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Novel Affinity Chromatography Processes for the Purification of Plasmid DNA Using Small Aromatic Molecules

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To my parents and sister

Somewhere, something incredible is waiting to be known.

Carl Sagan

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List of publications

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I. **Aromatic ligands for plasmid DNA chromatographic analysis and purification: an overview**
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III. **Purification of plasmid DNA from clarified and non-clarified *Escherichia coli* lysates by berenil pseudo-affinity chromatography**
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IV. **Negative pseudo-affinity chromatography for plasmid DNA purification using berenil as ligand**
C. Caramelo-Nunes, M.F. Gabriel, P. Almeida, J.C. Marcos, C.T. Tomaz
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V. **Specific recognition of supercoiled plasmid DNA by affinity chromatography using the intercalator DAPP as ligand**
C. Caramelo-Nunes, P. Almeida, J.C. Marcos, C.T. Tomaz
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VI. **Dynamic binding capacity and specificity of 3,8-diamino-6-phenylphenanthridine-Sepharose support for purification of supercoiled plasmid deoxyribonucleic acid**
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Papers from scientific conferences:

I. **Separation of plasmid DNA from non clarified lysate impurities using berenil as ligand in pseudo-affinity chromatography**
C. Caramelo-Nunes, M. Gabriel, P. Almeida, J.C. Marcos, C.T. Tomaz
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I. **A New Aproach For Affinity Chromatography Plasmid Purification Using Berenil As Ligand**
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V ANNUAL CICS SYMPOSIUM, July 6, University of Beira Interior, Covilhã, Portugal, 2010.

II. **Small natural ligands for plasmid DNA purification by affinity chromatography**
C. Caramelo-Nunes, T. Tente, P. Almeida, J.C. Marcos, C.T. Tomaz
8th European Symposium on Biochemical Engineering Science (ESBES), September 6-8, Bologna, Italy, 2010.

III. **Separation of plasmid DNA from non clarified lysate impurities using berenil as ligand in pseudo-affinity chromatography**
C. Caramelo-Nunes, M.F. Gabriel, P. Almeida, J.C. Marcos, C.T. Tomaz
Simpósio 2011 – Transferência de Conhecimento e Tecnologia, December 15-16, University of Beira Interior, Covilhã, Portugal, 2011.

IV. **Specific berenil-DNA interactions: an overall study in pDNA purification by pseudo-affinity chromatography**
C. Caramelo-Nunes, M.F. Gabriel, P. Almeida, J.C. Marcos, C.T. Tomaz
7th National Meeting on Chromatography, January 9-11, University of Porto, Porto, Portugal, 2012.

V. **Intercalator DAPP as a new affinity ligand for supercoiled pDNA chromatographic separation**
C. Caramelo-Nunes, P. Almeida, J.C. Marcos, C.T. Tomaz
32nd International Symposium on the separation of Proteins, Peptides and Polynucleotides, September 23-26, Istanbul, Turkey, 2012.

VI. **DAPP as affinity ligand: support characterization and chromatographic studies with pDNA**
C. Caramelo-Nunes, P. Almeida, J.C. Marcos, C.T. Tomaz
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Poster presentations resultant of the Doctoral work:

I. **Small natural ligands for plasmid DNA purification by affinity chromatography**
C. Caramelo-Nunes, T. Tente, P. Almeida, J.C. Marcos, C.T. Tomaz
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II. **Purification of plasmid DNA from non clarified lysates using berenil as ligand in pseudo-affinity chromatography**
C. Caramelo-Nunes, M.F. Gabriel, P. Almeida, J.C. Marcos, C.T. Tomaz
Affinity 2011, June 16-19, Tavira, Portugal, 2011.

III. **Purification of plasmid DNA from clarified cell lysates by a single chromatographic step using berenil as ligand**
C. Caramelo-Nunes, M.F. Gabriel, P. Almeida, J.C. Marcos, C.T. Tomaz
Preparative and Process Chromatography, July 10-13, Boston, USA, 2011.

IV. **DAPP-Sepharose affinity chromatography: supercoiled pDNA purification from clarified *E. coli* lysate solutions**
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Resumo alargado

As terapias moleculares que se baseiam na prevenção e tratamento de doenças por transferência genética, estão a tornar-se fortes alternativas aos tratamentos clássicos. A administração dos genes terapêuticos é feita recorrendo a veículos ou vetores que podem ser de origem viral ou não viral. Presentemente, os vetores virais ainda são considerados os mais eficientes para a administração genética, contudo os vetores baseados em DNA plasmídico (pDNA) estão a ganhar popularidade devido à sua simplicidade de produção e segurança, sendo o terceiro tipo de vetor mais usado em ensaios clínicos. Desta forma, a investigação relacionada com novos métodos para a obtenção de quantidades substanciais de pDNA de grau terapêutico tem aumentado bastante. O pDNA para fins terapêuticos deve estar em conformidade com as especificações das agências reguladoras, seguindo rigorosos critérios de qualidade em termos de uso de reagentes tóxicos ou de origem animal, bem como de pureza relativamente aos restantes constituintes dos lisados celulares. Contudo, as semelhanças existentes entre o pDNA e os seus contaminantes comuns, como proteínas, endotoxinas, DNA genómico e RNA, podem complicar a sua separação, pelo que o processo de purificação deverá ser extremamente eficiente.

A cromatografia líquida é o método mais utilizado para a purificação de pDNA, uma vez que é um processo relativamente simples, robusto, versátil e altamente reprodutível. Os métodos cromatográficos exploram propriedades do pDNA como tamanho, hidrofobicidade, carga e a afinidade das suas bases aos ligandos, para o separar das suas impurezas com a maior eficiência possível. A flexibilidade deste tipo de processo advém da grande variedade de suportes e ligandos que podem ser usados, tendo em conta as especificidades da molécula a separar. A identificação e seleção de ligandos de elevada seletividade e afinidade é presentemente uma prioridade no desenvolvimento de novos processos cromatográficos. De facto, a composição química dos suportes determina as interações estabelecidas com as moléculas alvo, permitindo uma interação preferencial com estas em detrimento das moléculas contaminantes.

Assim sendo, o objetivo deste trabalho foi desenvolver novos métodos cromatográficos para a purificação de pDNA de grau terapêutico, com o propósito de implementar métodos mais eficientes, mais simples, com menor custo e menor impacto ambiental. O ligando de "minor groove" berenil e o intercalante 3,8-diamino-6-phenylphenanthridine (DAPP) foram as moléculas escolhidas para serem testadas como ligandos cromatográficos. Ambos foram imobilizados numa matriz de Sepharose epoxi-ativada, em condições moderadas, sem recorrer a catalisador e com rácios de ligando:Sepharose relativamente baixos.

O berenil estabelece ligações não covalentes reversíveis com as bases do DNA, preferencialmente ao nível das sequências A-T. Liga-se principalmente através de interações hidrofóbicas, contudo, outras interações como as electrostáticas, van der Waals e pontes de

hidrogénio não podem ser negligenciadas. Com quantidades moderadas de sulfato de amónio no eluente, a matriz berenil-Sepharose mostrou ser bastante eficaz na separação e purificação de pDNA presente em lisados clarificados e não clarificados, usando três métodos distintos. Contudo, a separação da isoforma superenrolada das restantes não foi conseguida em nenhum dos casos. Porém, se a solução injetada for constituída por uma mistura purificada das isoformas superenrolada e circular aberta, estas são facilmente separadas. Usando o suporte berenil-Sepharose foi possível purificar dois plasmídeos de diferentes tamanhos a partir de lisados clarificados contendo cada uma deles. O mesmo se verificou quando o processo de clarificação foi substituído por um segundo passo cromatográfico. Em ambos os casos, as soluções de pDNA obtidas estavam de acordo com as especificações exigidas para um produto com aplicações terapêuticas. No entanto, os rendimentos obtidos apresentaram algumas diferenças importantes: os dois ensaios cromatográficos consecutivos originaram rendimentos relativamente baixos (33%), enquanto no método cromatográfico associado a um passo de clarificação foi obtido um rendimento superior para o pDNA de menor tamanho relativamente ao de maior dimensão (85% *vs* 45%). Estudos de cromatografia negativa foram também realizados com este suporte, os quais demonstraram algumas vantagens em termos de quantidade de sal utilizado e tempo total do método. Neste caso, o rendimento obtido foi bastante bom (87%) mas, apesar de as soluções de pDNA terem graus de pureza semelhantes às obtidas por cromatografia positiva, a redução da contaminação de DNA genómico não se revelou tão eficiente.

As moléculas de DAPP têm alguma especificidade para com as sequências A-T e ligam-se ao pDNA por introdução reversível e não covalente dos anéis aromáticos condensados entre dois pares de bases consecutivos, enquanto o grupo fenilo se introduz no "minor groove". Tal sugere que as interações hidrofóbicas têm um papel importante na ligação. Contudo, quando as moléculas de DAPP estão protonadas, estas ligam-se ao pDNA de forma muito mais forte devido ao estabelecimento de fortes interações electrostáticas. A ligação do pDNA à matriz DAPP-Sepharose é influenciada por variações de pH e pela presença de sal nas fases líquidas. Na verdade, a retenção total de todos os constituintes do lisado clarificado injetado só foi possível a um pH inferior ao pKa da molécula de DAPP (5,8), verificando-se que a presença de sal destabiliza essa mesma retenção. Assim, a eluição das espécies retidas foi facilmente conseguida por adição de pequenas quantidades de cloreto de sódio ao eluente. Todas estas características permitiram a purificação com sucesso de pDNA superenrolado, de diferentes massas moleculares, o qual foi obtido de acordo com as especificações das agências reguladoras. Uma vez mais, o rendimento para o pDNA de menor tamanho ultrapassou em muito o do pDNA de maiores dimensões (94% *vs* 65%). A capacidade dinâmica de ligação ao pDNA obtida para o suporte DAPP-Sepharose foi de 336,75 μg pDNA/mL gel, um valor aceitável considerando que é um suporte não comercial, com uma densidade de ligandos relativamente baixa (0,15 mmol DAPP/g Sepharose derivatizada). Para além disso, a

constante de dissociação (2,29 ± 0,195 x 10^{-7} M) constitui uma evidência importante de que esta matriz apresenta uma elevada afinidade para com o pDNA.

Em conclusão, o DAPP-Sepharose apresenta as características ideais para ser aplicado como suporte de afinidade na purificação de pDNA superenrolado de grau farmacêutico. Em comparação com a fase estacionária de berenil-Sepharose, usa quantidades de sal muito menores, tendo portanto um impacto económico e ambiental menor, ao mesmo tempo que permite a obtenção de pDNA com melhor qualidade e rendimento mais elevado. Para além disso, também permite a separação da isoforma superenrolada da linear e circular aberta, mesmo a partir de lisados complexos.

Desta forma, combinar um passo cromatográfico, usando o suporte DAPP-Sepharose, com processos optimizados de produção, extração e clarificação, pode ser extremamente vantajoso para a produção de pDNA. Para além disso, uma vez que a maior desvantagem deste suporte é a sua baixa capacidade para com o pDNA, que por sua vez está diretamente relacionada com a matriz usada, será extremamente interessante usar, como alternativa, outras matrizes mais estáveis e com macroporos interconectados que permitam uma elevada transferência de massa dos solutos.

Palavras-chave

Berenil-Sepharose, cromatografia de afinidade, cromatografia negativa de pseudo-afinidade, cromatografia de pseudo-afinidade, DAPP-Sepharose, DNA plasmídico superenrolado, intercalante, ligando de minor groove, pequenos ligandos do DNA, purificação de plasmídeos.

Abstract

Molecular therapies are gaining importance as an effective therapeutic approach for various types of diseases. The most efficient vectors used to introduce the therapeutic genes are of viral origin however, non-viral vectors based on pDNA are gaining popularity due to their superior safety and easy of production. These factors have increased the demand for high quantities of pharmaceutical grade plasmid DNA (pDNA). Therefore, the research for more efficient pDNA purification protocols has also increased. Moreover, the final pDNA product must meet stringent quality criteria established by the regulatory agencies.

Liquid chromatography is the method of choice for the purification of pDNA, since it is simple, robust, versatile and high reproducible. The most important features of a chromatographic procedure are the use of suitable stationary phases and ligands. As conventional purification protocols are being replaced by more sophisticated and selective procedures, the focus changes towards designing and selecting ligands of high affinity and specificity. In fact, the chemical composition of the chromatographic supports determines the interactions established with the target molecules, allowing their preferential retention over the undesirable ones.

With these facts in mind, the aim of this work was to develop new chromatographic methods for the purification of pharmaceutical grade pDNA, with the purpose of improving the overall procedures to more effective, simple, economic and environmental-friendly ones. The minor groove binder berenil and the intercalator 3,8-diamino-6-phenylphenanthridine (DAPP) where chosen to be used as ligands in pDNA chromatographic purification studies. They were immobilized to an epoxy-activated Sepharose matrix using a relatively mild curing method, without a catalyst and with quite small ligand:Sepharose weight ratios.

Berenil binds pDNA preferentially through hydrophobic interactions but other types of interaction contributions cannot be neglected. It was shown to be quite effective at separating and purifying pDNA from clarified and non-clarified cell lysates, using three different approaches, although isoform resolution was not obtained in either case. Using mild amounts of ammonium sulphate in the eluent, berenil-Sepharose support was able to purify distinct pDNA of two different sizes from clarified cell lysates. Moreover, the ability was continual when the clarification process was replaced by a second chromatographic run. In all cases plasmid solutions were in accordance to the specifications of a pharmaceutical product, however the yields were quite different: two consecutive chromatographic runs lead to lower recoveries (33%) and smaller pDNA molecules have higher recoveries using one run through the column (85% *vs* 45%). A negative chromatography approach was also performed with berenil-Sepharose, showing some advantages in terms of salt usage as well as procedure time. In this case the recovery yield was quite good (87%) and although pDNA solutions had a comparable purity to that obtained with the other approaches, the gDNA reduction was not so effective.

DAPP is slightly A-T specific and binds DNA through non-covalent, reversible stacking interactions of the condensed aromatic moiety into two successive base pairs, while the phenyl residue gets inserted into the minor groove. In addition, protonated DAPP molecules bind to DNA much strongly due to the generation of strong electrostatic interactions. Plasmid DNA binding to DAPP-Sepharose varies with pH and is affected by the presence of salt in the eluent. In fact, total retention of clarified lysate components was only possible with a pH below DAPP's free state pKa (5.8) and the presence of salt destabilizes that same retention. So, the elution of bound species was simply performed by adding small amounts of sodium chloride to the buffers. These features were successfully applied for purification of sc pDNA with two distinct sizes that were obtained according to the regulatory agencies specifications. Once more, the recovery yield of the smaller pDNA molecule was higher than the one obtained for the largest one (94% *vs* 65%).

In conclusion, DAPP-Sepharose showed exceptional characteristics to be used as an affinity support for the purification of pharmaceutical grade sc pDNA. In comparison with berenil-Sepharose, it uses much smaller amounts of salt, with less economic and environmental impact, while improving the quality and yield of the obtained plasmid fractions. Moreover, it is able to separate sc pDNA from linear and oc isoforms even in complex lysates.

Moreover, combining DAPP-Sepharose chromatography with other optimized production, extraction and clarification procedures, can offer a number of advantages for pharmaceutical pDNA purification. Also, since the most significant disadvantage of this DAPP-Sepharose support is the relatively low capacity for pDNA, which in turn is strongly related to the solid matrix used, other more stable stationary phases with low pressure drops and interconnected macropores, that allow a high mass transfer of solutes, are quite fascinating alternatives.

Keywords

Affinity chromatography, berenil-Sepharose, DAPP-Sepharose, intercalator, minor groove binder, negative pseudo-affinity chromatography, plasmid purification, pseudo-affinity chromatography, small DNA ligands, supercoiled plasmid DNA.

Thesis overview

This doctoral thesis is structured in five main chapters. The first chapter consists of a succinct literature revision closely related to the purposed objectives, in which the review article (Paper I) was included. The second chapter includes the global aims of this doctoral work and its development purpose. The third chapter includes the work developed during the PhD studies in form of original research articles, organized as follows:

Paper II - Specific berenil–DNA interactions: An approach for separation of plasmid isoforms by pseudo–affinity chromatography

Paper III - Purification of plasmid DNA from clarified and non-clarified Escherichia coli lysates by berenil pseudo-affinity chromatography

Paper IV - Negative pseudo-affinity chromatography for plasmid DNA purification using berenil as ligand

Paper V - Specific recognition of supercoiled plasmid DNA by affinity chromatography using the intercalator DAPP as ligand

Paper VI - Dynamic binding capacity and specificity of 3,8-diamino-6-phenylphenanthridine-Sepharose support for purification of supercoiled plasmid deoxyribonucleic acid

The fourth chapter includes the patent originated from the research work and finally, the fifth chapter summarizes all the concluding remarks obtained throughout the PhD work.

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Abbreviations

AC	Affinity chromatography
AEC	Anion-exchange chromatography
AT	Adenine - thymine
bp	Base pair
CsCl	Cesium chloride
DNA	Deoxyribonucleic acid
EtBr	Ethidium bromide
GC	Guanine - cytosine
gDNA	Genomic deoxyribonucleic acid
HAC	Hydroxypatite chromatography
HIC	Hydrophobic chromatography
IMAC	Immobilized metal-ion affinity chromatography
kbp	Kilo-base pair
mRNA	Messenger ribonucleic acid
oc	Open circular
pDNA	Plasmid deoxyribonucleic acid
RNA	Ribonucleic acid
RPIPC	Reverse-phase ion-pair chromatography
RPLC	Reverse-phase liquid chromatography
sc	Supercoiled
SDS	Sodium dodecyl sulfate
SEC	Size-exclusion chromatography
SiRNA	Small interfering ribonucleic acid
TAC	Thiophilic adsorption chromatography
THAC	Triple-helix affinity chromatography

Chapter 1

Introduction

1. Molecular therapies: an overview

The groundwork for molecular therapies was established in the late 70´s and early 80´s with the emergence of subcloning techniques of mammalian genes into prokaryotic plasmids and bacteriophages (Flotte, 2007). Inevitably, the following years testified the first clinical trials using quite different approaches (Cavazzana-Calvo *et al.*, 2000; Rosenberg *et al.*, 1990; Tang *et al.*, 1992). However, the great amount of tests that followed (Edelstein *et al.*, 2007) were greatly improved by the conclusion of the human genome project (McIlwain, 2000), since a better understanding of genetics and related diseases was achieved.

Molecular therapies aim at treating and preventing diseases by gene transfer, eliminating the cause instead of only treating the symptoms (Mountain, 2000). In theory, all diseases of genetic origin can be treated by gene therapy, administrating healthy copies of the mutated gene or promoting a therapeutic immune response with the administration of genes encoding specific antigens (Brown *et al.*, 2001). When a direct injection of DNA leads to an immune response sufficient enough to protect an organism from live infectious agents, the gene therapy approach is referred to as DNA vaccines (Kutzler *et al.*, 2008).
The production of any chosen transgene can be possible with gene therapy. It can be a protein which is deficient due to e.g. a genetic abnormality, or it can be a protein with a therapeutic effect (Gothelf *et al.*, 2010). The recipient organism or tissue acquires the ability to synthesize the therapeutic agent using the genetic information provided by the transferred transgene (Rome *et al.*, 2007), restoring the defective gene's function or initiating a new one (Stribley *et al.*, 2002). Thus, gene therapies require an extensive research to identify the genetic origin of a target disease.

The gene therapy process involves three steps: administration of DNA into the body, delivery of the genetic material from the administration site to the nucleus of target cells and finally the expression or output of the therapeutic gene product (Stribley *et al.*, 2002). The administration is performed by the introduction of an appropriate carrier or vector into the body, either locally (direct tissue injection) or into the blood stream (systemic delivery). Next, the delivery system has to "find" its target tissue, enter the cells, pass the cytoplasm and reach the nucleus. There, the therapeutic gene is transcribed and the resultant mRNA (messenger RNA) is translated into the therapeutic protein. Lastly, this protein acts on its receptor, inducing the biological effect, resulting in the expected therapeutic benefits (Rubanyi, 2001).
However, the successful implementation of a gene therapy treatment still has to overcome many challenges such as choosing the appropriate gene, select a suitable and safe delivery system, targeting the right tissue, controlling the host immune response against the

transferred gene and vector system, to avoid dangerous side effects or the destruction of the gene, achieving a stable transfection and an appropriate gene expression and finally, the obtained results must supersede those from conventional therapy (Stribley *et al.*, 2002; Worgall, 2005).

1.1. Approaches and strategies for gene therapy

There are two distinct types of gene therapy, the commonly used somatic gene therapy and germ-line gene therapy. Somatic gene therapy implicates the introduction and expression of recombinant genes into somatic cells, with the objective of treating an inherited or acquired disease. In this case, the modified DNA will not pass to the subsequent generations (Williams and Orkin, 1986). On the other hand, germ-like gene therapy affects the reproductive cells of an individual, eliminating an inherited disease and affecting all subsequent generations (Stribley *et al.*, 2002).

Regardless of the gene transfection technique, gene therapy can be divided into three different approaches, distinguished by where the final product will act (Gothelf *et al.*, 2010):

a) The intracrine approach, or single cell approach, in which the therapeutic product acts on the cells that produce it. In this case, every cell in a population must be targeted so that all therapy benefits can be achieved.
b) The paracrine approach, in which the transfected cells produce a therapeutic product that acts locally on neighbouring cells.
c) The systematic approach, in which normal cells are chosen to act as "factories" for the therapeutic product, and are transfected with the gene. The protein is then delivered to the blood stream where it can act systematically (Gothelf *et al.*, 2010).

Moreover, depending on the intended outcome, gene therapy may be classified as corrective or cytotoxic. The first strategy aims at the correction of a genetic defect by transferring the genes directly to the target cells. Differently, the cytotoxic strategy has the objective of destroying the target cells, using a cytotoxic pathway. For example, the therapeutic gene may encode a product that transforms a monotoxic prodrug into a toxic chemical, mediating not only the death of the cell that has taken up the gene but also the ones surrounding it (Stribley *et al.*, 2002).

1.2. Strategies for gene delivery

The administration of the therapeutic gene can be achieved through two distinct strategies, the *ex vivo* and *in vivo* approaches (Figure 1.1) (Stribley *et al.*, 2002; Thomas and Klibanov, 2003).

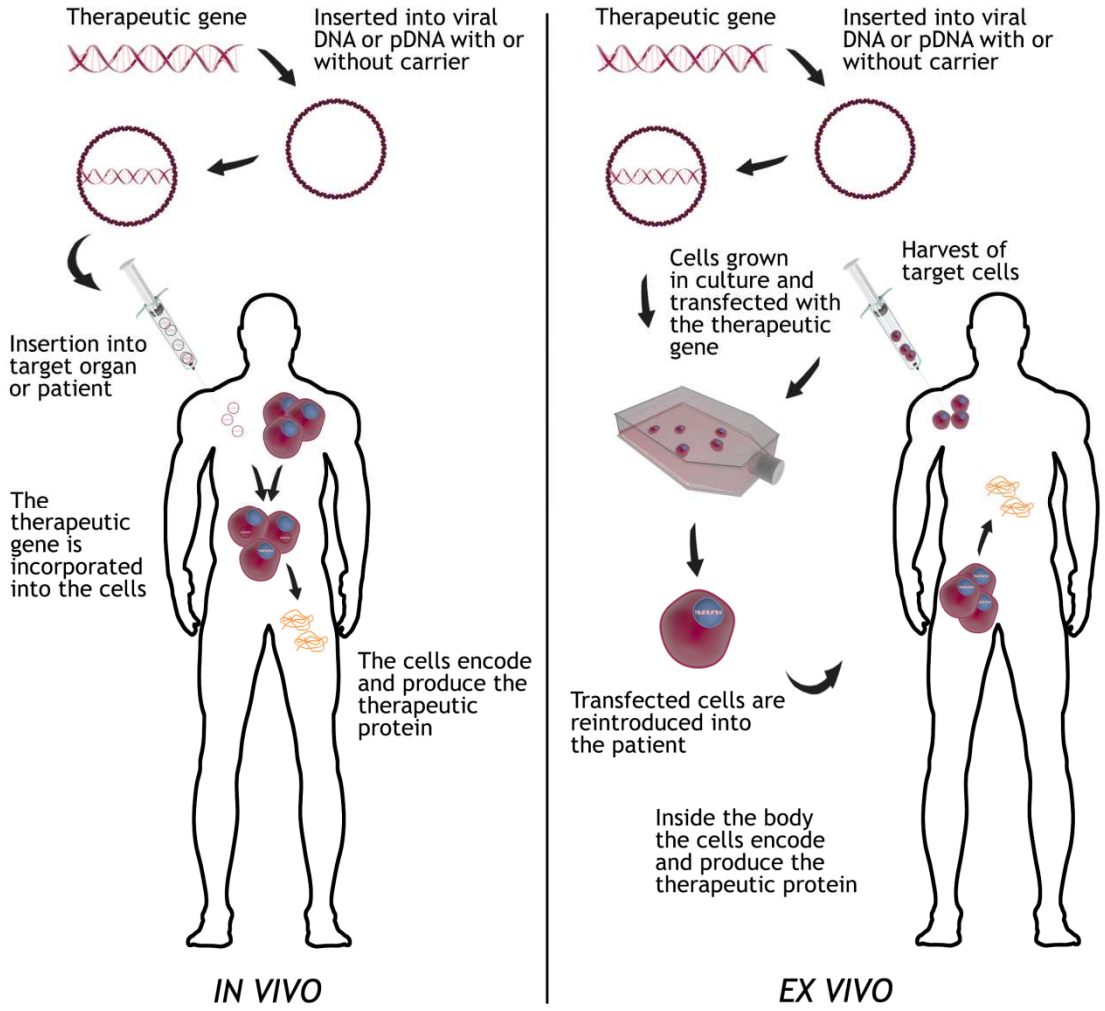

Figure 1.1. Strategies for gene administration (based on Stribley *et al.*, 2002).

In the *ex vivo* approach, the cells are harvested from patients after surgical biopsy or organ resection, the cells are cultured and the therapeutic genes are inserted *in vitro*. The transfected cells are carefully selected, expanded and reintroduced back into the patient. The effectiveness of this strategy depends on the permanent expression of the therapeutic gene. On the other hand, the *in vivo* approach is characterized for the direct injection of the genetic material into the target organ or patient. This is the only practicable option in tissues for which individual cells cannot be cultured in sufficient amounts or the cultured cells cannot be re-implanted. As opposed to the *ex vivo* strategy, permanent integration may not occur and only a temporary expression of the gene product takes place, since the sequences may undergo degradation or elimination from the cells (Stribley *et al.*, 2002; Thomas and Klibanov, 2003).

1.3. Gene delivery systems

The success of gene therapy greatly depends on the development of the appropriate carrier or vector. This vehicle has the purpose of carry the therapeutic gene into the intracellular

environment of the target cells (Stribley *et al.*, 2002). Gene delivery systems should be designed to protect the carried DNA from degradation and to efficiently transfer the genes to the target cells (Han *et al.*, 2000). Moreover, the vectors should be non-toxic to the target organism (Anderson, 1998). Currently used delivery systems can be divided into two main categories, viral and non-viral. Thus, there are some reports of the use of hybrid viral/non-viral vectors, produced from viral particles associated with DNA complexes (Curiel *et al.*, 1991), cationic lipid formulations (Fasbender et al., 1997) or synthetic polymers (Keswani, 2012). However, given the wide diversity of target cells, a single vector system is unlikely to be suitable for all gene therapy applications.

1.3.1. Viral based vectors

Viral vectors are the most commonly used vehicles in gene therapy (Johnson-Saliba and Jans, 2001), since viruses have evolved to become highly efficient at delivering their DNA to specific cell types while avoiding immunosurveillance (Robbins and Ghivizzani, 1998; Rubanyi, 2001). Viruses are not more than small DNA or RNA genomes surrounded by protein shells or capsids, which protect the genome and deliver it to specific cells (Evans and Lesnaw, 2002). They can efficiently recognize and enter cells, travel through the cytoplasm, translocate into the nucleus and express their genes in the host cell (Robbins and Ghivizzani, 1998; Rubanyi, 2001). Since viruses have limited genetic information, they greatly depend on the host cells machinery for their replication (Evans and Lesnaw, 2002).

Step one of designing a viral vector is to identify the viral sequences required for viral particle assembly, packaging of viral genome into the particles and delivery of the therapeutic gene. After that, some key genes are deleted from the viral genome to reduce the immuno- and pathogenicity of the vector. The residual viral genome and the therapeutic gene or transgene are then integrated into the vector structure (Pfeifer and Verma, 2001).

The most common gene therapy viral vectors are the adenoviruses, adeno-associated viruses, retroviruses, which include the lentiviruses, and the herpes simplex viruses. Also, poxviruses and alphaviruses have also been referred. Despite their success, there are many problems associated with viral vectors related to immunogenicity, insertional mutagenesis and latency (table 1.1).

Table 1.1. Characteristics of viral delivery systems

Viral system	Genetic material	Advantages	Disadvantages	References
Adenoviruses	Double - stranded DNA	Easy to produce at high titers; infects dividing and non-dividing cells; wide range of target cells; efficient gene transfer; high expression levels; can be administered *in vivo*	Host immune and inflammatory response; transient expression and effects	Robbins and Ghivizzani, 1998; Worgall, 2005; Wu and Ataai, 2000
Adeno-associated viruses	Single-stranded DNA	Almost no immunogenicity; efficient transfer; long-term gene expression; infects dividing and non-dividing cells; can be administered *in vivo*	Small transgene capacity; difficult to produce at high titers; risk of insertional mutagenesis	Evans and Lesnaw, 2002; Robbins and Ghivizzani, 1998; Wu and Ataai, 2000
Retroviruses (including lentiviruses)	Enveloped single-stranded RNA	Low immunogenicity; long-term expression; high transfection efficiency *ex vivo*; infects dividing cells and also non-dividing when lentiviral based	Low titers; possible insertional mutagenesis; low transfection efficiency *in vivo*; safety concerns regarding HIV	Mountain, 2000; Osten *et al.*, 2007; Wu and Ataai, 2000
Herpes Simplex	Enveloped double-stranded DNA	Large DNA insert capacity; tropism for neural tissue long-term expression; efficient transfer; infects non-dividing cells	Toxicity and immunogenicity; may become latent in neural tissues.	Robbins and Ghivizzani, 1998; Wu and Ataai, 2000
Poxviruses (Vaccinia)	Double-stranded DNA	Large DNA insert; stable; possible lyophilisation; no integration into the host genome	Host immune response	Evans and Lesnaw, 2002
Alphaviruses	Single-stranded RNA	High titer production; broad host range; extreme trangene expression; low immunogenicity	Transient expression	Lundstrom, 2003

1.3.2. Non-viral based vectors

Although less efficient, non-viral vectors have been developed to substitute viral vectors for their simplicity, ease of production and avoidance of immunogenicity and oncogenicity (Li and Ma, 2001). Viral vectors are based on plasmid DNA (pDNA) however, small interfering RNAs (SiRNAs) are emerging as an innovative way to incorporate the therapeutic genes (Oh and Park, 2009). Independently of the nucleic acid type, an efficient non-viral *in vivo* gene therapy approach must have a few optimized features, such as the solute for administration, the administration route, the design of the nucleic acid construct and selection of target cells (Nishikawa and Hashida, 2002).

Non-viral gene delivery systems consist mainly of four categories; a) direct naked DNA or RNA delivery, in the absence of a carrier and using a physical method such as injection, electroporation or gene gun, b) cationic lipids formulated into liposomes and complexed with DNA/RNA (lipoplexes), c) cationic polymers complexed with DNA/RNA (polyplexes) and d) DNA condensation with cationic peptides. A combination of both cationic lipids and cationic polymers complexed with DNA (lipopolyplexes) was also reported (Brown *et al.*, 2001; Li and Ma, 2001; Martin and Rice, 2007; Niidome and Huang, 2002; Oh and Park, 2009; Park *et al.*, 2006). Moreover, bacteria have also been used to transfer plasmid DNA into various cell types through a process called bactofection (Akin *et al.*, 2007).
Table 1.2 shows the main characteristics of these delivery systems.

Table 1.2. Characteristics of the most common non-viral delivery systems

Delivery system	Characteristics	Advantages	Disadvantages	References
Naked DNA	No carrier; gene transfer is performed using physical methods such as needle injection (DNA vaccines), gene gun, electroporation, ultrasound and hydrodinamic delivery with pDNA or SiRNA	Easy to produce, manipulate and use; very safe; high efficiency when electroporation or hydrodynamic methods are used	Very short duration of expression; inefficient transfection *ex vivo* and *in vivo*; rapid degradation; low efficiency for ultrasound and injection methods	Gao *et al.*, 2007; Li and Ma, 2001; Oh and Park, 2009
Cationic lipids	Include lipossomes, micelles, emulsion and solid lipid nanoparticles; use pDNA or SiRNA	No capacity limitations; low immunogenicity; efficient *ex* vivo transfection	Some toxicity; rapidly cleared from plasma; transient expression; inefficient *in vivo* transfection	Brown *et al.*, 2001; Mountain, 2000; Oh and Park, 2009
Cationic polymers	Polymers with protonated groups at physiological pH; use pDNA or SiRNA	Highly effective *ex vivo* transfection	Hard to transfect non-dividing cells; may induce citotoxicity and host immune response	Brown *et al.*, 2001; Park *et al.*, 2006; Oh and Park, 2009
Cationic peptides	Redox-sensitive thiols can be incorporated into the carriers; use pDNA	Generally have low cytotoxicity and immunogenicity; great biodegradability; endosomal escape	Some peptide choices may induce toxicity with modest transfection	Martin and Rice, 2007; Niidome and Huang, 2002

Non-viral systems are normally prepared by simply mixing a solution of nucleic acid encoding for the target gene with one of the aforementioned carriers. Particle size is usually 1-1000 nm range and they are administered short after preparation, since long-term stability had yet not been proven (Brown *et al.*, 2001).

Gene delivery carriers are designed to enable high functional delivery and gene expression and depending on their final goal, they can be divided into distinct groups, such as, those designed to release the therapeutic genes in the tissue to achieved a continuous or controlled expression, those designed to release the nucleic acid in the cytosol, the ones designed to enhance nucleic acid delivery to the cytosol or nucleus, those designed to target delivery to specific cell types and those forming condensed complexes with DNA/RNA to protect it from nucleases and other blood components (Niidome and Huang, 2002).

Many extra and intracellular barriers can hamper the administration performance and gene delivery with non-viral vectors. Among the extracellular barriers are the DNA degradation in plasma, DNA uptake by the reticuloendothelial system, transfection inhibited by mucus, inability to target DNA to specific organs and the oral route itself. The intracelular barriers count with lysosomal degradation of DNA, difficulties in translocation of DNA to the nucleus, endosome escape and troubles with cytoplasmatic stability of DNA (Brown *et al.*, 2001).

The focus of a great fraction of non-viral gene therapy studies is the vector optimization and its entrance mechanisms into the cell. However, not enough work has been directed to the nucleic acid fraction of the vector, commonly a plasmid DNA. Nevertheless, plasmid optimization may lead to prolonged expression levels and, therefore playing an important role in enhancing the transfection efficiency (van Gaal *et al.*, 2006).

1.4. Therapeutic uses for gene therapy

Independently of the vector, gene therapy approaches are becoming an important alternative for classic treatments. For this to happen successfully, the role of the therapeutic gene in disease pathophysiology must be clearly understood (Flotte, 2007). By July 2013, and according to the data published by the Journal of Gene Medicine, there were 1970 approved gene therapy clinical trials worldwide (http://www.abedia.com/wiley/index.html). Of those, the vast majority (64,2%) were addressed to cancer diseases (Figure 1.2).

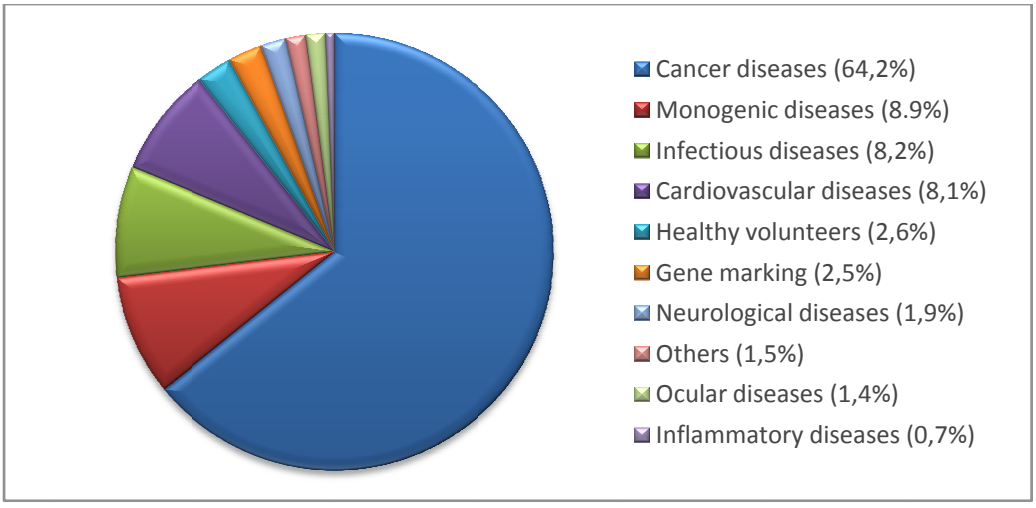

Figure 1.2. Clinical targets for gene therapy by July 2013
(adapted from http://www.abedia.com/wiley/index.html).

The therapeutic goal of any cancer treatment is to effectively kill most of cancer cells without damaging normal cells. For that purpose, gene therapy for cancer may consist of the administration of a copy of a mutated tumour suppressor gene, the administration of a gene encoding for an enzyme which activates an anti-cancer prodrug, the administration of a gene which encodes for an antigen designed to generate a protective immune response against

cancer cells or the administration of a gene that encodes for a toxin or an enzyme that converts a prodrug into a toxic metabolite (Alvarez *et* al., 2000; Schätzlein and Uchegbu, 2001). Even though gene therapy has found quite a challenge in efficiently generate high levels of direct killing of cancer cells, tumour regression is a promising reality (Templeton, 2009). Moreover, cooperation with other more established therapeutic modalities such as radiotherapy, can improve treatment efficiency, minimize treatment-associated toxicity and diminish cancer cell growth (Dutreix *et al.*, 2010).

Besides cancer, a wide range of other indications have approved gene therapy trials (Figure 1.2). With continuing research of the underlying genetic mechanisms of those and many other pathologies, the targets as well as the trials approved will most certainly grow spectacularly in the next few years.

2. Therapeutic plasmid DNA manufacture

Naked/plasmid DNA is the third most used vector in gene therapy trials (17,7%), surpassed only by retroviruses and adenoviruses (Figure 2.1) (http://www.abedia.com/wiley/index.html).

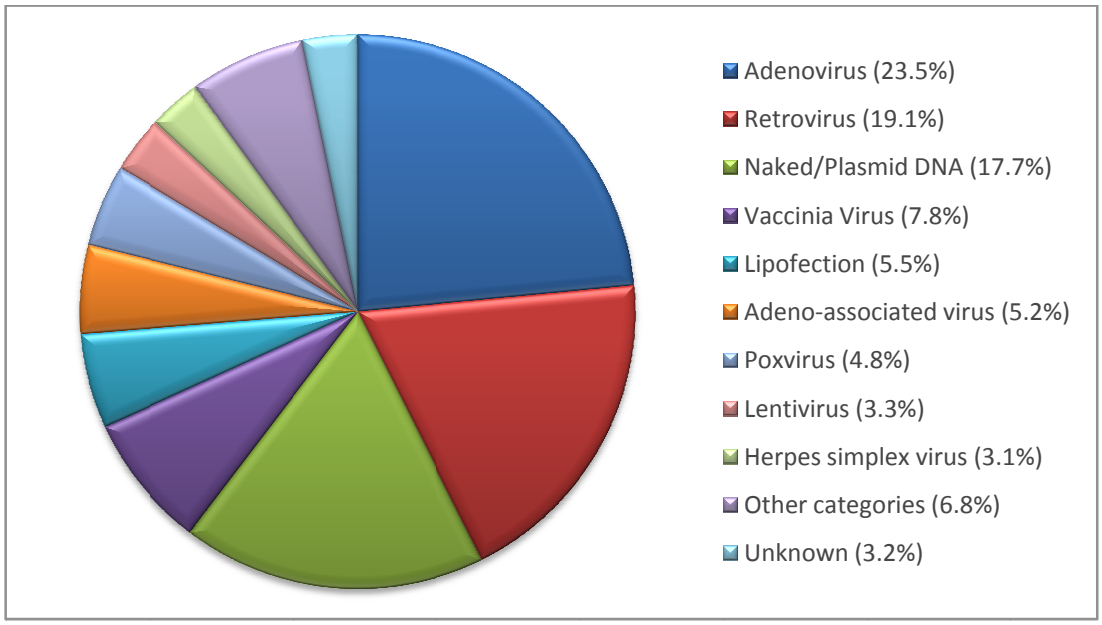

Figure 2.1. Vectors used in gene therapy clinical trials by July 2013 (adapted from http://www.abedia.com/wiley/index.html).

Since pDNA is an important alternative to viral based vectors (Li and Ma, 2001), the development of better production and purification processes is an urgent step for gene therapy success.

The therapeutic pDNA manufacture process may include several generic steps (Figure 2.2) such as plasmid construction, transfection into suitable bacterial host cells,

cultivation/fermentation, pDNA extraction by cell disruption (lysis), clarification, purification and polishing of pDNA solutions (Shamlou, 2003; Voß *et al.*, 2003).

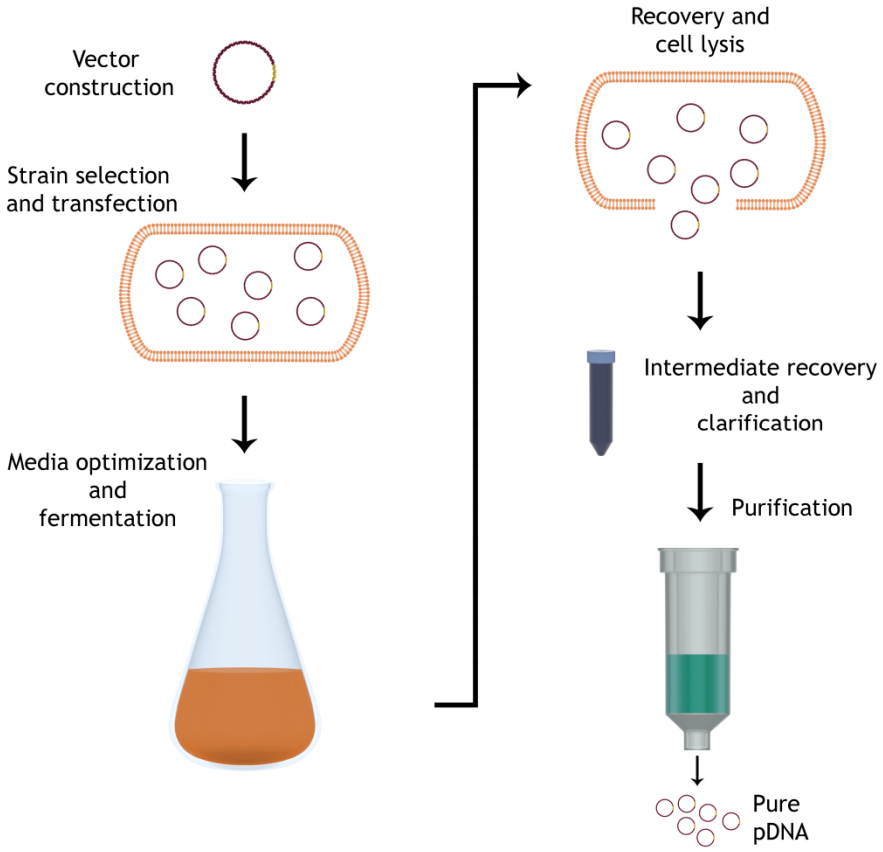

Figure 2.2. Steps in the manufacturing process of pDNA (based on Ferreira *et al.*, 2000 and Shamlou, 2003).

Moreover, to be used as a therapeutic product, pDNA must follow strict quality criteria in terms of purity and supercoiled (sc) content, established by the regulatory agencies (FDA, 2007). Table 2.1 shows FDA's recommendations for pharmaceutical grade pDNA.

Table 2.1. FDA specifications on impurities content for Plasmid DNA Vaccines (FDA, 2007).

Impurity	FDA Specifications
Protein (%)	Preferably < 1
RNA (%)	Preferably < 1
Endotoxins (EU/mg pDNA)	< 40
gDNA (%)	Preferably < 1
Open circular pDNA (%)	preferably < 20

Contamination of proteins in the final pDNA solutions may lead to biological reactions and immune responses, due to the production of hormones, cytokines and antibodies. Moreover, the presence of genomic DNA (gDNA) may encode oncogenes, which activation or deactivation may occur in recipient cells. In addition, endotoxins which are negatively charged lipopolydaccharides, are the most common cause of fever in humans, even at very small quantities (Ferreira, 2005).

pDNA represents approximately 1% of total bacterial cell content, just like gDNA. Endotoxins usually comprise 2% of total content, whereas RNA and proteins are the most common lysate components (more then 21%) (Atkinson and Mavituna, 1991).

2.1. Production and extraction of pDNA

Plasmids are double stranded, covalently closed DNA molecules that form a closed loop. Each strand of the pDNA molecule is a linear polymer of deoxyribonucleotides, linked by phosphodiester bonds. The winding of the two anti-parallel strands around each other and around a common axis, originates the right handed double helix structure, stabilized by stacking forces and by hydrogen bonds between AT and GC base pairs. Moreover, the inside of the double helix is highly hydrophobic due to close packing of the aromatic bases (Diogo *et al.*, 2005). A higher ordered structure or supercoiled (sc) is formed when the circular pDNA molecule coils in space, resulting in superhelix structures with different topological conformations (Ghanem *et al.*, 2013). Thanks to its coiled nature, pDNA is not a homogeneous product since different forms, derived from the sc isoform, can exist. In this cases, the supercoiling is lost either by a single or double strand break, originating open circular (oc) or linear isoform respectively (Voß *et al.*, 2003). The different pDNA isoforms are represented in figure 2.3.

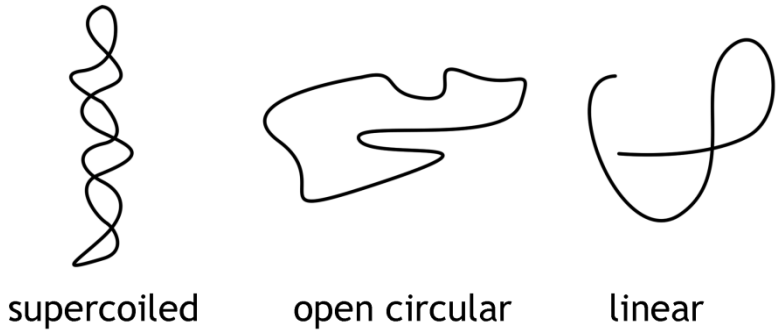

Figure 2.3. Different pDNA topologies.

Supercoiled pDNA is considered the most appropriate isoform for therapeutic applications, since it is more efficient at inducing gene transfer and expression (Cupillard *et al.*, 2005). Hence, linear and oc isoforms may be considered as lysate impurities (Shamlou, 2003).

The pDNA size can widely vary from 1 to over 1000 kbp. The molecules occur naturally in bacteria and can also be found in eukaryotic organisms. Due to the capability of replicating

autonomously within a suitable host, pDNA molecules are considered replicons (Ghanem *et al.*, 2013).

The clinical use of pDNA, functionality and bulk manufacture is deeply related to the organization of its genetic elements. Thus, pDNA molecules contain one unit responsible for its propagation in the microbial host and another unit that drives the expression of the therapeutic gene in the eukaryotic cells (Figure 2.4) (van Gaal *et al.*, 2006).

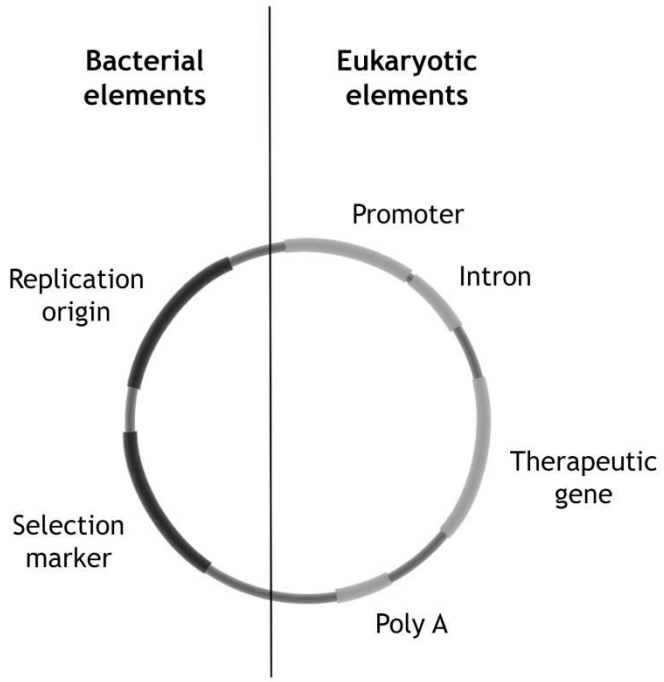

Figure 2.4. Illustration of a typical pDNA vector construct (based on Prather *et al.*, 2003 and van Gaal *et al.*, 2006).

The unit responsible for plasmid propagation in the microbial host contains a replication origin and a selection marker. The replication origin is a specific DNA sequence of 50-100 bp (e.g. ColE1 origin) to which the bacterial host-cell enzymes bind, initiating and regulating replication. This region allows the propagation and maintenance of the pDNA in host cells and its stable inheritance during bacterial growth (Bower and Prather, 2009; Glenting and Wessels, 2005; van Gaal *et al.*, 2006). Selection markers ensure stable inheritance of plasmids during bacterial growth since their presence only enables the propagation of the microbes hosting the pDNA (Glenting and Wessels, 2005; van Gaal *et al.*, 2006). Antibiotic resistance genes are the most common selection markers, and of those, kanamycin is the most popular (Carnes, 2005). Although a powerful selection mode, antibiotic resistance genes are discouraged by the regulatory agencies (EMEA, 2001), since plasmids carrying them can transform the recipient microflora by spreading the resistance genes (Glenting and Wessels, 2005). Thus, these concerns have spurred the development of alternative selection mechanisms such as repressor titration (Cranenburgh *et al.*, 2001).

The therapeutic gene expression unit should be designed to maximize a high level expression of the transgene upon entry into the eukaryotic host. The unit includes a promoter from which RNA polymerase initiates transcription, an enhancer which increases cells capacity to transcribe a gene with great efficiency, the transgene of interest, from which the therapeutic protein is encoded and other elements such as poly-A signal, and intron, a stop signal and various transcription factors (Prather *et al.*, 2003; van Gaal *et al.*, 2006).

The choice of microbial host strain can affect the quality of the final pDNA product (ICH, 1997). There are no specific guidelines on the most convenient genotypic or phenotypic characteristics of bacterial strains for pDNA productions. For reasons of efficiency, *E. coli* is usually the chosen production host (Glenting and Wessels, 2005). This preference is based on sustained pDNA stability, low probability of promoting genetic modifications of pDNA molecules, its high cellular density and its compatibility with subsequent purification steps (Vázquez *et al.*, 2005). However, as gram-negative bacteria, *E. coli* contain highly immunogenetic endotoxins in their outer membranes that can ultimately present a problem in pDNA purification (Glenting and Wessels, 2005). Among the most widely used *E. coli* strains are XL-1 *Blue*, TG1, DH10B, JM109 and DH5α (Vázquez *et al.*, 2005).

Once the chosen bacteria are transfected with the pDNA of interest, they are cultivated in shaking flasks or bioreactors with the appropriated medium. The main goal of a fermentation process for pDNA is to maximize the yield, improving at the same time, pDNA purity and quality (Carnes, 2005). Thus, the cultivation medium formulation can greatly affect the microbial process performance. Through type and ingredient concentration, it directly dictates the amount of biomass produced, influencing pDNA volumetric yield. Moreover, medium composition can directly bear on bacteria physiology, influencing their intricate regulatory systems and therefore, controlling plasmids copy number or specific yield. *E. coli* is a non-fastidious microorganism that grows in rich complex organic media as well as in salt-based chemically defined media, on the condition that a source of organic carbon is provided (Prather *et al.*, 2003). In fact, a high-cell density media commonly contain a carbon source, a nitrogen source and various salts and trace metals. Even though many media formulations are available, research aimed at improving those formulations continues to grow, with the objective of improving sc pDNA production yield and minimizing the levels of production process contaminants (O'Kennedy *et al.*, 2000; Xu *et al.*, 2005). Moreover, the effects of medium composition on pDNA production are closely related with that affecting pDNA segregational stability. The presence of an antibiotic is the factor contributing to that stability by enabling the selection for cells containing the desired plasmid (Prather *et al.*, 2003). Furthermore, the use of animal derived products in media formulation, particularly bovine products, is unacceptable because of the risk of prion or virus contamination (EMEA, 2001).

Fermentation and recovery of cells from the broth is followed by cell lysis, the first critical step in downstream processing (Ferreira *et al.*, 2000). During this step, bacteria are broken up and intracellular components are released. Therefore, it can determined the amount and quality of the pDNA produced for the subsequent purification steps and difficulties of those same steps, in terms of co-released impurities (O'Mahony *et al.*, 2005). Lysis can be performed using various procedures such as mechanical lysis, heat treatment and chemical lysis, in conjugation with unit operations like filtration or centrifugation (O'Mahony *et al.*, 2005; Paul *et al.*, 2008; Prazeres *et al.*, 1999; Voß *et al.*, 2003). However, the most popular procedure for cell disruption is alkaline lysis and its many variations. Originally described by Birnboim and Doly (Birnboim and Doly, 1979), it relies on the disruption of cells at high pH with sodium hydroxide, in the presence of sodium dodecyl sulphate (SDS), which destabilizes the cell wall's integrity and releases its cellular components. Subsequent neutralization with sodium acetate precipitates SDS together with denatured gDNA, proteins and cellular debris (Prather *et al.*, 2003). Nevertheless, the lysis step is rather critical for pDNA physical stability. In fact, some sc pDNA unwinds as a consequence of the alkali-promoted hydrogen bond disruption, being irreversible converted to oc pDNA (Chamsart *et al.*, 2001). Also, gDNA can be fragmented during alkaline lysis, which can make further downstream removal much more difficult (Prather *et al.*, 2003).

The lysate solutions obtained after lysis still contain great amounts of contaminants like RNA, proteins, gDNA and endotoxins, which have to be removed in subsequent purification steps.
Clarification and concentration steps are designed to remove a substantial amount of host proteins and nucleic acids and to reduce the volume of the process stream prior to the final purification steps (Prazeres *et al.*, 1999). Precipitation is the most popular intermediate pDNA recovery step. Its goal is to concentrate pDNA while removing low molecular weight nucleic acids and can be performed using polyethyleneglycol, isopropanol, cetyl trimethylammonium bromide, among others. Next, a large fraction of proteins, endotoxins and high molecular weight RNA are usually removed with an additional precipitation step using anti-chaotropic salts such as calcium chloride, ammonium acetate and ammonium sulphate. In alternative techniques such tangential flow filtration and aqueous two-phase systems can also be used as intermediate steps for pDNA recovery (Freitas *et al.*, 2009).

2.2. Purification of pDNA

The pDNA solutions obtained after intermediate recovery still contain a substantial amount of impurities that can only be removed using specific procedures.
Among the few existing downstream process methods, liquid chromatography (Figure 2.5) is the choice for process-scale therapeutic pDNA manufacturing (Diogo *et al.*, 2005).

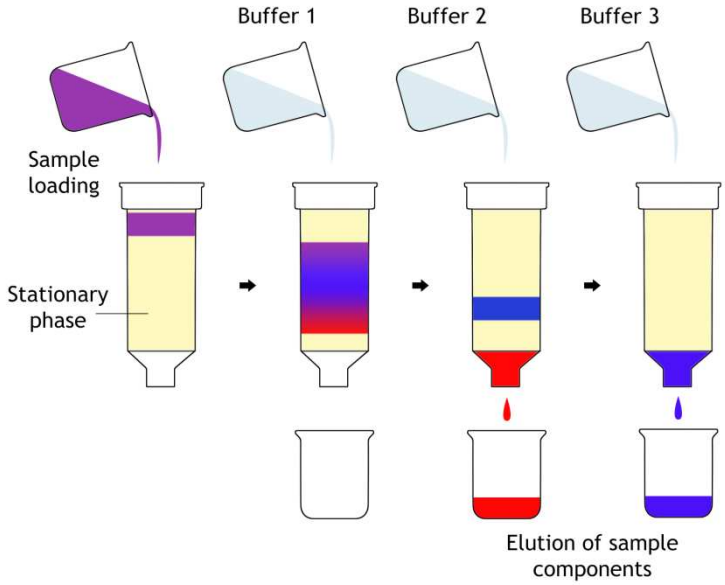

Figure 2.5. Basic illustration of a generic liquid chromatography process (Based on Diogo *et al.*, 2005). After packing the stationary phase into a column, the sample which is in the adequate buffer (usually the equilibration buffer - equilibration not shown), is loaded. The matrix is then washed with distinct buffers to enable the separated elution of the sample components.

Preparative or analytical, liquid chromatography is a simple, robust, versatile and high reproducible method, which explores pDNA properties such as size, hydrophobicity, charge, associability of the nucleotide bases to ligands and sc imposed topological constrains to isolate and purify pDNA from impurities (Ferreira *et al.*, 2000; Sousa *et al.*, 2012). Nevertheless, chromatographic methods face a few limitations related also to the characteristics of all molecules involved, namely pDNA and lysate impurities (Sousa *et al.*, 2008).

Chromatographic purification is a mandatory process in therapeutic pDNA production for the removal of impurities and assessment of the purity of pDNA solutions. The purification step should include the separation of the necessary sc pDNA from its other isoforms, together with the removal of important impurities such as proteins, gDNA, endotoxins and RNA (Ghanem *et al.*, 2013).

The successful use of chromatography relies on finding suitable and well characterized adsorbents. This indicates that mastering liquid chromatography requires a mix of skills, including proper material selection and characterization, as well as a good understanding of liquid-solid interactions (Unger *et al.*, 2010). The ideal stationary phase format should be solid, macroporous and chemical and physically stable, while exhibiting low nonspecific adsorption, with high binding capacity and mass transfer, maintaining good flow properties throughout processing. Also, it should be inexpensive, simple to use, reusable and stable to alkaline sanitization. Moreover, the capacity of the adsorbents is another crucial parameter to consider in pDNA chromatographic purification. Hence, the matrix pore size should be of

target biomolecules, to enable an easy access to immobilized ligands inside the pore, increasing the support's capacity. In fact, the matrix pore size is inversely correlated to its surface area, which directly affects the amount of immobilized ligand and thus, the binding capacity (Sousa *et al.*, 2012).

The flexibility and specific characteristics of chromatographic processes are due not only to the different stationary phases that can be used (Sousa *et al.*, 2012), but also to the various ligands that can be attached to the solid matrices. As a consequence, applying the right ligand is of extreme importance for the development of a successful purification method. Thus, the chemical composition of chromatographic supports determines the preferential interaction established with the target molecules, allowing their retention whereas undesirable molecules are eluted. So, the association of adequate ligands with solid supports takes advantage of higher selectivity and specificity for the target biomolecules (Sousa *et al.*, 2012).

2.2.1. Chromatographic methods for pDNA purification

Chromatographic techniques for pDNA purification, used in a single or in an integrated/combine mode, can be based on differences in size, charge, hydrophobicity and affinity of the different molecules in a mixture. As a result, chromatographic methodologies can be labelled as size-exclusion (SEC), anion-exchange (AEC), hydroxypatite (HAC), hydrophobic interaction (HIC), reverse-phase (RPLC), reverse-phase ion-pair (RPIPC), thiophilic adsoption (TAC) and affinity chromatography (AC) (Diogo *et al.*, 2005; Ferreira, 2005; Ghanem *et al.*, 2013).

Size-exclusion chromatography (SEC) distinguishes clarified lysate components on the basis of their size, and can be used alone or sequentially with other chromatographic methods such as, anion-exchange. The reduction of plasmid hydrodynamic radius due to supercoiling is the basis of its selective separation from distinct DNA molecules, RNA and other smaller impurities such as endotoxins and proteins. gDNA is excluded from the particles, eluting as the peak leading edge, followed by the relaxed and then by the sc pDNA isoform. Smaller RNA, oligonucleotides, endotoxins and salt molecules elute separately from the leading pDNA peak (Ferreira, 2005). SEC has some drawbacks such as limited capacity and selectivity for pDNA and since lysate solutions are complex mixtures of different molecules with various sizes, SEC's resolution capacity is quite limited. Consequently, this chromatographic modality is not a good choice for an initial pDNA purification step. It is however an ideal polishing step, enabling the separation of sc from oc pDNA and other impurity residues. Moreover, it enables a buffer exchange into an adequate formulation or storage buffer (Ghanem *et al.*, 2013).

In anion-exchange chromatography (AEC) strong ligands such as quaternary amines are typically coupled to polymeric matrices (Prazeres *et al.*, 1998). AEC is based on the interaction between the negatively charged phosphate groups on the DNA backbone and the

positively charged groups of the stationary phase. Despite the similarity of the overall charge and molecular weight of the different pDNA topologies, the different isoforms possess distinct conformations and consequently, different local charge densities. Therefore, the isoforms will have different retention times in an increasing salt gradient (Ghanem *et al.*, 2013). AEC is one of the most used techniques for pDNA separation, purification and quantification and many pDNA purification kits are exclusively based on this technology, offering rapid separations with no solvent requirements (Diogo *et al.*, 2005). Nevertheless, the major limitation of AEC is the lack of selectivity of the adsorbents, leading to co-elution of impurities, particularly endotoxins and high molecular weight RNA (Ferreira, 2005; Stadler *et al.*, 2004). This deficiency in selectivity makes the purification of pDNA very difficult to achieve in a single AEC step, so it is often used in series with other purification techniques like SEC (Diogo *et al.*, 2005).

Hydroxyapatite chromatography (HAC) belongs to mixed mode ion-exchange techniques due to the inclusion of both positively and negatively charge moieties. Nucleic acids bind to hydroxyapatite by coordination bonds between the phosphate moieties of the DNA backbone and the calcium sites on the surface of the matrix. HAC requires the use of a phosphate buffer for elution, since phosphate ions act as competing agents for the stationary phase binding sites. In the presence of urea, the method is quite effective at resolving RNA and pDNA mixtures. HAC was also effective at resolving isoform mixtures. However, it cannot operate directly with non-clarified alkaline cell lysates, since acetate ions interact with hydroxypatite, causing the dissolution of the medium (Diogo *et al.*, 2005).

Hydrophobic interaction chromatography (HIC) takes advantage of the higher hydrophobicity of single stranded nucleic acids and endotoxins, which interact more strongly with HIC supports than double stranded nucleic acids (Ghanem *et al.*, 2013). The pDNA elutes in the void volume, whereas impurities elute later, well separated from the pDNA peak. This behaviour can be explained by the fact that pDNA molecules have their hydrophobic bases packed and shielded inside the double helix, making the hydrophobic interactions with the support minimal. Differently, single stranded nucleic acids show a higher exposure of the hydrophobic bases, thus interacting more strongly with the hydrophobic ligands (Diogo *et al.*, 2005). The requirement of high salt concentrations can be a disadvantage, since its use is associated with economic and environmental impact (Biwer and Heinzle, 2004).

Reverse-phase chromatography (RPLC) is based upon the interaction of hydrophobic regions of molecules with non-polar immobilized ligands. Bound molecules are then eluted by adding organic modifiers to the eluent buffer, in increasing order of hydrophobicity (Ferreira, 2005). Therefore, nucleic acids of increasing size will be retained longer in the column. Moreover, single-stranded and partially desnaturated nucleotides, with higher base exposition, have higher retention times (Diogo *et al.*, 2005). Even though RPLC is an efficient method to

remove endotoxin contamination, it uses organic solvents, which are frequently toxic, mutagenic and volatile (Ghanem *et al.*, 2013; Stadler *et al.*, 2004).

Reverse-phase ion-pair chromatography (RPIPC) uses ion-pairing organic modifiers in the mobile phase to change selectivity and enable the separation and resolution of charged solutes. The stationary phase has a hydrophobic nature but the buffers contain a hydroorganic eluent, an amphiphilic ion and a small, hydrophilic counter ion. When charged solutes bind to the amphiphilic ion, its hydrophobicity effectively increases. The most popular ion-pair agent for pDNA purification is triethylamine (Diogo *et al.*, 2005). In similarity to RPLC, RPIPC also uses organic solvents, which is the major disadvantage of this chromatographic technique (Ferreira *et al.*, 2000).

Just like HIC, thiophilic adsorption chromatography (TAC) makes use of high concentrations of anti-chaotropic salts to establish hydrophobic interactions between the stationary phase and lysate components (Diogo *et al.*, 2005). The structural requirements to bind pDNA in a thiophilic support are the presence of an aromatic ring and a thiother moiety. Using TAC is possible to separate sc pDNA from the oc isoform and other lysate impurities such as RNA (Lemmens *et al.*, 2003).

Affinity chromatography (AC) is a unique method for the separation, identification and purification of biomolecules, based on a highly specific molecular recognition. It relies on a strong but reversible interaction between the target molecule and the immobilized ligand, similar to many interactions found in biological systems (Hage and Cazes, 2006; Jones, 2000; Scopes, 2000). That specific recognition involves a combination of various types of intermolecular forces, such as hydrogen bonding, electrostatic, hydrophobic and van der Waals interactions (Jones, 2000). AC is considered one of the most suitable purification strategies to specifically purify sc from oc pDNA and host impurities. The possibility of choosing the ideal ligand facilitates the establishment of specific interactions with nucleic acids, which can be manipulated by modifications in elution conditions (pH, ionic strength or temperature) to take advantage of ligand properties (Sousa *et al.*, 2012). Affinity methods have the advantage of eliminating additional steps, increasing yields and improving process economics (Sousa *et al.*, 2008).

Immobilized metal-ion chromatography (IMAC), boronate chromatography, triple-helix affinity chromatography (THAC), polymyxin B chromatography, protein-DNA chromatography and amino acid chromatography are all affinity methods used for pDNA purification (Diogo *et al.*, 2005; Sousa *et al.*, 2008).

IMAC matrices are charged with chelating agents, such as iminodiacetic acid or nitrilotriacetic acid, that coordinate transition metal ions like Ni(II) and Cu(II). The affinity binding between the metal ions and target molecules enable the removal of RNA and denatured pDNA, since

pDNA is not retained in the column. However, the separation between pDNA and gDNA is only possible if the gDNA structure is destabilized (Ghanem *et al.*, 2013; Sousa *et al.*, 2008).

Boronate chromatography uses the great affinity that phenylboronic acid has towards diol compounds. Thus, since only RNA and endotoxins have such *cis*-diol groups (Liu, 2006), this chromatographic technique can be applied to reduce those contaminations in pDNA-containing lysates (Firozi *et al.*, 2010; Singh and Willson, 1999).

THAC is based on the formations of Hoogsteen hydrogen bonds between AT base pairs forming T-AT triplexes, and between a protonated cytosine and GC, forming GC-C^+ triplexes. Target pDNA is then captured via an intermolecular triplex formation with the immobilized oligonucleotide and can be recovered by washing the stationary phase with a mild alkaline buffer which destabilizes the above mentioned Hoogsteen bonds (Ghanem *et al.*, 2013).

Polymyxin B affinity chromatography makes use of the ability of antibiotic polymyxin B to recognize the lipid structure of endotoxins. Thus, it has been explored to reduce endotoxin contamination from pDNA lysate solutions however, besides the poor yields obtained, polymyxin B is neuro and nephrotoxic (Diogo *et al.*, 2005; Sousa *et al.*, 2008).

Protein affinity chromatography is based on the immobilization of proteins or protein complexes to specially recognize a DNA motif. An example of such ligands is the complex formed between a zinc finger DNA binding domain and glutathione S-transferase. In this way, a target pDNA containing the recognition sequence can easily bind to the glutatione matrix, thus enabling the discrimination of distinct plasmids based on their sequence. However, the isolation of pDNA from gDNA is limited (Diogo *et al.*, 2005; Sousa *et al.*, 2008).

Finally, amino acid-DNA affinity chromatography takes advantage of the affinity that arginine and histidine have towards pDNA. The efficient recovery of pure sc pDNA is possible using both approaches, even though their method specificities are quite different. Arginine affinity chromatography has the advantage of low salt usage and higher recovery yields. The use of amino acid affinity ligands represents a promising pDNA purification approach, since they combine the selectivity of a natural occurring biological interaction with the simplicity of a single small molecule (Sousa *et al.*, 2008).

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4. Paper I

Aromatic ligands for plasmid DNA chromatographic analysis and purification: an overview

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Abstract

The aromatic ring systems are among the most stable chemical structures known and in combination with many other chemical groups, they can originate an extraordinary variety of molecules, with interesting chemical and physical properties. Many aromatic molecules have been applied for the purification of various biomolecules such as proteins, carbohydrates and nucleic acids. Combining aromatic chromatography with optimized production, extraction and clarification procedures, can offer a number of advantages for pharmaceutical plasmid DNA (pDNA) purification. This review focuses on pDNA chromatographic purification and analysis using aromatic ligands. The goal is to give an updated view of all existing aromatic ligands, their main characteristics, applicability and technical features of the chromatographic methods in which they have been applied. Also, a critical assessment of each method is performed as well as a comparison of the different procedures, their key features and limitations.

Keywords: Aromatic ligands, pDNA purification, analytical chromatography, preparative chromatography.

Contents

1. Introduction

Over the last two decades molecular therapies, such as gene therapy and DNA vaccines, have become promising therapeutic approaches for a wide range of human diseases [1-4]. Besides the results obtained in treating many disturbances like retinal diseases [5], hemophilia [6], heart diseases [7] and cancer [8-10], molecular therapies are also used as a support to radiotherapy [11]. However, a major challenge of these therapies is the safe and efficient delivery of the therapeutic gene to the target site. A good strategy is the application of non-viral vectors, since they are widely recognized as safe and with few side effects [10,12]. Various plasmid DNA (pDNA) vectors have been successfully applied in veterinary products as well as in human clinical trials [3]. As consequence, the demand for high quantities of pure pDNA has experienced a fast growth over the years. Nevertheless, to be used as a therapeutic product, pDNA must meet strict quality criteria, established by the regulatory agencies [13] as shown in Table 1.

Table 1. FDA specifications on impurities content for Plasmid DNA Vaccines.

Impurity	FDA Specifications[13]
Protein (%)	Preferably <1
RNA (%)	Preferably <1
Endotoxins (EU/mg pDNA)	< 40
gDNA (%)	Preferably <1

Many techniques have been applied for the purification of pDNA however liquid chromatography is considered one of the most efficient and potent procedures to obtain pure pDNA [14]. It is a technique that offers equal performance in both preparative and analytical scales, can be automated and is highly flexible [15] mainly due to the various stationary phases that can be used. The specific characteristics of the different chromatographic approaches are the result of the different ligands attached to the solid matrices [14]. As consequence, applying the right ligand is a crucial step for the development of a successful purification system. Both natural and synthetic molecules can be used as ligands for the various types of chromatography. It is however quite common to find an aromatic ring in the structure of those molecules. Aromatic compounds such as triazinyl-based reactive dyes [16], synthetic peptides [17], phenyl boronic acids [18], amino acids [19,20] and a therapeutic diamidine compound [21] have been successfully used to purify proteins, carbohydrates and nucleic acids (among others).

This review is focused on the aromatic ligands for pDNA chromatographic purification and analysis. The goal is to give an updated view on the several aromatic ligands, their advantages and limitations.

2. Aromatic ligands for pDNA analysis and purification

In the early nineteenth century, chemists recognized the difference between aliphatic and aromatic compounds [22], however it was only in the 1930's that Erich Hückel set the criteria for aromaticity [23-25]. To fall into these criteria, a compound must be cyclic, has to contain one *p* orbital in each atom of the ring, and has to be planar or nearly planar so that all *p* orbitals overlap in a continuous manner. Finally, the ring has to include a closed loop of (4*n*+2) π-electrons, where *n* is a whole number (Hückel molecular Orbital Theory - HBO) [26]. Nevertheless, some of these restrictions are no longer absolute, since e.g. mobile electrons may circulate in the ring plane and σ rather than π orbitals maybe involved [27].

The most well-known aromatic compound is the six-membered ring benzene (*n* of Hückel's rule corresponds to 1), in which our knowledge of aromatic chemistry is based. However, many other cyclic molecules are considered aromatic molecules.

The aromatic compounds have large resonance energies and are much more stable than similar unsaturated non-aromatic molecules like alkenes. These compounds typically undergo aromatic electrophilic and nucleophilic substitution reactions but not electrophilic addition reactions, since the stabilization of the rings would no longer be maintained. The substitution reactions are not only a characteristic of the benzene ring, but also of other aromatic compounds [28].

Next, a detailed view and analysis of all pDNA aromatic ligands (Fig.1) will be presented, with particular emphasis to their performance as chromatographic ligands.

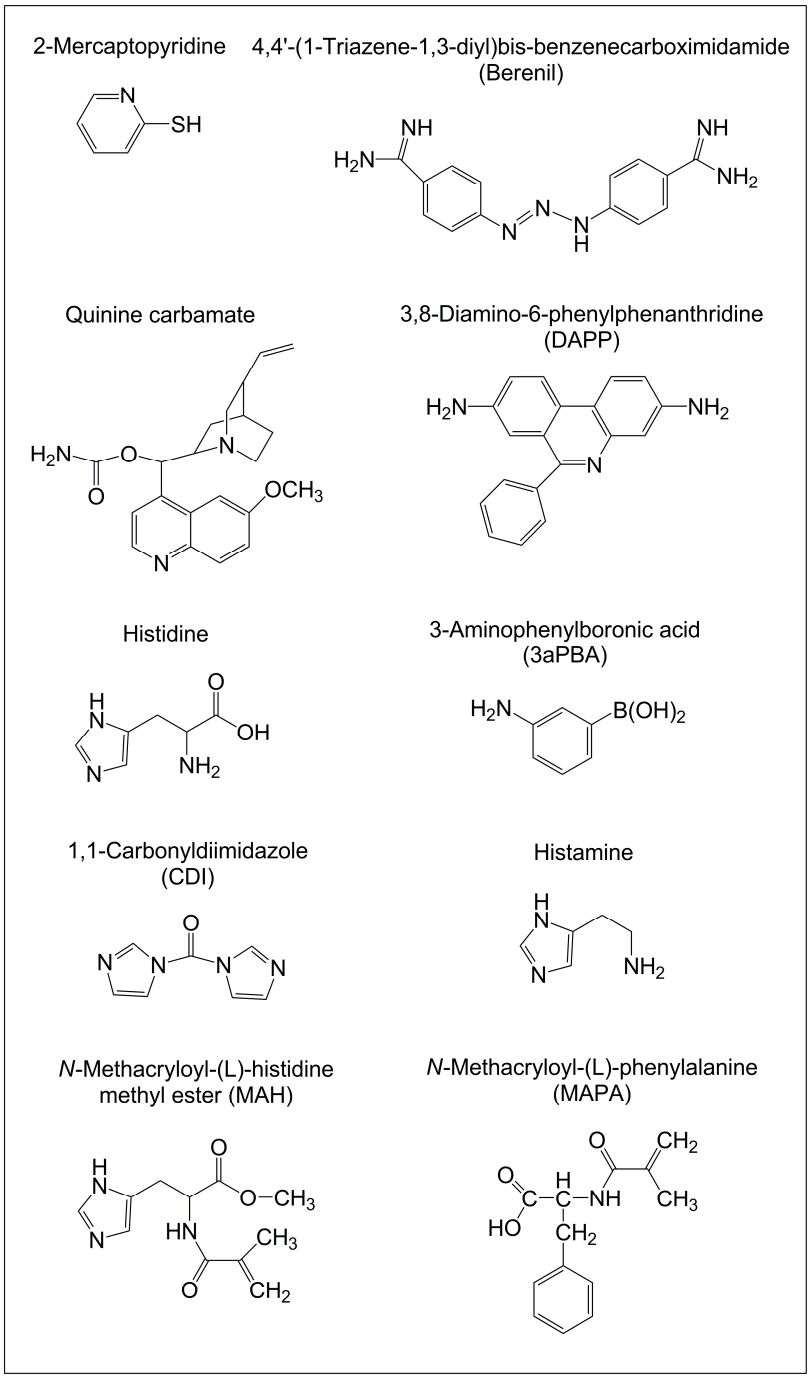

Fig. 1 - Aromatic ligand structures (unprotonated forms).

2.1. Aromatic thiophilic ligands

Thiophilic ligands are thioethers with the general formula HS-R or R'-S-R (also R'-S_2-R and R'-SO_2-R) where R can be aliphatic or aromatic and R' is usually aliphatic and works as a spacer arm [29]. These ligands were developed and used for the first time by Porath and co-workers for protein fractionation [30,31]. The general structure of their thiophilic adsorbent (T-Gel) can be represented as M-O-$CH_2CH_2SO_2CH_2CH_2$-S-R where M is the matrix polymer and R is a small aliphatic residue [30]. However, adsorbents with an aromatic R residue also have thiophilic properties and are also used in chromatographic procedures. In 1988, Porath *et al.*

reported the use of 3-(2-pyridylsulfido)-2-hydroxypropyl, 3-(2-pyridyloxy)-2-hydroxypropyl, 3-(phenylsulfido)-2-hydroxypropyl and 3-(phenyloxy)-2-hydroxypropyl agarose for protein adsorption [32]. More recently, the highly selective antibody ligand 4-mercaptoethyl pyridine (MEP) was used in IgG binding and elution studies by hydrophobic charge-induction chromatography [33].

In 2003, Lemmens and co-workers reported for the first time the use of aromatic thiophilic ligands for pDNA purification [34]. The thiophilic interaction chromatography method was integrated in a 3-step purification process: preceded by a group separation through size exclusion chromatography to separate pDNA from RNA and followed by anion-exchange chromatography to remove potential endotoxin traces. These procedures enabled the purification of supercoiled (sc) pDNA, from clarified *E. coli* alkaline lysates, suitable for molecular therapy applications [34]. First, different ligands, aliphatic and aromatic with or without the thioether moiety, immobilized onto Sepharose were tested for the selective separation of sc and open circular (oc) isoforms. For that purpose, high concentrations of ammonium sulphate in the eluent buffer were used. The results indicated that only the ligands containing an aromatic ring and a thioether moiety could retain the sc pDNA in the column [34]. Those are the structural requirements to bind sc pDNA in a thioether support, something very similar to what was reported for antibody separation by hydrophobic charge-induction chromatography [35]. With that in mind, Lemmens *et al*. successfully separated the sc from oc isoform using 2-mercaptopyridine (Fig. 1), 2-pyridine ethanethiol and benzenethiol as ligands [34]. Moreover, enhancing the electronegativity of the ring substituents affects the salt concentration needed for pDNA retention, since both isoforms bind stronger to the support with less salt in the eluent [34,36]. Besides that, the resolution between sc and oc isoforms is also improved when the electronegativity of the ligands is higher. Increasing the hydrophilicity of the ligand will promote the binding between its electronegative ring substituents and the more accessible hydrophilic phosphate groups of sc pDNA [36].

Hence, 2-mercaptopyridine was the ligand selected for the purification of sc pDNA from clarified cell lysates. This was accomplished using two different approaches, the referred 3-step process developed by Lemmens *et al*. [34] and a procedure developed by Sandberg *et al.*, that did not include the anion-exchange polish step [36]. The interaction between these ligands and polynucleotides requires high concentrations of water structuring salts. Also, the interaction is structure-dependent. It is possible that the aromatic ring interacts with sc pDNA through an intercalating hydrophobic π-π interaction, while the sulphur atom is responsible for electron-donating/charge transfer interactions with the nucleotides [34]. In both studies, the authors reported the purification of sc pDNA from oc pDNA and other lysate impurities such as endotoxins, RNA and genomic DNA (gDNA). Nonetheless, Sandberg and co-workers did not perform a sc pDNA sample quality analysis to support those conclusions. In the study by Lemmens *et al.*, the anion-exchange step removed a great amount of endotoxins [34].

However this step is absent in Sandberg *et al*. study, which raises the concern that endotoxins might be present in the sc pDNA samples. Moreover, information about the recovery of sc pDNA was not presented in both studies.

2-Mercaptopyridine was also tested as ligand in a hydrophobic-type chromatographic process by Lin and co-workers [37]. They synthesized a biporous adsorbent based on crosslinked GMA (poly(glycidyl methacrylate) and EDMA (ethylene dimethacrylate) containing 2-mercaptopyridine as immobilized ligand. Even though a pure pDNA sample was collected after the chromatographic run, the authors were unable to separate the sc isoform from the oc. Therefore, they highlighted their separation method on the possibility of implementing a high throughput pDNA production process. A great amount of feedstock sample was loaded at an elevated flow rate and a similar profile to that obtained with a normal injection was observed, within a short period of time and with high column efficiency [37]. However, these fine characteristics are a result of the biporous stationary phase rather than a result of the ligand *per se*. The authors concluded that the plasmid sample was separated from main impurities such as proteins and RNA, with complete recovery [37]. Nonetheless, it would be important to test the pDNA sample for endotoxin contamination, since it is an important and potentially dangerous impurity.

Another study by Bonturi *et al*. tested 2-mercaptopyridine immobilized on an agarose matrix for sc pDNA purification using three different buffer solutions [38]. Sodium citrate and potassium phosphate were used as ammonium sulphate alternatives (also tested for comparative purposes). For all cases, the species with more affinity towards the matrix were impurities such as RNA [38]. After pDNA quantification, gDNA, proteins and endotoxin analysis of sc pDNA samples, it was concluded that only with potassium phosphate buffer was possible to achieve a sc fraction in accordance with the requirements of the regulatory agencies. According to the authors, this is true regarding only the RNA and endotoxin impurities [38]. Although the best recovery yield and sc pDNA selectivity were achieved using sodium citrate buffer, in this case, the purity of the sc fraction was extremely low when compared to the 98.8% obtained using potassium phosphate buffer (with recovery of 68.5% of pDNA). With this salt it was possible to establish a good pDNA purification process, eliminating at the same time some commonly used preliminary recovery steps such as isopropanol and ammonium sulphate precipitation [38].

In conclusion, thiophilic aromatic pDNA chromatography is characterized by the use of heterocyclic aromatic ligands with a major hydrophobic character combined with a thiophilic site. This type of chromatography is clearly controlled on the basis of salt concentration. The most commonly used salt is ammonium sulphate however substantial amounts of this salt present an environmental problem [39]. The purification process developed by Bonturi *et al*. [38] overcomes this problem by using potassium phosphate and is perhaps the best approach

for pDNA purification using an aromatic thiophilic ligand. However, phosphates still have an environmental impact and need to be removed from wastewaters [39,40]. On the other hand, the injected sample is a neutralized lysate and not the result of an isopropanol or ammonium sulphate precipitation step, which are two of the most common intermediate recovery steps. These approaches show some disadvantages since isopropanol production has oil or natural gas as precursors and ammonium sulphate has a high eutrophication potential [41]. Even though the sc sample had a small contamination of oc pDNA and the recovery value was not very impressive, Bonturi *et al*. have performed an extensive quality analysis of the recovered pDNA sample [38].

2.2. Therapeutic compounds

2.2.1. Aromatic diamidine berenil

Berenil or 4,4'-(1-Triazene-1,3-diyl)bis-benzenecarboximidamide is an aromatic diamidine with the formula $C_{14}H_{15}N_7$ (Fig. 1). Basically, it is formed by two amidine groups, each one bound to a phenyl residue which in turn is linked to a central triazene group [42]. Its synthesis was reported for the first time in 1955 by Jensch as an interesting compound against blood parasites [43]. Since then, berenil has been used as an anti-trypanossomal agent [44-46] or in other applications related to its activity as an inhibitor of topoisomerase [47,48] and other enzymes [49]. Recently, because of its great affinity towards DNA [50], berenil was used for the first time as ligand for pDNA purification [21].

With a binding stoichiometry of 1:1 per nucleotide base pair [51,52], berenil establishes reversible non-covalent interactions with the floor and walls of the DNA minor groove (Fig. 2A), with a preference for A-T sequences [53-55]. However, berenil can also bind to RNA, and under the right conditions, it exhibit intercalative properties [42]. This intercalative behaviour is associated with the ability to also recognize and interact with CG - rich sites. The binding to these sites is stereochemically feasible and is possible that berenil can pseudo-intercalate into the major groove, establishing favourable hydrophobic interactions, or can partially intercalate into the minor groove. Therefore, berenil is able to bind both A-T and C-G base pairs, however the binding to A-T is stronger [56]. While van der Waals are the predominant forces in the C-G binding, the interaction with A-T sequences is mostly favoured by strong electrostatic interactions [56-58], enabled by the presence of two possibly charged terminal amino groups. Moreover, the preference for A-T sequences can also be associated with lower DNA structural perturbations [57].

Berenil-AT binding is sequence-specific [53,59,60], since the binding affinity can greatly differ between distinct arrangements of A-T base pairs [53]. Thus, berenil can bind to A-T through two slightly distinct ways: symmetrically bound to the minor groove, with both amidine groups linked through hydrogen bonds [61] or with one of the amidine groups bound through a water molecule bridge (Fig. 2B) [62]. However, hydrogen bonds and electrostatic interactions

are not the only forces responsible for berenil-DNA binding, since hydrophobic contacts between the phenyl rings and the hydrophobic regions of the DNA backbone play an important role. Additionally, the polar triazene group gets inserted between the two phosphodiester moieties of the backbone [62].

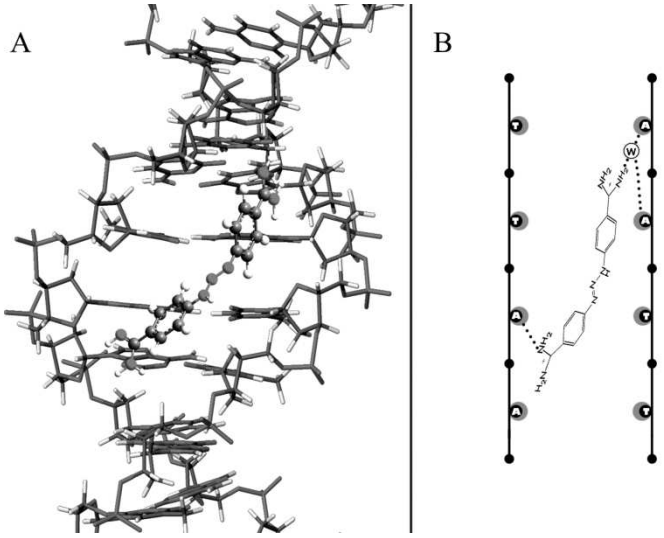

Fig. 2 - Schematic representation of berenil – DNA complex: A - binding with the minor groove and B - representation of the hydrogen bonds (dashed lines) where W represents a water molecule. Figure adapted and based on the studies from Laughton *et al*. [57] and Brown *et al*. [62].

Berenil has a high pDNA binding affinity that is highly dependent on sodium chloride concentration. However, in the presence of ammonium sulphate the affinity constant variation is not so significant and the values are smaller [50]. This implies that the hydrophobic interactions are not so affected by changes in salt concentration, displaying only a slight increase by raising the concentration of the antichaotropic salt used.

Berenil was immobilized onto an epoxy-activated Sepharose matrix and tested using two distinct approaches [21], one using an unique chromatographic step with clarified lysate solutions of pDNA with two different molecular weights (6.05 and 12.361 Kbp) and other approach using non-clarified lysates and replacing the clarification step by a second chromatographic run. For both cases, a pure sample was obtained in agreement with regulatory agencies specifications (Table 1). For the first approach, the recovery yield for the smaller and larger plasmids was 85% and 45% respectively. On the other hand, the yield obtained with two chromatographic runs was quite low (33%) [21]. Despite the fact that the isoforms separation [21] was not obtained when a lysate solution was used, the berenil-Sepharose matrix was able to separate a mixture of pure sc and oc isoforms. In this case, sc pDNA was strongly retained and eluted only when the salt was removed from the buffer solution [50]. The presence of great quantities of highly hydrophobic species, like RNA, may interfere in the matrix ability to differently interact with the two pDNA isoforms. Given that

these isoforms have a similar hydrophobicity, they elute at the same time and RNA molecules stay tightly bound to the support.

The results showed that berenil-Sepharose is able to successfully separate small and large pDNA molecules from host impurities. These have a stronger interaction with the support since they are the last species to be eluted [21]. This behaviour and the use of moderate ammonium sulphate concentrations is an indication that hydrophobic interactions play an important role in the binding between berenil and lysate species however, other polyelectrolyte and non-polyelectrolyte contributions cannot be neglected [50]. In fact, taking into account the nature of the interactions between non-imobilized berenil and pDNA, a stronger contribution of hydrogen bonds and electrostatic interactions would be expected. At working pH 8 the binding strongly depends on both charged ends of the berenil molecule. Still, one of those ends is involved in the binding with the Sepharose matrix, reducing the electrostatic contribution to the overall interaction energy. This is why the apparently weaker hydrophobic interactions are of great importance for the success of this berenil chromatographic method [50].

2.2.2. Quinine derivatives

Quinine is a plant alkaloid extracted from the bark [63], leafs [64] and young seedlings [65] of Cinchona trees [64]. Its empirical formula is $C_{20}H_{24}N_2O_2$, containing two fused-ring systems, an aromatic quinoline and a bicyclic quinuclidine [66].
In acid solution, quinine is used as a fluorescent standard [67]. However, its use in medicine has gained much more attention. For many years, quinine was extensively used to treat various forms of malaria [68]. Besides that, quinine has been employed as ligand in chiral stationary phases for the resolution of racemic mixtures [69]. Other alkaloids such as quinidine and quinine derivative molecules, like quinine carbamate (Fig. 1), have also been applied as ligands for chromatographic enantioseparation [70,71]. The remarkable ability of these alkaloids to resolve chiral mixtures is a consequence of their different functional groups with distinct enantiodiscriminating properties [72].
In the light of this information, Mahut *et al*. studied the applicability of quinine carbamate as ligand for the separation of sc pDNA topoisomers [73,74]. Using different plasmid molecules, the authors resolved a mixture of sc topoisomers, each with a different degree of negative supercoiling [73].

Various stationary phases based on quinine (aromatic and non-aromatic) were tested to study the structural characteristics for sc pDNA topoisomer recognition. It was observed that the carbamate group is crucial for topoisomer separation. Also, the presence of a quinoline ring reduces the ligand flexibility which is related to a superior topoisomer resolution [74]. By choosing quinine carbamate to be immobilized onto a silica matrix, the authors designed a low molecular weight selector that recognizes adjacent structures in the DNA groove [73]. It

is known that quinine behaves as a DNA intercalator [75] or at least, a partial intercalator [76]. Interestingly however, the distance between the tertiary amine and the carbamate moiety of quinine carbamate is similar to some DNA groove binders. It was concluded that unlike quinine, the carbamate derivative does not behave as an intercalator since upon binding, it does not induce structural changes in the DNA strand [73,77]. While the space between the amine and the carbamate group is responsible for the geometrical differentiation [73], the quinoline ring is responsible for some conformational stability [78]. Then, the key elements for topoisomer selectivity are the presence of a rigid and stable weak anion-exchange H-aceptor site, an H-donor site to allow specific hydrogen bonding with A-T sequences and a specific distance between these two moieties. These structural demands for molecular recognition are an important feature of affinity-like matrices [74]. Moreover, this stationary phase seems to have a good loading capacity and produces individual topoisomers in a short period of time [73].

The quinine carbamate ligand was used to analyse the topological changes of two plasmid molecules during fermentation. pDNA was first isolated through size-exclusion chromatography, collected and finally injected onto the quinine carbamate column [73]. Using the same chemoaffinity ligand, quinine carbamate, but changing the buffer conditions, Mahut *et al*. were able to create a switching method to achieve isoform or sc topoisomers separation [79]. When applying an increasing salt gradient of sodium chloride in a sodium phosphate buffer, a sc topoisomer separation was achieved. Moreover, no matter what changes are made in the buffer, the elution order is always the same: oc, linear and sc [79]. This constant behaviour is a result of the specific interactions between pDNA isoforms and the ligand through multiple contacts, which allows the selective recognition of the different species [74,79].

A pH gradient from 7.0 to 7.8 using the same quinine carbamate ligand led to full recovery of all isoforms due to repulsive charge induce elution. When a pH gradient was combined with an organic modifier gradient the sc isoform was eluted in a single peak, with an excellent resolution towards the other isoforms [79]. If sodium chloride is present in the elution buffer, the topoisomers are individually recognized by the matrix due to the different compaction states that lead to different available charges and consequently to a different interaction with the matrix [79]. A similar behaviour is observed with temperature changes. At high temperatures the selectivity for the three isoforms increases and the retention of sc pDNA is higher [79]. The authors were able to conclude that the binding between pDNA and the ligand is entropy-driven.

Apparently, the method developed by Mahut *et al*. with quinine carbamate as ligand is not suitable for one step pDNA purification, since it required a previous size-exclusion chromatography step to recover the molecule from the lysate. However it is the unique

procedure described until now for topological analysis of topoisomers. Since isoform separation led to full recoveries [79], the method may be appropriate for analytical chromatography of plasmid sample composition (pDNA quality analysis). The best procedure to apply for this purpose is the pH gradient, since the alternative uses environment unfriendly solvents not convenient for large scale uses [79].

2.3. DAPP: a phenanthridine derivative

The phenanthridine derivative 3,8-diamino-6-phenylphenanthridine (DAPP) (Fig. 1) is a nonquaternary analogue of ethidium bromide. DAPP has a phenanthridine core, which is a nitrogen heterocyclic compound formed of three fused aromatic rings. A phenyl and two amine groups are bound to that core and the phenyl group is the only that deviates from the planar molecular system [80]. DAPP has been used as structuring part of polymeric matrices [81], as a fluorescent dye [82] and recently, as a ligand in pDNA affinity chromatography [83].

The binding of DAPP to DNA shares a few similarities to that of ethidium bromide, and when the molecule is protonated, the binding is quite strong [84]. DAPP is also an intercalator, which means that DNA unwinds upon binding. Intercalation affects the nucleic acid flexibility, distorting the base-pair arrangement, which can facilitate the introduction of another intercalator molecule into the base-pair step [85,86]. Ethidium like molecules are slightly A-T specific and bind DNA through non-covalent, reversible stacking interaction of the condensed aromatic moiety into two successive base pairs [80,85]. Still, the phenyl residue gets inserted into the minor groove. Moreover, hydrogen bonds have a small contribution to the binding, which suggests that the hydrophobic term may have a major role in the binding. However, when protonated, DAPP molecules bind to DNA much strongly than in the neutral form, whether in the free state [80] or immobilized onto a Sepharose matrix [87]. This is possible since the stabilization energy of DAPP-DNA is substantially larger when the molecules are protonated [80], due to the generation of electrostatic interactions [88]. At pH values below DAPP's free state pK_a (5.8) additional strong attraction forces between the negative charge of DNA and the positive charge on the phenanthridine ring increase the intermolecular coulombic interaction [89]. When DAPP is bound to a Sepharose matrix, DAPP-DNA binding strength also varies with pH changes (Fig. 3). The matrix was able to retain all pDNA molecules (Fig. 3D) only with a pH value of 5, since a higher value led to semi retention (pH of 6) (Fig. 3C) or no retention at all (pH values of 7.4 and 8) (Fig. 3A and 3B). Interestingly, the protonation of immobilized DAPP molecules was evident from changes in gel colour with different pH values [87].

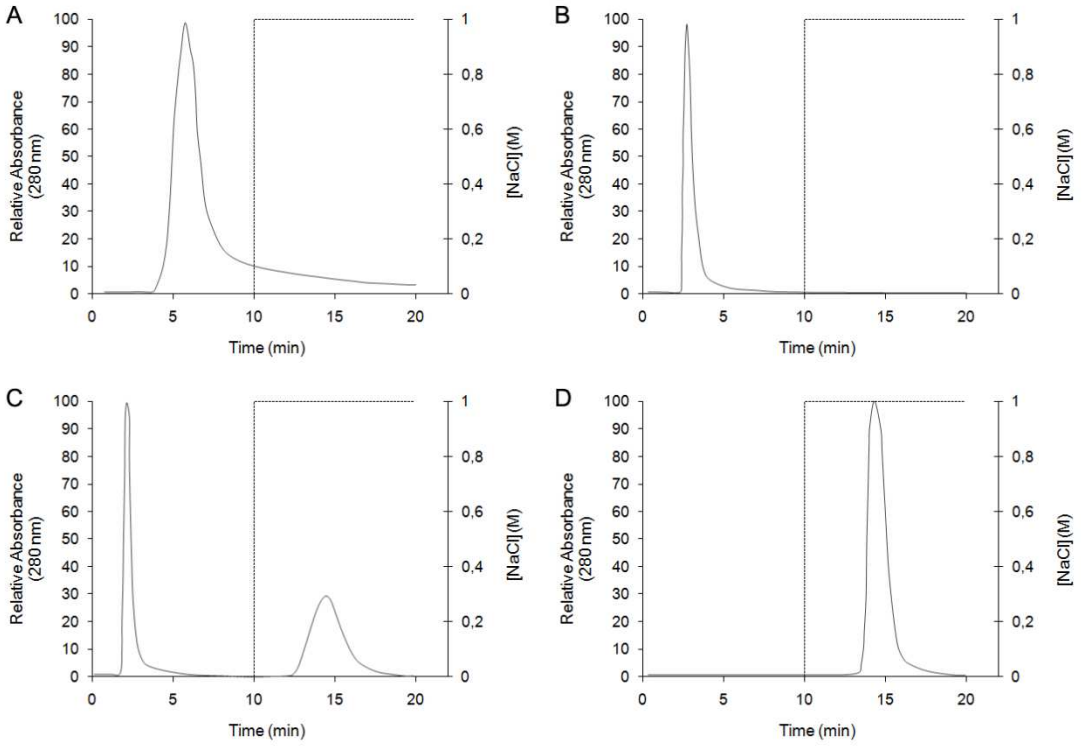

Fig. 3 - Retention of pDNA on the DAPP-Sepharose column using buffers with different pH values. An injection of pDNA (oc + sc) was performed varying only the loading buffer: (A) Tris-HCl 10 mM pH 8; (B) PBS 1x pH 7.4; (C) Acetate 10 mM pH 6 and (D) Acetate 10 mM pH 5. A solution of 1M of NaCl in the buffers was used in the elution.

The pH is not the only variable that affects DAPP-DNA stabilization. The presence of salt strongly opposes the binding between DNA and DAPP, destabilizing previously formed complexes [89]. This weakening of DAPP-DNA interaction with salt is also visible when DAPP is immobilized onto a polymeric matrix [87] which can be an advantage. Using an acetate buffer, pH 5 with no salt, all pDNA isoforms were retained in the DAPP-Sepharose due to strong electrostatic interactions. As expected, adding salt to the buffer led to the elution of all the species [87]. Salt effects result from a redistribution of ions around each molecule involved in the binding and its magnitude depends on salt concentration [89].

Using DAPP-Sepharose was possible to separate the sc pDNA from the less active oc and linear isoforms [87]. This affinity support was capable of retaining all species without any added salt to the eluent, and in only two elution steps was possible to collect a pure sc pDNA fraction [83], showing the utmost affinity towards this isoform. Clarified lysates from two distinct pDNA with different molecular weights (6.05 and 12.361 Kbp) were injected and all species were collected with stepwise isocratic elution (Fig. 4).

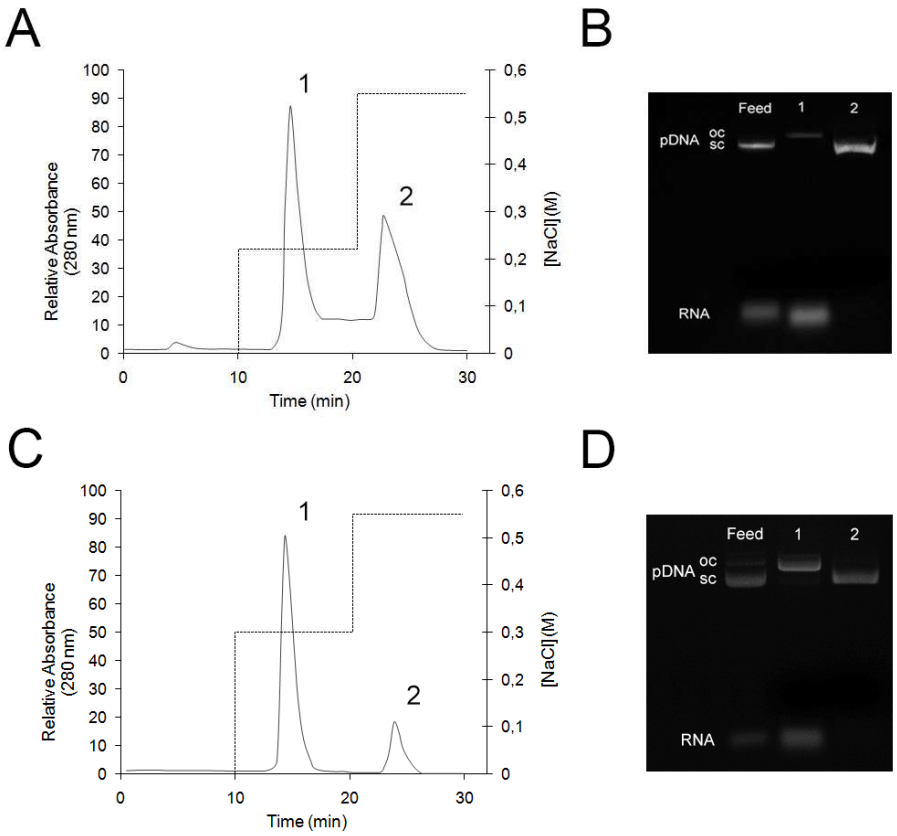

Fig. 4 - Supercoiled pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp) chromatographic separation from host cell impurities present in clarified feed solutions, using DAPP-Sepharose support. Agarose gel electrophoresis analysis of pDNA fractions. Peak 1 and electrophoresis lane 1: impurities eluted with 0.22 M of NaCl for pVAX1-LacZ and 0.3 M NaCl for pCAMBIA-1303, in 10 mM sodium acetate buffer pH 5; Peak 2 and electrophoresis lane 2: sc pDNA fractions collected after elution with 0.55 M NaCl in 10 mM sodium acetate buffer pH 5. The clarified lysate was also run in the agarose gel for comparative purposes (lane feed).

Moreover, the separation and purification performance of the support increases with temperature [83]. In similarity to what was observed with berenil-Sepharose [21], the recovery yield was lower for the larger plasmid molecule (65%). However, the 94% recovery obtained for the smaller molecule was quite extraordinary [83].

The maximum dynamic binding capacity (DBC) of DAPP-Sepharose for pDNA was 336.75 µg pDNA / mL gel, that is an acceptable value for a non-commercial support, with such a modest ligand density (0.15 mmol DAPP/g derivatized Sepharose). Moreover, the adsorption of pDNA to the support increases with increasing plasmid concentration, but stays almost unaffected by flow rate. Furthermore, the dissociation constant obtained ($2.29 \pm 0.195 \times 10^{-7}$ M) is a good evidence for the great affinity displayed towards pDNA [83].

The DAPP-Sepharose enables the obtainment of a sc pDNA sample in accordance with regulatory agencies specifications, with quite acceptable process losses and using small quantities of salt in the eluent [83]. By taking advantage of DAPP's pK_a, the process does not require salt in the binding buffer and has no environmental impact [39].

2.4. Simple phenyl derivatives

2.4.1. Phenyl HIC ligands

Simple commercial phenyl stationary phases are basically a phenyl group bound to a matrix (e.g. silica or Sepharose) through an alkyl spacer arm. Phenyl ligands can be applied for the purification of a vast number of molecules and biological structures. As a single step or integrated in multi-step purification protocols, phenyl derivatized matrices have been applied for the purification of endotoxins [90], viruses [91], proteins [92] and oligonucleotides [93], as well as in fractionation and enrichment of complex biomolecule mixtures [94]. Also diphenyl ligands have been used for immunoglobulin purification [95].

The good results obtained with phenyl ligands are due to π-π and hydrophobic interactions [96]. Thus, phenyl stationary phases are always associated with hydrophobic interaction chromatography (HIC) [97] where the adsorption of hydrophobic biomolecules is enhanced with increasing concentrations of a strong water structuring salt. On the other hand, adsorption is weakened when those concentrations are decreased [96]. As expected, this profile is very similar to the one observed with thiophilic aromatic ligands [34,36].

Simple phenyl ligands can be applied in preparative [98] or analytical chromatography [99]. Diogo *et al*. established a simple method both for quality control and quantification of pDNA in different types of solutions [99]. Using a commercial phenyl-Sepharose column, the authors were able to separate double-stranded pDNA from more hydrophobic impurities with a good resolution. pDNA purity was determined from the analytical chromatogram by comparing the estimated peak areas of pDNA and its impurities. Moreover, plasmid concentration of any sample can be calculated using a standard calibration curve. This method is easy to perform, fast, reproducible and capable of handling deeply contaminated samples [99], reasons that justify its wide use [21,100,101]. However, besides using ammonium sulphate in the eluent, the method is incapable of separating pDNA isoforms and quantify the sc pDNA alone [99] as opposed to a method developed with the non-aromatic amino acid arginine [102].
Negative phenyl HIC chromatography can also be used to purify pDNA on a preparative level. From all HIC ligands, phenyl is the one that shows the best selectivity for pDNA separation and purification [103]. Furthermore, stability of pDNA molecules is maintained during phenyl-HIC chromatography [104]. Existing phenyl-HIC supports may differ in the matrix (Sepharose, polystyrene/divinyl benzene, GMA and EDMA and epoxy membranes) but they all share the use of high salt concentrations in the equilibration buffer [98,105-108]. Membrane adsorbers can be integrated in hydrophobic downstream processing of pDNA using both alkyl and phenyl

ligands. However, phenyl membranes showed the highest capacity and pDNA selectivity. Nonetheless, pDNA recovery was lower than the one obtained with alkyl membranes [108].

Using a hydrophobic biporous resin with couple phenyl groups, Li *et al*. were able to semi-purify pDNA molecules with almost 100% of recovery. By reducing the feedstock volume, the authors stated that a pure plasmid fraction was obtained. However, the wide pore phenyl adsorbent was unable to separate pDNA isoforms [98] and the quality analysis was somehow incomplete, since the endotoxins and gDNA content in plasmid fractions was not analysed.
A phenyl-Sepharose support was also used to purify pDNA molecules from clarified lysate impurities. Results indicated that this stationary phase was able to separate pDNA from proteins, RNA and gDNA with a 51% yield [106]. Nevertheless, the endotoxin concentration in the plasmid fraction was not calculated and the sc pDNA was not isolated from the other isoforms.

Phenyl-Sepharose ligands have also been used as HIC stationary phases in an integrated multi-step chromatographic pDNA purification process [105,107]. When combined with hollow-fiber tangential filtration and anion-exchange membrane chromatography, the bioprocess efficiently separated pDNA from more hydrophobic RNA using high flow rates [105]. However, it would have been important to analyse the pDNA fraction for other impurities, since biopharmaceutical pDNA must follow stringent quality criteria in terms of purity (Table 1). In a more complex method phenyl-HIC was preceded by tangencial flow filtration and ion exchange chromatography, and followed by size exclusion chromatography [107]. This scaled-up, tandem like procedure enabled the large-scale production of a pDNA fraction that met the requirements for pharmaceutical use. HIC was integrated in this process mainly for endotoxin and RNA reduction, since they are more hydrophobic than pDNA. However, the time that the entire process takes to purify a plasmid sample can represent a slight drawback. Moreover, the obtained sample is composed of only 75% of sc pDNA and the final yield is not very impressive (48%) [107], possibly due to the high number of chromatographic steps.

None of the mentioned phenyl methods is able to separate pDNA isoforms. Although that is not an imperative criteria and greatly depends on the pDNA final use, sc pDNA is the most active and vector-efficient isoform, and should represent a great percentage of the final product [109]. Apparently, using ammonium sulphate in the buffer solutions does not lead to isoform separation. Using an alternative salt such as sodium citrate makes the process more environmentally friendly [39] however, sc isoform purification may be equally difficult to achieve [38,103]. A phenyl-agarose support was tested for sc pDNA purification with two different salts, sodium citrate and potassium phosphate. Using the later salt, resolution between isoforms was only partial, recovery was extremely low (38%) and purity of sc pDNA fraction was not acceptable (75.5%). Unfortunately, sodium citrate did not provide much

better results. Even though the pDNA fraction contained only the sc isoform (59.1% recovery) with acceptable traces of proteins, gDNA and endotoxins, the purity was set around 42% due to RNA contamination [38].

The results obtained by Freitas *et al*. were slightly different [103]. From the various phenyl-Sepharose supports, the best results were obtained with high substitution phenyl-Sepharose 6 fast flow (PheFF-HS). The best sc resolution was observed using sodium citrate concentrations above 1.2M and employing the lowest loadings of clarified lysate. When 1M of salt was used a better pDNA purity and recovery was achieved but sc/oc resolution was lost [103]. Higher sodium citrate concentrations promote phenyl-Sepharose/pDNA binding due to the synergetic action of the trivalent citrate and the monovalent sodium cation. Citrate structures water molecules, whereas sodium ions bind to DNA grooves, removing the water located around DNA molecules and eventually leading to a higher compaction of the coil [110]. This ultimately led to the isoform separation observed with salt concentrations above 1.2M [103] since the bases of oc and sc pDNA have a different exposure. Overall, replacing ammonium sulphate by sodium citrate represents a good economic and environmental strategy for phenyl-HIC pDNA chromatography. However, it seems that a little more work is needed to make this alternative approach superior to the established ammonium sulphate methods, or even to other methods using different ligands.

More recently, a commercial phenyl membrane was applied to study the impact of plasmid size on the interaction of pDNA with the hydrophobic support [111]. Using ammonium sulphate in the eluent and plasmids with three different sizes it was showed that the interaction strength between them and the HIC membrane depends on plasmid size. In fact, the larger the plasmid hydrodynamic diameter, the higher the conductivity drop required to disturb the hydrophobic interaction. Differences in the interaction strength were also detectable between oc and sc isoforms, allowing their separated elution. The hydrophobic interaction strength between oc isoforms and the membrane remained unchanged with pDNA size variations. On the other hand, the interaction with sc pDNA depends on plasmid hydrodynamic diameter. The pooled sc fractions were efficiently recovered with a very good yield however, a complete resolution between isoforms was apparently not achieved. Moreover, the genomic DNA content in pDNA pools was not determined [111].

2.4.2. Phenyl boronate ligands

Boronic acids have been applied as affinity ligands for the purification of various biomolecules [18]. The base molecule for these ligands is phenylboronic acid or $PhB(OH)_2$, which is a boronic acid with a phenyl substituent. The molecule is mostly planar, having an electronic delocalization (π-π interaction) between the aromatic ring and the dihydroxyboryl group [112], and can originate many derivatives by phenyl substitution. Phenylboronic acids are

strong Lewis bases due to boron's ability to donate electrons [113] and, depending on the phenyl substituent, they can have a pK_a between 4.5 and 8 [114].

In 1880 Michaelis and Becker prepared for the first time the precursor of phenylboronic acid, dichlorophenyl boronate [115]. Since then, phenylboronic acid and its derivatives have been widely used in different applications, such as biomolecule delivery systems [116] and other pharmaceutical agents [113], catalysts [117] and reagents in a variety of synthesis [118-120], development of saccharide sensors [121] and adsorbers [122,123], stationary phases for protein [124] and glycoprotein purification [125,126], for cell immobilization [127], for nucleoside [128] and nucleotide adsorption [129] and for nucleic acids adsorption and purification [130,131].

Many of these applications are a result of the great affinity that phenylboronic acid displays for diol compounds [132] or polyol compounds such as saccharides [133]. Such compounds containing 1,2-*cis*-diol groups are the ones that bind most strongly to the boronate portion of phenylboronic acids through reversible covalent ester formation [18,132]. Among the lysate solution components, only RNA and endotoxins have 1,2-*cis*-diol groups, a feature absent from the deoxyribose backbone of DNA. Since endotoxins are lipopolysaccharides, they have *cis*-diol groups that can strongly bind to boronate ligands. On the other hand, RNA molecules have only one *cis*-diol group at the 3'-end, which can cause a weaker binding with those ligands [18]. Therefore, the use of a phenylboronate ligand can be a way to readily separate DNA from RNA and endotoxins.

Secondary interactions such as ionic interactions, hydrogen bonding, hydrophobic interactions and coordination interactions can also provide additional selectivity towards the phenyl boronate ligand. Under basic conditions, the boronate is hydroxylated and gets transformed into a tetrahedral anion which can produce couloumbic interactions with ionic analytes. Moreover, the hydroxyl groups bound to the boron atom are active sites for hydrogen bonding. In addition, since the ligand contains a phenyl group, it can establish hydrophobic interactions with the analytes. Finally, under acidic conditions, coordination interactions can be established since the boron atom of the uncharged boronate has an empty orbital, serving as an electron receptor [18].

The ligand 3-aminophenylboronic acid (3aPBA) (Fig. 1) was chosen for pDNA clarification in the performed studies [108,131,134-136]. This *meta*-amino substituent forms a reversible five-membered ring complex with the sugar portion of nucleotides and saccharides, being usually coupled to the stationary phases through its anilino group [18].

Gomes and co-workers tested the ability of 3aPBA coupled to controlled porous glass (CPG) beads to clear *E. coli* impurities directly from alkaline lysates [134,135]. No special treatment

was performed in the preparation of lysate solutions. Potassium and acetate ions concentration (of neutralization buffer) in the lysate proved to be of great importance for plasmid recovery, a main reason why no isopropanol precipitation was performed. Moreover, pre-treatment of lysate solutions with RNase showed to be prejudicial for column performance. Water was chosen as eluent over the commonly used magnesium chloride solutions since it delivered the best plasmid recovery as well as the best protein and RNA removal [135]. Results showed that the support was not only able to bind *cis*-diol bearing species like RNA and endotoxins, but also gDNA and proteins. Nevertheless, pDNA did not interact with the solid phase and eluted in the flowthrough [134,135]. Before these results, it was believed that boronate could only form esters with *cis*-diols at basic conditions, when hydroxylated and with a tetrahedral configuration [18]. Interestingly, RNA and endotoxins were best bound to the matrix at an acidic pH [135], below the pK_a value of the ligand (8.8) [137]. Elution of these bound molecules was performed by adding a competing *cis*-diol bearing species to the eluent, which proved that reversible covalent bonds were indeed the major interactions involved in the binding [134]. Since proteins and gDNA are unable to sterify with boronate hydroxyl groups, its binding mechanism is a quite more difficult to explain. Due to the alkaline lysis, gDNA is mostly single-stranded with its bases exposed. The nitrogen atoms of those bases possess one lone pair of electrons that they can share with boron while it has a vacant p orbital (acidic pH). These types of charge-transfer interactions are most likely the ones responsible for the binding of proteins to the matrix [135], since they also have atoms with lone electron pairs in their amino acid residues.
This boronic acid method can process alkaline lysates without the need of isopropanol precipitation or sample conditioning. Moreover, it uses water in the injection buffer and has an excellent pDNA recovery (96.2%). Despite the great advantages over many other methods the purification of pDNA is not effective, since the process delivers a pDNA sample with a poor purity level [134,135].

A scale up of the chromatographic method presented similar features, advantages and unfortunately, similar disadvantages. Using the same buffer conditions, the adsorbent volume was increased in ten-fold, maintaining an optimal lysate loading/adsorbent volume ratio of 1.3. The method is simple, fast, reproducible and gave an excellent pDNA recovery (93-96%) however, the recovered pDNA is not a pure sample (gDNA and endotoxin analysis were not performed in this study) [131]. Moreover, all pDNA isoforms were eluted at the same time in the flowthrough, since the ligand is not able to discriminate between them.

The 3aPBA ligand was also tested with slightly different conditions, immobilized onto an epoxy-activated affinity membrane [108]. However, pDNA purification was also not achieved, and as opposed to the work of Gomes *et al*. [135], this membrane method did not show any specific selectivity towards *cis*-diol containing species [108]. The different buffer conditions and sample conditioning may be at the heart of this important discrepancy between methods.

In spite of 3aPBA chromatography not being able to produce a pharmaceutical grade pDNA fraction, its ability to greatly reduce RNA and endotoxin contamination can place it as an important intermediate recovery step. It can successfully replace commonly used operations such as isopropanol and ammonium sulphate clarification steps, since it is simpler, environmentally friendly and enables good recoveries. In this perspective, Firozi *et al*. took advantage of 3aPBA special ability to bind polisaccharides to remove specific endotoxin contamination from pDNA preparations by a multistep process [136].

2.5. Imidazole derivatives

Imidazole ($C_3N_2H_4$) is a heterocyclic five-membered ring system with two nitrogen atoms in the aromatic ring [138]. It is usually classified as a heterocyclic aromatic amine, since it has six π electrons in a planar and fully conjugated ring. Both nitrogen atoms have an unshared electron pair but only one is incorporated into the aromatic π system. The other is responsible for the basic properties of the compound, and its protonation produces a resonance-stabilized cation [26,139]. The imidazole system is an important structural feature of histidine (amino acid) and histamine (biogenic amine) (Fig. 1) [140]. Moreover, imidazole has been applied as an important starting compound in chemical reactions [141], a pharmacological tool [142], as a competing agent in chromatography [143] and proton solvent [144].

2.5.1. Amino acid histidine and derivative molecules

2.5.1.1. Simple histidine ligands

Histidine is one of the twenty naturally occurring proteinogenic amino acids and has an imidazole group, one amine and one carboxyl group in its structure (Fig. 1). The imidazole motif is an ionisable group with a pK_a near neutral pH, attributing a side chain pK_a of 6.5 to the amino acid [140]. When neutral, histidine can coordinate metal ions and establish H-bonds with many molecules. On the other hand, when histidine is positively charged, it can still establish hydrogen bonds, forming salt bridges with negatively charged molecules [145].

Histidine interacts with proteins, amino acids and various metallic cations through different types of interactions. These are, in a crescent strength order: π-π stacking interactions, since imidazole is able to interact with aromatic side chains of other molecules; hydrogen bonds due to the fact that imidazole is a hydrogen bond donor and acceptor; hydrogen π interactions, since the polar hydrogen of histidine can form hydrogen-π bonds with other aromatic amino acids in "T" orientation; cation-π interactions between the imidazole ring and metallic cations or organic cations and finally, coordinate interactions, since the basic nitrogen atom in the imidazole moiety has a lone electron pair, it can coordinate with many metallic cations [146].

Due to its binding versatility, histidine can be used in many applications. A special importance is given to its use in many chromatographic processes such as an affinity tag for protein purification using metal ion affinity chromatography [147], as ligand for purification of proteins [148], antibodies [19,149], oligossacharides [150], RNA [151,152] and sc pDNA [20].

Purification of sc pDNA was performed using a commercial L-histidine agarose gel, in the presence of high salt concentrations [20]. Besides providing a pDNA sample according to regulatory agencies specifications, this support was also able to separate the sc pDNA from the less active oc isoform [20,153]. The predominant interactions between histidine and pDNA are the ring stacking/hydrophobic interactions. This is due to the high salt concentrations used to bind the molecules to the support, and also due to the weakening of interaction when imidazole is used as a competing agent [143]. Moreover, histidine interacts preferentially with guanine (and adenine in a smaller level), not only because of the preferential bifurcated H-bond with its N7 and O6 atoms, but also because of its ability to establish extensive ring-stacking interactions [154]. However, the binding mechanism between L-histidine and the various nucleic acids involves not only hydrophobic interactions, but also other biorecognition interactions such as cation-π and electrostatic interactions between the positively charged amino acid and the phosphate groups of the DNA backbone [143]. Moreover, increasing the temperature was proved to negatively affect sc pDNA - histidine interaction, but not the interaction with oc pDNA, RNA and gDNA [143,154,155]. In contrast, the increase of the molecular mass of the polynucleotides seems to increase sc pDNA affinity towards the histidine matrix [156].

Although the yield of the histidine chromatographic process was quite below the expectations (45%), sc pDNA was successfully purified from the lysate impurities. Moreover, the obtained sc pDNA led to higher transfection efficiency than the pDNA purified with a commercial kit [20]. The pDNA maximum capacity calculated for this commercial support was of 530 μg pDNA/mL gel, decreasing when the temperature and/or flow rate were increased [157].

In an overall evaluation, this histidine-chromatographic process has only the disadvantage of using great amounts of ammonium sulphate in the binding and elution buffers. Thus, the collected pDNA sample has a high ionic strength [20] and has to be desalted before further use.

2.5.1.2. Histidine derivative molecules

Histamine (Fig. 1) is a neurotransmitter derived from the decarboxylation of histidine [140]. Histamine was also used as ligand for the chromatographic purification of pDNA and the major forces involved are electrostatic and hydrophobic interactions. This novel multimodal histamine monolith support has an excellent dynamic binding capacity of 2.7 mg/mL [158], which is a promising feature for a chromatographic matrix. A successful separation of a

mixture of sc and oc pDNA isoforms was obtained with different buffer conditions. However, when a clarified lysate was injected at high ammonium sulphate concentrations, the eluted isoforms were not completely resolved [158]. Moreover, the authors did not give any information concerning pDNA separation from lysate impurities (RNA, proteins, gDNA and endotoxins).

A similar conclusion is drawn after analysing the methods developed by Perçin *et al*. where an *N*-methacryloyl-(L)-histidine methyl ester (MAH) was used as a pseudospecific ligand [159,160]. MAH derivative polymers have been used in various other applications, especially as metal-chelating ligands for enzyme immobilization [161], and purification of antibodies [162], enzymes [163] and other proteins [164]. For pDNA adsorption studies, the polymer [poly(hydroxyethyl methacrylate-*N*-methacryloyl-(L)-histidine methyl ester or PHEMAH] was prepared by polymerizing hydroxyethyl methacrylate (HEMA) with MAH [159]. The MAH ligands were tested in two different stationary phase forms: a PHEMAH affinity cryogel [159] and PHEMAH magnetic nanoparticles [160]. The preliminary pDNA adsorption studies showed that both matrices have very good pDNA adsorption capacities. These capacities decrease with increasing salt concentration and when temperature was below or over 25ºC [159,160]. Moreover, for PHEMAH magnetic nanoparticles, pDNA adsorption was also pH dependent and the maximum value was observed at pH 5 [160], a value much higher than the one obtained for the cryogel matrix (154 mg/g vs 13.5 mg/g) [159,160]. Clearly, for both cases, ionic interactions play a major role in pDNA adsorption. Also, other non-specific hydrophobic interactions between PHEMAH and pDNA may intervene as well. Even though both matrices are able to adsorb a great percentage of pDNA [159,160], the chromatographic studies conducted with the cryogel matrix are not enough to conclude if pDNA is indeed separated from RNA [159]. Moreover, both cryogel and nanoparticle matrices cannot be extensively used since they start losing the adsorption capability after a few assays [159,160]. Their great advantage over other solid supports is the intrinsic physical nature of both cryogels and magnetic nanoparticles, since they allow rapid separations, with high binding rate, due to a large surface area and virtually no mass transfer resistance [159,160].

2.5.2. Carbonyldiimidazole ligands

The compound 1,1-Carbonyldiimidazole (CDI) with the molecular formula $(C_3H_3N_2)_2CO$ is basically formed by two imidazole molecules bridged by a carbonyl group (Fig. 1). Carbonyldiimidazole is an important reagent in organic synthesis [141] and it is also widely used in preparation and activation of stationary phases [165,166]. Despite CDI was not commonly applied as the ligand itself for purification processes, Sousa *et al*. used a CDI poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith disc as stationary phase for sc pDNA purification [167]. The CDI ligand enabled not only the separation of sc pDNA from the less active oc isoform [167,168], but also from other lysate impurities such as RNA, proteins, endotoxins and gDNA [167]. In similarity to what was observed when the histidine

ligand was used [20], the hydrophobic interactions are also the major force involved in the binding between CDI and pDNA. Hydrogen bonds between the non-protonated nitrogen atoms of the imidazole ring and nucleic acid bases, water mediated hydrogen bonds and electrostatic interactions can also contribute to the selectivity of the different molecules [167].

The calculated dissociation constant ($4.81\pm0.21x10^{-8}$ M) confirmed that the support has a good affinity towards pDNA. The dynamic binding capacity (max ≈ 3.38 mg/mL) improved with the increase of sc pDNA concentration and with the decrease of the flow rate. Nevertheless, the separation efficiency of plasmid isoforms remained unchanged with flow rate variations [168]. Using a CDI monolith support with the described buffer conditions is possible to recover 74.7 % of sc pDNA, according to the requirements of the regulatory agencies. Moreover, the obtained sc fraction successfully transfected a high number of cells (59%) [167]. This chromatographic method has the advantage of purifying sc pDNA in a fast and efficient manner due, not only to the CDI ligand, but also to the outstanding mass transfer properties typical of the monolith matrix. It has, however, the disadvantage of using high quantities of ammonium sulphate in phosphate buffers [167], two salts with a high environmental impact [39].

2.6. Phenylalanine derivatives

Like histidine, phenylalanine, which structure comprises one phenyl ring, is one of the naturally occurring amino acids. After its hydroxylation to tyrosine, phenylalanine is the precursor of acetyl-CoA, dopamine, epinephrine and norepinephrine [140]. Phenylalanine derivatives were also applied as ligands for the purification of biomolecules such as proteins [169,170] and pDNA [171]. *N*-methacryloyl-(L)-phenylalanine (MAPA) (Fig.1) was used as a monomer for the preparation of two distinct cryogels, one using a conventional cryogelation process [P(HEMA-MAPA)] and the other using a freeze-drying step [P(HEMA-MAPA)-FD]. P(HEMA)-FD was also prepared for comparative purposes [171]. pDNA adsorption capacities of those cryogels showed that MAPA is crucial for pDNA adsorption. Moreover, the higher capacity value obtained for P(HEMA-MAPA)-FD (45.31 mg/g) was a result of the higher surface area of that matrix, enabled by the nanospines created in the freeze-drying step [171].

Plasmid purification studies were performed using the P(HEMA-MAPA)-FD cryogel and injecting lysate solutions with sodium sulphate in the buffer. A good resolution between impurities and pDNA was obtained (process efficiency of 80%), since the later was eluted after removing the salt from the eluent [171]. This is an indication that the main interactions involved in MAPA-pDNA binding are hydrophobic. In fact, pDNA adsorption increased with increasing salt concentration due to the salting-out effect, causing a decrease in pDNA solubility and an increased diffusion towards the cryogel surface. Moreover, the adsorption capacity also increased with temperature [171].

The described method has interesting advantages, mainly due to the matrix nature, however the presented results are still quite preliminary and a more complete purity analysis of collected pDNA must be performed.

2.7. Summary and comparison of the ligands key features

All the previously enumerated ligands share the presence of, at least, one aromatic ring in their structure (Fig. 1). However, their interaction with pDNA is quite different, as well as the method in which they were applied.

Table 2 shows a brief summary of those features on the ligand application for pDNA chromatographic purification.

Table 2. Summary of key method characteristics using each aromatic ligand

Ligand	DNA interaction	Essential chromatographic method characteristics	pDNA purification efficiency	Ref.
2-mercaptopyridine	Possibly a pseudo-intercalator Hydrophobic interactions	Moderate-high ammonium sulphate or potassium phosphate usage Suitable for integrated or as "stand alone"* process	Moderate yield May separate sc pDNA from impurities	[34,36-38]
Berenil	Minor-groove binder Hydrophobic and electrostatic interactions, hydrogen bonds	Moderate ammonium sulphate usage Suitable for "stand alone" process	Good yield Efficient pDNA purification from impurities No isoforms separation	[21,50]
Quinine Carbamate	Minor-groove binder Hydrogen bonds	Small sodium chloride usage Not suitable for "stand alone" process	No yield information No efficient sc pDNA purification	[73,74,79]
DAPP	Intercalator Electrostatic interactions when protonated Small contribution of hydrogen bonds	Small sodium chloride usage for elution DBC unaffected by flow rate Suitable for "stand alone" process	Excellent yield Efficient sc pDNA purification from impurities	[83,87]
Phenyl	Hydrophobic interactions	Moderate-high ammonium	Moderate-high recoveries	[38,98,99,103, 105-107,111]

		sulphate or sodium citrate usage Applied for analytical or preparative chromatography Suitable for integration in a multi-step process	(preparative) May purify sc pDNA using membrane adsorbers Efficient analytical method (no isoforms separation)	
3aPBA	No interaction	1,2-cis-diol preference (RNA and endotoxins) Reduced or no salt usage Suitable for intermediate step: RNA and endotoxin reduction	Excellent yield No efficient pDNA purification	[108,131,134-136]
Histidine	Hydrophobic interactions Small contribution of electrostatic interactions	High ammonium sulphate usage Suitable for "stand alone" process	Low yield Efficient sc pDNA purification from impurities	[20,153,154]
Histamine	Hydrophobic and electrostatic interactions	High ammonium sulphate usage Excellent DBC	No information available	[158]
MAH	Ionic interactions Small contribution of hydrophobic interactions	Low-moderate sodium chloride usage Good DBC	No information available	[159,160]
CDI	Hydrophobic interactions Small contribution of electrostatic interactions and hydrogen bonds	High ammonium sulphate usage Suitable for "stand alone" process Good DBC	Good yield Efficient sc pDNA purification from impurities	[167,168]
MAPA	Hydrophobic interactions	Low-moderate sodium sulphate usage Excellent DBC	No isoforms separation Not enough information about pDNA quality	[171]

*"Stand alone" is to be interpreted as a unique chromatographic process after pDNA extraction or non-chromatographic clarification

Depending on the buffer composition, the interactions between pDNA and the immobilized aromatic ligand can deeply vary [21,38,79,83]. Aromatic ligands have a strong hydrophobic nature, however the change in salt composition, concentration or buffer pH value can enhance the formation of other interactions with DNA, such as strong electrostatic interactions [83,87] or hydrogen bonds [73,74,79]. Nevertheless, most of the ligands still interact with pDNA through strong hydrophobic interactions (Table 2). For that to happen successfully, moderate to high quantities of a "salting out" salt must be used, which can be a major drawback for large scale applications, both in economic and environmental standards.

After the analysis of all developed methods (Table 2), it is noticeable that some of them need more work before its widespread application. The use of histamine [158], MAH [159,160], MAPA [171] and quinine carbamate [73,74,79] are examples of that. Moreover, the use of alternative salts to ammonium sulphate with thiophilic [38] and phenyl ligands [38,103] seems to be an excellent strategy, although the work developed is still somehow incomplete.

The described chromatographic methods that can efficiently deliver a purified pDNA sample are the ones using berenil [21], DAPP [83], histidine [20] and CDI [167] (Table 2). However, the berenil-Sepharose support is not able to separate sc pDNA from other isoforms when present in a complex lysate solution and only the purification of total pDNA can be achieved [21]. Hereupon, considering that CDI and histidine have the need for great quantities of salt [20,167], the use of DAPP [83] as a chromatographic ligand appears to be the most promising approach (Table 2). Additionally, in this particular case, sc pDNA purification is achieved with an outstanding recovery and slightly faster than some other methods [83].

3. Conclusions and future perspectives

In this review was presented an overview of aromatic ligands used for pDNA chromatography. The focus was not only directed for ligand-DNA interactions, but also for ligand key characteristics, applications and an experimental review of the methods in which they were applied. Aromatic ligands are very versatile molecules due to the unique characteristics derived from the presence of the aromatic ring and also due to all ring substituent possibilities. These features make them an interesting choice for a great variety of chromatographic applications.

The phenanthridine derivative DAPP showed the better purification results and the most economic, environmentally friendly and fast procedure. Nevertheless, all the other ligands present admirable and unique features. In this case, it would be interesting to test different buffer conditions and different immobilization matrices. Even for those successfully applied for pDNA purification, changing the polymer matrix could help to solve some drawbacks such as low capacity, diffusivity and even resolution.

As a final point, combining aromatic chromatography with optimized production, extraction and clarification procedures, can offer a number of advantages for pharmaceutical pDNA purification.

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Chapter 2

Global aims

The aim of this doctoral work was to develop new chromatographic strategies for plasmid DNA (pDNA) purification, with the goal of improving the overall procedures to be more effective, simple, economic and environmental-friendly.

The screening and development of new chromatographic ligands for pDNA purification is of the utmost importance, since this biomolecule is in close relation to the future success of molecular based therapies.

Choosing the specific ligand is one of the critical steps in the establishment of a chromatographic process. Therefore, it is important to take into account several molecule characteristics such as structure, size, charge, toxicity and ultimately, pDNA binding specificities and affinity. The ideal chromatographic conditions (buffer composition, ionic strength, pH and temperature) must be carefully studied since they are related to the chosen ligand and can enable the specific interactions between that ligand and pDNA. Moreover, the pDNA therapeutic product must meet strict quality criteria in terms of impurities levels. Therefore, choosing the loading and elution conditions must also take into account the similarities and differences between pDNA and host *Escherichia coli* impurities such as genomic DNA, proteins, RNA, and endotoxins.

With these global aims in mind, the development of the work followed the subsequent tasks:

a) Selection of the most promising molecules to be used as ligands in the subsequent purification steps.

b) Immobilization of the selected molecules onto chromatographic matrices using suitable curing methods. Quantitative and qualitative characterization of the matrices using several specific techniques.

c) Retention studies with pure pDNA and impact of the mobile phase composition influence on the chromatographic behaviour and separation of pDNA isoforms.

d) Evaluation of support's capacity to separate and purify pDNA from lysate impurities, as well as assess if the purity of pDNA solutions is in conformity to the specifications of regulatory agencies.

Chapter 3

Paper II

Specific berenil–DNA interactions: an approach for separation of plasmid isoforms by pseudo-affinity chromatography

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Specific berenil–DNA interactions: An approach for separation of plasmid isoforms by pseudo-affinity chromatography

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ABSTRACT

Small molecules, like some antibiotics and anticancer agents that bind DNA with high specificity, can represent a relevant alternative as ligands in affinity processes for plasmid DNA (pDNA) purification. In the current study, pDNA binding affinities of berberine, berenil, kanamycin, and neomycin were evaluated by a competitive displacement assay with ethidium bromide using a fluorimetric titration technique. The binding between pDNA and ethidium bromide was tested in different buffer conditions, varying the type and the salt concentration, and was performed in both the absence and presence of the studied compounds. The results showed that the minor groove binder berenil has the higher pDNA binding constant. Chromatographic experiments using a derivatized column with berenil as ligand showed a total retention of pDNA using 1.3 M ammonium sulfate in eluent buffer. A selective separation of supercoiled and open circular isoforms was achieved by further decreasing the salt concentration to 0.6 M and then to 0 M. These results suggest a promising application of berenil as ligand for specific purification of pDNA supercoiled isoform by pseudo-affinity chromatography.

Molecular therapies such as gene therapy and DNA vaccines are increasingly being established as promising and potent alternatives to classical treatments [1]. Both methodologies are based on vector-mediated introduction of a therapeutic nucleic acid molecule in patient-selected cells. Although a great amount of work has been reported using viral vectors, its safety is a major concern in human studies [2]. Nonviral vectors, such as plasmid DNA (pDNA),[1] are emerging as a better alternative in terms of both safety and ease of production [3]. The expected wide application of these methodologies requires the large-scale production and purification of plasmids. Classical protocols for purification of plasmids used in molecular biology are widely available [4]; however, besides the fact that they are not suitable to be applied in large-scale manufacturing, they frequently use toxic reagents that prevent their use for therapeutic product purification [5].

Recent years have witnessed increasing research efforts in the development of new methods for plasmid purification. Most of these are based on chromatographic processes [6], but other procedures using precipitation [7] and aqueous two-phase systems [8] have also been described. Although affinity methods seem to be very adequate for purification purposes, they have not been explored extensively. Successful cases report the use of proteins as ligands [9]; however, besides the cost of the ligands, the target plasmid needs to bear a sequence with high affinity for these molecules, and this limits and complicates the application of these methodologies. Therefore, the use of other ligands, both cheaper and sequence independent, is required. The use of affinity chromatography (AC) using arginine [10], histidine [11], and lysine [12] as ligands for separation of pDNA isoforms, and for selective purification of supercoiled (sc) pDNA from clarified cell lysates, has been reported. Although amino acid based AC is a promising approach for DNA purification, some drawbacks need to be overcome, including low capacity of available supports and low recovery yields [13].

Several molecules, such as certain antibiotics and anticancer agents, bind DNA with high specificity [14]. Due to the complex structure of double-helical DNA, different binding modes are possible. Besides covalent binding, there are several classes of specific or unspecific noncovalent binding modes such as minor groove binding, intercalation between base pairs, bisintercalation, major groove binding, and a combination of the above [15]. Because the first two are specific for DNA but sequence independent, molecules that bind this way should be very promising for developing affinity purification methods [16]. Examples of these DNA binding molecules include berberine, berenil, kanamycin, and neomycin.

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[1] *Abbreviations used:* pDNA, plasmid DNA; AC, affinity chromatography; sc, supercoiled; EtBr, ethidium bromide; FPLC, fast protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; oc, open circular; MGB, minor groove binder.

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Neomycin and kanamycin belong to the family of antineoplasic drugs produced by *Streptomyces* genera [17,18]. Neomycin binds to guanine bases in the deep major groove of the double helix in an intercalative manner [19]. On the other hand, berenil is a member of the aromatic diamidine class of DNA binding agents that reversibly binds preferentially to DNA minor groove at central AATT sequence [20]. Finally, berberine is a major isoquinoline alkaloid present in a number of clinically medical plants [21] that binds to the double helix with high affinity; nevertheless, the binding mode is not completely clear. The most likely way of binding is a partial intercalation, where a portion of the ligand molecule intercalates into the double helix and the other portion protrudes into the minor groove at the AT-rich sequences [22,23].

More than a new and unexplored approach, the application of small DNA binders as ligands for pDNA affinity purification seems to be able to improve and simplify currently available protocols and could, in the near future, be implemented in the large-scale purification of plasmid vectors for molecular therapies.

In this work, we report the evaluation of DNA binding affinities of berberine, berenil, kanamycin, and neomycin using a model plasmid (pVAX1–LacZ). The binding affinities were determined by a competitive displacement assay with ethidium bromide (EtBr) using a fluorimetric titration technique. The most promising ligand was then used in sc pDNA isoform purification studies by a pseudo-affinity-type chromatography.

Materials and methods

Reagents and stock solutions

EtBr was obtained from AMRESCO (Cochran, OH, USA). Kanamycin was obtained from Calbiochem (San Diego, CA, USA). Berenil, neomycin, berberine, and 1,4-butanediol diglycidyl ether were purchased from Sigma–Aldrich (St. Louis, MO, USA). All salts used were of analytical grade.

Sodium chloride and ammonium sulfate solutions were prepared in different concentration in Tris–HCl buffer (0.05 M, pH 7.5) for the fluorimetric measures and in Tris–HCl buffer (10 mM, pH 8.0) for the chromatographic experiments. A stock solution of EtBr (solution A) in deionized water was prepared with an approximate concentration of 3.0×10^{-4} M. The exact concentration of this solution was determined by diluting a small portion 100-fold and measuring the absorbance at 480 nm ($\varepsilon = 5600\ M^{-1}\ cm^{-1}$). Working solutions were obtained by appropriate dilution with Tris–HCl buffer. Solution B was prepared by diluting solution A with buffer to a concentration of 3.0×10^{-6} M.

Plasmid production and purification

An *Escherichia coli* DH5α strain harboring 6.05 kpb plasmid pVax1–LacZ (Invitrogen, Carlsbad, CA, USA) was cultured overnight in Luria–Bertani medium supplemented with 30 μg/ml kanamycin at 37 °C. Cell growth was carried out at the same temperature in an Erlenmeyer flask with Terrific Broth medium supplemented with 30 μg/ml kanamycin. The growth was suspended at late log phase ($OD_{600} \approx 10.0$). Cells were recovered by centrifugation and stored at −20 °C. pDNA was purified using a Qiagen Plasmid Midi Kit (Hilden, Germany) according to the manufacturer's instructions. Plasmid quantification was made by measuring the absorbance at 260 nm.

Determination of fluorometer settings

Fluorescence measurements were carried out on a FluoroMax-4 Horiba spectrofluorometer (Edison, NJ, USA) using a cell of 1 cm path length. A cuvette was filled with 2.2 ml of buffer (0.05 M Tris–HCl + 0.5 M NaCl, pH 7.5), 0.10 ml of plasmid solution (1.5×10^{-3} M in buffer), and 50 μl of solution A and mixed well. The cuvette was then placed in the fluorometer and the excitation wavelength was set to 525 nm, the excitation slit to 5 nm, and the emission slit to 3 nm. The emission wavelength was set to the value that gave the maximum intensity (599 nm).

Determination of EtBr binding parameters to DNA

The proportionality constant for free EtBr (k_f), the proportionality constant for EtBr bound to the DNA (k_b), and the binding constant of DNA–EtBr (K) were determined experimentally according to the method described by Strothkamp and Strothkamp [24].

Determination of EtBr binding to DNA in presence of studied compounds

The binding constant of EtBr to pDNA in the presence of the four different ligands (K_{obs}) was determined according to the method described by Strothkamp and Strothkamp [24]. A starting aliquot of 75 μl of ligand solution (100 mM neomycin and kanamycin in buffer; 1.0 mM berberine and berenil in deionized water) was added to the plasmid solution, and the fluorescence intensity was measured. This solution was titrated with aliquots of solution A. The titration was repeated with 150-, 225-, 300-, and 375-μl aliquots of ligand solution. The method for determination of k_f, k_b, and K_{obs} was repeated for seven different concentrations (0.5–2.0 M) of sodium chloride and ammonium sulfate in buffer. The assays were repeated three times, and the median of each group of constants was determined for each ligand.

Analysis of Scatchard plots and binding modes

The analysis of the Scatchard plots was made according to Strothkamp and Strothkamp [24]. The influence of the four compounds on pDNA–EtBr binding was determined by measuring the reduction of fluorescence intensity. The amount of EtBr bound per unit of DNA was determined using a simple binding model. The data were fitted to the Scatchard equation $r/C_f = nK - rK$, where r is the ratio of bound EtBr to DNA base pair concentration, C_f is the concentration of free EtBr, n is the maximum value of r, and K is the intrinsic binding constant of the EtBr [24]. The change of fluorescence was calculated considering the ligands as competitive inhibitors. Adding a competitive ligand to a Scatchard analysis produces the relationship $1/K_{obs} = (K'/K)C'_f + 1/K$, where K_{obs} is the slope of the straight line r/C_f according to r, for each concentration of ligand, K' is the binding constant of the ligand to DNA, and C'_f is the concentration of free ligand. By plotting the values of $1/K_{obs}$ against concentration of ligand, it is possible to fit a straight line, giving a line with slope K'/K from which one can calculate the value of K' [24].

Preparation of berenil–sepharose support

Sepharose CL-6B (Amersham Biosciences, Uppsala, Sweden) was epoxy-activated according to the method described by Sundberg and Porath [25] and coupled to berenil. Initially, Sepharose CL-6B was washed with large volumes of deionized water. To 5 g of moist gel was added 5 ml of 0.6 M NaOH solution containing 50 mg of $NaBH_4$ and 5 ml of 1,4-butanediol diglycidyl ether. The slurry was swirled at 25 °C for 8 h using a bath with orbital agitation. The epoxy-activated gel was then washed in a sintered glass funnel with large volumes of deionized water. The gel was then suction-filtered to near dryness, and 2 g was added to 4 ml of a 2.0 M sodium carbonate solution containing 500 mg of berenil. After swirling for

16 h at 70 °C, the derivatized Sepharose was washed with large volumes of deionized water and 70% ethanol solution to remove the excess of ligand and sodium carbonate. The derivatized gel was stored at 4 °C in deionized water.

Chromatographic method

Chromatography studies were performed in a fast protein liquid chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden) at room temperature. A 3-ml column was packed with the berenil-derivatized support and initially tested with different mobile phases, namely 10 mM Tris–HCl buffer (pH 8.0) with 0.1 to 3.0 M sodium chloride or 0.5–3.0 M ammonium sulfate concentrations. Prior to the sample application, the column was equilibrated with 1.3 M ammonium sulfate in 10 mM Tris buffer (pH 8.0) at a flow rate of 1 ml/min. Plasmid samples (25 µl) of 900 µg/ml in equilibration buffer were then injected at the same flow rate. The elution of bonded pDNA was carried out by decreasing the ionic strength to 0 M (10 mM Tris buffer, pH 8.0). To promote the selective elution of bonded species, the ionic strength was first decreased to 0.6 M ammonium sulfate in 10 mM Tris buffer (pH 8.0) and afterward to 0 M. The absorbance was monitored at 280 nm. Fractions were pooled according to the chromatograms obtained and were concentrated and desalted using Vivaspin concentrators (Vivascience) and analyzed by gel electrophoresis (100 V for 40 min.) using 1% agarose gel in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0) in the presence of 0.5 µg/ml EtBr. After the chromatographic runs, the column was washed with at least 5 bed volumes of deionized water.

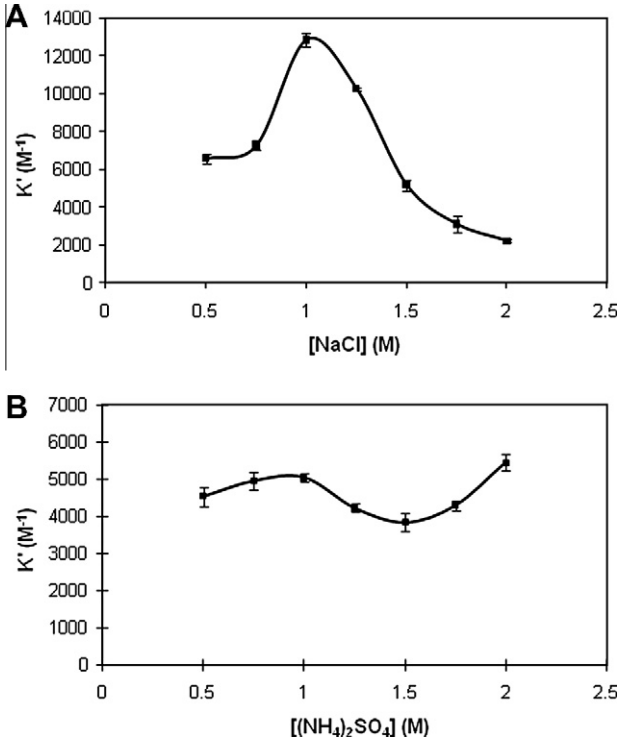

Fig.1. Variation of the binding constant (K') of berenil with sodium chloride (A) and ammonium sulfate (B) concentrations. Results show means ± standard deviations (n = 3).

Results

Analysis of pDNA–ligand binding affinity

The binding of berberine, berenil, kanamycin, and neomycin to DNA plasmid pVAX1–LacZ was studied by a fluorimetric titration technique. The influence of sodium chloride in this interaction was evaluated using seven different concentrations of salt (0.5–2.0 M) in 0.05 M Tris (pH 7.5). The highest affinity was found for 1.0 M sodium chloride, with neomycin being the only exception, for which 0.5 M gave the highest affinity constant (67.36 M^{-1}). Therefore, the best binders at the concentration of 1.0 M sodium chloride were berenil (12849 M^{-1}) and berberine (7110.86 M^{-1}), with affinity constants approximately two orders of magnitude higher than kanamycin (33.25 M^{-1}) and neomycin (55.73 M^{-1}).

Overall, the relative order of affinity was berenil > berberine > neomycin > kanamycin. Berenil presents a wide variation of the affinity constant with salt concentration, with values ranging from 2223 to 12849 M^{-1} (Fig. 1A). For the other compounds tested, the variation observed was much slighter (data not shown).

The affinity of berenil to pDNA was also tested in the presence of ammonium sulfate, which enhances hydrophobic interactions. The variation of K' with this salt (Fig. 1B) showed a different behavior and magnitude, with values ranging from 3833 to 5453 M^{-1}. The maximum value was found with a salt concentration of 2.0 M but was much smaller than the one obtained with sodium chloride (12849 M^{-1}). Moreover, there was a broad amplitude of K' values obtained with sodium chloride compared with ammonium sulfate, where K' showed small variations with salt concentration (Fig. 1).

Chromatographic studies

Chromatographic studies were performed to test berenil as an affinity ligand because this compound showed the highest pDNA binding constant. Moreover, berenil structure makes it a natural candidate to be directly bonded onto an activated support, unlike berberine, which must be submitted to structure modifications in order to introduce suitable functional groups to make the immobilization possible. Thus, the derivatized support for the chromatographic studies was prepared by covalent coupling of berenil on Sepharose CL-6B, previously activated using 1,4-butanediol diglycidyl ether to introduce epoxy groups in the hydroxylic polymer agarose (Fig. 2). Nevertheless, the epoxidation of berenil established through the amine moiety of amidine, the imine linkage, or even both cannot be disregarded.

Initially, the retention profile of pDNA on berenil–Sepharose CL-6B column was studied under the influence of different sodium chloride concentrations (0.1–3.0 M) in eluent buffer, considering the results obtained with the fluorescent measurements. However, the pDNA was not retained on the column in these conditions. On the other hand, the experiments performed with ammonium sulfate showed that the maximum retention of pDNA on the column was obtained with 1.3 M salt and the elution was performed simply by decreasing the salt concentration in eluent buffer to zero. To determine the specific binding conditions of sc and open circular (oc) isoforms, several binding/elution experiments were performed. The data showed that the epoxidated berenil support interacts differently with the plasmid isoforms. In fact, oc plasmid was eluted from the column with the decrease of the ionic strength of the buffer to 0.6 M ammonium sulfate, whereas the sc isoform remained bound, eluting only when the salt concentration in eluent was decreased to zero (Fig. 3).

The analysis of the elution fractions by agarose gel electrophoresis confirms that peak 2 eluted with 0.6 M ammonium sulfate corresponds to the oc isoform (Fig. 3, lane 2), whereas peak 3 was attributed to the sc isoform (Fig. 3, lane 3). Control experiments using a nonderivatized column, submitted to the same coupling preparation process but in the absence of berenil, did not

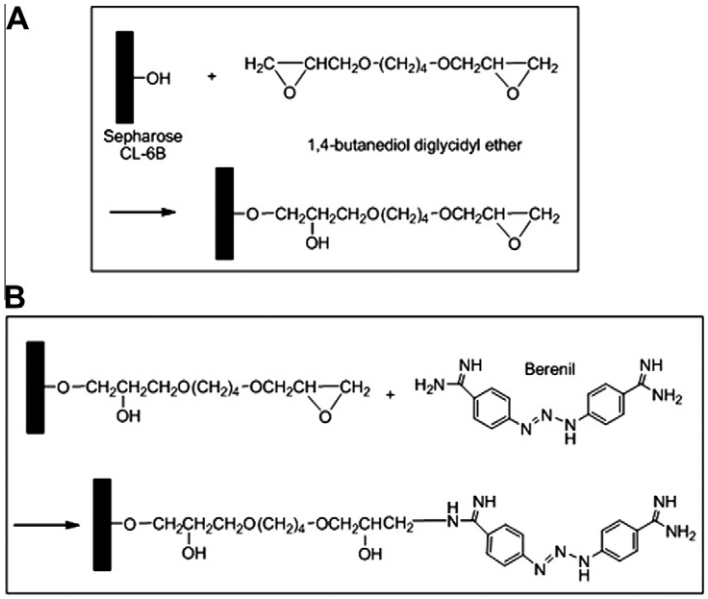

Fig.2. Epoxy activation of sepharose CL-6B (A) and derivatization with berenil (B).

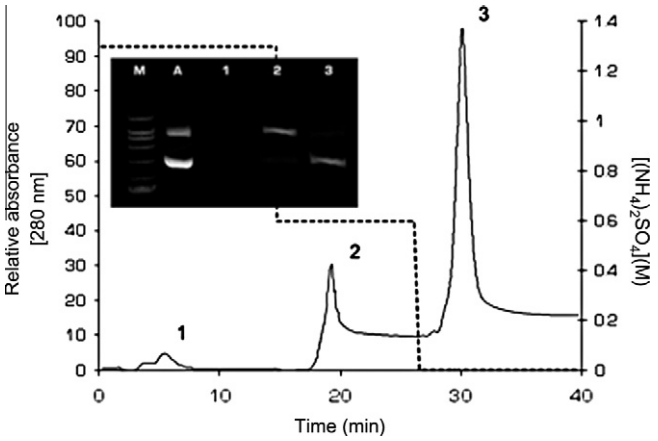

Fig.3. Chromatographic separation of pDNA (25 μl, 900 μg/ml) isoforms on berenil-derivatized column and agarose gel electrophoresis analysis of the eluted fractions. Lane M: molecular weight markers; lane A: pDNA sample injected onto the column (oc + sc); lane 1: fraction collected after injection with 1.3 M $(NH_4)_2SO_4$; lane 2: sample collected from the first peak (oc) eluted with 0.6 M $(NH_4)_2SO_4$; lane 3: sample collected from the second peak (sc) eluted with 0 M $(NH_4)_2SO_4$.

result in any measurable retention of pDNA on the column even using a different salt concentration in the eluent.

Discussion

Differences between interaction types

The magnitude of the binding constants (K') varies widely for the different compounds. All showed a competitive behavior in relation to EtBr, especially berenil and berberine, which have the highest K' for pDNA. Considering the binding mode of each of the four compounds, results showed that whereas for the minor groove binder (MGB) berenil the pDNA association constant was in the range of 10^4 M, for the intercalators neomycin and kanamycin it was in the range of 10^1 M. The partial intercalator berberine showed an association constant value in the range of 10^3 M. The results suggest that the MGB is the most promising compound for use as ligand in sc isoform purification from the other pDNA isoforms, and rationalized thinking could indicate that in sc the access of the ligands to the minor groove should be easier than to the space between the base pairs.

Groove binding differs from intercalation in at least one significant way. Structural evidence suggests that there is little change in the DNA conformation on binding of a groove binding agent. In general, groove binding consists of only two steps; the first would correspond to the transfer of the ligand from solution to the DNA minor groove, and in the second step the ligand can form noncovalent molecular interactions with DNA groups accessible in the groove that influence the excellent structural fit of these ligands [26,27]. In contrast, intercalators bind DNA by unwinding the helix and unstacking the base pairs [15], consuming energy to form the intercalation cavity [28] and thereby justifying the lower binding constants of these compounds.

Nature of pDNA–free ligand interaction

Both partial intercalator and MGB tested in this study feature aromatic ring systems that are hydrophobic in nature. Transfer of these rings from aqueous solution into the interior of the minor groove of DNA should be energetically favorable [26]. So, it is expected that the binding of these ligands with pDNA would have a large hydrophobic contribution, but those might not be the only interactions involved in the binding. The results indicate that the binding of intercalators or MGB to DNA is salt dependent, but only at high salt concentrations (1 M or higher for both salts) is it possible to observe strong binding affinities. It seems that the predominant forces involved in pDNA–ligand binding are hydrophobic interactions [29]; however, the electrostatic contribution due to a salt effect alone cannot be ignored. It is also important to consider the type of salt used to study the interaction because a salt like sodium chloride in low concentrations increases the electrostatic interactions, whereas ammonium sulfate in high concentrations enhances the hydrophobic interactions.

A more comprehensive study was dedicated to the binding of berenil to DNA because this compound showed the highest K'. First, it is important to establish the interactions involved in berenil binding to DNA. The DNA binding affinity of this ligand has been attributed to several factors such as electrostatic interactions with the negative electrostatic potential specifically associated with AT

sequences [30], hydrophobic contacts between the phenyl rings and the hydrophobic regions of the backbone, and the triazene group near the polar phosphodiester groups [31]. The amidines form hydrogen bonds with thymine and/or adenine acceptor groups of the bases at the floor of the groove [32].

At working pH values (7.5 and 8.0), both amidine groups of berenil are protonated (pK_a of 11.0), providing electrostatic contributions to the complex energetics through phosphate interactions [33]. The relative importance of these interactions in determining the binding of berenil cations to DNA is reflected in the salt dependence of the constant [34], but only with a salt that enhances the electrostatic interactions. Our results showed that berenil K' changed considerably with the variation of sodium chloride concentration, whereas this constant is not greatly affected by ammonium sulfate concentration (Fig. 1). So, at least one of the putative binding modes of berenil to pDNA depends more on the charged ends, which can be explained by the presence of strong electrostatic interactions [35] and high constant values.

The nonpolyelectrolyte contribution to berenil–pDNA binding should not change appreciably with the ionic strength. This means that hydrogen bonding and van der Waals interactions will not vary significantly [35]. On the other hand, with ammonium sulfate in the eluent, the binding of berenil to pDNA depends more on the hydrophobic interactions than on the electrostatic ones.

Interaction between pDNA and berenil bonded to the support

After the fluorimetric titration measurements, a higher retention of pDNA using sodium chloride over ammonium sulfate could be expected. In addition, the variation of binding constant values, with different concentrations of sodium chloride, suggested a simple method for binding and elution of the plasmid from derivatized support in pseudo-affinity chromatography by a simple decrease of buffer salt concentration. Instead, the experimental studies showed that retention of the pDNA was achieved only using a relatively high ammonium sulfate concentration in the buffer solution. The high salt concentration increases the binding of biopolymers to the stationary phase by hydrophobic interactions, which are attenuated on reducing the ionic strength of the eluent, in the order of decreasing the hydrophobic character [36].

Many causes may be behind this peculiar behavior of berenil after immobilization onto the matrix. It is expected that berenil binds to epoxy groups through mainly one of the amidine groups, the same one that is responsible for hydrogen bonds and electrostatic interactions with DNA bases [32]. With sodium chloride, the strongest forces involved in the binding are the electrostatic interactions dependent on both charged ends of berenil [34,35]. So, the fact that one of those ends is involved in the binding to epoxy groups of the support is clearly going to negatively affect the binding of berenil to pDNA. Reducing or removing the charge of the molecule results in a systematic reduction of the overall binding because the electrostatic contribution to the total interaction energy is expected to be lowered [30]. Besides this fact, the contribution of the hydrogen bonds to the binding [32] is different for both ends of the molecule. At one end of the ligand, the amidine group establishes direct hydrogen-bonded contact with the DNA base. However, the other amidine group does not make direct interactions with DNA; instead, a water molecule mediates between them [31]. This water-mediated contact is likely to be weaker than the direct one [37], so it is possible that berenil cannot establish the binding with only one end performing the hydrogen-bonded contact, in part because of the stereochemical hindrance but mainly because that end might not establish direct and strong contacts with the groove. Therefore, the reasons for the lack of retention of pDNA on the derivatized column using sodium chloride may include (i) the decrease of the electrostatic energy contribution to the binding by decreasing the charge of the molecule and (ii) the impediment of establishing enough direct, and therefore more efficient, hydrogen bonds.

With ammonium sulfate, the electrostatic interactions and hydrogen bonds are not as crucial to the binding as the hydrophobic interactions [31]. So, apparently the fact that one amidine group was bonded to the epoxy groups of the support was not an impediment to the binding of berenil to pDNA. It appears that the region of berenil molecule that establishes hydrophobic contacts with pDNA remained able to interact with the nucleic acid molecule. These studies allow us to conclude that the apparently weaker hydrophobic interactions are enough to promote the binding of pDNA to immobilized berenil.

In addition, a difference in retention behavior of the two pDNA isoforms was observed. Because phosphate and sugar groups are equally exposed in both isoforms, the observed selectivity must be due to stronger interactions of berenil with the bases of the groove of sc pDNA but not with the bases of oc isoform. In fact, as a consequence of deformations induced by torsional strain, the bases of the sc isoform are more exposed than the ones of the oc isoform [38]. Moreover, the DNA structure is sensitive to the composition and concentration of the ion atmosphere. In solution, salt cations surrounding the double helix can neutralize the anionic charges of the sc DNA molecule so that the repulsions of the chains will decrease. When the salt concentration is higher, the repulsion is smaller [39]. Thus, at high salt concentrations, the sc DNA molecule adopts a highly compact and bent interwound state [40], promoting an overexposure of the bases. At low salt concentrations, the sc DNA molecule is much more open and loosely interwound [40], weakening the binding between the ligand and the less exposed bases and promoting the elution of the sc isoform.

The control experiments unequivocally identified berenil bonded onto the activated Sepharose as the ligand responsible for the retention and separation of the two pDNA isoforms. Moreover, the retention and separation of pDNA isoforms was achieved using a lower salt concentration compared with other pseudo-affinity ligands [38], thereby avoiding the drawbacks of the use of high salt concentrations in chromatographic processes [13].

Conclusions

In the current study, four small DNA ligands (berenil, berberine, neomycin, and kanamycin) were analyzed for their binding affinity to pDNA using a fluorimetric titration technique. Among the ligands evaluated in a free form, berenil was found to have the highest affinity constant for pDNA. A different binding mode may be the reason for the stronger binding affinity of berenil compared with the other ligands because it is an MGB in opposition to the other three intercalators. The essential mechanism seems to involve not only hydrophobic interactions between the ligand and the pDNA molecules but also other nonpolyelectrolyte and polyelectrolyte contributions important to the binding.

The influence of the type and salt concentration on the ligand interaction with pDNA demonstrated that the binding affinity is influenced by both parameters. The results demonstrated that the interactions between berenil and pDNA in the presence of ammonium sulfate are enough to promote a total retention of this biopolymer and a selective separation of the oc and sc isoforms. In fact, one of the most remarkable results of the current work is related to the specific recognition of the sc isoform by this ligand. So far as we know, this is the first study showing the use of berenil as an efficient alternative to existing ligands for sc plasmid isoform purification. In addition, the retention and separation of pDNA isoforms was achieved using a lower salt concentration compared with other pseudo-affinity ligands, thereby favoring the environmental sustainability.

The promising results obtained using berenil as ligand indicate that this could be an excellent choice for the future purification of sc pDNA from *E. coli* host impurities present in the clarified lysates for further application in gene therapy and DNA vaccines.

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Paper III

Purification of plasmid DNA from clarified and non-clarified *Escherichia coli* lysates by berenil pseudo-affinity chromatography

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ABSTRACT

In this study, berenil was tested as a ligand, specifically to purify plasmids of different sizes pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp) from clarified *Escherichia coli* alkaline lysates. For this purpose, chromatographic experiments were performed using Sepharose derivatized with berenil. The results showed that both pDNA molecules are completely purified using lower amounts of salt in the eluent than those previously reported for other pseudo-affinity and hydrophobic interaction chromatography based processes. Total retention of all lysate components was achieved with 1.3 M ammonium sulphate in the eluent buffer and pDNA elution was obtained by decreasing the salt concentration to 0.55 M. All impurities were eluted after decreasing the concentration to 0 M. The recovery yield for pCAMBIA-1303 (45%) was lower than that obtained for pVAX1-LacZ (85%), however the larger pDNA showed a higher purity level. Purification of pVAX1-LacZ was also performed using non-clarified *E. coli* process streams, replacing the clarification step with a second chromatographic run on the berenil-Sepharose. Using the same binding and elution conditions as before, a pure plasmid sample was obtained with a 33% yield and with all host impurity levels in accordance with the requirements established by the regulatory agencies. These results suggest that this chromatographic method is a promising alternative to purify pDNA for therapeutic use.

1. Introduction

Molecular therapy approaches using non-viral vectors, such as plasmid DNA (pDNA) for the introduction of therapeutic genes, are fast becoming the preferred method to treat various types of diseases [1–3]. Thus, therapeutic pDNA is an emerging biotechnology product with great potential for use in human and animal healthcare. More than 400 clinical trials relating to gene therapy or DNA vaccines are currently being conducted worldwide using these vectors (http://www.wiley.com/legacy/wileychi/genmed/clinical/). In addition four DNA vaccine products have already been approved for veterinary application [4–6]. Accordingly, the expected far reaching application of these vectors in the future requires the large-scale production and purification of pDNA. In recent years, there has been an increased effort in the research and development of new methods for plasmid purification that meet strict quality criteria in terms of purity, efficacy and safety as required by the regulatory agencies. The critical contaminants of pDNA preparations are similar in size (genomic DNA (gDNA)), negatively charged (RNA, gDNA and endotoxins) and have similar hydrophobicity (endotoxins), which can complicate their separation [7]. The maximum levels of gDNA, host proteins and RNA in the final product should preferably be under 1% (w/w). The levels of endotoxins found on lysate solutions after disruption of the outer membrane of *Escherichia coli* by alkaline lysis should not exceed 40 EU/mg plasmid [8]. Moreover, the purification method should not comprise the use of organic reagents, mutagenic and toxic compounds and animal derived enzymes [9].

The process for pDNA preparation involves the production in *E. coli* cells by fermentation followed by an alkaline lysis step [10,11], a concentration with isopropanol and a pre-purification/clarification with ammonium sulphate [10]. The further downstream processing aims to eliminate impurities such as gDNA, low molecular weight RNA, residual proteins and endotoxins. Chromatography is the most suitable method for this purpose [10,12], however there are still several drawbacks that remain to be solved, like the poor selectivity of anion-exchange supports [13]. The use of affinity ligands can be a simple and efficient approach to overcome this problem [14,15]. Affinity and

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pseudo-affinity chromatography use the specificity and bio-recognition properties of the ligands to separate pDNA molecules from the impurities found in cell lysates [16].

Several molecules (certain antibiotics and anticancer agents, for example), bind DNA with high specificity [17] by different binding modes. Besides covalent binding there are several classes of specific and unspecific noncovalent binding modes, such as minor groove binding, intercalation between base pairs, bisintercalation, major groove binding and a combination of the above [18]. Berenil (1,3-bis(4-phenylamidinium) triazene) is used as an anti-trypanosomal agent in veterinary applications [19] and is a member of the aromatic diamidine class of DNA binding agents, which reversibly and preferentially binds to the DNA minor groove in the central AATT sequence [20,21]. Berenil was recently applied as ligand for the specific separation of the supercoiled (sc) plasmid isoform from the less active open circular (oc) isoform [15]. The DNA binding affinity of this ligand has been attributed to several factors: electrostatic interactions with the AT sequences, hydrophobic contacts between the phenyl rings and the hydrophobic regions of the DNA backbone and hydrogen bonds between the amidines and thymine and/or adenine acceptor groups of the bases at the floor of the groove [15].

This study reports a pseudo-affinity chromatographic technique to purify pDNA directly from clarified and non-clarified *E. coli* lysate solutions. This approach was tested for the purification of pDNA molecules with different sizes (pVAX1-LacZ and pCAMBIA-1303, with 6.05 Kbp and 12.361 Kbp, respectively).

2. Materials and methods

2.1. Materials

Sepharose CL-6B was obtained from Amersham Biosciences (Uppsala, Sweden). Berenil and 1,4-butanediol diglycidyl ether were purchased from Sigma–Aldrich (St. Louis, MO, USA). All salts were of analytical grade.

2.2. Bacterial culture

E. coli DH5α strain harbouring 6.05 Kbp plasmid pVAX1-LacZ (Invitrogen, Carlsband, CA, USA) and *E. coli* XL1 blue strain harbouring 12.361 Kbp plasmid pCAMBIA-1303 (Cambia, Brisbane, Australia) were cultured overnight in Luria Bertani agar (Lennox) medium (Laboratorios Conda, Madrid, Spain) supplemented with 30 μg/mL of kanamycin at 37 °C. *E. coli* DH5α strain was grown at the same temperature in an orbital shaker with Terrific Broth medium (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 30 μg/mL kanamycin. The XL1blue strain cells were grown in similar conditions using Luria Bertani medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0). Both cell strains were harvested by centrifugation at the end of the exponential growth phase and stored at −20 °C until use. Plasmid-free *E. coli* cells were also grown in the absence of antibiotic, under the same conditions as previously described.

2.3. Lysis and primary isolation

The plasmid harbouring cells were lysed using a modification of the alkaline method proposed by Sambrook et al. [22]. Centrifugation of a 250 mL sample of the cell broth was performed at 5445 × *g* for 30 min at 4 °C with a Sigma 3–18 K centrifuge. The supernatants were discarded and the bacterial pellets were resuspended in 20 mL of 50 mM glucose, 25 mM Tris–HCl and 10 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0). The cells were lysed by adding 20 mL of a 200 mM NaOH, 1% (w/v) sodium dodecyl sulphate solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated by gently adding and mixing 16 mL of prechilled 3 M potassium acetate (pH 5.0). The precipitate was removed by a double centrifugation at 20,000 × *g* for 30 min at 4 °C with a Beckman Allegra 25 R centrifuge. The plasmid in the supernatant was precipitated after the addition of 0.7 volumes of isopropanol and a 30 min incubation period on ice. The pDNA was recovered by centrifugation at 16,000 × g for 30 min at 4 °C. The pellets were then redissolved in 1 mL of 10 mM Tris–HCl buffer (pH 8.0). A fraction of this solution was subjected to a clarification step. For this purpose, and after optimization studies, solid ammonium sulphate was dissolved in the pDNA solutions up to a final concentration of 2.0 M, for the pVAX1-LacZ solution, and 2.5 M for the pCAMBIA-1303 solution, followed by a 15 min incubation period on ice. Precipitated proteins and RNA were then removed by centrifugation at 10,000 × *g* for 20 min at 4 °C. The supernatant was recovered and its nucleic acid concentration quantified by measuring the absorbance at 260 nm.

2.4. Preparation of berenil-Sepharose support

Sepharose CL-6B was epoxi-activated according to the method described by Sundberg and Porath [23] and coupled to berenil as previously described [15]. The orange derivatized gel thus obtained was stored at 4 °C in deionized water.

2.5. Preparative chromatography

Chromatographic studies were performed in a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden) at room temperature. A 10 cm × 10 mm column (Amersham Biosciences, Uppsala, Sweden) was packed with 2 mL berenil-derivatized (Fig. 1) gel and initially tested with different ammonium sulphate concentrations (0.2–1.5 M) in the mobile phase. Prior to sample application, and after achieving the optimal conditions of binding and elution, the column was equilibrated with 1.3 M ammonium sulphate in 10 mM Tris–HCl buffer (pH 8.0) at a flow rate of 1 mL/min.

2.5.1. Injection of clarified samples

Clarified samples (25 μL) were loaded onto the column in equilibration buffer at a flow rate of 1 mL/min. To promote the selective elution of bonded species, the salt concentration was first decreased to 0.55 M ammonium sulphate in 10 mM Tris–HCl buffer (pH 8.0) and then to 0 M. The absorbance was continuously monitored at 280 nm. Fractions were pooled according to the chromatograms obtained, concentrated and desalted using Vivaspin concentrators (Vivaproducts, Littleton, MA, USA) and kept for further analysis as described below.

2.5.2. Injection of non-clarified samples

Non-clarified samples (25 μL) with a nucleic acid concentration of approximately 600 μg/mL in equilibration buffer were loaded onto the column at 1 mL/min flow rate. The elution was performed as described above for clarified samples. A second chromatographic step was then performed in the same binding and elution conditions, injecting the pDNA fraction, pooled after the first run and concentrated to an approximate nucleic acid concentration of 600 μg/mL. Fractions were pooled according to the obtained chromatograms, then concentrated and desalted using Vivaspin concentrators (Vivaproducts, Littleton, MA, USA) and kept for further analysis as described below. After the chromatographic runs, the column was washed with at least 5 bed volumes of deionized water.

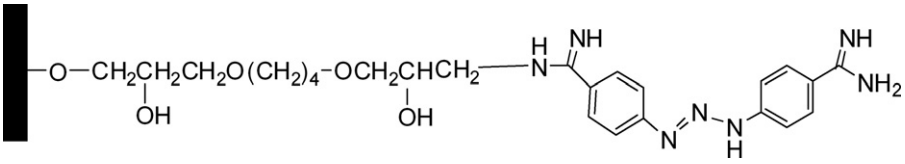

Fig. 1. Schematic representation of the berenil-Sepharose support. Berenil is covalently bonded to the epoxy arm.

2.6. Agarose gel electrophoresis

Pooled fractions were analyzed by horizontal electrophoresis (100 V for 40 min) using 1% and 0.8% agarose gel in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) in the presence of 0.5 μg/mL ethidium bromide. The gels were visualized in a UVITEC Cambridge system (UVITEC Limited, Cambridge, UK).

2.7. Analytical chromatography

The pDNA of feed samples were injected onto the berenil column and the fractions pooled after the chromatographic runs. The concentration and purity of the extracts was assessed by high-performance liquid chromatography (HPLC), according to the method described by Diogo et al. [24]. A 4.6/100 mm HIC (hydrophobic interaction chromatography) Source 15 PHE PE column (Amersham Biosciences, Uppsala, Sweden) was connected to a Waters HPLC system (Waters Corporation, Milford, MA, USA) and equilibrated with 1.5 M ammonium sulphate in 10 mM Tris–HCl buffer (pH 8.0). Samples (20 μL) were injected and eluted at a flow rate of 1 mL/min. After injection, the elution occurred with the equilibration buffer for 3 min, at which point the elution buffer was immediately changed to Tris–HCl 10 mM (pH 8.0) without ammonium sulphate. This condition was maintained for 6 min in order to elute bound species. The column was then re-equilibrated for 7 min with the equilibration buffer to prepare the column for the next run. The absorbance of the eluate was continuously recorded at 254 nm. The concentration of pDNA in each sample was calculated using a calibration curve constructed with pDNA standards (2.5–400 μg/mL), purified using the Qiagen plasmid midi kit (Hilden, Germany), according to the manufacturer's instructions. Plasmid quantification was achieved by measuring the absorbance at 260 nm, assuming an absorbance of 1.0 for a solution of 50 μg/mL. The degree of purity was defined as the percentage of the pDNA peak area in relation to the total area of all chromatographic peaks.

2.8. Protein analysis

Protein concentration of the samples was measured using the micro-bicinchoninic acid (BCA) assay from Pierce (Rockford, IL, USA), according to the manufacturer's instructions and 50 μL of each sample was added to 200 μL of BCA reagent in a microplate and incubated for 30 min at 60 °C. Absorbance was measured at 595 nm in a microplate reader. The calibration curve was prepared using bovine serum albumin standards (0.025–1 mg/mL).

2.9. Endotoxin analysis

Analysis of endotoxin contamination on both feed samples and pDNA fractions pooled after chromatography was performed using the ToxinSensor™ Chromogenic LAL Endotoxin Assay kit from GenScript (GenScript USA Inc., Piscataway, NJ, USA) which had a detection level of 0.005 EU/mL.

2.10. Genomic DNA analysis

Genomic DNA contamination in purified plasmid solutions and in feed samples was assessed using real-time polymerase chain reaction (PCR) in a iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), according to the method described by Martins et al. [25]. Sense (5′-ACACGGTCCAGAACTCCTACG-3′) and antisense (5′-GCCGGTGCTTCTTCTGCGGGTAACGTCA-3′) primers were used to amplify a 181-bp fragment of the 16S rRNA gene. PCR amplicons were quantified by following the change in fluorescence of the DNA binding dye Syber Green (Bio-Rad, Hercules, CA, USA). *E. coli* genomic DNA was purified with the Wizard gDNA purification kit (Promega, Madison, WI, USA) and used to generate a standard curve ranging from 0.005 to 50 μg/mL. Negative controls, with no template, were run at the same time as the standards.

3. Results and discussion

3.1. Clarification of pDNA lysates by salt precipitation

RNA is the main contaminant of non-clarified cell lysates, and since it is structurally similar to pDNA [7], its separation can represent a true challenge. One common procedure for RNA removal is the precipitation with high salt concentrations, using ammonium sulphate as the clarifying agent. This precipitation step is very effective in reducing the amounts of high molecular weight RNA and proteins, and equally effective in improving intermediate pDNA recovery, thus representing an excellent complement to any further purification step [26].

Standard ammonium sulphate precipitation uses 2.5 M of salt concentration to clarify crude cell lysates, which has a great environmental impact due to its high eutrophication potential [26]. However, Freitas et al. [27] concluded that precipitation with high salt concentration is not always needed. Based on these conclusions, optimization studies were performed to select the best conditions for high pDNA recovery and purity by analyzing the influence of salt concentration on RNA removal. Ammonium sulphate, in a concentration range from 1.3 M to 2.5 M, was added to the non-clarified pDNA suspensions and the precipitated proteins and RNA were then removed by centrifugation. The obtained samples were injected onto the berenil-Sepharose column (with the optimized conditions of binding and elution) and the purity of eluted pDNA fractions in terms of RNA contamination was evaluated by HPLC analysis.

The data obtained showed that for the 6.05 Kbp pDNA (pVAX1-LacZ), a concentration of 2.0 M ammonium sulphate in the precipitation step was enough to achieve a pure sample after the chromatographic step using berenil as ligand (Table 1). Precipitation with a salt concentration of 1.9 M and lower, led to a similar recovery yield, but resulted in impure pDNA samples. On the other hand, when the clarifying step was performed with ammonium sulphate concentrations above 2.0 M, all the pDNA samples were 100% pure, with similar recovery yields. In contrast, for pCAMBIA-1303 (12.361 Kbp) a higher ammonium sulphate concentration (2.5 M) was needed to obtain a 100% HPLC pure sample after the same chromatographic process (Table 1). Using a salt concentration of 2.0 M in the clarification step, the purity of pDNA samples was set

Table 1
Dependence of sample purity (HPLC) and recovery yield with ammonium sulphate concentration used in the clarification step.

Plasmid	$(NH_4)_2SO_4$ (M)	Sample purity (%)	Approximated yield (%)
pVAX1-LacZ	1.3	66	85
	1.9	80	
	2.0	100	
	2.5	100	
pCAMBIA-1303	2.0	20	45
	2.25	40	
	2.5	100	

around 20% increasing to more than 40% with 2.25 M of ammonium sulphate. It is known that large size plasmids are more susceptible to shear forces and, in general, smaller pDNA concentrations were obtained after alkaline lysis [28]. Thus, for pCAMBIA-1303, the pDNA/RNA ratio in non-clarified solutions is smaller and possibly because of that, higher ammonium sulphate concentrations are required to maximize plasmid purification and recovery from the highly RNA contaminated solutions.

3.2. Berenil-Sepharose pseudo-affinity chromatography

3.2.1. Injection of clarified pDNA solutions

Recently, berenil-Sepharose pseudo-affinity support (Fig. 1) was successfully applied for the separation of sc plasmid isoform from the less active oc isoform, showing a great affinity for pDNA [15]. Therefore, the ability of this support to purify pDNA directly from clarified *E. coli* cell lysates was exploited in the present study. Moreover, the applicability of this process to purify pDNA molecules with different sizes was also tested. For this purpose, solutions of pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp), obtained separately after clarification with ammonium sulphate, were used as feedstock for the pseudo-affinity chromatography studies. Several binding-elution experiments were performed to achieve optimal buffer conditions to separate pDNA from the lysate impurities, namely RNA. These experiments showed that the chromatographic conditions are the same for both plasmids: total retention of all lysate components was achieved using 1.3 M of ammonium sulphate in the binding buffer and elution of pDNA was obtained by a simple decrease of salt concentration to 0.55 M. The more hydrophobic impurities, such RNA, were eluted only when the concentration was decreased to 0 M. Fig. 2 shows the chromatograms after injection of pVAX1-LacZ and pCAMBIA-1303 samples onto the berenil-Sepharose column. Both plasmids showed a comparable separation performance represented by similar chromatograms, which demonstrates the reproducibility of the chromatographic process for plasmid molecules of different sizes. The chromatograms are characterized by a first small system peak, followed by a sharp higher peak of pDNA and a smaller one of strongly retained species such as RNA. As shown by agarose gel electrophoresis (Fig. 2) and confirmed by HPLC, RNA was completely separated from pDNA molecules (electrophoresis lane 1 in Fig. 2 for both plasmids) and was eluted in the peak 2. All cell impurities are retained in the column for longer when compared with pDNA (Fig. 2). In these double-stranded molecules, the hydrophobic bases are packed and shielded inside the helix and thus interaction with the support ligands is smaller, eluting first. On the other hand, the single-stranded RNA molecules are retained more strongly in the support because the hydrophobic bases are largely exposed [29].

The binding between the berenil ligand and the lysate components results not only from hydrophobic interactions between the phenyl rings of berenil and the hydrophobic regions of the backbone of the molecules, but also from other more specific interactions that are responsible for the great affinity that the support shows for those molecules [15].

An epoxy activated Sepharose gel obtained using the same experimental conditions minus the berenil ligand was used for the control experiments (results not shown). A distinct pattern from that which was originally observed with the berenil-Sepharose support was obtained: total retention was not observed at 1.3 M of ammonium sulphate and the lysate components were not separated by decreasing the salt concentration. Accordingly, these experiments unequivocally identified berenil bonded onto the activated Sepharose as the ligand responsible for the retention and separation of pDNA molecules. Moreover, the column cleaning and the high number of chromatographic runs did not cause any change in the chromatographic performance of the derivatized gel.

3.2.2. Injection of non-clarified pVAX1-LacZ solution

The ability of the berenil-Sepharose to separate and purify pDNA directly from non-clarified *E. coli* process streams was also tested. This was accomplished without the ammonium sulphate clarification step. In a first run, the feedstock sample was injected onto the berenil-Sepharose column with 1.3 M of ammonium sulphate in the eluent. The obtained chromatographic profile is shown in Fig. 3A, as is the agarose gel electrophoresis analysis of the pDNA eluted fractions. The electrophoresis shows that the pDNA fraction still contains a slight RNA contamination, even though the great majority of this contaminant clearly elutes in the second

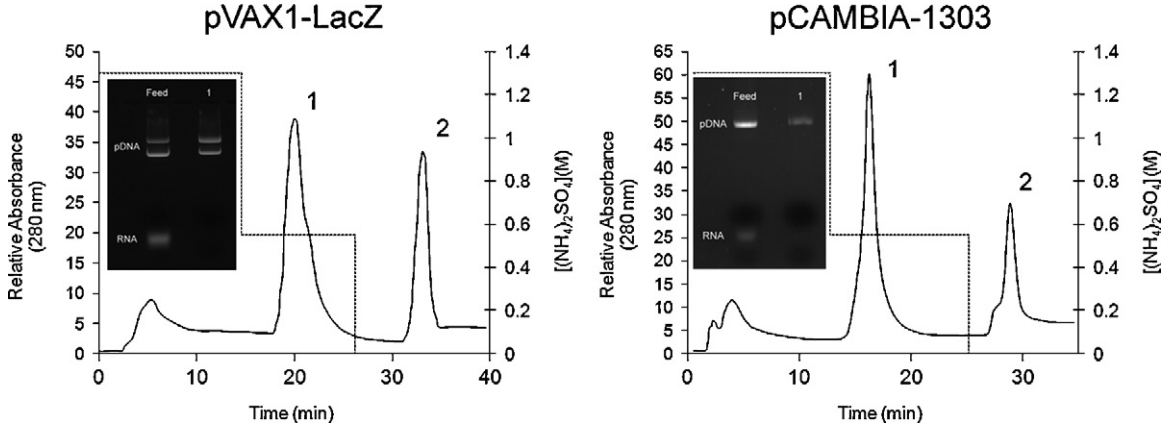

Fig. 2. Chromatographic separation of clarified feed solution (25 μL) of pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp) from the host cell impurities on berenil-Sepharose support. Agarose gel electrophoresis analysis of the pDNA fractions. Peak 1 and electrophoresis lane 1: pDNA fractions collected after elution with 0.55 M $(NH_4)_2SO_4$; Peak 2: impurities eluted with 0 M $(NH_4)_2SO_4$. The clarified lysate was also run in the agarose gel for comparative purposes (lane feed).

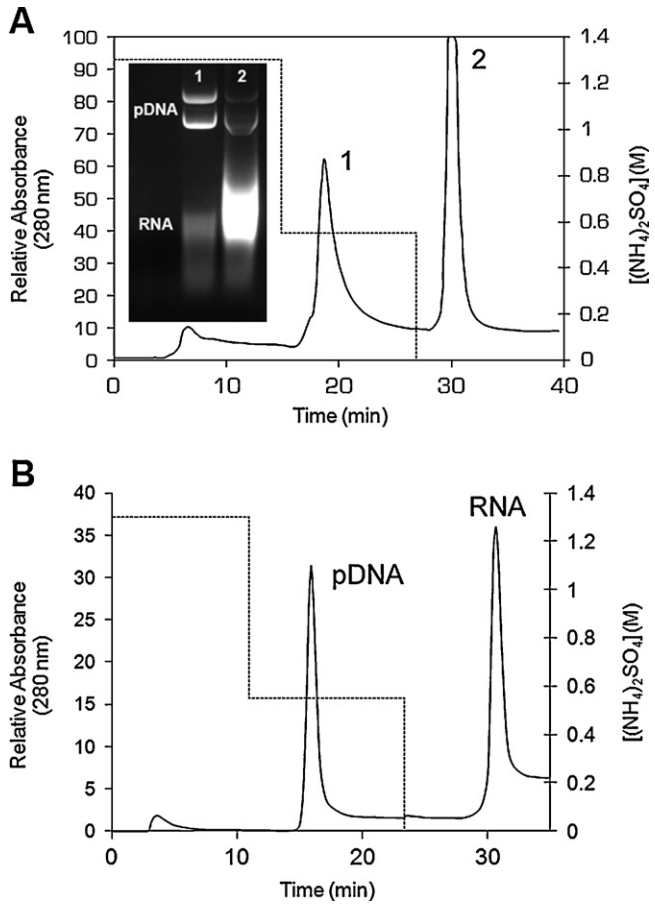

Fig. 3. Chromatographic separation of non-clarified pVAX1-LacZ samples on berenil-Sepharose column: (A) After one run through the support and agarose gel electrophoresis analysis of the eluted fractions: Peak 1 and electrophoresis lane 1: pDNA fraction eluted with 0.55 M $(NH_4)_2SO_4$; Peak 2 and electrophoresis lane 2: RNA eluted with 0 M $(NH_4)_2SO_4$. (B) After the second run through the berenil support.

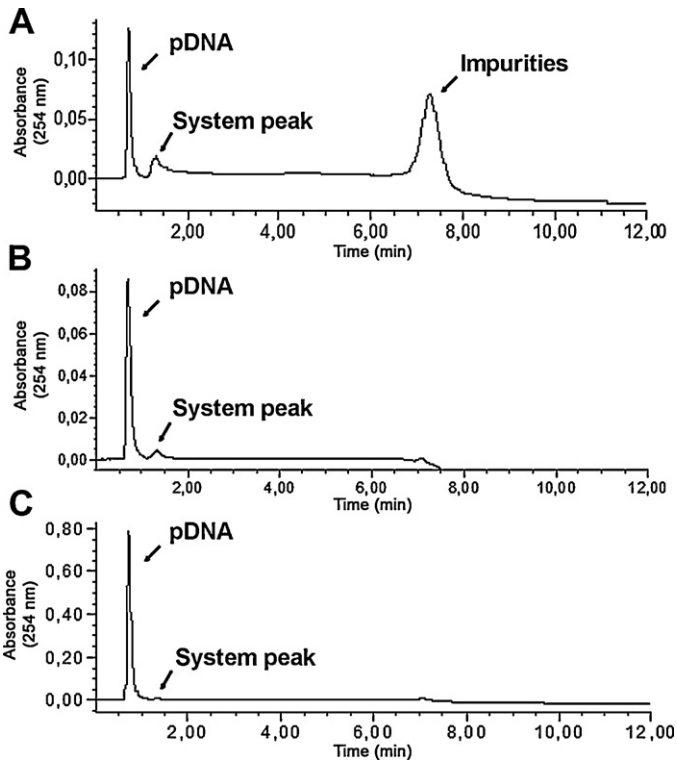

Fig. 4. HPLC analysis of different pVAX1-LacZ samples: (A) *E. coli* clarified lysate diluted 1:20. (B) pDNA fraction collected after the chromatographic run. (C) 400 μg/mL pVAX1-LacZ standard for comparative purposes. The chromatograms from HPLC analysis of pCAMBIA-1303 samples were similar to those here represented.

peak. In the second run, the pDNA fraction obtained after the first chromatographic step was concentrated and injected onto the berenil-Sepharose column using the same buffer conditions. The obtained chromatogram (Fig. 3B) showed two well defined peaks, the first one corresponding to the elution of pDNA after decreasing the salt concentration to 0.55 M and a second peak corresponding to the all contaminants, eluted after decreasing the salt concentration to 0 M.

3.3. Plasmid quality and purity assessment

The performance of pDNA purification processes using the berenil-Sepharose was examined by the determination of yield and purity (Table 2). Besides HPLC analysis of RNA contamination, the purity of the recovered plasmid fractions was also determined by quantification of proteins (BCA assay), endotoxins (Chromogenic Limulus amoebocyte lysate Endotoxin assay) and gDNA (real-time PCR) (Table 3).

3.3.1. Clarified pDNA solutions

For the clarified samples, agarose gel electrophoresis (Fig. 2) and HPLC analysis (Fig. 4) revealed that the plasmid pools were RNA free. The analytical chromatogram shown in Fig. 4A represents the clarified lysate injected onto the berenil-Sepharose column. Clearly a high percentage of the sample was constituted by impurities, namely RNA. In this feed sample, the HPLC pDNA purity was 23% (Table 2) for pVAX1-LacZ and 7% for pCAMBIA-1303, however after the chromatographic step, both plasmid samples were 100% pure (Table 2) and free of RNA. The analytical chromatogram for the obtained pDNA (Fig. 4B) shows a single plasmid peak, confirming the agarose gel electrophoresis results. The HPLC purification factors (Table 2) are higher than those described for similar chromatographic procedures [30,31], particularly for pCAMBIA-1303 which has an impressive value of 14.3. HPLC analysis also showed a 85% recovery of pVAX1-LacZ (Table 2). This value is similar to the 84% obtained with an affinity support by Sousa et al. [31], but slightly higher than the 70% yield obtained with a pure hydrophobic chromatographic matrix [29]. Nevertheless, the recovery yield of pCAMBIA-1303 was much lower than for the smaller pDNA molecule (Table 2). This may be due to the fact that larger plasmids are more unstable and degrade more easily during the extraction and purification procedures, since they are more sensible to shear forces [28] and more susceptible to losses during the chromatographic step.

The quantitative analysis of impurities in injected feeds and pDNA fractions is shown in Table 3. The BCA protein assay indicated that both plasmid pool solutions have undetectable levels of proteins. Real-time PCR analysis of pooled plasmid fractions showed a great reduction of gDNA content after the berenil-Sepharose chromatographic step. A gDNA decrease of 3653-fold was achieved for pVAX1-LacZ and an impressive 554,472-fold reduction was achieved for pCAMBIA-1303 (Table 3). Berenil-Sepharose support is thus extremely efficient to separate gDNA from heavily contaminated pDNA solutions. Genomic DNA from *E. coli* is double-stranded, but becomes mostly single-stranded during alkaline lysis. During this process, the complementary strands of gDNA are completely separated and partially cleaved. The resulting gDNA molecules show a high exposure of their hydrophobic bases

Table 2
HPLC analysis of purity and recovery yield of two plasmids with different sizes: pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp).

Plasmid	Process step	Purity (%)	Purification factor	Plasmid mass (μg)	Yield (%)
Clarified pVAX1-LacZ	Injected feed	23	–	11.1	–
	pDNA fraction	100	4.3	9.4	85
Clarified pCAMBIA-1303	Injected feed	7	–	11	–
	pDNA fraction	100	14.3	5	45
Non-clarified pVAX1-LacZ	Injected feed[a]	4.4	–	7.5	–
	pDNA fraction[b]	100	22.7	2.5	33

[a] Corresponding to the first run feed.
[b] pDNA fraction collected after two runs through the berenil-Sepharose column.

Table 3
Quantitative analysis of the pDNA, proteins, RNA, endotoxins and gDNA in injected feed and plasmid samples after berenil-Sepharose chromatography. For the non-clarified pVAX1-LacZ the feed corresponds to the initial non-clarified lysate solution and the pDNA fraction to the sample collected after the second chromatographic run.

Plasmid	Impurity	Feed	pDNA fraction[a]	Reduction factor (fold)
Clarified pVAX1-LacZ	pDNA (μg/mL)	1223.8	37.4	–
	Protein (μg/mL)	66	Undetectable	–
	RNA (mass%)	77	Undetectable	–
	Endotoxins (EU/mL)	2.71	0.136	20
	gDNA (μg/mL)	364.9	9.99×10^{-2}	3653
Clarified pCAMBIA-1303	pDNA (μg/mL)	278.9	25	–
	Protein (μg/mL)	Undetectable	Undetectable	–
	RNA (mass%)	93	Undetectable	–
	Endotoxins (EU/mL)	1.967	0.099	20
	gDNA (μg/mL)	6.82	1.23×10^{-5}	554,472
Non-Clarified pVAX1-LacZ	pDNA (μg/mL)	680	8.22	–
	Protein (μg/mL)	1170	Undetectable	–
	RNA (mass%)	95.6	Undetectable	–
	Endotoxins (EU/mL)	2.03	0.099	21
	gDNA (μg/mL)	726.7	2.4×10^{-3}	302,792

[a] Values extrapolated from results obtained with several injections.

[28] and can thus interact more strongly with berenil, eluting in the second peak (Fig. 2).

The chromatographic process described here was also very effective in reducing the endotoxin levels from plasmid solutions. A decrease of 20-fold was obtained for both types of plasmid molecules (Table 3) demonstrating that endotoxins bind to the berenil support more strongly than pDNA.

The quality analysis of pDNA showed that pVAX1-LacZ and pCAMBIA-1303, purified with the berenil-Sepharose support, meet the specifications of the regulatory agencies, namely FDA (Table 4) [8]. Both RNA and proteins (preferably <1%) are undetectable in the final plasmid solutions and the endotoxin levels are ten times lower than the maximum required by FDA (40 EU/mg pDNA). Genomic DNA content was the only parameter that greatly varies among pDNA feed solutions and plasmid pools, being much higher for pVAX1-LacZ. Nevertheless, both small and higher size plasmids are under FDA specifications (preferably <1%) (Table 4).

3.3.2. *Non-clarified pVAX1-LacZ solution*

Starting from a highly contaminated non-clarified lysate (Table 2) and after two consecutive chromatographic runs on the berenil-Sepharose column, the pVAX1-LacZ fraction became 100% free from RNA, with a purification factor of almost 23. Nevertheless, the yield (33%) is lower than expected. As before, the BCA protein assay showed that the pDNA pool had undetectable levels of proteins (Table 3), despite the feed solution being highly contaminated (1170 μg/mL).

Real-time PCR analysis of both feed and plasmid fraction showed a remarkable 302,792-fold decrease in gDNA contamination (Table 3). In regard to endotoxin contamination, a 21-fold reduction was achieved, an analogous value to that obtained with only one run through the berenil-Sepharose column. The comparison between FDA specifications and pDNA sample composition collected after the second chromatographic run shows that all values are in accordance to the requirements for molecular therapy products (Table 4).

In an overall analysis, the berenil-Sepharose chromatographic process meets all the requirements to be used as a pDNA purification step. This new chromatographic support enables pDNA purification using a lower salt concentration, compared with other processes that use hydrophobic or pseudo-affinity ligands [16,29], showing a better purification performance and yield for plasmids with similar size.

Table 4
Comparison of plasmid samples composition with FDA specifications.

Impurity	FDA specifications [8]	pVAX1-LacZ collected sample	pCAMBIA-1303 collected sample	pVAX1-LacZ collected sample after two runs
Protein (%)	Preferably <1	Undetectable	Undetectable	Undetectable
RNA (%)	Preferably <1	Undetectable	Undetectable	Undetectable
Endotoxins (EU/mg pDNA)	<40	4	4	12
gDNA (%)	Preferably <1	0.27	5×10^{-5}	0.03

4. Conclusions

In this study, a new chromatographic process using berenil as ligand was developed and applied for the purification of pDNA molecules with different sizes: pVAX1-LacZ (6.05 Kbp) and pCAMBIA-1303 (12.361 Kbp). Both types of plasmids were successfully separated from host impurities showing that the method can be applied to small plasmids as well as to larger ones.

An optimization of the clarification step was also performed. Precipitation with 2.0 M, a value lower than the 2.5 M usually used, is enough to achieve pVAX1-LacZ with a high recovery and purity. However pCAMBIA-1303 was only completely purified using 2.5 M of salt, perhaps due to its larger size. The purification of pCAMBIA-1303 showed a lower yield, however, its purity is superior to that obtained with pVAX1-LacZ. This could be interesting since future requirements for multigene vectors, including extensive control regions, may require the production of larger plasmids.

Despite the loss in yield, the replacement of the clarification step with ammonium sulphate by a second chromatographic run indicated real advantages such as reduction in salt usage and procedure steps. Even though gDNA decontamination is more efficient when applying two runs through the support, the association of a clarification step with a chromatographic run with berenil-Sepharose gel is more effective in removing endotoxins.

This pseudo-affinity chromatographic method showed to be very effective in purifying plasmid DNA from *E. coli* cell lysate impurities, using small amounts of salt in the overall process. The essential separation mechanism seems to involve not only differential hydrophobic interactions between the ligand, pDNA molecules and host impurities, but also other important and specific contributions for the affinity binding. This purification method seems to be more environmentally friendly and less costly, and can be used as a main purification process of pharmaceutical grade pDNA.

Acknowledgements

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Paper IV

Negative pseudo-affinity chromatography for plasmid DNA purification using berenil as ligand

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ABSTRACT

The present study, reports the utilization of berenil as ligand in a negative pseudo-affinity chromatographic step to purify the plasmid pVAX1-LacZ from *Escherichia coli* clarified lysates. The chromatographic support was prepared by coupling berenil to epoxy-activated Sepharose and was qualitatively and quantitatively characterized using scanning electron microscopy, Fourier transformed infrared spectroscopy and elemental analysis. The clarified lysate was loaded onto the berenil-Sepharose support with 0.55 M of ammonium sulphate in the eluent, achieving the immediate elution of plasmid DNA. The impurities tightly bound to the support, were eluted after decreasing the salt concentration to 0 M. The overall process enabled the recovery of 87% of loaded plasmid DNA with a HPLC purity of »99% and according to FDA specifications. This method represents an alternative approach to the previous utilization of the same chromatographic pseudo-affinity support in a positive mode. It uses lower amounts of salt and one-step chromatographic procedure, resulting in smaller operating time and costs and representing an alternate procedure for plasmid DNA purification.

Keywords: berenil-Sepharose; negative pseudo-affinity chromatography; plasmid purification; small DNA ligands.

1. Introduction

The research efforts in developing new plasmid purification methods have seen substantial growth in the past few years, due to the rapid advances of molecular therapies [1,2]. The resulting purified plasmid molecules must meet strict quality criteria in terms of purity, efficacy and safety, as established by the Regulatory Agencies [3]. However, similarities between its common contaminants and pDNA can cause difficulties in their separation process [4]. The use of affinity and pseudo-affinity ligands can represent an efficient way to overcome this problem [5,6]. The high specificity of these ligands is due to the strong interaction with pDNA, and is a result of a combination of different types of intermolecular binding forces [7]. Berenil or 1,3-bis(4-phenylamidinium) triazene is an aromatic diamidine that was recently applied as ligand for the purification of pDNA from clarified and non-clarified *Escherichia coli* process streams by pseudo-affinity chromatography [6]. The DNA binding affinity of this minor groove ligand has been attributed to electrostatic interactions with the adenine and thymine sequences, hydrophobic contacts between the phenyl rings and the hydrophobic regions of the DNA backbone and also to hydrogen bonds between the amidines and thymine and/or adenine acceptor groups of the bases at the floor of the groove [8].

The present study reports a single step purification of pDNA directly from clarified *E. coli* lysates by negative berenil pseudo-affinity chromatography. Due to its ease and expedition in obtaining the desired products, negative chromatography was successfully applied to purify other biomolecules [9-11]. The target products, such as pDNA molecules, do not interact with the ligands and are obtained in the flowthrough, while the contaminants are retained in the column and can be recovered in the elution steps [12].

Thus, this method aims to make some improvements over existing one [6], since it reduces operation time and costs, providing a DNA sample similar in quality, but with a better yield . Moreover, it is a more environmental friendly process, since it uses smaller amounts of salt.

2. Materials and Methods

2.1. Materials

Sepharose CL-6B was obtained from Amersham Biosciences (Uppsala, Sweden). Berenil and 1,4-butanediol diglycidyl ether were purchased from Sigma-Aldrich (St. Louis, MO, USA). All salts used were of analytical grade.

2.2. Preparation and characterization of berenil-Sepharose support

Sepharose CL-6B was epoxy-activated according to the method described by Sundberg and Porath [13] and coupled to berenil as previously described [8]. Fourier transformed infrared

spectroscopy (FT-IR) spectra of the berenil derivatized gel and of epoxy-activated Sepharose (with no berenil) were acquired on a Thermo Scientific Nicolet iS10 FT-IR spectrometer (Thermo Scientific, Waltham, MA). Spectra were recorded at 4 cm^{-1} (128 scans) over the 400-4000 cm^{-1} range with and without baseline corrections. Bands were given in cm^{-1}. In this case three replicates of FT-IR analysis were performed. The microanalyses of both Sepharose matrices were performed in triplicate using a Carlo-Erba CHNS-O AE-1108 Elemental Analyser (Thermo Scientific, Waltham, MA). Scanning electron microscopy (SEM) images of both matrices were acquired in a Hitachi S-2700 with a UHV Dewar detector (Rontec EDX) (Tokyo, Japan). The samples were magnified 150, 450 and 4000x. Before all analyses, the samples were well-dried at 50ºC, in the presence of phosphorus pentoxide.

2.3. Bacterial culture, lysis and primary isolation

Escherichia coli DH5α strain harbouring 6.05 Kbp plasmid pVax1-LacZ (Invitrogene, Carlsband, CA, USA) and plasmid-free DH5α cells were cultured and stored as previously described [6]. The plasmid harbouring cells were then lysed using a modification of the alkaline method proposed by Sambrook *et al*. [14] also as described before [6].

2.4. Preparative Chromatography

A 10 cm x 10 mm column (Amersham Biosciences, Uppsala, Sweden) was packed with 2 mL berenil-Sepharose support and connected to a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden). It was then equilibrated with 0.55 M ammonium sulphate in 10 mM Tris-HCl buffer, pH 8.0 at a flow rate of 1mL/min and at room temperature. Clarified samples (25 µL) in equilibration buffer, with a nucleic acid concentration of 600 µg/mL, were loaded onto the column at the same flow rate and temperature. The absorbance was continuously monitored at 280 nm. After elution of unbound species (pDNA) with the equilibration buffer, the ionic strength was decreased to 0M to elute strongly bound impurities. Fractions were pooled according to the chromatograms obtained, concentrated and desalted using Vivaspin concentrators (Vivascience) and kept for further analysis. After the chromatographic runs, the column was washed with at least 5 bed volumes of deionised water. The complete assay was replicated twelve times.

2.5. Analysis of Plasmid Quality and Purity

Plasmid pooled fractions and feed sample were then analysed by various methods as previously described [6]: agarose gel electrophoresis, high-performance liquid chromatography (HPLC) to measure pDNA concentration and purity, micro-bicinchoninic acid (BCA) assay from Pierce (Rockford, USA) to measure protein concentration, endotoxin contamination analysis by the ToxinSensor™ Chromogenic LAL Endotoxin Assay kit from GenScript (GenScript USA Inc., New Jersey, USA), with a detection level of 0.005 EU/mL, and real-time polymerase chain reaction (PCR) to determine the gDNA contamination.

3. Results and Discussion

3.1. Berenil-Sepharose support characterization

Berenil was coupled to epoxy-activated Sepharose using a mild curing method, in the absence of a catalyst, and with a 1:4 weight ratio of ligand:Sepharose. This ratio was used to be possible to compare the results with those obtained in our previous work [6,8]. The derivatized support, as well as the epoxy-activated Sepharose with no berenil, were characterized by FT-IR spectroscopy, SEM and elemental analysis (EA).

Representative SEM micrographs of 150, 450 and 4000x magnifications performed on both matrices (Fig. 1), did not present any significant differences between them. This observation demonstrates the retention of the original Sepharose morphology and its physical properties after having been subjected to the curing process, which is crucial to its application as a chromatographic support.

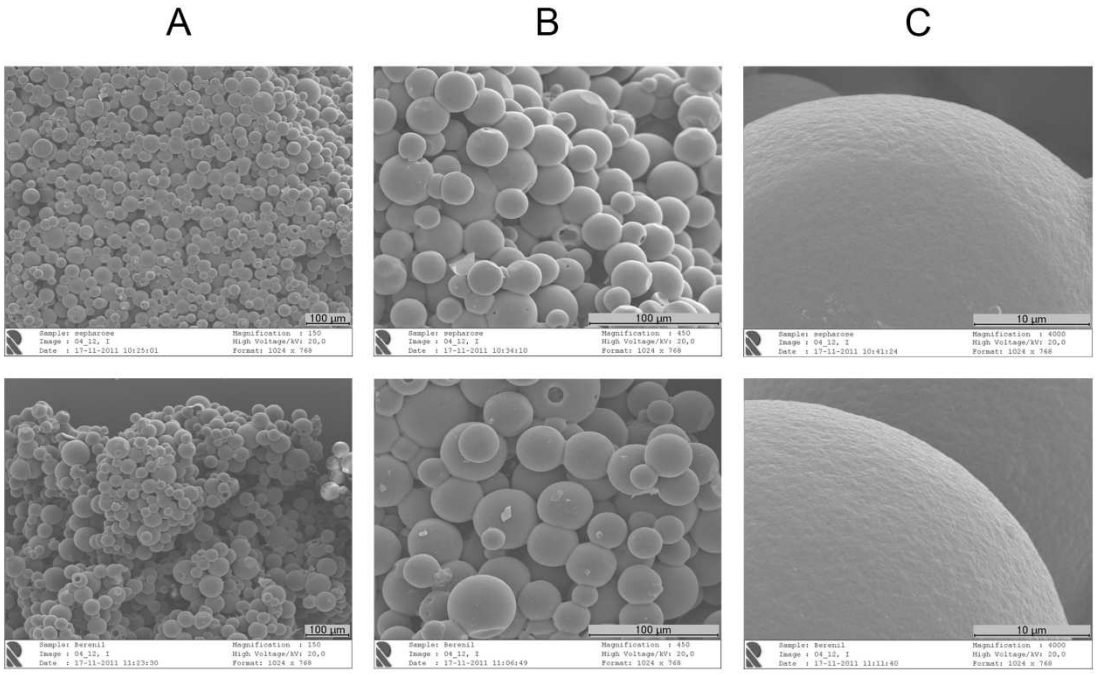

Fig. 1 - Representative SEM micrographs of Sepharose beads at 150x (A), 450x (B), and 4000x (C) magnification. Top row: non derivatized epoxy-activated Sepharose and bottom row: berenil-Sepharose.

The FT-IR spectra of berenil-Sepharose and epoxy-Sepharose are shown in figure 2. As the remaining spectrum for both cases is very similar, only the wavelength range from 1200 to 2000 cm^{-1} is presented. The berenil-Sepharose spectrum revealed a new band at 1606 cm^{-1} which could be the result of the in-plane skeletal vibrations of berenil aromatic rings (Fig. 2) [15].

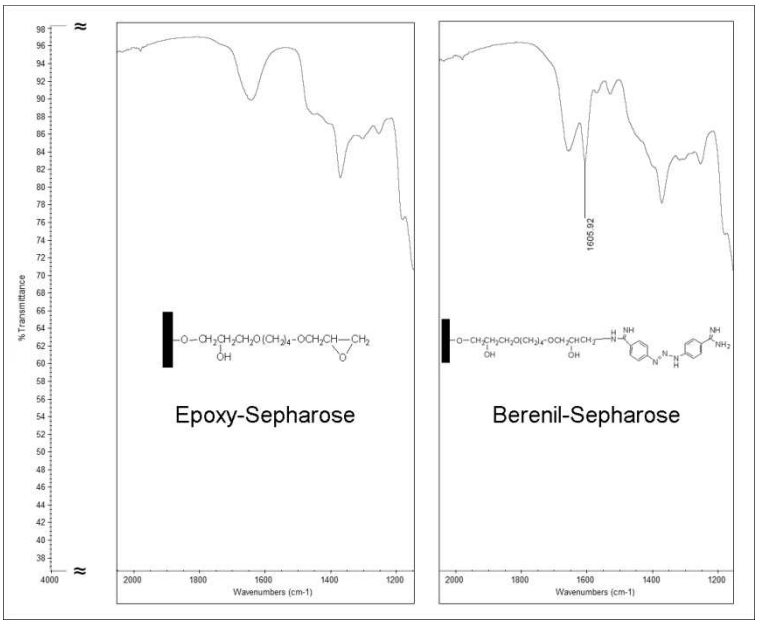

Fig 2. FT-IR spectra of berenil-Sepharose and epoxy-Sepharose. Only the wavelength range from 1200 to 2000 cm^{-1} is presented for both cases. The presented spectra were recorded without baseline corrections, since no changes were observable to the segment presented when those corrections were performed.

Since this band is absent in the epoxy-Sepharose spectrum (Fig. 2) it appears to demonstrate strong qualitative evidence that berenil was indeed coupled to the Sepharose matrix.

The amount of berenil bound to Sepharose (Q, mmol berenil/g derivatized Sepharose) was determined by EA using Eq. (1), where %N is the nitrogen percentage, assuming that all of the nitrogen present in the sample comes exclusively from berenil [16].

Q = %N/(1.4x7) (mmol berenil/g derivatized Sepharose) (1)

The results showed that the amount of berenil bound to Sepharose was 0.17 mmol berenil/g derivatized Sepharose (87.64 mg berenil/g derivatized Sepharose). The relatively low ligand density obtained could be advantageous, since a very dense layer of immobilized ligands can hinder the access of an isolated ligand molecule onto the buried binding sites of pDNA [7,17]. The amount of nitrogen determined in a sample of beaded epoxy-Sepharose was below the detection level (0.07%).

3.2. Berenil-Sepharose negative chromatography

The feedstock sample was injected onto the berenil-Sepharose column with 0.55M of ammonium sulphate in the eluent, under the previous optimized conditions for elution of pDNA [6]. Figure 3 shows the chromatographic profile and the agarose gel electrophoresis analysis of the pDNA eluted fractions.

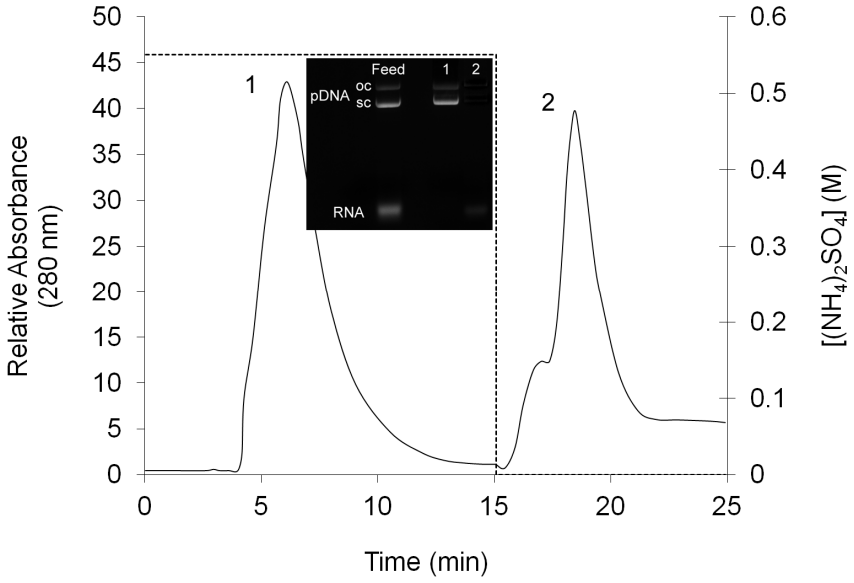

Fig. 3 - Chromatographic separation of pVAX1-LacZ clarified feed solution (25 µL, 600 µg/mL) from the host cell impurities on berenil-Sepharose and agarose gel electrophoresis analysis of the pDNA eluted fractions. Peak 1 and electrophoresis lane1: pDNA fractions collected after injection with 0.55 M $(NH_4)_2SO_4$; Peak 2 and electrophoresis lane2: impurities eluted with 0 M $(NH_4)_2SO_4$. The clarified lysate was also run in the agarose gel for comparative purposes (lane feed).

The elution of pDNA molecules occurs immediately after injection (peak 1 of Fig. 3) indicating that at this salt concentration there is no interaction between pDNA and berenil-Sepharose matrix. Next, when the ammonium sulphate concentration was decreased to zero, all the retained contaminants eluted into a single peak (peak 2 of Fig. 3). Similarly to previous work with berenil [6], the resolution between pDNA isoforms was not observed, even with higher salt concentrations [6]. Nevertheless, the procedure described here lead to a faster recovery of purified pDNA with a lower salt concentration.

3.3. Plasmid quality and purity assessment

The pDNA fraction was successfully separated from the RNA present in the lysate solution, as shown by agarose gel electrophoresis and confirmed by HPLC analysis (lane 1 in Fig. 3). The clarified lysate injected onto the berenil-Sepharose column has a high percentage of impurities (77%), namely RNA. HPLC analysis also proved that it was possible to recover 87% of the loaded plasmid with a purity of »99%, corresponding to a purification factor of 4.3. Both values are higher than those obtained for similar chromatographic procedures [6,11].

Proteins were not detectable in the pDNA pool, though a residual concentration of 66 µg/mL was detected in the feed solution. The elimination of endotoxins was very significant (from 2.71 EU/ml in the feed to 0.099 EU/ml in the pDNA pool) and the gDNA content in the plasmid fraction was dramatically reduced from 364.90 µg/mL to 0.37 µg/mL after the

chromatographic step. Table 1 shows the comparison between pDNA sample composition and FDA specifications.

Table 1. Comparison of purified plasmid sample composition with FDA specifications

Impurity	FDA Specifications [3]	pVax1-LacZ collected sample*
Protein (w/w %)	Preferably <1	Undetectable
RNA (w/w %)	Preferably <1	Undetectable
Endotoxins (EU/mg pDNA)	< 40	3
gDNA (w/w %)	Preferably <1	1.1

* Values resultant from three replicates except from endotoxins for which two replicates were performed

These values are very similar to the ones obtained in a previous work [6] however, in the present case, the endotoxin contamination value was reduced in 1 EU/mg pDNA. In addition to the time and cost cutback, this endotoxin reduction is very important since these molecules are major pDNA contaminants which elicit strong inflammatory responses in mammals [18] being a major concern for pDNA therapeutic uses. On the other hand, although the gDNA content (1,1%) is higher than the previously obtained value [6] and also slightly higher than the reference one (1%), this is not a mandatory value.

The negative pseudo-affinity process developed in this work is an attractive method of producing pure plasmid DNA. When compared to other similar chromatographic approaches [5,6,11], it shows a comparable pDNA quality, but higher yield, less elution steps and smaller use of ammonium sulphate. Consequently, this method has reduced costs and can represents an eco-friendly alternative to the existing pseudo-affinity and hydrophobic processes [6,11,19,20].

4. Conclusions

The present work describes a new approach for pDNA purification by negative chromatography using the pseudo-affinity berenil-Sepharose support. The berenil-Sepharose was prepared using a rather mild curing method and with a 1:4 weight ratio of ligand:Sepharose, resulting in a ligand density of 0.17 mmol berenil/g derivatized Sepharose. Additionally, FT-IR indicated the presence of berenil's aromatic rings in the support, which confirms the covalent bond between Sepharose and berenil. SEM analysis showed the preservation of the original beaded Sepharose morphology and therefore its mechanical properties after the derivatization process. The overall purification process using the berenil-Sepharose enabled the recovery of 87% of loaded pDNA with HPLC purity of »99% and according to FDA specifications. Additionally, this method represents an interesting and

promising alternative to previously developed chromatographic method, with less operating time and costs.

Acknowledgments

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Paper V

Specific recognition of supercoiled plasmid DNA by affinity chromatography using the intercalator DAPP as ligand

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Short communication

Specific recognition of supercoiled plasmid DNA by affinity chromatography using the intercalator DAPP as ligand

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ABSTRACT

Small molecules that bind DNA with high specificity present a promising opportunity for application as chromatographic ligands for plasmid DNA (pDNA) purification. This research used the intercalator 3,8-diamino-6-phenylphenanthridine (DAPP) as an immobilized ligand for the specific separation of supercoiled (sc) pDNA by affinity chromatography. The results showed that the protonated DAPP-Sepharose support has a great affinity for sc pDNA isoform, separating it from the less active open circular and linear isoforms. All pDNA isoforms were retained in the column using 10 mM acetate buffer pH 5. Selective elution of oc and linear isoforms was achieved with 0.22 M of sodium chloride in the same buffer. Finally, increasing the concentration to 0.55 M led to the elution of the sc isoform. The binding of pDNA to DAPP-Sepharose varies in function of pH, and the stability of the protonated DAPP–DNA complex decreases with increasing salt concentration.

1. Introduction

Molecular therapies such as gene therapy and DNA vaccines have been attracting increasing attention as alternative therapeutic procedures [1,2]. The main challenge for molecular therapies is the development of an effective method to deliver the therapeutic gene to the selected cells. Plasmid DNA (pDNA) plays an important role in non-viral vector gene delivery [3] as a third generation biopharmaceutical product with great potential in human and animal healthcare. However, pDNA must meet strict quality criteria in terms of contaminant levels and supercoiled (sc) pDNA content [4]. Thus, the approach to purification plays a significant role in the pDNA manufacturing processes.

Small molecules that bind DNA with high specificity can represent a promising application as chromatographic ligands for pDNA purification. The minor groove binder berenil was recently applied as a ligand for the separation of sc pDNA isoform from the open circular (oc) isoform [5]. The binding of many other small molecules to RNA and DNA, such as actinomycin C1 (D), actinomin, berberine, DAPI, methylene blue, Hoechst, acridine orange and sanguinarine, among others, has been the subject of several studies [6,7]. The DNA intercalator 3,8-diamino-6-phenylphenanthridine (DAPP) is one of those molecules that binds strongly to DNA [8,9]. DAPP is a planar aromatic system (Fig. 1), where only the phenyl group deviates from the molecular plane. This ethidium like molecule binds to DNA double helix via a non-covalent stacking interaction of its condensed aromatic rings with DNA base pairs, while the phenyl residue gets inserted into the minor groove. The hydrogen bonding between DAPP amino groups and the DNA's sugar-phosphate backbone plays a less important role in the binding [10].

DAPP has the structural requirements to be immobilized onto a chromatographic matrix, namely two amino groups which in the presence of a bis-epoxide spacer arm can bound onto the hydroxyl groups of agarose. Therefore, this work reports the development of a new support with DAPP as ligand, and its applicability for the specific separation of sc pDNA isoform from the oc and linear isoforms by affinity chromatography.

2. Materials and methods

2.1. Materials

Sepharose CL-6B was obtained from Amersham Biosciences (Uppsala, Sweden). DAPP and 1,4-butanediol diglycidyl ether were purchased from Sigma–Aldrich (St. Louis, MO, USA). All salts were of analytical grade.

Abbreviations: sc, supercoiled; oc, open circular; DAPP, 3,8-diamino-6-phenylphenanthridine.

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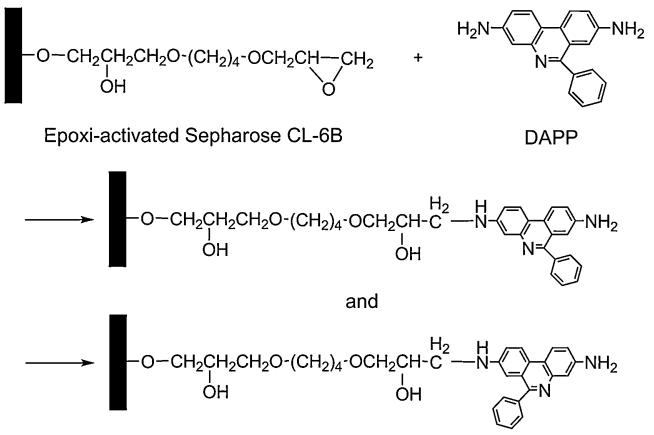

Fig. 1. Derivatization of epoxy-activated Sepharose with DAPP (two binding hypothesis for the unprotonated form are shown).

2.2. Plasmid production and purification

Escherichia coli DH5α strain harbouring 6.05 Kbp plasmid pVax1-LacZ (Invitrogene, Carlsband, CA, USA) was cultured as previously described [5]. Plasmid DNA was purified using the Qiagen plasmid midi kit (Hilden, Germany) according to the manufacturer's instructions. Linear plasmid isoform sample was prepared by enzymatic digestion of the purified pDNA with the Hind III enzyme for 2 h, at 37 °C. Plasmid quantification was made by measuring the absorbance at 260 nm.

2.3. Preparation of DAPP-Sepharose support

Sepharose CL-6B was epoxy-activated according to the method of Sundberg and Porath [11] and coupled to DAPP (Fig. 1) in accordance to the process described before [5]. 3 g of dry epoxy-activated Sepharose was added to 4 mL of a 2.0 M sodium carbonate solution containing 500 mg of DAPP. After swirling for 16 h at 70 °C, the derivatized Sepharose was washed with large volumes of deionised water and 70% ethanol solution to remove the excess of ligand and sodium carbonate. Several additional steps were performed to check for leakage of the ligand and to prove the safety of this DNA intercalator. Since DAPP-Sepharose support presents a strong colour it was verified by visible spectrophotometric analysis if any ligand leakage was occurring. The derivatized support was washed meticulously several times and the colour never faded. It was also left in water and ethanol at 4 °C for almost six months and the liquid did not present any measured ligand molecules. After more than one hundred injections the colour of the support remained the same and the ligand was never spectrophotometrically detected in the final plasmid solutions.

2.4. Chromatographic method and pH studies

A 10 cm × 10 mm column (Amersham Biosciences, Uppsala, Sweden) was packed with 2.5 mL DAPP-derivatized gel (Fig. 1), connected to a Fast Protein Liquid Chromatography (FPLC) system (one collimation lens) (Amersham Biosciences, Uppsala, Sweden) and tested with samples of oc and sc isoforms. Different mobile phases with different salt concentrations (0–2.0 M sodium chloride or 0.1–2.0 M ammonium sulphate) were used to select the best binding/elution conditions, namely: 10 mM Tris-HCl buffer pH 8; 1× PBS buffer pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and 10 mM acetate buffer pH 6 and pH 5 (10 mM sodium acetate and acetic acid). After that, 25 μL of plasmid samples (600 μg/mL of a mixture of oc, linear and sc isoforms) were injected at the same flow rate (1 mL/min) with 10 mM acetate buffer pH 5. The species with lower affinity to the derivatized support were eluted with 0.22 M NaCl in the same buffer. The elution of strongly bound species was then achieved with a second isocratic elution step by increasing the NaCl concentration to 0.55 M. The absorbance was monitored continuously at 280 nm. In all cases, fractions were pooled according to the chromatograms obtained and analyzed by gel electrophoresis (110 V) using 1% agarose gel in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA at pH 8.0) in the presence of 0.5 μg/mL ethidium bromide. The gels were visualized in a UVITEC Cambridge system (UVITEC Limited, Cambridge, UK).

3. Results and discussion

Initial experiments were performed to find the best buffer conditions to achieve total retention of pDNA molecules on the DAPP-Sepharose column. A pH 8 buffer (Tris–HCl 10 mM) was tested without ammonium sulphate or sodium chloride however pDNA was completely eluted immediately after the sample injection (Fig. 2A). A total retention was only obtained with high salt concentration (2.1 M of ammonium sulphate, results not shown) which could have both economic and environmental negative impact.

At initial buffer conditions (pH 8) the DAPP-Sepharose gel presented a dark orange colour, changing to red on washing the column with water. As this colour change could only be a result of a pH variation, it became interesting to explore this behaviour more closely. DAPP has a pKa value of 5.8 in the free state [12] but it can change if the molecule is bonded to other molecules such as DNA [9]. Thus, a screening for the best pDNA retention conditions was made using different buffers with three distinct pH values (7.4, 6.0 and 5.0). When the PBS 1× buffer with a pH of 7.4 was used (Fig. 2B), the gel remained orange and pDNA was not retained in the column. In contrast, when the acetate buffer 10 mM, pH 6 was used without salt, the gel turned to a slightly darker red colour and a partial retention of pDNA was observed (Fig. 2C). However, total retention of pDNA was only observed when the pH was decreased to a value below DAPP's pKa (5.8), using acetate buffer pH 5. Plasmid DNA was then eluted with 1 M of sodium chloride in the same buffer (Fig. 2D).

The binding of pDNA to DAPP-Sepharose is very sensitive to pH variations and at low pH values, the electrostatic attraction between the oppositely charged surface of DNA and the support dominates [13]. At pH 5, the gel was of an intense dark red colour due to the protonated phenanthridine moiety [14] meaning that the pKa of DAPP bonded to Sepharose did not change substantially from the free form. The protonation of the phenanthridine groups in DAPP molecules was evident from the colour changes shown by the gel at different pH values. A progressive darkening of the gel from dark orange to red and then to dark red was observed as the pH decreased from 8 to 5. Being protonated, DAPP can more easily bind to the negatively charged pDNA molecules. In the protonated form, the additional strong attraction between the positive charge on the phenanthridine ring and the negative charges on the DNA backbone substantially increases the intermolecular coulombic interaction [9]. Thus, the positively charged intercalator molecules bind to pDNA much more strongly than in the neutral form. This is mostly due to electrostatic contributions, substantially increasing the stabilization energy of the complex [10]. However, the binding of the charged DAPP molecule to DNA becomes progressively weaker with increasing bulk salt concentration. High salt concentrations strongly oppose the formation of the protonated DAPP–DNA complex, destabilizing it [9]. This is very interesting given that pDNA was totally retained in the

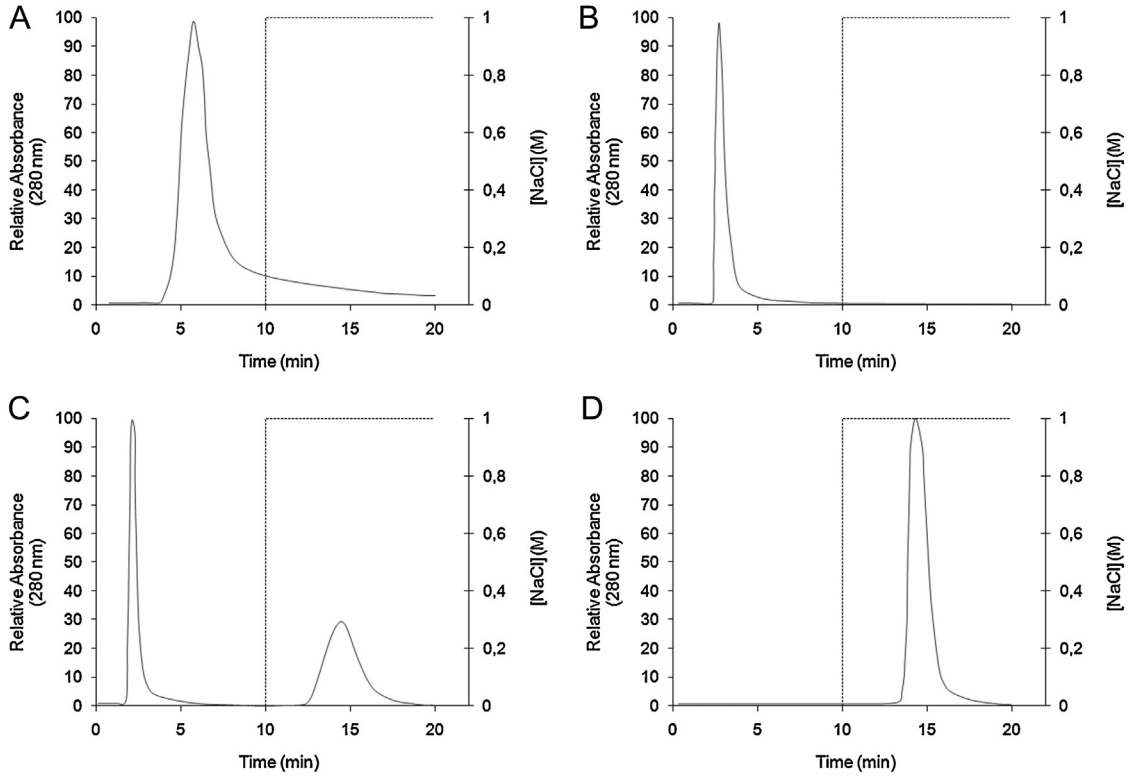

Fig. 2. Retention of pDNA on the DAPP-Sepharose column using buffers with different pH values. An injection of 25 μL (600 μg/mL) of a pDNA (oc + sc) solution was performed varying only the loading buffer: (A) Tris–HCl 10 mM pH 8; (B) PBS 1× pH 7.4; (C) acetate 10 mM pH 6 and (D) acetate 10 mM pH 5. A solution of 1 M of NaCl in the buffers was used in the elution.

column with no salt in the loading buffer and the elution was performed simply by addition of a small amount of sodium chloride.

Moreover, after a total retention of pDNA, a selective elution of oc and linear isoforms was achieved (peak 1/lane 1) by increasing sodium chloride concentration to 0.22 M (Fig. 3). Finally, increasing the concentration of sodium chloride to 0.55 M led to the elution of the sc isoform (peak 2/lane 2). After analysing the electrophoresis bands (Fig. 3), it was concluded that oc and linear isoforms were not detected in the sc fraction (lane 2 in Fig. 3), which indicates that the

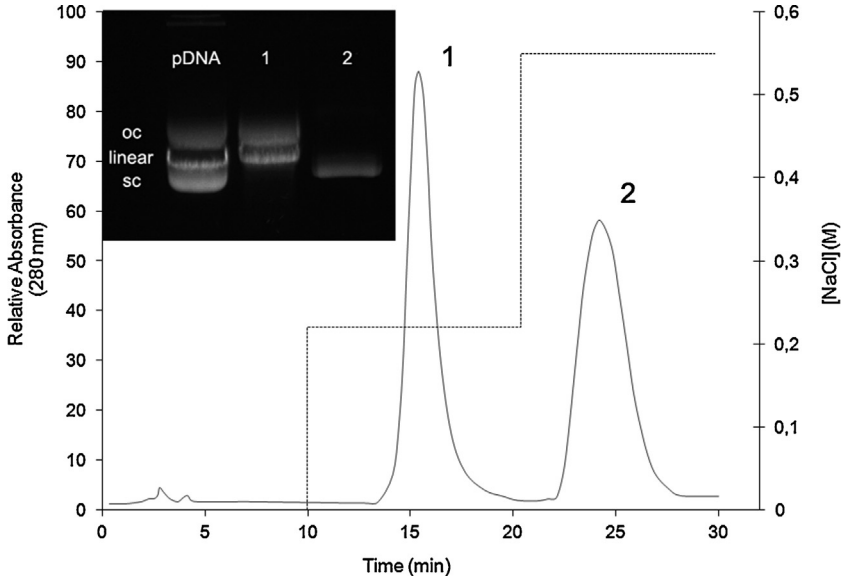

Fig. 3. Chromatographic separation of pDNA (25 μL, 600 μg/mL) isoforms on DAPP-Sepharose and agarose gel electrophoresis of the peak fractions. Lane pDNA: sample of pDNA injected onto the column (mixture of oc and sc isoforms, plus the linear isoform obtained from the enzymatic digestion of the plasmid with Hind III). Lane 1: oc and linear pDNA fraction collected from peak 1 using as eluent acetate buffer 10 mM pH 5 with 0.22 M NaCl. Lane 2: sc pDNA fraction collected from peak 2 using acetate buffer 10 mM pH 5 with 0.55 M NaCl as eluent.

sc pDNA amount in peak 2 is near 100%. These results show that the DAPP-Sepharose matrix has a great affinity towards the sc pDNA isoform. The fact that pDNA is completely retained in the column without any added salt to the binding buffer (Fig. 3) indicates the presence of strong interactions with charged phosphate groups of the DNA backbone [15]. However, phosphate and sugar groups are equally exposed in all isoforms. Therefore, the observed differences in retention may be due to the higher exposure of the bases of sc isoform, consequence of deformations induced by torsional strain [16].

This new affinity DAPP-Sepharose matrix enables sc pDNA selective separation from the other isoforms, using a lower amount of salt than some other chromatographic processes [5,16].

4. Conclusions

In conclusion, the present work shows that the DAPP-Sepharose affinity support can be successfully applied in the selective separation of sc pDNA isoform from the other less active isoforms. This is achieved using small amounts of salt in the eluent, favouring the economic and environmental sustainability of the method we report here. These promising results indicate that DAPP can be an excellent choice as a ligand for the purification of sc pDNA molecules from *E.coli* lysate solutions.

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Paper VI

Dynamic binding capacity and specificity of 3,8 - diamino - 6 - phenylphenanthridine - Sepharose support for purification of supercoiled plasmid DNA

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Dynamic binding capacity and specificity of 3,8-diamino-6-phenylphenanthridine-Sepharose support for purification of supercoiled plasmid deoxyribonucleic acid

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ABSTRACT

Affinity chromatography represents a sole technique in purification of different biomolecules. The specific recognition between affinity ligands and target biomolecules has a major role in the specificity of the process. Therefore, choosing the right ligand is a crucial step for the development of a successful purification system. This work describes the application of the DNA intercalator 3,8-diamino-6-phenylphenanthridine (DAPP) as a chromatographic affinity ligand for the specific separation and purification of supercoiled plasmid DNA (pDNA). The support was prepared by coupling DAPP onto an epoxy-activated Sepharose matrix, using mild conditions and resulting in a ligand density of 0.15 mmol DAPP/g derivatized Sepharose. The characterization of DAPP-Sepharose support in terms of dynamic binding capacity was achieved by studying the effect of plasmid DNA concentration and flow rate on pDNA adsorption. The maximum capacity value of 336.75 μg pDNA/mL gel was obtained at 1 mL/min with a pDNA concentration of 150 μg/mL. Moreover, the values did not vary significantly with the variation of flow rate. In addition, the DAPP-Sepharose showed a high affinity towards pDNA as quantified by the respective dissociation constant ($K_d = 2.29 \pm 0.195 \times 10^{-7}$ M). The support was also tested for the purification of two plasmid molecules with different sizes (pVAX1-LacZ and pCAMBIA-1303, with 6.05 kbp and 12.361 kbp, respectively) from clarified *Escherichia coli* lysate solutions. Total retention of all lysate components was achieved without any added salt to the eluent buffer. The selective elution of impurities and supercoiled pDNA was accomplished simply by the addition of small amounts of salt to the buffer solution. The yield for pCAMBIA-1303 was 65% and for pVAX1-LacZ was 94%, with all host impurity levels in accordance with the requirements established by the regulatory agencies.

1. Introduction

Affinity chromatography (AC) represents a unique method in separation, identification and purification of biomolecules, based on a highly specific molecular recognition. It relies on a strong but reversible interaction between the target molecule and the immobilized ligand, similar to many interactions found in biological systems, like antibody-antigen or enzyme-substrate binding [1–3]. That specific recognition involves a combination of various types of intermolecular forces, such as hydrogen bonding, electrostatic, hydrophobic and van der Waals interactions [2].

Since the introduction of AC by Cuatrecasas *et al.* [4] in 1968, many molecules such as proteins, nucleic acids (RNA and plasmid DNA), antibodies, sugars, glicoproteins, hormones and even viruses have been separated/purified using this methodology [5–7]. This technique has the advantage of the specific nature of the underlying interactions, which results in high resolution and selectivity that potentially avoids multi-purification steps, increasing yields and improving the process economics. However, the biological origin, cost and stability of the ligands may be critical [5,8,9].

Ligands are crucial components in AC, since they have a major role in the specificity and stability of the purification systems. The success of an affinity based separation is mostly dictated by the type of ligand used. As conventional purification protocols are being replaced by more sophisticated and selective procedures, the focus changes towards designing and selecting ligands of high affinity and specificity [5,7,8]. During the last decade, many ligands were

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developed and studied to improve plasmid DNA (pDNA) purification [5,10,11]. However, the purification of pharmaceutical grade pDNA is particularly challenging, since its critical contaminants are similar in size, charge, hydrophobicity and base exposition [10]. The final product must meet strict quality criteria in terms of purity, efficacy and safety, established by the regulatory agencies [12]. Moreover, the purification method has to ensure that the structural and functional stability of the most active conformation of pDNA (supercoiled – sc) is maintained, as well as its content (>97%) regarding the less active linear and open circular (oc) isoforms [13].

Our research group recently developed a pseudo-affinity chromatographic method for the separation and purification of pDNA from lysate solution impurities, using the minor groove binder berenil as ligand [11]. After these studies, the DNA intercalator 3,8-diamino-6-phenylphenanthridine (DAPP) was applied as an affinity ligand to specifically separate the sc pDNA from the oc and linear isoforms [14]. DAPP is a planar aromatic system where only the phenyl group deviates from the molecular plane [15]. This molecule binds strongly to DNA double helix [16,17] via a non-covalent stacking interaction of its condensed aromatic rings with DNA base pairs, while the phenyl residue gets inserted into the minor groove. The hydrogen bonding between DAPP amino groups and DNA's sugar-phosphate backbone plays a less important role in the binding [15]. The binding is very sensitive to pH variations. When the pH of the buffer solution was below DAPP's pKa (5.8), the ligand was protonated and all negatively charged molecules were retained in the column [14]. In the protonated form, the additional strong attraction between the positive charge on the phenanthridine ring and the negative charges on the DNA backbone substantially increases the intermolecular coulombic interaction [17]. Thus, the positively charged intercalator molecules bind to pDNA much more strongly than in the neutral form, since with higher pH values partial or no retention was observed [14].

The present work reports the chromatographic purification of sc pDNA molecules of different sizes (pVAX1-LacZ and pCAMBIA-1303, with 6.05 kbp and 12.361 kbp, respectively), directly from clarified *Escherichia coli* lysate solutions, using DAPP as ligand. Moreover, this support was structurally characterized and the pDNA adsorption was studied regarding pDNA concentration and flow rate, to determine how these conditions affect its dynamic binding capacity (DBC).

2. Materials and methods

2.1. Materials

Sepharose CL-6B was obtained from Amersham Biosciences (Uppsala, Sweden). DAPP and 1,4-butanediol diglycidyl ether were purchased from Sigma–Aldrich (St. Louis, MO, USA). All salts used were of analytical grade.

2.2. Preparation of DAPP-Sepharose support

Sepharose CL-6B was epoxy-activated according to the method of Sundberg and Porath [18] and coupled to DAPP as previously described [14,19]. Initially, Sepharose CL-6B was washed with large volumes of deionised water. Then 5 g of moist gel were mixed with 5 mL of 0.6 M NaOH solution containing 50 mg of $NaBH_4$ and 5 mL of 1,4-butanediol diglycidyl ether. The slurry was swirled at 25 °C for 8 h using a bath with orbital agitation. The epoxy-activated gel was then washed in a sintered glass funnel with large volumes of deionised water. The gel was then suction-filtered to near dryness. The dry epoxy-activated Sepharose (3 g) was then added to 4 mL of a 2.0 M sodium carbonate solution containing 500 mg of DAPP. After swirling for 16 h at 70 °C, the derivatized Sepharose was washed with large volumes of deionised water and 70% ethanol solution to remove the excess of ligand and sodium carbonate. The derivatized gel was stored at 4 °C in deionised water.

2.3. Characterization of DAPP-Sepharose support

Fourier transformed infrared spectroscopy (FT-IR) spectra of derivatized DAPP gel and of epoxy-activated Sepharose (with no DAPP) were acquired on a Thermo Scientific Nicolet iS10 FT-IR spectrometer (Thermo Scientific, Waltham, MA). Spectra were recorded at 4 cm^{-1} (128 scans) over the 400–4000 cm^{-1} range without baseline corrections. Bands are given in cm^{-1}. Scanning electron microscopy (SEM) images of both derivatized DAPP gel and epoxy-activated Sepharose were acquired in a Hitachi S-2700 with a UHV Dewar detector (Rontec EDX) (Tokyo, Japan). The samples were magnified 150, 450 and 4000×. The microanalyses of both matrices were performed in triplicate using a Carlo-Erba CHNS-O AE-1108 Elemental Analyser (Thermo Scientific, Waltham, MA). Before all analyses, the samples were well-dried at 50 °C, in the presence of phosphorus pentoxide.

2.4. Bacterial culture

E. coli DH5α strain harbouring the 6.05 kbp plasmid pVAX1-LacZ (Invitrogen, Carlsband, CA, USA) and *E. coli* XL1 blue strain harbouring the 12.361 kbp plasmid pCAMBIA-1303 (Cambia, Brisbane, Australia) were cultured overnight in Luria Bertani agar (Lennox) medium (Laboratorios Conda, Madrid, Spain) supplemented with 30 μg/mL of kanamycin at 37 °C. *E.coli* DH5α strain was grown at the same temperature in an orbital shaker with Terrific Broth medium (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 30 μg/mL kanamycin. The XL1 blue strain cells were grown in similar conditions using Luria Bertani medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0). Both cell strains were harvested by centrifugation at the end of the exponential growth phase and stored at −20 °C until use. Plasmid-free *E. coli* cells were also grown in the absence of antibiotic, under the same conditions as previously described.

2.5. Lysis, primary isolation and plasmid production

The plasmid harbouring cells were lysed using a modification of the alkaline method proposed by Sambrook *et al.* [20]. Centrifugation of a 250 mL sample of the cell broth was performed at 5445 × *g* for 30 min at 4 °C with a Sigma 3-18K centrifuge. The supernatants were discarded and the bacterial pellets were resuspended in 20 mL of 50 mM glucose, 25 mM Tris–HCl and 10 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0). The cells were then lysed by adding 20 mL of a 200 mM NaOH, 1% (w/v) sodium dodecyl sulphate (SDS) solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated by gently adding and mixing 16 mL of prechilled 3 M potassium acetate (pH 5.0). The precipitate was removed by a double centrifugation at 20000 × *g* for 30 min at 4 °C with a Beckman Allegra 25 R centrifuge. The plasmid in the supernatant was precipitated after the addition of 0.7 volumes of isopropanol and a 30 min incubation period on ice. The pDNA was recovered by centrifugation at 16,000 × *g* for 30 min at 4 °C. The pellets were then redissolved in 1 mL of 10 mM sodium acetate buffer (pH 5.0). Next, the clarification of the lysates for both plasmid molecules was achieved by dissolving solid ammonium sulphate in the solutions up to a final concentration of 2.5 M, followed by a 15 min incubation period on ice. Precipitated proteins and RNA were then removed by centrifugation at 10,000 × *g* for 20 min at 4 °C. The supernatants were recovered and the nucleic acid concentration quantified by

measuring the absorbance at 260 nm. Plasmid DNA with a sc isoform percentage over 90% was also obtained using the Qiagen plasmid midi kit (Hilden, Germany) according to the manufacturer's instructions.

2.6. Dynamic binding capacity

The DBC characterization of the DAPP-Sepharose support for pDNA was conducted at varying flow rates (0.5, 1 and 1.5 mL/min) and DNA concentrations (50, 100 and 150 μg/mL) at room temperature and using the model plasmid pVAX1-LacZ. A 10 cm × 10 mm column (Amersham Biosciences, Uppsala, Sweden) was packed with 0.4 mL DAPP-derivatized gel and connected to an ÄKTA Purifier system (GE Healthcare Biosciences, Uppsala, Sweden), consisting of a compact separation unit and a personal computer with Unicorn control system Version 5.11. Since the binding conditions of pDNA onto the DAPP-Sepharose support were already achieved [14], the column was equilibrated with 10 mM sodium acetate buffer pH 5 either with or without 1 M NaCl (non binding and binding conditions respectively). The same buffer was used for dissolving the pDNA purified with the Qiagen plasmid midi kit, and for the non-binding studies, the ionic strength was corrected by dissolving the necessary amount of NaCl. Plasmid solutions were loaded into the column at the same pH and temperature conditions. For both flow rate and pDNA concentration studies, the conditions were run in duplicate. Elution of bound plasmid was achieved with 1 M NaCl in the buffer solution. The DBC was calculated from the breakthrough curves at 50% of maximal absorbance value.

2.7. Preparative chromatography

A 10 cm × 10 mm column (Amersham Biosciences, Uppsala, Sweden) was packed with 2.5 mL DAPP-derivatized gel and connected to a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden). It was then equilibrated with 10 mM sodium acetate buffer pH 5 at a flow rate of 1 mL/min and at room temperature (26 °C). 25 μL of clarified samples (600 μg/mL of nucleic acids) of plasmid molecules (pVAX1-LacZ or pCAMBIA-1303) in equilibration buffer were then loaded onto the column at the same flow rate and temperature. The species with lower affinity to the derivatized support were eluted with 0.22 M NaCl in the same buffer for pVAX1-LacZ and 0.3 M NaCl for pCAMBIA-1303. The elution of strongly bound species was then achieved with a second isocratic elution step by increasing the NaCl concentration to 0.55 M for both plasmid molecules. The absorbance was monitored continuously at 280 nm. The influence of the temperature on the chromatographic runs was studied using a water-jacket column and injecting 25 μL of pVAX1-LacZ clarified samples. The runs were performed at 3 °C and 15 °C, using the same buffer conditions described before. All fractions were pooled according to the obtained chromatograms, concentrated and desalted using Vivaspin concentrators (Vivaproducts, Littleton, MA, USA) and kept for further analysis as described below. After the chromatographic runs, the column was washed with at least 5 bed volumes of deionised water.

2.8. Agarose gel electrophoresis

Pooled fractions were analyzed by horizontal electrophoresis (100 V for 40 min) using 1% and 0.8% agarose gel in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) in the presence of 0.5 μg/mL of GreenSafe. The gels were visualized in a UVITEC Cambridge system (UVITEC Limited, Cambridge, UK).

2.9. Analytical chromatography

Feed samples (clarified lysate) were injected onto the DAPP-Sepharose column and the fractions were pooled after the chromatographic runs. The pDNA concentration and purity of the extracts was assessed by high-performance liquid chromatography (HPLC), according to the method described by Diogo *et al.* [21]. A 4.6/100 mm HIC Source 15 PHE PE column (Amersham Biosciences, Uppsala, Sweden) was connected to a Waters HPLC system (Waters Corporation, Milford, MA, USA) and equilibrated with 1.5 M ammonium sulphate in 10 mM Tris–HCl buffer (pH 8.0). Samples (20 μl) were injected and eluted at a flow rate of 1 mL/min. After injection, the elution occurred with the equilibration buffer for 3 min, at which point the elution buffer was immediately changed to Tris–HCl 10 mM (pH 8.0) without ammonium sulphate. This condition was maintained for 6 min in order to elute bound species. The column was then re-equilibrated for 7 min with the equilibration buffer to prepare the column for the next run. The absorbance of the eluate was continuously recorded at 254 nm. The concentration of pDNA in each sample was calculated using a calibration curve constructed with pDNA standards (2.5–400 μg/mL) purified using the Qiagen plasmid midi kit. Plasmid quantification was achieved by measuring the absorbance at 260 nm, assuming an absorbance of 1.0 for a solution of 50 μg/mL. The degree of purity was defined as the percentage of the pDNA peak area in relation to the total area of all chromatographic peaks.

2.10. Protein analysis

Protein concentration of feed samples and pDNA fractions pooled after chromatography was measured using the micro-bicinchoninic acid (BCA) assay from Pierce (Rockford, IL, USA), according to the manufacturer's instructions. 50 μL of each sample were added to 200 μL of BCA reagent in a microplate and incubated for 30 min at 60 °C. Absorbance was measured at 595 nm in a microplate reader. The calibration curve was prepared using bovine serum albumin standards (0.01–1 mg/mL).

2.11. Endotoxin analysis

Analysis of endotoxin contamination on feed samples and pDNA fractions pooled was performed using the ToxinSensor™ Chromogenic LAL Endotoxin Assay kit from GenScript (GenScript USA Inc., Piscataway, NJ, USA) which had a detection level of 0.005 EU/mL.

2.12. Genomic DNA analysis

Genomic DNA contamination in both pDNA solution and feed sample was assessed using real-time polymerase chain reaction (PCR) in a iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), according to the method described by Martins *et al.* [22]. Sense (5′-ACACGGTCCAGAACTCCTACG-3′) and antisense (5′-GCCGGTGCTTCTTCTGCGGGTAACGTCA-3′) primers were used to amplify a 181-bp fragment of the 16S rRNA gene. PCR amplicons were quantified by following the change in fluorescence of the DNA binding dye Syber Green (Bio-Rad, Hercules, CA, USA). *E. coli* genomic DNA was purified with the Wizard gDNA purification kit (Promega, Madison, WI, USA) and used to generate a standard curve ranging from 0.005 to 50 μg/mL. Negative controls, with no template, were run at the same time as the standards.

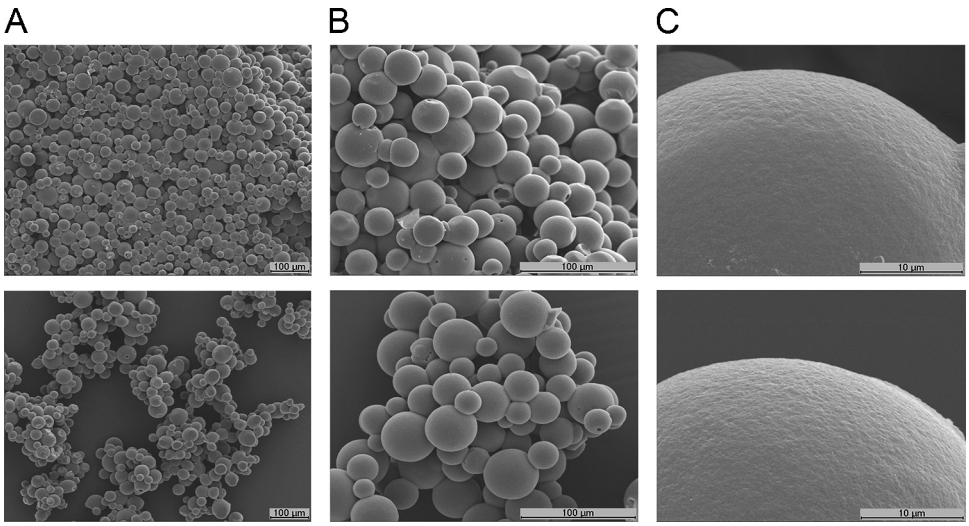

Fig. 1. Representative SEM micrographs of Sepharose beads at 150× (A), 450× (B), and 4000× (C) magnification. Top row: epoxy-activated Sepharose with no DAPP and bottom row: DAPP-Sepharose matrix.

3. Results and discussion

3.1. DAPP-Sepharose support characterization

Following our previous work with berenil as a pseudo-affinity ligand [11,19], a novel affinity support was synthesized by immobilizing the intercalator DAPP to an epoxy-activated Sepharose matrix. The curing method was rather mild, without the use of a catalyst and with a DAPP:Sepharose ratio of 1:6 (g/g). The resulting support was then characterized using SEM (Fig. 1), elemental analysis (EA) and FT-IR spectroscopy.

The amount of DAPP bound to Sepharose (Q, mmol DAPP/g derivatized Sepharose) was determined by EA using Eq. (1), where %N is the nitrogen percentage, assuming that all of the nitrogen present in the sample comes exclusively from DAPP [23].

$$Q = \frac{\%N}{1.4 \times 3} \quad (\text{mmol DAPP/g derivatized Sepharose}) \tag{1}$$

Results showed that the amount of DAPP bonded to Sepharose was 0.15 mmol DAPP/g derivatized Sepharose (42.8 mg DAPP/g derivatized Sepharose). The relatively low ligand density obtained could be favourable to pDNA binding, since a high density is not always desirable to achieve a better selectivity towards the target molecules. A very dense layer of immobilized ligands can hinder the access of an isolated ligand molecule to the buried binding sites of pDNA and therefore prevent the recognition between them [2,24,25]. The amount of nitrogen was also determined in a sample of beaded epoxy-Sepharose but without DAPP. In this case, the %N was below the detection level (0.07%).

The surface morphology and structure of the Sepharose beads before and after the immobilization process were analyzed by SEM (micrographs of 150, 450 and 4000× magnification) and no significant differences were found between them (Fig. 1). The maintenance of the original Sepharose morphology and its physical properties after the curing process are crucial to DAPP-Sepharose application as a chromatographic support.

DAPP-Sepharose FT-IR spectrum revealed an additional adsorption band at 1621 cm^{-1} comparing to non-derivatized epoxy-Sepharose (spectra not shown). This band could indicate the presence of amines and their characteristic N—H bending band or can be attributed to the in-plane skeletal vibrations of DAPP aromatic rings. The assignment is complex because they both occur in the same region [26] however, since this band is absent in the epoxy-Sepharose spectrum it appears as strong qualitative evidence of a covalent bond between DAPP and the Sepharose matrix.

3.2. Dynamic binding capacity

The performance of pDNA-ligand adsorption can be characterized by DBC which represents the adsorption capacity of the column for the target molecule at a certain breakthrough. Determining the DBC of a stationary phase is essential for the purification process optimization. DBC is defined as the amount of target molecules that bind to the matrix under standard flow conditions and should be determined under specific flow and load characteristics [27,28]. It is calculated based on the amount that can be loaded before significant product levels are measured in the flowthrough (the breakthrough point). The collected information about the support performance is useful for assessing loading conditions and column lifetime [29,30]. In order to determine the DBC of DAPP-Sepharose for pDNA, breakthrough curves were performed at different flow rates (0.5, 1 and 1.5 mL/min) and pDNA concentrations (50, 100 and 150 µg/mL). Plasmid solutions were loaded through the column in binding (without NaCl) and non binding (with 1 M NaCl) conditions. All the obtained curves have a similar sigmoidal shape (Fig. 2) and DBC was calculated at 50% breakthrough. DBC was calculated according to Eq. (2):

$$\text{DBC} = C\frac{V - V_0}{V_b} \quad (\text{mg pDNA/mL gel}) \tag{2}$$

where C is the pDNA concentration in the feedstock (µg/mL), V is the volume of pDNA solution loaded at 50% breakthrough (mL), V_0 is the void volume of the column at 50% breakthrough (mL), and V_b is the packed bed volume of DAPP-Sepharose (mL).

Breakthrough curves and the results obtained at 50% breakthrough are shown in Fig. 2 and Table 1 respectively. The DBC values range from 27.38 to 336.75 µg pDNA/mL gel, in function of the

Table 1
Effect of flow rate and pDNA concentration on the DBC (µg pDNA/mL gel) of pDNA to the DAPP-Sepharose. DBC was estimated at 50% breakthrough.

Flow rate (mL/min)	pDNA concentration (µg/mL)		
	50	100	150
0.5	105.38	250.75	330
1	103.25	208.13	336.75
1.5	110.44	221.50	27.38

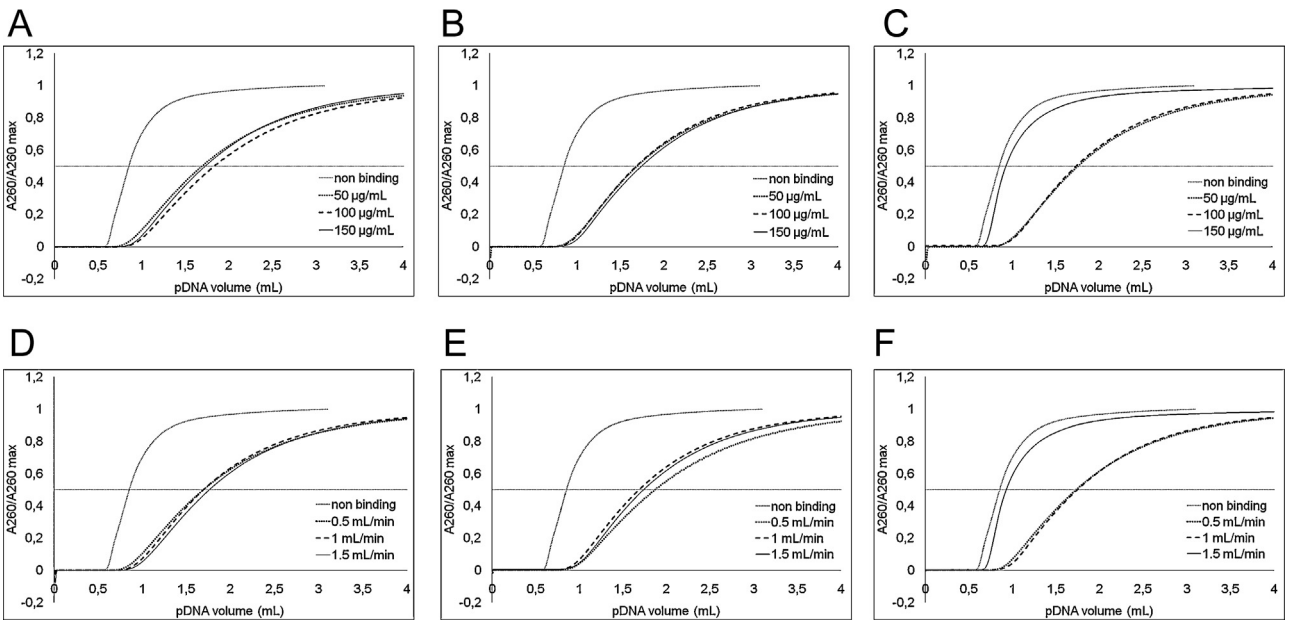

Fig. 2. Effect of pDNA concentration and flow rate on breakthrough curves. (A), (B) and (C) – assays performed at 0.5, 1 and 1.5 mL/min respectively, with different feed concentrations; (D), (E) and (F) – assays performed using 50, 100 and 150 μg/mL of pDNA respectively, with different flow rates.

flow rate and pDNA concentration (Table 1). The maximum capacity value was obtained at 1 mL/min with a pDNA concentration of 150 μg/mL, standing in the same range of other affinity agarose based supports [31].

Fig. 2D/E/F shows that the slope of the breakthrough curves is very similar and almost unaffected by flow rate (Table 1) which is in agreement with a few monolith based studies [32–34] but diverging from results obtained with a bead based histidine–agarose support [31]. For a pDNA concentration of 50 μg/mL, the maximum and minimum values are 110.44 and 103.25 μg pDNA/mL gel respectively. A similar behaviour was found for the other concentrations except for the highest concentration and flow rate since this value greatly deviates from the predictive pattern (Table 1). The flow rate dependence of the DBC is a central criterion for productivity, optimization, throughput, and stationary phase application [35]. Therefore, a flow-unaffected DBC is particularly important for the purification of molecules on a preparative and industrial level, since productivity is a crucial feature. The results presented in Table 1 show that DBC increases with increasing pDNA concentration, except in the case of the highest flow rate. For 1 mL/min flow rate, DBC starts in 103.25 μg pDNA/mL gel for 50 μg/mL of pDNA, then increases to 208.13 μg pDNA/mL gel when the pDNA concentration rises up to 100 μg/mL and reaches the maximum value of 336.75 μg pDNA/mL gel for a pDNA concentration of 150 μg/mL (Table 1). A similar pattern is found in previous plasmid-based works using CDI monolithic chromatography [36], membrane chromatography [37] and histidine chromatography [31]. Upon an increase in pDNA concentration, the chains undergo significant changes in their thermodynamic, dynamic, and structural behaviour. At high pDNA concentrations, the coils become squeezed due to mechanical deformations, decreasing their radius of gyration and reducing in size [33]. Therefore, in this more compact form, each adsorbed DNA molecule covers a smaller surface area, leading to higher DBC values.

The capacity of the stationary phase significantly depends on the accessible active surface. The studies of Benčina and co-workers showed that an 11× decrease in group density resulted in a 30% decrease in capacity [34]. Consequently, the DBC of the support can be improved by increasing the ligand density. In our case, this could be achieved using a higher DAPP:Sepharose ratio when preparing the support (see Section 3.1). Although the ligand density has a major impact on the recovery, it is more advantageous to work at the group density providing maximum recovery in the purification process [34].

With the purpose of quantitatively evaluate the column loading and determine the dissociation constant (K_d) between DAPP-Sepharose support and pDNA, frontal analysis was used [38]. This method assumes a Langmuirian behaviour and is based on the following relationships between K_d and the chromatographic parameters:

$$V - V_0 = \frac{m_L}{K_d + C} \tag{3}$$

where m_L is the total number of available binding sites in the column, V the volume (mL) of pDNA solution loaded at 50% breakthrough, V_0 the void volume (mL) of the column at 50% breakthrough and C the pDNA concentration (μg/mL) in the feedstock. Equation (3) can be rearranged giving the following form:

$$\frac{1}{C(V - V_0)} = \left(\frac{K_d}{m_L}\right)\left(\frac{1}{C}\right) + \left(\frac{1}{m_L}\right) \tag{4}$$

According to Eq. (4), a plot of $1/[C(V-V_0)]$ versus $1/C$ should yield a straight line with a slope corresponding to K_d/m_L and ordinate intercept corresponding to $1/m_L$ [38]. The interaction of pDNA and DAPP-Sepharose support was quantified using the breakthrough curves obtained in the experiments testing different pDNA concentrations (50, 100 and 150 μg/mL). Using Eq. (4), the m_L value was found to be $1.83 \pm 0.17 \times 10^{-10}$ mol and K_d was $2.29 \pm 0.195 \times 10^{-7}$ M. The optimal range of K_d for affinity chromatography lies between 10^{-4} and 10^{-8} M. The risk of irreversible molecule adsorption and denaturation are minimized when the K_d value is in this range. At lower K_d values the risk of pDNA damage increases and at higher K_d values the interaction with surface ligands may not be strong enough to retain the pDNA molecules [2]. The obtained K_d value is in the range of these optimal values and very close to 10^{-8} M, revealing a good affinity interaction between DAPP and pDNA.

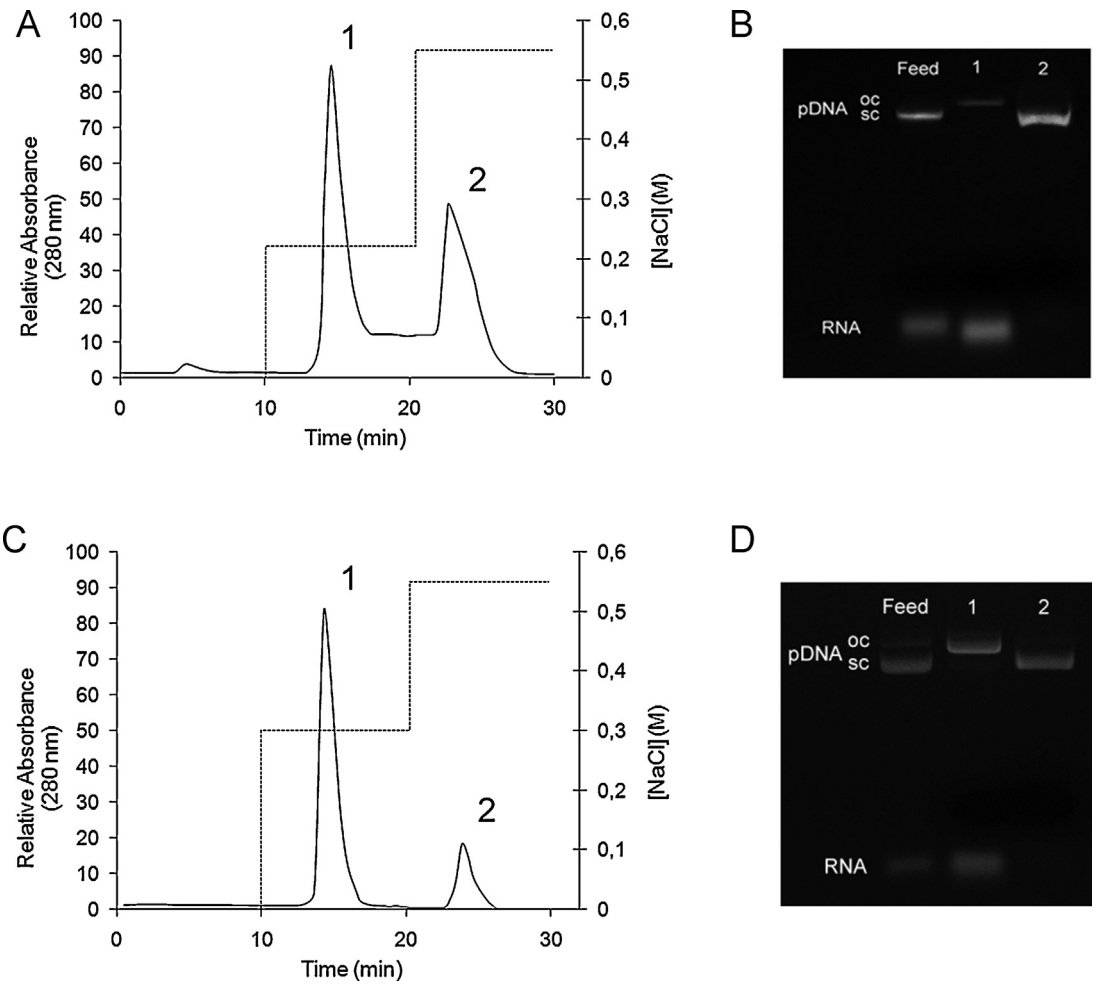

Fig. 3. Supercoiled pVAX1-LacZ (6.05 kbp) and pCAMBIA-1303 (12.361 kbp) chromatographic separation (25 μL) from host cell impurities present inclarified feed solutions, using DAPP-Sepharose support. Agarose gel electrophoresis analysis of pDNA fractions. Peak 1 and electrophoresis lane 1: impurities eluted with 0.22 M of NaCl for pVAX1-LacZ and 0.3 M NaCl for pCAMBIA-1303, in 10 mM sodium acetate buffer pH 5; Peak 2 and electrophoresis lane 2: sc pDNA fractions collected after elution with 0.55 M NaCl in 10 mM sodium acetate buffer pH 5. The clarified lysate was also run in the agarose gel for comparative purposes (lane feed).

3.3. DAPP-Sepharose affinity chromatography

The DAPP-Sepharose was previously used as an AC stationary phase for sc pDNA specific separation from the less active oc and linear isoforms [14]. The present work reports the subsequent application of this stationary phase in purification of sc pDNA from clarified cell lysate solutions of two plasmid molecules with different sizes: pVAX1-LacZ and pCAMBIA-1303 with 6.05 kbp and 12.361 kbp, respectively. After the equilibration of the column with 10 mM sodium acetate buffer pH 5, each of the clarified feedstock samples were injected separately using the same buffer. Fig. 3 shows the chromatographic profile and the agarose gel electrophoresis analysis of the eluted fractions for the studied plasmid molecules. In both cases, although there was no salt in the eluent buffer, a total retention of all lysate components was achieved (Fig. 3A and C). Elution of the species was simply performed by adding small quantities of sodium chloride to the eluent. For the smaller plasmid molecule pVAX1-LacZ, elution of the slightly bound impurities was achieved by adding 0.22 M of NaCl to the eluent (Fig. 3A). The impurities of pCAMBIA-1303 feed were eluted

Table 2
HPLC analysis of purity, recovery yield and quantitative analysis of pDNA, proteins, RNA, endotoxins and gDNA in feed and plasmid samples after DAPP-Sepharose AC. All measures were done for the two different sized plasmids: pVAX1-LacZ (6.05 kbp) and pCAMBIA-1303 (12.361 kbp).

Parameter	pVAX1-LacZ		pCAMBIA-1303	
	Feed	sc pDNA fraction[a]	Feed	sc pDNA fraction[a]
Purity (%)	54	100	21	100
Purification factor	–	1.90	–	4.80
Yield (%)	–	94	–	65
pDNA (μg/mL)	177.21	43.17	218.32	25.29
Protein (μg/mL)	259	Undetectable	216	Undetectable
RNA (mass%)	46	Undetectable	79	Undetectable
Endotoxins (EU/mL)	2.03	0.14	1.97	0.14
gDNA (μg/mL)	13.34	0.36	6.82	0.14

[a] Values extrapolated from results obtained with several injections.

Table 3
Comparison of plasmid samples composition with FDA specifications.

Impurity	FDA specifications [12]	pVAX1-LacZ collected sample	pCAMBIA-1303 collected sample
Protein (%)	Preferably <1	Undetectable	Undetectable
RNA (%)	Preferably <1	Undetectable	Undetectable
Endotoxins (EU/mg pDNA)	<40	3	5
gDNA (%)	Preferably <1	0.82	0.55

with 0.3 M NaCl in the eluent (Fig. 3C). For both cases, oc pDNA and RNA elute together into the first peak, which is confirmed by electrophoresis images (peak 1/lane 1 of Fig. 3A/B and C/D). Finally, strongly bound sc pDNA elutes after increasing NaCl concentration to 0.55 M (peak 2/lane 2 of Figs. 3A/B and 4C/D). After analysing the electrophoresis bands (Figs. 3B and 4D), it was concluded that oc pDNA isoform and RNA were not detected in both sc fractions (lane 2 in Fig. 3B and D). These results show that DAPP-Sepharose has a greater affinity towards the sc pDNA isoform than to the other species of the lysate solutions. The influence of temperature in pDNA purification using DAPP-Sepharose support was also studied. Besides the room temperature (26 °C) assays, additional chromatographic runs were also performed at 3 °C and 15 °C. Results show that at low temperatures (3 °C) the support loses its capability to separate pDNA from RNA, since a small amount of that impurity appears in the sc pDNA fraction after elution with 0.55 M of NaCl (results not shown). The results obtained at 15 °C were more similar to those obtained at room temperature, however a residual amount of RNA still elutes with sc pDNA. At room temperature, RNA molecules elute completely in the oc fraction, with no contamination of the sc isoform. These experimental observations led to the conclusion that the column performance is enhanced by increasing the temperature of the assays. This effect of temperature on the column efficiency may be due to a decrease in solvent/eluent viscosity, resulting in an increased of solute diffusivity which improves mass transfer through the stationary phase [39].

An epoxy-activated Sepharose gel obtained using the same curing method but without DAPP was used for the control experiments (results not shown). A completely different chromatographic pattern was observed when using this matrix with the optimized buffer conditions: a total retention of the species was not obtained without salt and the sc pDNA was not separated. Accordingly, these experiments unequivocally identified DAPP bonded onto Sepharose as the ligand responsible for the retention and separation of pDNA molecules. Moreover, the high number of chromatographic runs performed did not cause any changes in the chromatographic performance of the derivatized gel as well as in plasmid recovery.

3.4. Plasmid quality and purity assessment

The quality of the final sc pDNA product was determined by performing agarose gel electrophoresis, HPLC analysis, quantification of proteins (BCA assay), endotoxins (Chromogenic Limulus amoebocyte lysate Endotoxin assay) and gDNA (real-time PCR) (Table 2). Agarose gel electrophoresis (Fig. 3B and D) proved that the sc pDNA fraction of both plasmid molecules was separated from the oc isoform, and along with HPLC analysis, confirmed that RNA was fully isolated from the sc fractions. The HPLC purity of both plasmid pools was 100%, corresponding to a purification factor of 1.9 for pVAX1-LacZ and 4.8 for pCAMBIA-1303 (Table 2). The recovery yield of pVAX1-LacZ plasmid pool (94%) was higher than the one of pCAMBIA-1303 (65%). This is possibly due to the fact that larger plasmids are more unstable and degrade more easily during the extraction and purification procedures, since they are more sensible to shear forces [33] and more susceptible to losses during the chromatographic step. Furthermore, the 94% recovery yield for sc pVAX1-LacZ is much higher than the one obtained with a similar chromatographic process (79%) using the amino acid arginine as affinity ligand [40] or when compared with the recovery obtained with a CDI monolith as stationary phase (74%) [41]. Results from the BCA protein assay indicated that the obtained plasmid solutions had undetectable levels of proteins (Table 2). Real-time PCR analysis of pooled fractions showed a great reduction of gDNA content after the DAPP-Sepharose chromatographic step. Moreover, the endotoxins level was also extremely low (Table 2).

Quality analysis of final pooled fractions showed that the AC process here presented is able to produce pDNA that meets the specifications of the regulatory agencies, namely FDA (Table 3) [12]. Proteins and RNA (preferably <1%) are undetectable in the final solutions and the endotoxin levels are much lower than the maximum required by FDA (40 EU/mg pDNA). Genomic DNA content for both small and higher size plasmids is also under FDA specifications (preferably <1%).

4. Conclusions

Sepharose derivatized with DAPP was prepared and used as an AC stationary phase for sc pDNA separation and purification. The preparation of this support used a mild curing method without a catalyst, and with a 1:6 weight ratio of ligand:Sepharose, resulting in a ligand density of 0.15 mmol DAPP/g derivatized Sepharose (42.8 mg DAPP/g derivatized Sepharose). Moreover, FT-IR indicated the presence of DAPP linked to Sepharose and SEM analysis showed the preservation of the original beaded Sepharose morphology after the derivatization process. The performance of pDNA-ligand adsorption was characterized by DBC, which leads to a maximum value of 336.75 μg pDNA/mL gel obtained at 1 mL/min with a pDNA concentration of 150 μg/mL. Furthermore, the K_d value of $2.29 \pm 0.195 \times 10^{-7}$ M shows that DAPP-Sepharose can be applied as affinity chromatographic support.

This new AC stationary phase was used to purify sc pDNA molecules with different sizes from clarified *E. coli* lysate solutions. The chromatographic process used a buffer without salt to retain all species, whereas the elution was carried out by adding small amounts of sodium chloride to the eluent. The purification process using DAPP-Sepharose AC enabled the recovery of 94% of loaded pVAX1-LacZ (6.05 kbp) and 65% of pCAMBIA-1303 (12.361 kbp). The final product presents a HPLC purity of 100% and is in accordance with FDA specifications. The overall process uses low amounts of salt in the eluent and provides a high quality sc pDNA in a single chromatographic step.

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Chapter 4

Patent

RESUMO

Suporte cromatográfico de afinidade para purificação de DNA plasmídico superenrolado utilizando 3,8-diamino-6-fenilfenantridina (DAPP) como ligando

A presente invenção refere-se a uma nova fase estacionária para a purificação de DNA plasmídico (pDNA) superenrolado, a partir de lisados celulares, utilizando um processo de cromatografia de afinidade. A fase estacionária consiste numa matriz de Sepharose epoxi-ativada à qual foram imobilizadas moléculas de um intercalante do DNA, DAPP ou 3,8-diamino-6-fenilfenantridina, que apresenta uma elevada afinidade com o pDNA. Usando este novo suporte, o método cromatográfico pode compreender as seguintes fases: preparação do suporte cromatográfico DAPP-Sepharose, preparação da amostra biológica para extração do pDNA e purificação do pDNA superenrolado aplicando o suporte DAPP-Sepharose numa técnica de cromatografia de afinidade.
Desta forma, a presente invenção é útil para a obtenção rápida e eficiente de pDNA superenrolado de elevado grau de pureza, para fins terapêuticos.

DESCRIÇÃO

Suporte cromatográfico de afinidade para purificação de DNA plasmídico superenrolado utilizando 3,8-diamino-6-fenilfenantridina (DAPP) como ligando

Domínio da invenção

A presente invenção diz respeito a um suporte inovador para a purificação de DNA plasmídico superenrolado, para aplicação terapêutica, por um processo de cromatografia de afinidade. A molécula específica utilizada como ligando, o 3,8-diamino-6-fenilfenatridina (DAPP), apresenta uma elevada afinidade para o pDNA superenrolado, permitindo a sua eficaz separação dos contaminantes que usualmente estão presentes nas misturas a purificar, bem como de isoformas menos ativas como a circular aberta e linear. Esta invenção pertence ao domínio da Biotecnologia/Engenharia Bioquímica.

Sumário da invenção

O objectivo da presente invenção é a preparação de pDNA superenrolado de alta qualidade, através do uso de uma nova fase estacionária em cromatografia de afinidade. Para isso, tira-se partido da elevada afinidade que a molécula DAPP apresenta para com o DNA, utilizando-se a mesma como ligando numa matriz cromatográfica.
A presente invenção é útil para a obtenção de DNA plasmídico de elevado grau de pureza, para utilização futura como vetor terapêutico em Terapia Génica ou Vacinas de DNA.

Estado da técnica

As terapias moleculares como a Terapia Génica e as Vacinas de DNA têm sido estabelecidas, de forma crescente, como alternativas promissoras aos tratamentos médicos clássicos. Ambas as metodologias se baseiam na introdução de uma molécula terapêutica de ácido nucleico nas células ou tecidos a tratar, através da utilização de um vetor. O organismo ou tecido recetor adquire a capacidade de sintetizar, ele próprio, o agente terapêutico através da informação recebida. Desta forma, o objetivo é reparar o genoma, substituindo a região defeituosa pela correcta, introduzindo novos genes, ou modificando a expressão dos existentes.

Inicialmente, os vetores virais apresentavam-se como os mais populares e aqueles com os quais estava a ser desenvolvido mais trabalho de investigação. No entanto, a segurança deste tipo de vetores tem sido ultimamente posta em causa, pois para além de poderem desencadear uma resposta imunitária aguda, levando à neutralização do vírus e destruição do DNA terapêutico, existe ainda a possibilidade da ativação de oncogenes.
Desta forma, vetores não virais como o pDNA estão a emergir como potentes alternativas, tanto em termos de segurança, como em simplicidade de utilização, facilidade de produção em larga escala e baixo custo. Assim, é de esperar que nos próximos anos exista um aumento cada vez maior na procura de grandes quantidades de pDNA de pureza elevada.
De um modo geral, o processo de manufatura de pDNA inclui um passo de construção do plasmídeo, com o gene alvo a reparar, crescimento celular, geralmente em *Escherichia coli*, extração por lise celular, recuperação primária e vários passos de purificação, que visam a separação do pDNA de outros componentes celulares como o RNA, o DNA genómico, proteínas e endotoxinas.

Nos últimos anos tem-se observado um grande esforço no desenvolvimento de novos métodos para a purificação de pDNA, usando inovadores suportes cromatográficos, a maioria dos quais aplicados em processos de alta resolução. Foram ainda descritos outros procedimentos como a precipitação seletiva, ultrafiltração ou sistemas de duas fases aquosas.
Têm sido igualmente utilizados vários tipos de cromatografia para purificar o pDNA, baseados nas diferenças de tamanho, carga, hidrofobicidade e afinidade de diferentes moléculas numa mistura. Apesar de bastante adequados e promissores, os métodos baseados na afinidade ainda não foram devidamente explorados. A cromatografia de afinidade e de pseudo-

afinidade é definida como uma técnica de cromatografia líquida, que faz uso de interações biológicas específicas para a separação e análise de diferentes espécies numa amostra. A utilização desta abordagem cromatográfica requer que, primeiro, se obtenha um agente de ligação, conhecido como ligando de afinidade, o qual interage seletivamente com a espécie desejada e que esse ligando seja depois imobilizado num suporte sólido, ficando pronto a ser utilizado na separação e quantificação de biomoléculas. Desta forma, o ligando, neste caso a molécula de DAPP, é o fator chave que determina o sucesso de qualquer método de afinidade.

Estão descritos casos de sucesso para a purificação de pDNA utilizando proteínas como ligandos (Hasche e Voß, 2005) contudo, para além do seu custo e tamanho elevados, o plasmídeo alvo teria de conter uma sequência de elevada afinidade para essas moléculas, o que limita e complica a aplicação desta metodologia. Assim sendo, torna-se necessário utilizar outros ligandos, de preço mais acessível e pequeno tamanho. Tendo isto em conta, foram desenvolvidas técnicas de cromatografia de afinidade e pseudo-afinidade usando diferentes tipos de ligandos que, apesar de bastante promissores, apresentam ainda algumas desvantagens que deverão ser ultrapassadas, como a baixa capacidade dos suportes e o baixo rendimento dos processos (Sousa *et al.*, 2008).

Pequenas moléculas que apresentam uma elevada afinidade e especificidade para com o pDNA representam uma alternativa interessante aos ligandos existentes. O ligando de "minor groove" berenil foi já usado com sucesso para separar a isoforma superenrolada do pDNA da menos ativa circular aberta (Caramelo-Nunes *et al.*, 2011) bem como para purificar o pDNA das impurezas que estão presentes nos lisados celulares (Caramelo-Nunes *et al.*, 2012). O intercalante 3,8-diamino-6-fenilfenatridina (DAPP) é uma pequena molécula que apresenta uma elevada afinidade para com o DNA (Misra and Honig, 1995). Assim, esta apresenta-se como uma alternativa promissora a ser usada como ligando em processos de cromatografia de afinidade que visam a purificação de pDNA a partir de misturas celulares complexas.

As patentes US6730781 (B1) e US2004157244 (A1) dizem respeito a métodos cromatográficos de purificação de DNA plasmídico para uso terapêutico. A presente invenção destaca-se das anteriormente referidas pelo facto de usar apenas um passo cromatográfico com o suporte DAPP-Sepharose após a clarificação dos lisados celulares, para obtenção de pDNA de elevado grau de pureza. Consiste assim num método menos dispendioso em termos custos e tempo de operação, por ser mais simples, rápido e usar uma menor quantidade de reagentes.

Descrição geral da invenção

A presente invenção refere-se a um novo suporte cromatográfico, que faz uso da elevada afinidade que a molécula DAPP tem para com o DNA, sendo por isso adequado para a purificação de pDNA. O DAPP liga-se à dupla cadeia de DNA através do encaixe dos seus anéis

aromáticos entre os pares de bases do ácido nucleico, enquanto que o grupo fenilo é encaixado no "minor groove". Apesar de menos importantes, também se estabelecem pontes de hidrogénio entre os grupos amina do DAPP e os grupos fosfato do DNA. No seu conjunto, estas interações permitem a separação eficiente dos outros constituintes dos lisados celulares, como o RNA, endotoxinas, DNA genómico (gDNA) e proteínas. A diferente afinidade do ligando DAPP para o pDNA superenrolado e para os diferentes contaminantes permite a sua separação seletiva, já que a fração constituída pelos contaminantes é recolhida em primeiro lugar, enquanto o pDNA superenrolado permanece ligado à coluna. Este é posteriormente eluído, com um excelente grau de pureza, por aumento da força iónica do eluente. Para além da separação dos contaminantes referidos, verifica-se também a separação da isoforma superenrolada das restantes menos ativas como a circular aberta e linear. Estas eluem no primeiro pico em conjunto com o RNA, proteínas, gDNA e endotoxinas. Desta forma, o suporte DAPP-Sepharose apresenta uma maior afinidade para com o pDNA superenrolado.

O suporte cromatográfico foi preparado por ligação covalente do DAPP à Sepharose CL-6B, previamente ativada com 1,4-bis-(2,3-epoxipropóxido) de butilo, para introdução de grupos epóxido no polímero de agarose hidroxílico. Durante a imobilização, um dos grupos amina da molécula de DAPP reage com um grupo epóxido do suporte, estabelecendo desta forma a ligação covalente.

Apesar de já terem sido descritos vários suportes para a purificação de pDNA por cromatografia de afinidade, o aqui proposto apresenta vantagens em termos de consumo de reagentes, tempo dispendido e rendimento do processo ao qual está associado.
Em termos gerais, o processo é composto pelos seguintes passos:

1. Preparação do suporte cromatográfico DAPP-Sepharose. A Sepharose CL-6B é inicialmente epoxi-ativada, resultando a incorporação de braços espaçadores epóxidos que permitirão a posterior ligação da molécula de DAPP;
2. Preparação da amostra biológica para extração do pDNA. Após o crescimento celular, a amostra obtém-se por um método de lise celular alcalina e consequente precipitação com isopropanol. Depois de um passo de centrifugação, o *pellet* de amostra (principalmente ácidos nucleicos e proteínas) ressuspende-se numa solução tampão de acetato de sódio, pH 5 (pH de trabalho). A amostra de DNA plasmídico obtida é submetida a um passo de clarificação, no presente caso por precipitação dos contaminantes com uma quantidade adequada de sulfato de amónio. No final, a amostra é lavada com o mesmo tampão sem sal de forma a adequar a força iónica da solução para o passo seguinte;
3. Purificação do pDNA superenrolado por cromatografia de afinidade usando o suporte DAPP-Sepharose:

a) Ligação de todos os constituintes do lisado celular ao suporte cromatográfico. Injecta-se uma amostra do lisado obtido anteriormente (com clarificação), na coluna ligada a um sistema de cromatografia líquida, empacotada com o suporte DAPP-Sepharose e previamente equilibrada com o tampão adequado sem sal. Neste passo, o pDNA e as impurezas ligam-se à coluna na sua totalidade.
b) Eluição das impurezas e outras isoformas de pDNA. Ao introduzir uma concentração adequada de cloreto de sódio (NaCl) no tampão, as impurezas, bem como as isoformas linear e circular aberta, são eluídas e separadas do pDNA;
c) Obtenção do pDNA superenrolado. Com um único passo de eluição, após aumento da concentração de cloreto de sódio no tampão, obtém-se a fração de pDNA superenrolado com um grau de pureza bastante elevado.

Descrição das figuras

Figura 1: Cromatograma correspondente à separação do plasmídeo pVAX1-LacZ das impurezas do lisado celular utilizando o suporte DAPP-Sepharosee após ligação de todos os constituintes celulares com tampão acetato 10 mM pH 5, no qualo pico (1) corresponde às impurezas eluídas com 0,22 M de NaCl no mesmo tampão e o pico (2) corresponde ao pDNA superenrolado eluído após aumento da concentração de NaCl para 0,55 M.

Figura 2: Cromatograma correspondente à separação do plasmídeo pCAMBIA-1303 das impurezas do lisado celular utilizando o suporte DAPP-Sepharosee após ligação de todos os constituintes celulares com tampão acetato 10 mM pH 5, no qual o pico (3) corresponde às impurezas eluídas com 0,3 M de NaCl no mesmo tampão e o pico (4) corresponde pDNA superenrolado eluído após aumento da concentração de NaCl para 0,55 M.

Figura 3: Gel de electroforese em agarose, onde são analisadas as frações de pVAX1-LacZ recolhidas ao longo do processo cromatográfico, na qual a linha (5) representa a amostra de lisado clarificado injetado, a linha (6) representa as impurezas e pDNA circular aberto recolhidas após eluição com tampão acetato, pH 5, com 0,22 M de NaCl e a linha (7) representa a fração de pDNA superenrolado recolhida após eluição com tampão com 0,55 M de NaCl.

Figura 4: Gel de electroforese em agarose, onde são analisadas as fracções de pCAMBIA-1303 recolhidas ao longo do processo cromatográfico, na quala linha (8) representa a amostra de lisado clarificado injetado, a linha (9) representa as impurezas e pDNA circular aberto recolhidas após eluição com tampão acetato, pH 5, com 0,3 M de NaCl e a linha (10) representa a fração de pDNA superenrolado recolhida após eluição com tampão com 0,55 M de NaCl.

Descrição detalhada da invenção

A presente invenção diz respeito a um suporte cromatográfico inovador para a purificação de DNA plasmídico, o qual faz uso da elevada afinidade que a molécula de DAPP tem para com o DNA e que compreende os seguintes passos:

1. Preparação do suporte cromatográfico DAPP-Sepharose. Numa primeira fase, a Sepharose CL-6B comercial é epoxi-ativada através de uma reacção com 1,4-bis-(2,3-epoxipropóxido) de butilo, em condições específicas de temperatura e agitação. O gel resultante é depois adicionado a uma solução de DAPP e carbonato de sódio em condições de temperatura e agitação específicas, permitindo a ligação covalente do ligando ao suporte epoxi-ativado;

2. Preparação da amostra biológica para extração do pDNA. Duas estirpes de *Escherichia coli*, a DH5α e a XL1blue contendo respectivamente o plasmídeo pVAX1-LacZ de 6,05 Kpb e o plasmídeo pCAMBIA-1303 de 12,361 Kpb, são cultivadas em condições adequadas de meio de cultura, temperatura e pH. Após o processo de lise alcalina das células de *E. coli*, procede-se a um passo de concentração por precipitação com isopropanol sob concentração e condições adequadas. As amostras são recuperadas por centrifugação e os *pellets* resultantes redissolvem-se num volume adequado de solução tampão (acetato 10 mM, pH 5). Os lisados de ambos os plasmídeos são clarificados, neste caso, por adição de sulfato de amónio sólido até uma concentração de 2,5 M, sendo o precipitado resultante posteriormente removido por centrifugação. As amostras de lisado clarificado são depois lavadas com tampão acetato sem sal de forma a adequar a força iónica da solução para o passo seguinte. Este passo pode ser realizado usando diferentes tipos de culturas, meios, processos de lise e clarificação, de acordo com os plasmídeos a purificar e com a disponibilidade e conveniência de materiais para cada caso. O método de preparação das amostras aqui descrito é apenas um exemplo, podendo ser aplicados outros processos;

3. Purificação do pDNA superenrolado por cromatografia de afinidade usando o suporte DAPP-Sepharose. Este tipo de cromatografia explora vários tipos de interações entre o ligando cromatográfico e os constituintes dos lisados celulares, nomeadamente o pDNA. Especificamente, explora diferenças na carga e hidrofobicidade, bem como outras ligações específicas, entre o pDNA de dupla cadeia, o DNA genómico e os ácidos nucleicos de cadeia simples (RNA, oligonucleótidos e formas desnaturadas de pDNA) que constituem a grande maioria das impurezas e as endotoxinas (lipopolissacarídeos). Este processo compreende os seguintes passos:

    a. Ligação de todos os constituintes do lisado celular ao suporte cromatográfico - Injeta-se uma diluição da amostra biológica clarificada (obtida no passo 3) numa coluna empacotada com o suporte DAPP-Sepharose, através de um sistema de

cromatografia líquida. A coluna deverá ter sido previamente equilibrada com uma solução tampão acetato 10 mM, pH 5. Nestas condições, o pDNA e contaminantes ficam retidos na coluna;

b. Obtenção do pDNA e separação dos contaminantes celulares - Após retenção de todos os constituintes dos lisados no suporte cromatográfico, procede-se à eluição dos contaminantes e isoformas de pDNA menos ativas usando o tampão acetato 10 mM, pH 5 com 0,22 M de NaCl no caso do pVax1-LacZ e 0,3 M para o pCAMBIA-1303. O pDNA superenrolado é depois eluído num passo direto, por aumento da concentração de NaCl no tampão para 0,55 M, em ambos os casos. As frações de ambas as moléculas de plasmídeo recolhidas são constituídas por pDNA de elevado grau de pureza. Seguidamente procede-se ao re-equilíbrio do suporte com a solução tampão acetato 10 mM pH 5 sem sal, para preparar a coluna para uma nova injeção. A amostra de pDNA apresenta um teor salino relativamente baixo, podendo ser dessalinizada por um método apropriado.

Exemplos de aplicação

Exemplo 1:

Purificação por afinidade do plasmídeo pVAX1-LacZ (6,05 Kpb) a partir de lisado clarificado de Escherichia coli utilizando o suporte cromatográfico DAPP-Sepharose

Células de *Escherichia coli* foram cultivadas em meio LB (250 ml) a 37 ºC e 250 rpm até uma densidade ótica de aproximadamente 10. A amostra de lisado clarificado foi preparada por lise alcalina das células segundo o método de Sambrook e Maniatis (Sambrook *et al.*, 1989). O lisado obtido foi submetido depois a uma precipitação com 0,7 volumes de isopropanol e incubado em gelo durante 30 minutos. O pDNA, RNA e outros constituintes como as proteínas, foram recuperados por centrifugação a 16.000 g durante 30 minutos, a 4 ºC. O *pellet* foi depois redissolvido em 1 mL de solução tampão acetato 10 mM, pH 5. O pDNA presente nessa solução foi clarificado por adição de sulfato de amónio sólido até uma concentração de 2,5 M. A solução foi incubada em gelo durante 15 minutos e centrifugada a 10.000 g durante 20 minutos, descartando-se o *pellet* resultante. A amostra foi depois lavada com tampão acetato sem sal de forma a adequar a força iónica da solução para o passo seguinte.

As moleculas de DAPP foram imobilizadas, por ligação covalente, a Sepharose CL-6B previamente epoxi-ativada segundo o método descrito por Sudberg e Porath (Sundberg e Porath, 1974). A 5 g de Sepharose CL-6B foram adicionados 5 mL de solução de hidróxido de sódio 0,6 M, contendo 50 mg de borohidreto de sódio, e 5 mL de 1,4-bis-(2,3-epoxipropóxido) de butilo. A mistura foi agitada a 25 ºC durante 8 horas num banho de agitação orbital. O gel epoxi-ativado foi depois lavado com grandes volumes de água desionizada. Após ter sido filtrado até se apresentar quase seco, 3 g de gel foram adicionados a 4 mL de uma solução 2 M de carbonato de sódio contendo 0,5 g de DAPP. Após ter sido agitada durante 16 horas a 70

ºC, num banho de agitação orbital, a Sepharose derivatizada foi lavada com grandes volumes de água desionizada e solução de etanol a 70%.

Os estudos cromatográficos foram realizados num sistema de FPLC (Fast Protein Liquid Chromatography) à temperatura ambiente. Uma coluna de 10 x 10mm foi empacotada com 3mL de suporte derivatizado e equilibrada com solução tampão acetato 10 mM pH 5 sem sal, a um fluxo de 1 mL/min. 25 µL de amostra de lisado clarificado com uma concentração de 600 µg de ácidos nucleicos/mL em tampão de equilíbrio foram injectados a um fluxo de trabalho de 1 mL/min. A absorvância foi continuamente monitorizada a 280 nm. As espécies contaminantes foram eluídas usando tampão acetato 10 mM pH 5, com 0,22 M de cloreto de sódio. O pDNA superenrolado foi eluído, num único passo, aumentando a concentração de sal para 0,55 M, no mesmo tampão. Recolheram-se frações de 0,5 mL em tubos de 1,5 mL. As frações de pDNA foram recolhidas segundo o cromatograma obtido (figura 1), concentradas e dessalinizadas usando para isso concentradores com um MWCO de 10.000 da Vivaspin (Vivascience). A análise das mesmas foi realizada por eletroforese em gel de agarose 1%, com 0,5 µg/ml de GreenSafe. Na figura 3 apresenta-se a análise das frações de pDNA recolhidas em gel de agarose 1%. A linha (5) corresponde à amostra de lisado clarificado, a linha (6) à fração de impurezas recolhidas após eluição com tampão acetato 10 mM, pH 5, com 0,22 M de cloreto de sódio e a linha (7) à fração de pDNA superenrolado recolhida após aumento da concentração de sal para 0,55 M.

As frações de pDNA recolhidas foram analisadas tendo em conta diferentes técnicas analíticas, tais como, a já referida eletroforese em gel de agarose, High Preformance Liquid Chromatography (HPLC), Real-Time PCR, análise da concentração de proteínas e análise do índice de endotoxinas. A massa de DNA plasmídico no lisado e amostra foi determinada por HPLC concluindo-se que o rendimento do passo cromatográfico ronda os 94%. Da mesma forma se verificou que a amostra apresenta um grau de pureza, por HPLC, de 100%, confirmando os resultados da eletroforese em gel de agarose, ou seja, a ausência de formas plasmídicas menos ativas ou desnaturadas e de RNA. A ausência de proteínas na amostra de pDNA foi confirmada através do método de Pierce® BCA. Os níveis de contaminação por DNA genómico (0,82%) foram determinados recorrendo à técnica de Real-Time PCR, verificando-se que estão abaixo do nível recomendado pelas agências reguladoras para as preparações a utilizar em terapias moleculares (1% massa gDNA/(massa gDNA + Massa pDNA)). Por sua vez, os níveis de endotoxinas na amostra foram determinados através do método de Limulus Amebocyte Lysate (LAL) Cromogénico e correspondem a 3 EU/mg de pDNA, encontrando-se muito abaixo do valor máximo aceite pelas agências reguladoras (< 40 EU/mg de pDNA).

Exemplo 2:

Purificação por afinidade do plasmídeo pCAMBIA-1303 (12,361 Kpb) a partir de lisado clarificado de Escherichia coli utilizando o suporte cromatográfico DAPP-Sepharose

Células de *Escherichia coli* foram cultivadas em meio LB (250 ml) a 37ºC e 250 rpm até uma densidade ótica de aproximadamente 10. A amostra de lisado clarificado foi preparada por lise alcalina das células segundo o método de Sambrook e Maniatis (Sambrook *et al.*, 1989). O lisado obtido foi submetido depois a uma precipitação com 0,7 volumes de isopropanol e incubado em gelo durante 30 minutos. O pDNA, RNA e outros constituintes como as proteínas, foram recuperados por centrifugação a 16.000g durante 30 minutos, a 4ºC. O *pellet* foi depois redissolvido em 1 ml de solução tampão acetato 10 mM, pH 5. O pDNA presente nessa solução foi clarificado por adição de sulfato de amónio sólido até uma concentração de 2,5 M. A solução foi incubada em gelo durante 15 minutos e centrifugada a 10.000g durante 20 minutos, descartando-se o *pellet* resultante. A amostra foi depois lavada com tampão acetato sem sal de forma a adequar a força iónica da solução para o passo seguinte.

O DAPP foi imobilizado, por ligação covalente, a Sepharose CL-6B previamente epoxi-ativada segundo o método descrito por Sudberg e Porath (Sundberg e Porath, 1974). A 5 g de Sepharose CL-6B foram adicionados 5 mL de solução de hidróxido de sódio 0,6 M, contendo 50 mg de borohidreto de sódio, e 5 mL de 1,4-bis-(2,3-epoxipropóxido) de butilo. A mistura foi agitada a 25ºC durante 8 horas num banho de agitação orbital. O gel epoxi-ativado foi depois lavado com grandes volumes de água desionizada. Após ter sido filtrado até se apresentar quase seco, 3 g de gel foram adicionados a 4 mL de uma solução 2 M de carbonato de sódio contendo 0,5 g de DAPP. Após ter sido agitado durante 16 horas a 70ºC, num banho de agitação orbital, a Sepharose derivatizada foi lavada com grandes volumes de água desionizada e solução de etanol a 70%.

Os estudos cromatográficos foram realizados num sistema de FPLC (Fast Protein Liquid Chromatography) à temperatura ambiente. Uma coluna de 10 x 10mm foi empacotada com 3mL de suporte derivatizado e equilibrada com solução tampão acetato 10 mM pH 5 sem sal, a um fluxo de 1 mL/min. 25 µL de amostra de lisado clarificado com uma concentração de 600 µg de ácidos nucleicos/mL em tampão de equilíbrio foram injetados a um fluxo de trabalho de 1 mL/min. A absorvância foi continuamente monitorizada a 280 nm. As espécies contaminantes foram eluídas usando tampão acetato 10 mM pH 5, com 0,3 M de cloreto de sódio. O pDNA superenrolado foi eluído, num único passo, aumentando a concentração de sal para 0,55 M, no mesmo tampão. Recolheram-se frações de 0,5 mL em tubos de 1,5 mL. As frações de pDNA foram recolhidas segundo o cromatograma obtido (figura 2), concentradas e dessalinizadas utilizando para isso concentradores com um MWCO de 10.000 da Vivaspin (Vivascience). A análise das mesmas foi realizada por eletroforese em gel de agarose 0,8%, com 0,5 µg/mL de GreenSafe. Na figura 4 apresenta-se a análise das frações de pDNA

recolhidas em gel de agarose 0,8%. A linha (8) corresponde à amostra de lisado clarificado, a linha (9) corresponde à fração de impurezas recolhidas após eluição com tampão acetato 10 mM, pH 5, com 0,3 M de cloreto de sódio e a linha (10) à fração de pDNA superenrolado recolhida após aumento da concentração de sal para 0,55 M.

As fracções de pDNA recolhidas foram analisadas tendo em conta diferentes técnicas analíticas, tais como, a já referida eletroforese em gel de agarose, High Preformance Liquid Chromatography (HPLC), Real-Time PCR, análise da concentração de proteínas e análise do índice de endotoxinas. A massa de DNA plasmídico no lisado e amostra foi determinada por HPLC concluindo-se que o rendimento do passo cromatográfico ronda os 65%. Da mesma forma se verificou que a amostra apresenta um grau de pureza, por HPLC, de 100%, confirmando os resultados da eletroforese em gel de agarose, ou seja, a ausência de formas plasmídicas menos ativas ou desnaturadas e de RNA. A ausência de proteínas na amostra de pDNA foi confirmada através do método de Pierce® BCA. Os níveis de contaminação por DNA genómico foram determinados recorrendo à técnica de Real-Time PCR, verificando-se que estão abaixo (0.55%) do nível recomendado pelas agências reguladoras para as preparações a utilizar em Terapias Moleculares (1% massa gDNA/(massa gDNA + Massa pDNA)). Por sua vez, os níveis de endotoxinas na amostra foram determinados através do método de Limulus Amebocyte Lysate (LAL) Cromogénico e correspondem a 5 EU/mg de pDNA, encontrando-se muito abaixo do valor máximo aceite pelas agências reguladoras (< 40 EU/mg de pDNA).

Exemplo 3:

A invenção aqui apresentada pode ser aplicada a nível industrial em larga escala, aumentando para isso as quantidades de gel imobilizado com DAPP. Este método irá permitir a purificação rápida e de baixo custo de pDNA para uso terapêutico. As quantidades de cloreto de sódio usadas são relativamente baixas, para além de se tratar de um sal com um potencial eutrófico baixo.
A este suporte cromatográfico podem ser aplicados lisados bacterianos após qualquer tipo de obtenção e clarificação, não estando condicionado aos apresentados anteriormente: lise alcalina e clarificação por precipitação com sulfato de amónio.

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DESENHOS

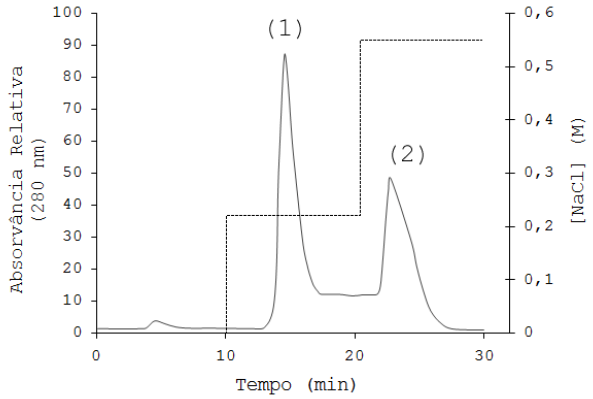

Figura 1

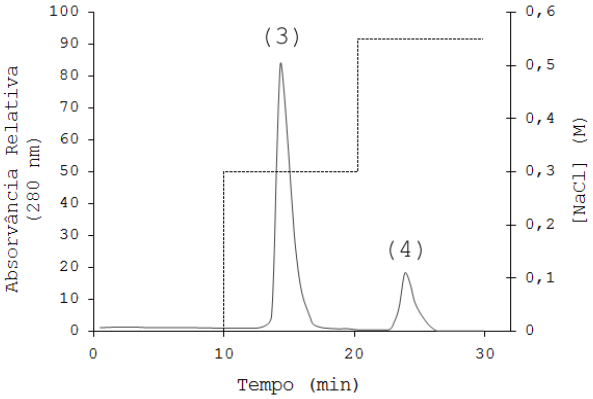

Figura 2

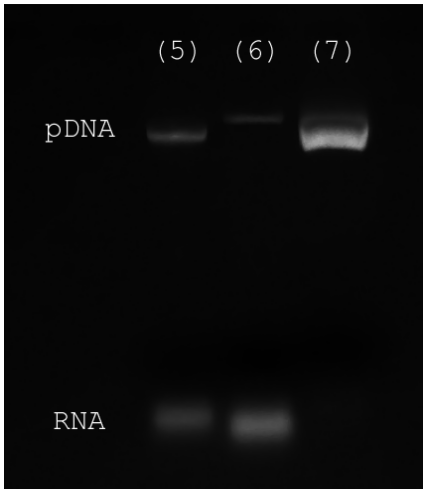

Figura 3

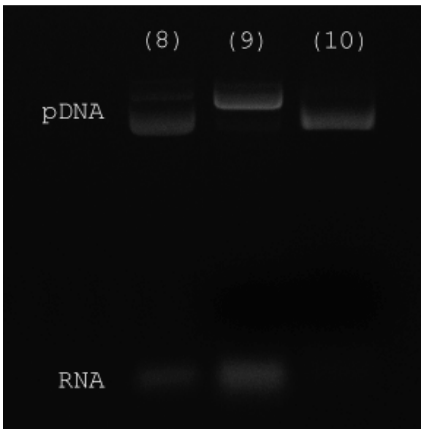

Figura 4

REIVINDICAÇÕES

1 – Suporte cromatográfico de afinidade DAPP-Sepharose, a aplicar na purificação de DNA plasmídico superenrolado a partir de lisados celulares, caraterizado por ser parte integrante de um método de purificação de pDNA superenrolado que compreende os seguintes passos:

a) Imobilização das moléculas de DAPP numa matriz de Sepharose epoxi-ativada;
b) Preparação das amostras de lisado celular por um método de lise das células bacterianas e clarificação dos lisados brutos;
c) Ligação do pDNA e dos restantes constituíntes do lisado celular ao suporte DAPP-Sepharose após injeção das amostras clarificadas, utilizando tampão acetato 10 mM, pH 5 sem sal;
d) Eluição simultânea dos contaminantes e isoformas menos ativas usando tampão acetato 10 mM, pH 5 com 0,22 M de cloreto de sódio para a amostra de pVAX1-LacZ e 0,3 M de cloreto de sódio para a amostra de pCAMBIA-1303;
e) Eluição e obtenção das amostras de pDNA superenrolado livres de contaminantes por aumento da concentração de cloreto de sódio para 0,55 M em tampão acetato 10 mM, pH 5.

2 - Suporte cromatográfico de afinidade DAPP-Sepharose, a aplicar na purificação de DNA plasmídico superenrolado a partir de lisados celulares, de acordo com a reivindicação 1 caraterizado por constituir uma fase estacionária com elevada afinidade e seletividade para com o pDNA superenrolado presente numa mistura complexa de contaminantes celulares, uma vez que é o último constituínte a ser eluído após adição de uma pequena quantidade de sal ao eluente.

3 - Suporte cromatográfico de afinidade DAPP-Sepharose, a aplicar na purificação de DNA plasmídico superenrolado a partir de lisados celulares, de acordo com as reivindicações 1 e 2 caraterizado por poder ser aplicado para a purificação de diferentes moléculas de DNA plasmídico, com diferentes pesos moleculares.

4 - Suporte cromatográfico de afinidade DAPP-Sepharose, a aplicar na purificação de DNA plasmídico superenrolado a partir de lisados celulares, de acordo com as reivindicações 1 e 2 caraterizado por poder ser aplicado no tratamento de soluções complexas de lisados celulares de diferentes estirpes bacterianas.

5 - Suporte cromatográfico de afinidade DAPP-Sepharose, a aplicar na purificação de DNA plasmídico superenrolado a partir de lisados celulares, de acordo com as reivindicações 1, 2, 3 e 4 caraterizado por permitir a separação e remoção de impurezas como o RNA, proteínas, endotoxinas e DNA genómico, das frações de pDNA superenrolado, com obtenção deste com um elevado grau de pureza.

6 - Suporte cromatográfico de afinidade DAPP-Sepharose, a aplicar na purificação de DNA plasmídico superenrolado a partir de lisados celulares, de acordo com as reivindicações 1, 2, 3 e 4 caraterizado por elevados rendimentos de recuperação do pDNA superenrolado.

7 - Suporte cromatográfico de afinidade DAPP-Sepharose, a aplicar na purificação de DNA plasmídico superenrolado a partir de lisados celulares, de acordo com as reivindicações 1, 2, 3, 4, 5 e 6 caraterizado por constituir uma fase estacionária adequada para a purificação de pDNA superenrolado à escala industrial.

Chapter 5

Concluding remarks and future trends

This work was performed with the objective of developing new and more efficient chromatographic processes for the purification of therapeutic grade pDNA.
Since non-viral pDNA based vectors are gaining importance in gene therapy clinical trials, there is a need to produce this therapeutic product in large quantities under the requirements of the regulatory agencies, and also with low economic and environmental impacts. However, this is not always easy, since the similarities between pDNA and the major lysate contaminants may difficult their separation. Moreover, the use of great quantities of salt in the eluent buffers may become a major drawback for large scale applications.
With this in mind, two small molecules known for binding DNA with considerable affinity, the diamidine berenil and the phenantridine derivative DAPP, where chosen to be used as ligands in pDNA chromatographic purification studies. They were both immobilized onto epoxy-activated Sepharose matrix using a relatively mild curing method, without a catalyst and with quite small ligand:Sepharose weight ratios.

Essentially, berenil establishes reversible non-covalent interactions with the floor and walls of the DNA minor groove with a preference for A-T sequences. It binds pDNA preferentially through hydrophobic interactions however, other contributions cannot be neglected, since they help to understand the binding profile obtained in this study. The pDNA binding affinity of berenil is quite influenced by salt type and concentration. Even though non-immobilized berenil showed a higher binding constant in the presence of low sodium chloride concentrations, the use of ammonium sulphate proved to be crucial in promoting total retention of all species in the berenil-Sepharose matrix. After injecting a mixture of the two pDNA isoforms (sc and oc) using average amounts of ammonium sulphate, they were separately obtained through two step wise elution by decreasing the salt concentration. In fact, sc pDNA was obtained after entirely removing the salt from the eluent buffer.
However, this isoform resolution is lost when complex lysate solutions are loaded. Possibly, the presence of great quantities of highly hydrophobic species like RNA, may interfere in the matrix ability to differently interact with the two pDNA isoforms. Given that these isoforms have similar hydrophobicity, they elute at the same time and RNA molecules stay tightly bound to the support.

The potential of berenil-Sepharose matrix to separate pDNA molecules from lysate impurities was explored using three different approaches: using one berenil chromatographic step with clarified lysate solutions of two distinct plasmids with different weights; loading a non-clarified lysate solution and performing two consecutive chromatographic step runs; and finally, loading a clarified lysate solution using a negative chromatography approach. For all

cases, the obtained pDNA sample was in agreement with the specifications for therapeutic pDNA.

The first approach led to the successful separation of both plasmids from host impurities showing that the method is quite reproducible and can be applied to plasmids of different weights. Interestingly, the purity of the larger pDNA molecule is superior, which could be appealing since future requirements for multigene vectors, including extensive control regions, may require the production of larger plasmids. However, the purification of the smaller plasmid showed a higher yield (85% vs 45%). This may be due to the fact that larger plasmids are more sensible to shear forces, degrading more easily during extraction and purification procedures.

The replacement of the ammonium sulphate clarification by a second berenil-Sepharose chromatographic step showed a few advantages such as reduction in salt usage and procedure steps, despite the recovery yield (33%) suffered an important reduction probably due to a dilution effect. Even though applying these classical positive approaches with berenil-Sepharose already made use of mild quantities of salt, the negative approach further diminishes salt usage and reduces procedure time. The recovery yield is quite good (87%) and although pDNA solutions have a comparable purity to that obtained with the other approaches, gDNA reduction is not so effective. Apparently, the total retention step is important to enhance the hydrophobic interactions between the berenil matrix and the bases of that mostly single-stranded host nucleic acid (due to alkaline lysis).

The intercalator DAPP is slightly A-T specific and binds DNA through non-covalent, reversible stacking interactions of the condensed aromatic moiety into two successive base pairs, while the phenyl residue gets inserted into the minor groove. However, when protonated, DAPP molecules bind to DNA much strongly than in the neutral form, whether in the free state or immobilized onto a Sepharose matrix, due to the generation of strong electrostatic interactions. Moreover, DAPP's protonation is visible through colour changes even when attached to the solid matrix.

The pDNA binding strength to DAPP-Sepharose varies with pH and is affected by the presence of salt in the eluent. In fact, total retention of pDNA molecules and other lysate components was only possible with a pH below DAPP's free state pKa (5.8) and the presence of salt destabilizes that same retention. Also, the studies performed showed that the binding affinity increases with temperature. Therefore, after retention of the species with a pH 5 buffer with no salt, the elution was performed simply by adding small amounts of sodium chloride to the buffers. By using this approach it was possible to achieve the purification of the sc pDNA isoform of plasmid molecules with two different sizes. This more active isoform was the last to elute only after the elution of the impurities.

The collected sc pDNA solutions were in accordance to the regulatory agencies specifications and, in similarity to what was observed with the berenil-Sepharose matrix, the recovery yield

was lower for the larger plasmid molecule (65% vs an impressive 94% for the smaller molecule).

The maximum dynamic binding capacity of DAPP-Sepharose for pDNA was 336.75 μg pDNA/mL gel, which is quite acceptable considering that it is a non-commercial support with such a low ligand density (0.15 mmol DAPP/g derivatized Sepharose). Of course that, in this particular case, improving the ligand density could be advantageous, since the capacity of the stationary phase significantly depends on the accessible active surface area. Still, this beaded-based support showed an interesting behaviour with flow rate variations, since its pDNA capacity stays unaffected by them. This behaviour is particularly important for the purification of molecules on a preparative and industrial level, since flow rate dependence of the dynamic binding capacity is a crucial feature for productivity, optimization, throughput, and stationary phase application. Moreover, the dissociation constant obtained ($2.29 \pm 0.195 \times 10^{-7}$ M) is an important evidence of the affinity of this matrix towards pDNA.

Hence, DAPP-Sepharose shows outstanding characteristics to be used as an affinity support for the purification of pharmaceutical grade sc pDNA. In comparison with berenil-Sepharose, it uses much smaller amounts of a salt with no environmental impact, while improving the quality of the obtained plasmid fraction. DAPP-Sepharose is able to separate sc pDNA from linear and oc isoforms even in complex lysates.

Moreover, combining DAPP-Sepharose chromatography with other optimized production, extraction and clarification procedures, can offer a number of advantages for pharmaceutical pDNA purification. Also, since the most significant disadvantage of this DAPP-Sepharose support is the relatively low capacity for pDNA, which in turn is strongly related to the solid matrix used, other more stable stationary phases with low pressure drops and interconnected macropores, that allow a high mass transfer of solutes, are quite fascinating alternatives.
In addition, the large scale application of DAPP as ligand for pDNA chromatographic purification is especially promising, either with Sepharose or with any other solid matrix.