were covalently attached to a HiTrap-NHS column (Amersham Biosciences). Columns were equilibrated with a high concentration of NaCl and used to purify plasmids, containing the corresponding sequences [$(GAA)_{17}$, $(GGA)_{15}$, $(GA)_{25}$]. Plasmid binding via triplex formation was slow (2 h, RT). Plasmid elution was carried out by raising pH to 9.0 in order to deprotonate cytosines, and thus, dissociate the triple helix. The best results were obtained with a 21-mer oligonucleotide $(CTT)_7$ attached to via a $(CH_2)_{12}$ linker [108]. The yield decreased when increasing the percentage of cytosines in the third strand because CG-C^+ triplets are destabilised by the presence of adjacent CG-C^+ [108]. Another interesting conclusion was that a 20-mer oligonucleotide gives a higher capture efficiency than a 32-mer [115]. Yields were also higher when longer target double strands (51 versus 21 nucleotides) were used to form triplexes with a 21-long recognition oligonucleotide [108]. This result was attributed to the higher number of possible

Table 9
Affinity-related chromatography of pDNA

Type	Stationary phases	Supplier	Refernece s
THAC	Oligo-Sephacryl S1000	Non-commercial	[106,107]
	Oligo-HiTrap NHS	Non-commercial	[108]
PDNA-AC	Zinc finger-glutathione *S*-transferase-gluthatione Sepharose	Non-commercial	[109]
IMAC	Iminodoacetic acid (IDA) resin charged with Cu(II)		[110]
EndoAC	Polymyxin B-Sepharose	Non-commercial	[112,113]

hybridisation positions for the first case. After optimisation of several parameters, the best yield obtained was around 50%. Another interesting observation is that the purified fraction contained a higher percentage of SC pDNA than the starting material. Other authors [114] have reported an opposite behaviour, probably indicating that the preferential binding of a specific isoform might be a function of the target sequence or position on the plasmid [106]. Plasmid purified in one step from a cleared lysate by using this method appeared as a single peak in HPLC analysis. No RNA was found by agarose gel electrophoresis and gDNA and endotoxin contamination was decreased by two orders of magnitude. The authors also showed that the performance of the process could be improved by inserting THAC into a DNA purification process involving classical purification steps. In this case, it would be possible to obtain pDNA with very low *E. coli* gDNA levels (0.01%) and with endotoxin levels suitable for intravenous injection. This production process would yield high-quality pharmaceutical-grade pDNA for human clinical trials.

THA batch adsorption was later described by Schluep et al. [106]. The triplex-forming oligonucleotide 5′-TCT TCT TTC CTC TTT, was immobilised by its 5′-end on hydrazide functionalized Sephacryl S-1000 (20 kbp exclusion limit) chromatographic beads via a hydrophilic 16 atom spacer arm. Due to the size-exclusion limit of the support, about 34% of the ligands are expected to be accessible to the plasmid [106]. Several batch adsorption experiments were performed, using pure pDNA. A bead capacity of 28 μg/ml for pDNA and pDNA yields up to 62% were obtained. Isolation of pDNA from clarified lysates with an efficient removal of RNA, gDNA and protein was also successful. Furthermore, a certain enrichment of the SC pDNA form was observed by agarose gel electrophoresis. Again, this type of preferential binding might be a function of the target sequence or target position in the plasmid [106].

3.4.2. Protein–DNA affinity chromatography

Protein–DNA interactions have also been explored in affinity chromatography of pDNA. A bi-functional protein was prepared by fusing a zinc finger (ZF) DNA binding domain with glutathione *S*-transferase (GST) as the N-terminal domain [109]. The ZF protein is a consensus sequence ZF protein that binds to the sequence 5′-GGG-GCG-GCT-3′, while the GST domain is able to bind to matrix immobilised glutathione. In this way, a complex can be formed between a target pDNA, containing the recognition sequence and glutathione-Sepharose. The protein–pDNA complexes can then be recovered from glutathione-Sepharose by competitive elution with reduced glutathione buffer. The authors proved that this protein-based affinity linker was able to distinguish between plasmids containing the recognition sequence and pUC19, even though this later plasmid contained a DNA sequence with a 7-bp homology with the recognition sequence [109]. Furthermore, the referred ligand was also able to isolate pDNA directly from clarified cell lysates. The relatively low yield obtained was attributed to the restricted access and mobility of the fusion protein within the Sepharose matrix.

3.4.3. Immobilised metal affinity chromatography

Immobilised metal affinity chromatography (IMAC) has been recently applied to pDNA purification, using an iminodiacetic acid (IDA) resin charged with Cu(II) [110]. The resin was found to bind exposed purine bases in solution. In this way, denatured DNA and RNA bind to the IMAC column whereas the pDNA is not retained.

3.4.4. Boronate affinity chromatography

One of the few differences between RNA and DNA which can serve as a basis of separation is the presence of a vicinal 2′,3′ *cis*-diol at the 3′ end of RNA molecules [111]. Boronate ligands (e.g. *m*-aminophenylboronic acid) bound to chromatographic matrices are able to recognise and bind RNA molecules via this feature, which is absent from the deoxyribose backbone of DNA. Boronate affinity chromatography (BAC) has been used essentially in applications in which RNA is the desired product (e.g. ribozyme science and rRNA probe methods). Although, the use of BAC has not been described in the context of pDNA purification, its application as a way of reducing the RNA content in pDNA-containing clarified or pre-purified lysates should be straightforward [111].

3.4.5. Polymyxin B affinity chromatography

Polymyxin B is a cationic polypeptide antibiotic that has a bactericidal activity against gram-negative bacteria. This activity is based on the ability to disorganize the bacterial cell wall due to the specific interaction of polymyxin B with the lipidic structure of endotoxins (Lipid A) [116]. This ability has been explored by affinity chromatography with polymyxin B linked to Sepharose to significantly reduce endotoxin contamination (200–100,000-fold) in pDNA solutions [112,113]. However, poor yields were obtained as a consequence of non-specific ionic interactions between pDNA and polymyxin B [112]. Another disadvantage of the technique is related with the neuro- and nephrotoxicity of polymyxin B and stimulation of monocytes to release interleukin-1 [116].

4. Selection of chromatographic techniques

The selection of one or more chromatographic techniques for the purification of SC pDNA is determined by the nature and distribution of the residual impurities and contaminants, as well as by the anticipated pDNA dosage [117]. Table 10 shows the main advantages and disadvantages of the several chromatographic techniques presented and their applicability on the context of pDNA purification processes.

SEC is a unique method for pDNA purification because it offers the possibility of separating these molecules from high- and low-molecular-mass nucleic acids impurities in the same step (endotoxins, RNA, oligonucleotides, proteins, genomic

Table 10
Advantages and disadvantages of chromatographic modes for pDNA separation and purification

Type	Advantages	Disadvantages	Applications
SEC	Separates endotoxins, gDNA, high- and low-M_r RNA, oligonucleotides, proteins Fractionates pDNA isoforms	Low feed volumes and concentrations Long chromatographic runs for high resolution media Product dilution Limited scale-up at a manufacturing scale	Final polishing step Group separation Endotoxin removal Isoform fractionation
AEC	Concentration of pDNA Rapid and simple Efficient removal of low charge density impurities Some stationary phases separate pDNA isoforms	Resolution of SC pDNA from gDNA, other isoforms and multimers is difficult Co-elution of pDNA with endotoxins and high M_r RNA Low capacity Elution of pDNA in high salt	pDNA capture pDNA concentration quantitation
HAC	HA is a naturally occurring material Separates several impurities from pDNA Separates pDNA isoforms	Elution of pDNA in high phosphate concentrations Acetate ions cause dissolution of the stationary phase	pDNA capture
RPLC and RPIPC	Elution of pDNA in a volatile solvent Separates several impurities from pDNA Separates pDNA isoforms	Loss of pDNA integrity Safety concerns related to the use of solvents Long chromatographic runs	pDNA capture quantitation
HIC	Separates endotoxins and single stranded nucleic acids Some stationary phases separate pDNA isoforms Mild mobile phases Rapid chromatographic runs	In some cases pDNA is eluted in high salt In some cases pDNA elutes in the flow through and is diluted	pDNA or impurity capture pDNA concentration quantitation
TAC	Separates pDNA from impurities and isoforms Concentration of pDNA	Elution of pDNA in a highly concentrated salt solution	pDNA capture pDNA concentration
THAC	Highly specific One step purification Concentration of pDNA	Expensive Low versatility Low capacity	pDNA capture pDNA concentration
Polymyxin B	Specificity	Non-specific binding of pDNA Toxicity of polymyxin B	Endotoxin removal

DNA and non-SC pDNA isoforms). In the case of pharmaceutical applications, SEC is also a very useful method to exchange the product into an appropriate formulation buffer and to remove any contaminating salts and/or residual metals allowing for the careful control of counter-ion in the final drug. It can be used to obtain small amounts of pDNA for Phase-I and Phase-II clinical trials with a single chromatographic step [117]. Typical disadvantages of SEC include restricted loading (<2% column volume), loss in resolution at higher loading and a significant dilution of the pDNA product with the consequent requirement of a final concentration step. Furthermore, high resolution is usually obtained at the expense of very long run times. Due to these characteristics, SEC is typically chosen as the last step in a pDNA purification process. An interesting exception has been reported recently where a high loading (30%) SEC step with Sepharose 6 FF is used prior to TAC and AEC [24].

AEC media bind pDNA strongly due to the high charge density of these molecules. The technique is very rapid and simple, allowing for the injection of high feed volumes and for the elution of highly concentrated pDNA. AEC is ideal for removing oligoribonucleotides and some proteins. Although, AEC of pDNA has been limited by the low capacity of stationary phases available commercially, a significant number of efforts have been directed towards the design of stationary phases (e.g. “superporous” beads, monoliths, membranes) more appropriate to handle large pDNA molecules. Furthermore, the isolation of the SC isoform from other pDNA variants has been described a number of times. However, several types of high M_r, negatively charged molecules, such as pDNA concatamers, RNA, gDNA, non-SC pDNA isoforms and LPS, often smear across the SC peak. Co-elution of pDNA and LPS is particularly critical if a therapeutic application is envisaged. This lack of selectivity decreases binding capacity for pDNA and results in poor resolution and purity. Nevertheless, the use of RNase to degrade host RNA and increase resolution, which was once mandatory, has now been abandoned in a number of processes that resort to other prepurification strategies. Another limitation of AEC is related to the necessity of removing the high salt load following chromatography. In summary, AEC is particularly useful as a first step for pDNA capture and purification, but its weakness in terms of selectivity usually calls for subsequent chromatographic steps.

RPLC of pDNA generally requires a number of prepurification operations, since RPLC by itself is not capable of delivering a pure product. When using RPLC for pDNA purification, a loss of pDNA structural integrity can arise

from the addition of organic solvents to the mobile phase. Furthermore, many of the organic solvents are toxic, volatile or even explosive, and thus, special measures must be taken to guarantee the safety of personal and facilities [12]. Additionally, when processing pDNA, long elution times and diluted fractions are generally obtained. The fact that pDNA is sometimes obtained in a volatile solvent may be advantageous, since it allows the formulation of the final product using routine post-chromatographic methods (rotary evaporation, lyophilization) that do not induce alterations of the SC structure [19].

HIC of pDNA constitutes an advance over the more common AEC. In fact, HIC is able to separate pDNA from LPS, denatured gDNA, RNA and denatured pDNA. Furthermore, separation of pDNA isoforms is possible with some stationary phases. An advantage of HIC over RPLC is the use of less harsh mobile-phases that do not cause denaturation of biomolecules. If conditions are used that promote pDNA binding, HIC becomes a high-throughput method that is compatible with high load and high concentration of pDNA. However, since the hydrophobicity of pDNA is very small, binding to stationary phases requires the use of high salt that must then be removed from the final pDNA pool with an additional operation.

Affinity chromatography is a powerful technique for the one-step purification of pDNA from a clarified lysate. Large volumes of lysate can be processed and pools with high concentration of pDNA are obtained. However, since each ligand in a stationary phase targets a specific base-sequence, the versatility of AC is low. This translates into high costs, which render the technique unfeasible as a pilot or process-scale purification step.

5. Future trends

A large number of chromatographic techniques has been exploited for the purpose of pDNA purification. However, there are still several problems that remain to be solved. Clearly, future research efforts will have to be focused towards the design of new stationary phases with an increased capacity for pDNA (e.g. "superporous" beads, beads with "hairy" surface, etc). The use of formats more adequate to handle large pDNA molecules, such as membranes and monoliths will likely become more and more important in pDNA chromatography, eventually surpassing the traditional bead format. The poor selectivity of some supports (e.g. anion-exchange) towards the separation of pDNA from impurities that raise concerns to regulatory agencies is still an important bottleneck. Imaginative solutions, such as the use of restricted access beads will have to be devised to improve this selectivity. The importance devoted to the separation of the different pDNA isoforms will also continue to push the development of stationary phases and chromatographic methodologies, both for preparative and analytical purposes. Scalability of the several chromatographic techniques to allow the industrial production of pDNA for therapeutic applications will become more important in years to come, since a large expansion in the market is expected with the emergence of the first clinically successful pDNA vectors.

Acknowledgements

This work was supported by FCT, the Portuguese Foundation for Science and Technology (POCTI/BIO/38509/2001).

References

[1] H.L. Davis, M.-L. Michel, R.G. Whalen, Ann. NY Acad. Sci. 772 (1995) 21.
[2] A. Mountain, Trends Biotechnol. 18 (2000) 119.
[3] F.D. Ledley, Hum. Gene Ther. 6 (1995) 1129.
[4] D.M.F. Prazeres, G.A. Monteiro, G.N.M. Ferreira, M.M. Diogo, S.C. Ribeiro, J.M.S. Cabral, in: M.R. El-Gewely (Ed.), Biotechnology Annual Review, Elsevier, Amsterdam, 2001, p. 1.
[5] D.M.F. Prazeres, G.N.M. Ferreira, G.A. Monteiro, C.L. Cooney, J.M.S. Cabral, Trends Biotechnol. 17 (1999) 169.
[6] R.R. Sinden, DNA Structure and Function, Academic Press, San Diego, CA, 1994.
[7] D.K. Summers, The Biology of Plasmids, Blackwell, Oxford, UK, 1996.
[8] H.C. Birnboim, J. Doly, Nucl. Acids Res. 7 (1979) 1513.
[9] D.S. Holmes, M. Quigley, Anal. Biochem. 114 (1981) 193.
[10] M.M. Diogo, J.A. Queiroz, G.A. Monteiro, D.M.F. Prazeres, Anal. Biochem. 275 (1999) 122.
[11] J. Sambrook, E.F. Fritisch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989.
[12] D.M.F. Prazeres, G.N.M. Ferreira, Chem. Eng. Process 43 (2004) 615.
[13] H.L. Davis, M. Schleef, P. Moritz, M. Mancini, J. Schorr, R.G. Whalen, Biotechniques 21 (1996) 92.
[14] G.N.M. Ferreira, J.M.S. Cabral, D.M.F. Prazeres, Biotechnol. Prog. 15 (1999) 725.
[15] M. Marquet, N.A. Horn, J.A. Meek, Bio. Pharm. (May) (1997) 42.
[16] M. Marquet, N. Horn, J.A. Meek, G. Budahazi, US Patent No. 5561064, 1 October 1996.
[17] N. Horn, J.A. Meek, G. Budahazi, M. Marquet, Hum. Gene Ther. 6 (1995) 565.
[18] 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.
[19] A.P. Green, G.M. Prior, N.M. Helveston, B.E. Taittinger, X. Liu, J.A. Thompson, Bio. Pharm. (May) (1997) 52.
[20] M.M. Diogo, S.C. Ribeiro, J.A. Queiroz, G.A. Monteiro, N. Tordo, P. Perrin, D.M.F. Prazeres, J. Gene Med. 3 (2001) 577.
[21] A.L. Lee, S. Sagar, US Patent No. 6197553, 6 March 2001.
[22] S. Nochumson, R. Durland, A. Yu-Speight, J. Welp, K. Wu, R. Hayes, US Patent No. 2001034435, 25 October 2001.
[23] R. Bhikhabhai, US Patent No. 6410274 B1, 25 June 2002.
[24] R. Lemmens, U. Olsson, T. Nyhammar, J. Stadler, J. Chromatogr. B 784 (2003) 291.
[25] G. Athas, Scientist 13 (1999) 20.
[26] H.E. Sussman, Scientist 13 (2003) 42.
[27] B. Atkinson, F. Mavituna (Eds.), Biochemical Engineering and Biotechnology Handbook, Macmillan, New York, 1991.
[28] N. Horn, M. Marquet, J.A. Meek, G. Budahazi, US Patent No. 5576196, 19 November 1996.

[29] G. Chandra, P. Patel, T. Kost, J. Gray, Anal. Biochem. 203 (1992) 169.
[30] M.S. Levy, I.J. Collins, J.T. Tsai, P.A. Shamlou, J.M. Ward, P. Dunnill, J. Biotechnol. 76 (2000) 197.
[31] D. Kendall, G.J. Lye, M.S. Levy, Biotechnol. Bioeng. 79 (2002) 816.
[32] D.W. Kahn, M.D. Butler, D.L. Cohen, M. Gordon, J.W. Kahn, M.E. Winkler, Biotechnol. Bioeng. 69 (2000) 101.
[33] A. Eon-Duval, G. Burke, J. Chromatogr. B 804 (2004) 327.
[34] A. Eon-Duval, R.H. MacDuff, C.A. Fisher, M.J. Harris, C. Brook, Anal. Biochem. 316 (2003) 66.
[35] D.M.F. Prazeres, T. Schluep, C. Cooney, J. Chromatogr. A 806 (1998) 31.
[36] V.I. Ugarov, T.R. Samatov, H.V. Chetverina, A.B. Chetverin, Biotechniques 26 (1999) 194.
[37] J.C. Murphy, J.A. Wibbenmeyer, G.E. Fox, R.C. Wilson, Nat. Biotechnol. 17 (1999) 822.
[38] A. Eon-Duval, K. Gumbs, C. Ellet, Biotechnol. Bioeng. 83 (2003) 544.
[39] G.N.M. Ferreira, J.M.S. Cabral, D.M.F. Prazeres, Bioseparation 9 (2000) 1.
[40] L.A. Ciccolini, P.A. Shamlou, N.J. Titchener-Hooker, J.M. Ward, P. Dunnill, Biotechnol. Bioeng. 60 (1998) 768.
[41] D.M. Fishman, G.D. Patterson, Biopolymers 38 (1996) 535.
[42] M.T. Tyn, T.W. Gusek, Biotechnol. Bioeng. 35 (1990) 327.
[43] C.G. Huber, J. Chromatogr. A 806 (1998) 3.
[44] G.N.M. Ferreira, J.M.S. Cabral, D.M.F. Prazeres, Biotechnol. Prog. 16 (2000) 416.
[45] J. Coffman, J. Eldering, A. Schwarz, E. Boschetti, Poster presented at recovery of biological products VIII, Tucson, AZ, 1996.
[46] I. Theodossiou, M. Sondergaard, O.R. Thomas, Bioseparation 10 (2001) 31.
[47] Perseptive-Biosystems, Biochemica 1 (1996) 9.
[48] K. Branovic, D. Forcic, J. Ivancic, A. Strancar, M. Barut, T. Gulija, R. Zgorelec, R. Mazuran, J. Chromatogr. B 801 (2004) 331.
[49] M.A. Teeters, S.E. Conrardy, B.L. Thomas, T.W. Root, E.N. Lightfoot, J. Chromatogr. A 989 (2003) 165.
[50] H.N. Endres, J.A.C. Johnson, C.A. Ross, J.K. Welp, M.R. Etzel, Biotechnol. Appl. Biochem. 37 (2003) 259.
[51] S. Zhang, A. Krivosheyeva, S. Nochumson, Biotechnol. Appl. Biochem. 37 (2003) 245.
[52] G.N.M. Ferreira, D.M.F. Prazeres, J.M.S. Cabral, Processo cromatográfico para purificação de DNA plasmídico utilizando suportes superporosos, PT 102,341, 1 January 2003.
[53] J. Stadler, R. Lemmens, T. Nyhammar, J. Gene Med. 6 (2004) 54.
[54] A. Ljunglof, P. Bergvall, R. Bhikhabhai, R. Hjorth, J. Chromatogr. A 844 (1999) 129.
[55] E. Thwaites, S.C. Burton, A. Lyddiatt, J. Chromatogr. A 943 (2001) 77.
[56] I. Theodossiou, O.R.T. Thomas, J. Chromatogr. A 971 (2002) 73.
[57] J.C. Murphy, G.E. Fox, R.C. Willson, J. Chromatogr. A 984 (2003) 215.
[58] N. Horn, G. Budahazi, M. Marquet, US Patent No. 5707812, 13 January 1998.
[59] A. Lyddiatt, D. O'Sullivan, Curr. Opin. Biotechnol. 9 (1998) 177.
[60] P.-E. Gustavsson, P.-O. Larsson, J. Chromatogr. A 734 (1996) 231.
[61] M. McCoy, K. Kalghatgi, N. Afeyan, J. Chromatogr. A 743 (1996) 221.
[62] R. Giovannini, R. Freitag, T.B. Tennikova, Anal. Chem. 70 (1998) 3348.
[63] W.-C. Tseng, F.L. Ho, T.-Y. Fang, S.Y. Suen, J. Memb. Sci. 233 (2004) 161.
[64] M. Bywater, R. Bywater, L. Hellman, Anal. Biochem. 132 (1983) 219.
[65] D. Micard, M. Sobrier, J. Couderc, B. Dastugue, Anal. Biochem. 148 (1985) 121.
[66] G. Ferreira, J. Cabral, D. Prazeres, J. Mol. Recognit. 11 (1998) 250.
[67] G. Ferreira, J. Cabral, D. Prazeres, Biotechnol. Technol. 11 (1997) 417.
[68] J. Gómez-Márquez, M. Freire, F. Segade, Gene 54 (1987) 255.
[69] T. Vo-Quang, Y. Malpiece, D. Buffard, P. Kaminski, D. Vidal, A. Strsberg, Biosci. Rep. 5 (1985) 101.
[70] A. Suominen, M. Karp, P. Mantsala, Biochem. Int. 8 (1984) 209.
[71] J. McClung, R. Gonzales, Anal. Biochem. 177 (1989) 378.
[72] E. Whisenant, B. Rasheed, Y. Bhatnagar, Nucleic Acids Res. 16 (1988) 5202.
[73] N. Moreau, X. Tabary, F. Le Goffic, Anal. Biochem. 166 (1987) 188.
[74] J. Hirabayashi, K.-I. Kasai, J. Chromatogr. A 893 (2000) 115.
[75] G. Raymond, P. Bryant, A. Nelson, J. Johnson, Anal. Biochem. 173 (1988) 125.
[76] J. Hirabayashi, K.-I. Kasai, Anal. Biochem. 178 (1989) 336.
[77] J. Hirabayashi, K.-I. Kasai, J. Chromatogr. A 722 (1996) 135.
[78] M. Colpan, D. Riesner, J. Chromatogr. 296 (1984) 339.
[79] Y. Onishi, Y. Azuma, H. Kizaki, Anal. Biochem. 210 (1993) 63.
[80] Y.-F. Maa, S.-C. Lin, C. Horváth, U.-C. Yang, D.M. Crothers, J. Chromatogr. 508 (1990) 61.
[81] M.M. Diogo, S. Ribeiro, J.A. Queiroz, G.A. Monteiro, P. Perrin, N. Tordo, D.M.F. Prazeres, Biotechnol. Lett. 22 (2000) 1397.
[82] G. Ferreira, J. Cabral, D. Prazeres, Pharm. Pharmacol. Commun. 5 (1999) 57.
[83] M. Schleef, in: A. Mountain, U. Ney, D. Schomburg (Eds.), Biotechnology. Recombinant Proteins, Monoclonal Antibodies and Therapeutic Genes,, vol. 5a, Wiley-VCH, Weinheim, 1999 (Chapter 20).
[84] R. Hines, K. O'Connor, G. Vella, W. Warren, Biotechniques 12 (1992) 430.
[85] M. Merion, W. Warren, Biotechniques 7 (1989) 60.
[86] W.-C. Tseng, F.L. Ho, J. Chromatogr. B 791 (2003) 263.
[87] P.-E. Gustavsson, R. Lemmens, T. Nyhammar, P. Busson, P.-O. Larsson, J. Chromatogr. A 1038 (2004) 131.
[88] H. Yamakawa, K.-i. Higashino, O. Ohara, Anal. Biochem. 240 (1996) 242.
[89] G.N.M. Ferreira, G.A. Monteiro, D.M.F. Prazeres, J.M.S. Cabral, Trends Biotechnol. 18 (2000) 380.
[90] C.S. Alberin, S.G. Franklin, US Patent No. 6406892, 18 June 2002.
[91] A. Colman, M.J. Byers, S.B. Primrose, A. Lyons, Eur. J. Biochem. 91 (1978) 303.
[92] T.R. Johnson, J. Ilan, Anal. Biochem. 132 (1982) 20.
[93] R. Giovannini, R. Freitag, Biotechnol. Bioeng. 77 (2002) 445.
[94] A.N. Best, D.P. Allison, G.D. Novelli, Anal. Biochem. 114 (1981) 235.
[95] S. Colote, C. Ferraz, J.P. Liautard, Anal. Biochem. 154 (1986) 15.
[96] M.P. Weiner, T.W. Thannhauser, J.H. Laity, M.E. Benning, D.P. Lee, H.A. Scheraga, Nucl. Acids Res. 16 (1988) 8183.
[97] D. McNeilly, US Patent No. 6214586, 10 April 2001.
[98] 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.
[99] T. Kitamura, S. Nakatani, US Patent No. 6441160 B2, 27 August 2002.
[100] M.M. Diogo, J.A. Queiroz, D.M.F. Prazeres, J. Chromatogr. A 998 (2003) 109.
[101] S. Iuliano, J.R. Fisher, M. Chen, W.J. Kelly, J. Chromatogr. A 972 (2002) 77.
[102] R.B. Sparks, J.H. Elder, Anal. Biochem. 135 (1983) 345.
[103] M.M. Diogo, J.A. Queiroz, D.M.F. Prazeres, Bioseparation 10 (2002) 211.
[104] P. Cashion, G. Sathe, A. Javed, J. Kuster, Nucl. Acids Res. 8 (1980) 1167.
[105] J.A. Queiroz, C.T. Tomaz, J.M.S. Cabral, J. Biotechnol. 87 (2001) 143.

[106] T. Schluep, C. Cooney, Nucl. Acids Res. 26 (1998) 4524.
[107] T. Schluep, C. Cooney, Bioseparation 7 (1999) 317.
[108] P. Wils, V. Escriou, A. Warnery, F. Lacroix, D. Lagneaux, M. Ollivier, J. Crouzet, J.-F. Mayaux, D. Scherman, Gene Ther. 4 (1997) 323.
[109] J. Woodgate, D. Palfrey, D.A. Nagel, A.V. Hine, N.K.H. Slater, Biotechnol. Bioeng. 79 (2002) 450.
[110] J. Murphy, D. Jewell, K. White, G. Fox, R. Willson, Biotechnol. Prog. 19 (2003) 982.
[111] N. Singh, R.C. Willson, J. Chromatogr. A 840 (1999) 205–213.
[112] P.M. Montbriand, R.W. Malone, J. Biotechnol. 44 (1996) 43.
[113] I.P. Wicks, M.L. Howell, T. Hancock, H. Kohsaka, T. Olee, D.A. Carson, Hum. Gene Ther. 6 (1995) 317.
[114] T. Ito, C.L. Smith, C.R. Cantor, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 495.
[115] H. Ji, L.M. Smith, Anal. Chem. 65 (1993) 1323.
[116] D. Petsch, B.F. Anspach, J. Biotechnol. 76 (2000) 97.
[117] M. Marquet, N.A. Horn, J.A. Meek, Bio. Pharm. (September) (1995) 26.