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Sciences

Affinity chromatography in plasmid DNA purification for therapeutic applications

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Dedicatory

To my family

The greatest pleasure in life is doing what other people say you cannot do.

Walter Bagehot

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III. **Selectivity of arginine chromatography in promoting different interactions using synthetic oligonucleotides as model**
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IV. **Biorecognition of supercoiled plasmid DNA isoform in lysine-affinity chromatography**
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II. **Separation of open circular and supercoiled plasmid DNA isoforms by a new chromatographic strategy with lysine-agarose support**
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A. Sousa, D. Bicho, C.T. Tomaz, F. Sousa, J.A. Queiroz
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Resumo alargado

A investigação do genoma e proteoma humano tem fornecido informações fundamentais em relação às funções dos genes, assim como ao impacto das mutações desses genes na saúde humana. O conhecimento contínuo e crescente destas áreas tem conduzido à implementação de técnicas efectivas que usam genes como biofármacos para o tratamento de determinadas doenças incuráveis. O desenvolvimento de qualquer estratégia terapêutica baseada na entrega de genes requer uma avaliação precisa de vários parâmetros, incluindo a garantia de que uma dada doença pode ser realmente tratada usando esta estratégia, a escolha e obtenção do gene terapêutico correcto, o controlo da expressão do gene em células de mamíferos e a selecção do sistema de entrega e administração mais adequado. Na verdade, a sequenciação completa do genoma humano combinado com a compreensão das propriedades de vários genes relacionados com determinadas doenças tem posicionado a terapia génica ao nível somático e a vacinação génica entre os processos biotecnológicos mais interessantes.

A terapia génica pode ser vista como um sistema de expressão de proteínas específicas usando as células dos próprios pacientes como mini-biorreactores. Esta estratégia terapêutica permite não só repor os genes defeituosos ou inexistentes dentro das células dos pacientes, mas também suplementar o organismo com a produção de proteínas que podem prevenir ou até mesmo tratar a doença alvo. Por outro lado, o desenvolvimento de novas vacinas génicas, que têm a capacidade de induzir respostas imunes anti-patogénicas ou anti-tumorais, tem sido explorado para promover tratamentos profilácticos ou terapêuticos inovadores contra doenças infecciosas e cancro. Assim sendo, a terapia génica e as vacinas de DNA têm evoluído firmemente nos últimos anos, tornando-se numa nova classe terapêutica com potencial para prevenir, corrigir ou modelar doenças adquiridas ou genéticas.

Embora os vectores virais sejam usados na maioria dos ensaios clínicos, os vectores não virais, particularmente o vector de DNA plasmídico (pDNA), têm atraído uma atenção considerável como biofármacos quer na terapia génica, quer nas vacinas de DNA, devido à sua baixa toxicidade e imunogenecidade, assim como a sua obtenção segura, fácil e económica. Sabendo que o sucesso da transferência de genes para células eucarióticas e subsequente expressão do gene de interesse contido no pDNA é estritamente afectado pelo processo de obtenção do vector, torna-se essencial o desenvolvimento de novas plataformas que permitam a recuperação simples do vector, com um elevado número de cópias e grau de pureza satisfatório. Na verdade, o pDNA é maioritariamente amplificado no hospedeiro *Escherichia coli* (*E. coli*) sob a conformação superenrolada (sc). Uma vez que esta isoforma é a única conformação do pDNA naturalmente intacta e sem danos estruturais, sendo considerada por isso a isoforma mais activa e eficiente na transfecção e expressão de genes

em células eucarióticas, torna-se extremamente importante desenvolver estratégias adequadas para o isolamento e purificação desta isoforma do vector plasmídico.

Existem várias técnicas que podem ser aplicadas para obter a conformação sc, isolando esta isoforma das topologias não efectivas do plasmídeo, permitindo ainda a eliminação das impurezas do hospedeiro, que caso estejam presentes no produto final em quantidades superiores às recomendadas pelas agências reguladoras podem causar efeitos adversos e respostas inflamatórias nos pacientes. No entanto, o grande desafio para o sucesso destas etapas passa pela dificuldade em diferenciar a isoforma sc das outras topologias do plasmídeo (ao nível estrutural) e das impurezas do hospedeiro que apresentam características comuns, tais como carga negativa (RNA, DNA genómico (gDNA) e endotoxinas), tamanho (gDNA, endotoxinas) e hidrofobicidade (endotoxinas). Comparando as várias estratégias cromatográficas já aplicadas na purificação da isoforma sc do pDNA, algumas delas apresentam mais vantagens do que outras devido à especificidade e selectividade promovidas por determinados suportes, utilizando condições de eluição suaves que evitam a perda de integridade desta biomolécula. Contudo, a cromatografia de afinidade usando aminoácidos como ligandos tem mostrado ser uma técnica adequada para obter a isoforma sc do pDNA de acordo com os critérios das agências reguladoras, eliminando as restantes topologias do plasmídeo e impurezas do hospedeiro num só passo cromatográfico. Assim sendo, o presente trabalho de doutoramento tem como objectivo principal o desenvolvimento e implementação de novas estratégias de cromatografia de afinidade que permitam melhorar e ultrapassar algumas limitações dos processos já desenvolvidos para a obtenção da isoforma sc do pDNA.

Inicialmente foram realizados estudos com oligonucleótidos sintéticos para compreender as interacções envolvidas entre os ácidos nucleicos e as matrizes de afinidade, assim como as condições de eluição que favorecem o bioreconhecimento da conformação sc do pDNA pelas matrizes de histidina- e arginina-agarose já usadas na purificação destes vectores. Os resultados obtidos nesses estudos fundamentais mostraram o envolvimento de várias interacções entre os ácidos nucleicos e as matrizes de aminoácidos estudadas, tais como interacções hidrofóbicas, interacções-π, pontes de hidrogénio, pontes mediadas por água, interacções electrostáticas e forças de van der Waals. Embora as interacções hidrofóbicas prevaleçam quando se usa a matriz de histidina e as interacções iónicas com a matriz de arginina, também se observou a presença de outras interacções que se tornaram dominantes em função das condições de eluição usadas, e preponderantes para o reconhecimento específico da isoforma sc. Estes resultados forneceram informações proveitosas para a implementação de novas estratégias cromatográficas de afinidade como por exemplo com a matriz de lisina, que é um aminoácido de constituição semelhante à arginina.

A cromatografia de afinidade com lisina resultou na purificação selectiva e eficiente do pDNA, apresentando uma homogeneidade superior a 97% da isoforma sc, e eliminando as impurezas do hospedeiro de *E. coli*, respeitando os requerimentos das agências reguladoras. O

rendimento da recuperação global do pDNA no final desta etapa cromatográfica foi de 55%. No entanto, a eficiência de transfecção de células eucarióticas (COS-7) com a amostra de pDNA sc final foi consideravelmente superior à eficiência obtida com uma amostra de plasmídeo na conformação circular aberta ou relativamente à amostra de pDNA nativo purificada através de um kit comercial.

Adicionalmente, foi realizado um estudo comparativo do comportamento de ligação e eluição dos vários ácidos nucleicos injectados individualmente nas três matrizes de aminoácidos anteriormente referidas, sob influência de diferentes condições de temperatura, composição e força iónica do tampão de eluição. As matrizes de histidina e lisina mostraram uma retenção preferencial pelos ácidos nucleicos com maior grau de exposição das bases nucleotídicas como por exemplo o RNA, parecendo que ambas as matrizes são indicadas para utilizar na purificação deste ácido nucleico. Tendo em conta que a matriz de lisina requer condições de eluição suaves, torna-se um método de purificação mais económico do que a histidina-agarose, principalmente para aplicar ao nível industrial. Por outro lado a arginina-agarose apresentou uma retenção preferencial pela isoforma sc do plasmídeo em condições cromatográficas suaves tornando-se um suporte adequado à purificação deste vector.

Embora as matrizes de afinidade com aminoácidos imobilizados permitam um reconhecimento específico e selectivo pela molécula de interesse, as limitações associadas aos suportes convencionais permanecem por resolver. Assim sendo, foi aplicado um suporte monolítico como uma alternativa cromatográfica para a purificação de pDNA, revelando boas propriedades de selectividade e transferência de massa, assim como elevada capacidade de ligação para o pDNA, permitindo uma rápida e eficiente separação das isoformas independentemente da taxa de fluxo aplicada. Tendo em conta que o monolito utilizado apresenta grupos funcionais de carbonildiimidazole e que as condições de eluição do pDNA são semelhantes às usadas na matriz de histidina-agarose, foi sugerido que o anel de imidazole presente neste disco monolítico é o principal responsável pelo reconhecimento específico da isoforma sc. A integração da cromatografia com monolitos no processo geral de obtenção do plasmídeo puro também resultou num aumento do rendimento global da etapa de recuperação de pDNA para 89%. A amostra final de pDNA sc foi obtida com um elevado grau de pureza, respeitando os critérios das agências reguladoras, que se reflectiu no aumento da eficiência de transfecção de células eucarióticas (59% com a linha celular COS-7). Este suporte monolítico além de evitar a degradação do pDNA devido ao reduzido tempo de contacto, também pode ser aplicado na purificação de plasmídeos com maiores dimensões sem grandes alterações da estratégia de purificação estabelecida.

Em conclusão, este projecto de investigação revelou que a cromatografia de afinidade baseada em aminoácidos é uma metodologia poderosa e versátil para a purificação de ácidos nucleicos, especialmente da topologia sc do pDNA, com um elevado grau de pureza, adequado para aplicações terapêuticas baseadas em DNA. Além da selectividade e

especificidade obtida com os ligandos de aminoácidos, a aplicação da tecnologia monolítica inovadora na purificação do pDNA forneceu grandes avanços relativamente à velocidade, resolução e capacidade da performance cromatográfica, revelando-se uma associação promissora para a tecnologia de purificação de plasmídeos. Assim, este trabalho reune informações úteis e valiosas relativamente à cromatografia de afinidade baseada em aminoácidos e dos suportes cromatográficos que podem ser aplicáveis futuramente, quer em processos preparativos quer analíticos da bioseparação do pDNA.

Palavras-chave

Aminoácidos, Bioreconhecimento, Cromatografia de afinidade, DNA plasmídico superenrolado, Eficiência de transfecção, Monolitos, Purificação

Abstract

The discovery of disease-related genes and the possibility to manipulate the gene set-up in some organisms has fostered the development of innovative human DNA therapeutics over the last years. Although viral vectors are used in the majority of the trials, non-viral vectors, particularly plasmid DNA (pDNA) vectors, are attracting considerable attention as biotherapeutics both in gene therapy or DNA vaccination, due to their lower immunogenicity, toxicity and also the economic, safer and easier manufacture. Nevertheless, it is well known that the success of gene transfer to cells and subsequent expression is strictly affected by the pDNA manufacturing process. The use of pDNA-based therapeutics relies on procedures that efficiently purify the most biologically active and effective topology, the supercoiled (sc) plasmid conformation. However, chromatographic purification of sc pDNA has specific problems which are mostly related to the structural nature of this biomolecule, the resemblance between pDNA topologies and also with some host impurities, as well as the lack of capacity and selectivity of the traditional bead adsorbents. Recently, sc pDNA purification strategies that use amino acids as immobilized ligands have yielded interesting results.

Thus, the present work intends to explore and understand the underlying interactions responsible for the biorecognition of sc conformation by the amino acids ligands already used for pDNA purification as well as to establish the elution conditions that favor the prevalence of some interactions on other. By performing some fundamental studies with oligonucleotides it was observed the involvement of several elementary interactions with the amino acids matrices studied, such as hydrophobic, ring-stacking, cation-π, water-mediated bonds, multiple hydrogen bonds, van der Waals and electrostatic interactions. Although hydrophobic interactions easily appear with histidine matrix or ionic interactions with arginine matrix, it was verified the presence of other interactions in function of the elution conditions used. These results were useful for the implementation of a new affinity chromatographic strategy with the amino acid lysine for efficiently and selectively purify the sc pDNA isoform. Lysine-affinity matrix allowed the elimination of the *E. coli* impurities as well as other ineffective plasmid isoforms present in a complex clarified lysate meeting all the regulatory requirements. The preferential retention of the nucleic acids with higher bases exposure indicates that this matrix can be more suitable for RNA purification. In accordance with the traditional supports limitations, the alternative monolithic chromatography revealed satisfactory affinity properties with excellent mass transfer and capacity characteritics, allowing a fast and efficient plasmid isoforms separation without flow rate dependence. The similar elution conditions with histidine-agarose and the presence of canbonyldiimidazole groups suggested that the imidazole ring present in this monolithic disk is the major responsible in the specific biorecognition of sc pDNA isoform. Integration of monolithic chromatography in the pDNA manufacturing process also increase the global yield of pDNA

recovery for 89%, with a purity degree according to the recommendations of the regulatory agencies that was reflected in the high transfection efficiency of sc pDNA sample on eukaryotic cells (59% in COS-7 cells).

Overall, this doctoral research work revealed that amino acid-based affinity chromatography is a powerful and versatile methodology for nucleic acids purification, mainly the sc pDNA topology, guaranteeing suitable purity degree for DNA-based therapies. Besides the selectivity and specificity obtained with amino acids affinity ligands, the application of the innovative monolithic technology in the pDNA purification field brought a great improvement of the speed, resolution and capacity to the chromatographic performance, being a promising association for plasmid purification technology. Hence, this work provided valuable and helpful information concerning amino acid-affinity chromatography and chromatographic supports that can be useful in the future pDNA bioseparation either for preparative and analytical processes.

Keywords

Affinity chromatography, Amino acids, Biorecognition, Monoliths, Purification, Supercoiled plasmid DNA, Transfection efficiency

Thesis Overview

This thesis is structured in four main chapters. The first chapter includes the general and intermediate purpose established for the development of this study. Subsequently, the second chapter consistes in a concise literature review related with the importance and application of the plasmid DNA in DNA-based therapies, the challenges implicit for its proper achievement as well as the different chromatographic supports and strategies developed for its purification (paper I). Thereafter, the third chapter presents the results obtained during the PhD course that were summarized in some original research papers organized as follows:

Paper II – Histidine affinity chromatography of homo-oligonucleotides. Role of multiple interactions on retention

Paper III - Selectivity of arginine chromatography in promoting different interactions using synthetic oligonucleotides as model

Paper IV - Biorecognition of supercoiled plasmid DNA isoform in lysine-affinity chromatography

Paper V - Impact of lysine-affinity chromatography on supercoiled plasmid DNA purification

Paper VI - Differential interactions of plasmid DNA, RNA and genomic DNA with amino acid-based affinity matrices

Paper VII - Performance of a non-grafted monolithic support for purification of supercoiled plasmid DNA

Paper VIII - Successful application of monolithic innovative technology using a carbonyldiimidazole disk to purify supercoiled plasmid DNA suitable for pharmaceutical applications

Finally, the fourth chapter summarizes the concluding remarks obtained during this research work, regarding the application and characterization of some chromatographic supports for plasmid DNA purification by affinity chromatography using in some cases amino acids as ligands, and the understanding of the biorecognition phenomena of the plasmids by these affinity ligands.

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Chapter 1

Global aims

The main scope of this work is the development of adequate strategies to efficiently purify plasmid DNA (pDNA) based on amino acids-affinity chromatography for DNA-based therapeutic applications. To obtain the most biologically active plasmid isoform, the supercoiled (sc) pDNA, firstly it is important to understand the specificity of the underlying interactions with the amino acids affinity ligands. This information can be useful to adjust and apply the best chromatographic conditions when new affinity matrices are explored to isolate sc pDNA isoform from impure and complex *Escherichia coli* (*E. coli*) extracts. Likewise, the study, characterization and implementation of modern chromatographic supports can improve the performance of chromatographic runs, namely with relation to speed and resolution, also increasing the binding capacity for the sc pDNA.

Therefore, the knowledge of sc pDNA physicochemical properties and its similarities with the *E. coli* host impurities in association with the preferential interactions that the affinity matrices promote with these compounds can be the key to efficiently achieve a final pDNA product suitable for clinical applications. According to these global aims, the work will be developed respecting the following intermediate tasks:

1. Development of a screening study of the binding/elution conditions of several pDNA topologies in the lysine-agarose and carbonyldiimidazole monolithic supports to test their ability to specifically recognize the sc pDNA isoform.

2. Study of the influence of elution conditions on pDNA isoforms and host nucleic acids, such as the temperature, flow rate and buffer composition (pH, ionic strength or presence of competitive agents), to characterize the general binding performance of the proposed supports.

3. Integration of the innovative chromatographic strategies in the global process of pDNA manufacturing, evaluating their capacity to selectively isolate and purify the sc pDNA isoform from a complex and impure *E. coli* extract.

4. Evaluation of the quality of the final pDNA product purified under the best chromatographic conditions, in conformity with the regulatory guidelines. Prediction of sc pDNA therapeutic applicability according its purity degree and transfection efficiency of eukaryotic cells.

Chapter 2

Introduction

1. DNA-based therapy: General considerations

During the advent of life science, biological questions have been raised mainly by studying the function of individual genes and gene products to understand the interaction among all the individual components of the cell (Ge *et al.*, 2003). This reductionist approach allows a comprehensive explanation of living system, which results of the complex networks of several gene products leading to the interconnectivity of cell components (genes, proteins, metabolites, etc) responsible for a particular phenomenon in an organism (metabolic activity, response to external stimuli, etc.) (Aggarwal and Lee, 2003).

Human genome and proteome-based research has provided valuable information in relation to the role and action of normal genes (Aggarwal and Lee, 2003), as well as the impact of their mutations on human health. The growing knowledge in this area has implemented the effective technologies using genes as drugs for the treatment of human diseases (Manthorpe *et al.*, 2005). The development of any gene-based therapy approach requires an accurate evaluation of a series of parameters, including the assurance that a given disease might in reality be treated by this strategy, followed by the choice of the correct therapeutic gene and its acquisition, the certification of gene expression in the mammalian cells, the selection of delivery system, and administration route.

Indeed, the full sequencing of the human genome combined with an increased understanding of the properties and characteristics of several disease-related genes has positioned somatic gene therapy and genetic vaccination amongst the most exciting and promising biotechnological progresses (Manthorpe *et al.*, 2005; Rolland, 2005). Gene therapy can be viewed as a system that aims the expression of specific proteins using the patient's own cells as mini-bioreactors. Gene therapy allows to replace not only the missing or defective gene within the patient's cells, but also to supplement the organism with the production of proteins that can prevent or treat the target disease (Rolland, 2005; Wang *et al.*, 2001). On the other hand, the advances of new genetic vaccines that have the ability to generate specific anti-pathogen or anti-tumor immune responses are being explored to provide innovative prophylactic or therapeutic treatments for infectious disease and cancer (Gurunathan *et al.*, 2000; Shiver *et al.*, 2002; Epstein *et al.*, 2004). Therefore, gene therapy and DNA vaccination have progressed steadily in recent years, becoming a new class of therapeutic strategies that have the potential to prevent, correct, or modulate genetic or acquired diseases. Thus, detailed information about these both DNA-based therapies and a brief summary of the clinical applications already implemented and still under development are discussed below.

1.1. Gene therapy

Genetic diseases normally result from a mutation on specific genes, suitably organized in chromosomes, which can affect the cellular mechanisms, suppressing the expression of the respective protein or leading to uncorrected or uncontrolled production. Gene therapy is considered a potential revolution in medicine because it aims the treatment or elimination of the cause of disease, whereas most current strategies treat the symptoms (Mountain, 2000). This therapeutic was originally developed with the purpose of supplying a missing cellular function by transferring a normal copy of the altered gene into the relevant and damaged cells, as it is schematized in Figure 1.

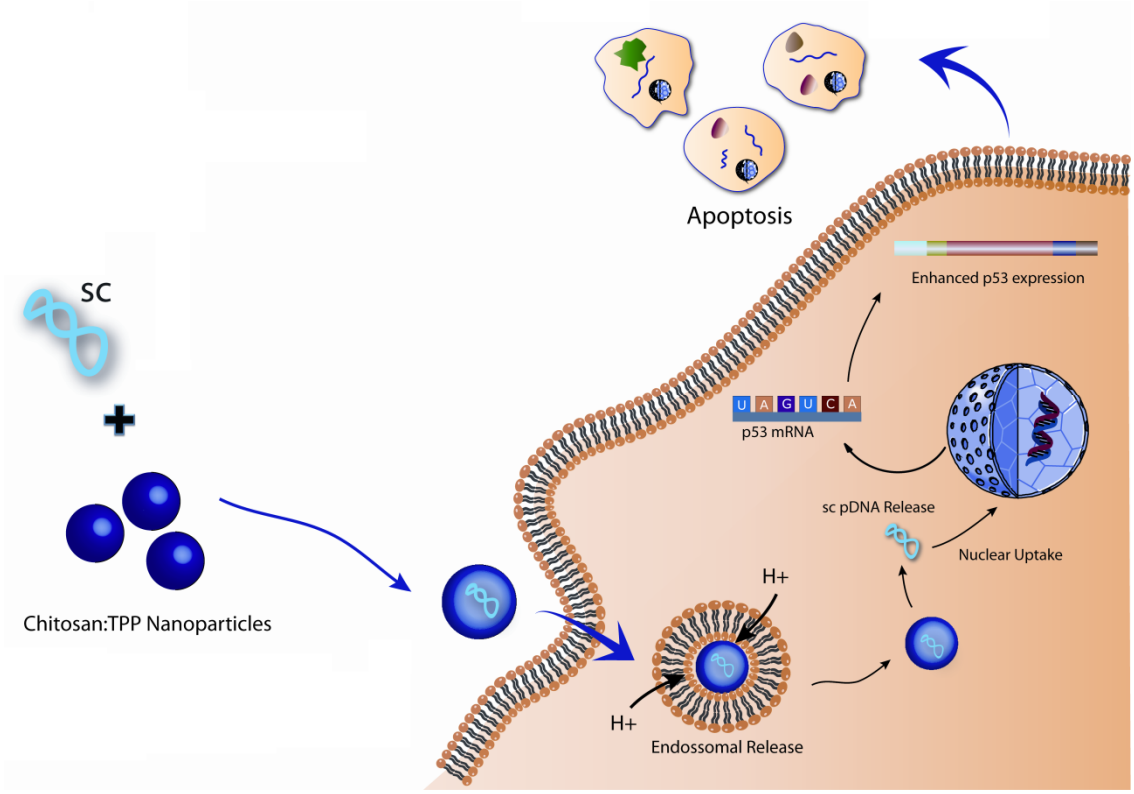

Figure 1. Depiction of an example of gene therapy, using a non-viral vector (supercoiled plasmid DNA) to express the p53 gene transported by nanoparticles (adapted from (Gaspar et al., 2011a)].

The concept "gene therapy" refers to the phenomenon of gene transfer mainly in somatic cells, both *in vivo* and *ex vivo*, based on the application of different nucleic acids for therapeutic purposes (Giacca, 2010). In the *ex vivo* approach, cells are removed from the patient for transfection, and the therapeutic entity comprises engineered cells. Although the gene transfer acquires higher efficiency with this strategy, there are notable disadvantages as the cellular immunogenicity and higher costs due to cell manipulation, manufacturing and quality-control difficulties for each patient. The *in vivo* approach involves direct administration of the gene-transfer vector to patients by different methodologies. It is therefore not patient-specific, thus conferring advantages of reduced costs, logistics and infrastructure requirements (Mountain, 2000; Giacca, 2010).

Gene therapy has become the research focus for many worldwide laboratories in pharmacy, medicine, biochemistry and chemical engineering, being a broad technological platform, applicable to a wide range of diseases. The first foreseen applications of gene therapy were the treatment of patients with inherited monogenic disorders with recessive inheritance. Over the last two decades, gene therapy has gained significant attention due to the potential solution for genetic disorders such as severe combined immunodeficiency, cystic fibrosis, Parkinson's disease, muscular dystrophies, lysosomal storage disorders, hemophilia, and several thousand other different conditions (Lee *et al.*, 2005; Manthorpe *et al.*, 2005; Giacca, 2010). These diseases are due to single gene defects and the pathologic phenotype becomes evident only when both alleles are defective.

The main objective in gene therapy is *in vivo* successful transfer of a defined DNA sequence into specific cells either to replace a defective gene, or to impart a new function to the cell in order to induce it to secrete a protein that has a putative therapeutic function to the targeted tissues, being considered in the beginning as an alternative method to traditional chemotherapy used in cancer treatment. The gene expression and delivery systems differ according to the application and are normally optimized in function of the target. For example, the treatment of diseases related to one gene dysfunction like hypercholesterolemia need the prolonged and continued expression, while for most cancer gene therapy strategies it is sufficient a short period of gene expression (El-Aneed, 2004).

The first human gene therapy trial was performed by Rosenberg and coworkers in 1989. They used a retrovirus to introduce the gene coding for resistance to neomycin into human tumor-infiltrating lymphocytes before infusing them into five patients with advanced melanoma (Edelstein *et al.*, 2004). This pioneering study demonstrated the feasibility of using retroviral gene transduction in humans and established a number of important prerequisites for further clinical gene transfer studies. From the wide range of diseases studied on gene therapy, cancer is the most investigated disorder due to its complex nature (Edelstein *et al.*, 2004). The purpose of many tested therapeutic genes on this field is to correct or eliminate cancerous lesions. The development of cancer cells is associated with multiple alterations on the genetic level of these cells. Oncogenes and tumor suppressor genes play a crucial role in cancer development, counterbalancing each other. Oncogenes enhance cell proliferation, while tumor suppressor genes induce apoptosis (programmed cell death). For that reason, anti-oncogenes and apoptotic genes were considered potential tools for cancer treatment (El-Aneed, 2004; Tangney *et al.*, 2006). In 2004, Gendicine was the first gene-based product approved by the Chinese regulatory agencies, which consists in an adenoviral vector that expresses a tumor suppressor gene (termed p53), inducing apoptosis of malign cells for the treatment of patients with head and neck squamous cell carcinoma (Manthorpe *et al.*, 2005; Wilson, 2005; Patil *et al.*, 2005). Figure 1 exemplifies the action mechanism of p53 gene in eukaryotic cells, but instead of an adenoviral vector it was applied a non-viral vector

associated with nanoparticles to delivery the genetic information of p53. Other examples of clinical trials already approved and under development are presented in section 1.3. Nevertheless, most of clinical studies for cancer therapy have involved gene addition rather than the correction or replacement of defective genes, which is technically more difficult.

1.2. DNA vaccination

Over the past century, the progress and widespread use of vaccines against infectious agents have been a great victory of medical science. Initially, the vaccination process, which can also be designed as genetic immunization, was based on administration of weakened or modified infectious agents to healthy individual only to prevent a specific disease, promoting immunity (Abdelnoor, 2001). One reason for the success of classical vaccines is that they are capable of inducing long-lived antibody responses, which are the principal agents of immune protection against most viruses and bacteria. There are several classic vaccines for human prevention, among which life-attenuated viruses (e.g. polio or measles), inactivated microorganisms (e.g. cholera bacteria), inactivated viruses (e.g. influenza), recombinant vaccines (e.g. HBV surface antigens), as well as pseudo-viral or virus particles produced in yeast, hybrid vectors (bacterial or viral) or synthetic peptides (Schleef and Schmidt, 2004). Some examples of currently available vaccines and its properties are summarized in Table 1.

Table 1. Comparision of the main vaccine technologies (adapted from (Liu, 2003)).

Traditional vaccines	**Characteristics**
Live attenuate viruses	Highly effective Potential risk for the patients Manufacturing challenge
Recombinant proteins	Potent antibody response Effective Non-native forms at times No induction of cytolytic T lymphocytes (CTL)
Viral vectors	Potential risk Resistance/pre-existing antibody Inflammation
DNA vaccines	Need for increased potency Designer immune responses (e.g. type of helper T cell) Specificity: avoid deleterious or diversional antigens Relative stability Safety Generic manufacturing Cost advantage

Despite these successes, vaccination against intracellular organisms that require cell-mediated immunity, such as the agents of tuberculosis, malaria, leishmaniasis, and human immunodeficiency virus (HIV) infection, are either not available or not uniformly effective (Liu, 2003). Millions of people, including children die each year from infectious emergent diseases for which there is no efficient vaccine. Owing to the substantial morbidity and mortality associated with these diseases worldwide, an understanding of the mechanisms involved in generating long-lived cellular immune responses arises like a critical point. For these reasons, a new form of vaccination, using DNA that contains the gene encoding for the antigen of interest, is under intensive investigation, because it can stimulate both humoural and cellular immune responses (Figure 2) (Gurunathan *et al.*, 2000; Liu, 2003).

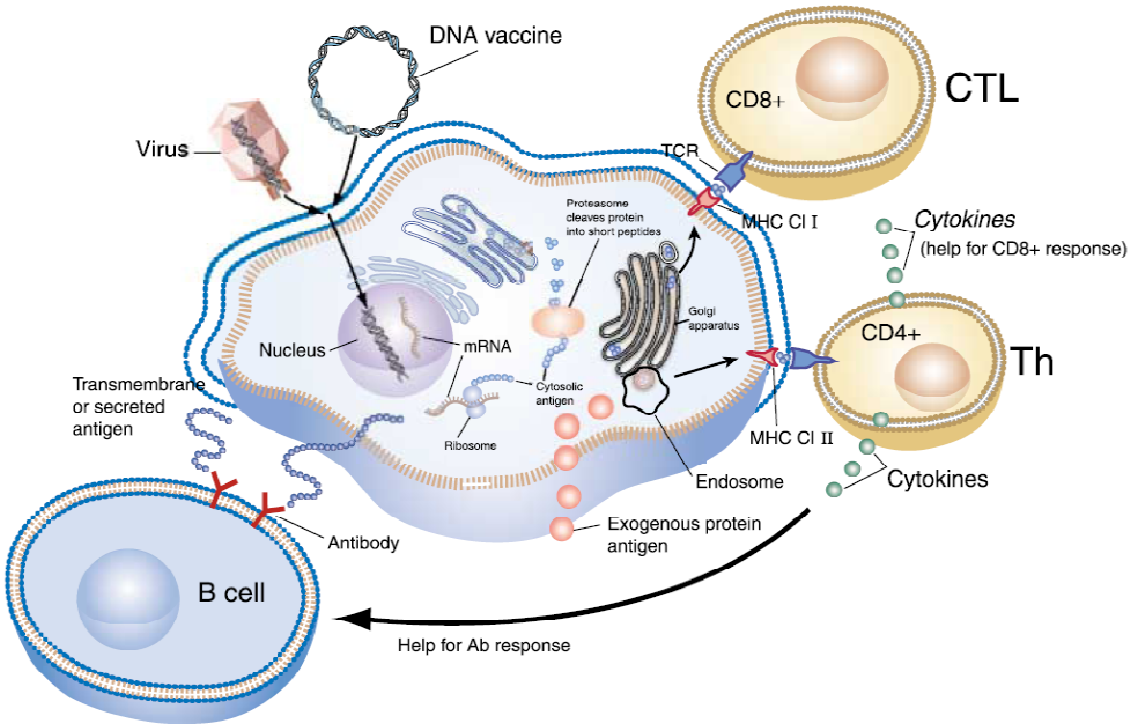

Figure 2. Depiction of the mechanisms of generation of antigen-specific humoural and cellular responses (Liu, 2003).

The DNA vaccines are normaly composed by a simple ring of DNA containing a gene that encodes the target antigen, and a promoter/terminator to make the gene expression in mammalian cells (Schleef and Schmidt, 2004; McDonnell and Askari, 1996). In the early 1990s it was verified that the vaccination of animal models with nucleic acid vaccines (normally constituted of a naked DNA plasmid) could provide several advantages compared to conventional vaccines (McDonnell and Askari, 1996). In opposition to recombinant viruses or live attenuated virus, the nucleic acid vectors code for a single protein of interest (antigen) and are not associated to any other proteins (Weide *et al.*, 2008). The main advantage of DNA vaccines is the capacity of generating all types of desired immunity, namely cytolytic T

lymphocytes, T helper cells and antibodies (Figure 2), while conventional vaccines only elicit antibody responses (Gurunathan *et al.*, 2000; Liu, 2003; McDonnell and Askari, 1996; Johnston *et al.*, 2002).

Figure 2 illustrates, in a simplified form, the intracellular and intercellular interactions required for an antigen to result in the generation of both cytotoxic and helper T-cell responses, and antibody generation. The gene encoded on DNA vaccines normally express an antigenic portion of the virus, such as a core protein or an envelope protein. Host cells take up the foreign DNA, express the viral gene, and construct the corresponding viral protein inside the cell. Either the proteins are expressed inside the antigen presenting cells or exogenous antigenic proteins are conducted into the endolysosomal degradation pathway (Liu, 2003). The protein is degraded into peptides that associate with major histocompatibility complex (MHC) Class II molecules that are then exhibited on the surface of the cell. Specific helper T cells (CD4+ T cells) recognize this antigen peptide/MHC Class II molecule complex and are activated to produce "help" in the form of cytokines. These cytokines have myriad activities including, depending upon the cytokine, helping B cells activate into antibody producing cells (humoural immunity), and helping cytolytic T lymphocyte responses (McDonnell and Askari, 1996). Activation of cytolytic T lymphocytes (CD8+ T cells) is generally dependent upon an antigen-processing pathway reserved for intracytoplasmic proteins that are degraded into peptides that associate with newly synthesized MHC Class I molecules. These complexes, when presented on the surface of antigen presenting cells in conjunction with co-stimulatory molecules, result in the activation of the proper CD8+ T cells (cell-mediated immunity). For antibody responses, B cells recognize and respond to antigens that are either present extracellularly, or exposed extracellularly as transmembrane proteins (Liu, 2003; McDonnell and Askari, 1996; Johnston *et al.*, 2002).

Given that antigenic protein is intracellularly synthesized in the transfected cells, the possibility of antigen to be presented by MHC class I and II molecules, inducing the potential antigen-specific CD8+ T cell responses that is the foremost mechanism of protection against intracellular pathogens, becomes the major advantage of DNA vaccines over protein vaccines at the immunological level (Wang *et al.*, 1998; Olsen, 2000). However, other advantages are related with DNA manufacturing, which can be easily obtained in large quantities with great purity, minimizing the risk of vaccine contamination with potential pathogens, being safety for a broad population administration (Liu, 2003). Additionally, DNA vaccines can also be more efficient and safe than some live attenuated vaccines because the manufacturing techniques can alter epitopes, reducing the antigenicity. Moreover, several antigenic genes could be included on one plasmid DNA molecule (multivalent vaccination), reducing the total number of vaccinations that must be administered, and the quality control is standardize and independent of the encoding genes (Manthorpe *et al.*, 2005). Furthermore, DNA vaccines also

provide the potential for longer-lived antigen exposure *in vivo*, which could lead to increased immunogenicity, and could be easily transported and stored because DNA provides more stability than other biological molecules (Rottinghaus *et al.*, 2003).

Vaccination progress revealed that DNA vaccines can be used not only for preventive immunization but also as a versatile method to control and treat cancer (Liao *et al.*, 2006; Weide *et al.*, 2008; Hung *et al.*, 2007). Tumor cells over-express specific antigens which allow them to be recognized and destroyed by the immune system. Injection of plasmid DNA encoding xenogeneic melanosomal differentiation antigens (e.g. tyrosinase) can promote antitumor immune responses leading to long-term tumor control and survival (Liao *et al.*, 2006). The implementation of multivalent vaccination by sequential immunization with DNA and recombinant protein (called heterologous prime-boost) arose to overcome some limitations such as safety, tolerability and enhance of immune responses when compared with the use of DNA or recombinant protein alone, suggesting an improved protective immunity (Epstein *et al.*, 2004). However, despite of prominent advances in this new vaccination field, the official acceptance of this technology for human therapeutic depends on the successful demonstration of its safety and efficacy in advanced clinical trials (Anderson and Schneider, 2007).

1.3. Clinical applications

Up to 2010, over 1708 clinical trials had been conduced or are currently ongoing. In order to be approved, each clinical experimentation of gene-based therapy (gene therapy or DNA vaccination) should successfully complete a series of sequential phases. After successfully testing the therapeutic approach in animal models, phase I trial can be started by human experimentation with a small number (10–20) of patients or healthy volunteers (Giacca, 2010). The purpose of this stage is to determine the pharmacokinetics of the compound under investigation (engineered of patient's target cells and delivery process of the vector, levels of therapeutic nucleic acid expression, duration of effect, etc.) and to evaluate the safety or at least to show a tolerable toxicity in relation to the foreseen therapeutic benefit. Phase II consists on application of defined dosage and modality of administration, conducted also with small number of patients, to analyze the efficacy. Due to the reduced number of patients that are required to conduct the clinical experiments, Phase I or I/II are the most performed (almost 80%) for different diseases (Figure 3 (A)) (Edelstein *et al.*, 2004; Giacca, 2010). When a Phase II trial provides encouraging results, a large Phase III trial is organized, involving a larger number of patients (usually one or a few hundred individuals), to confirm the previous efficacy data, to identify the most appropriate dosage, and monitor the appearance of adverse effects in a statistically significant sample. Finally, if the treatment modality overcomes Phase III, it becomes commercially available (Giacca, 2010).

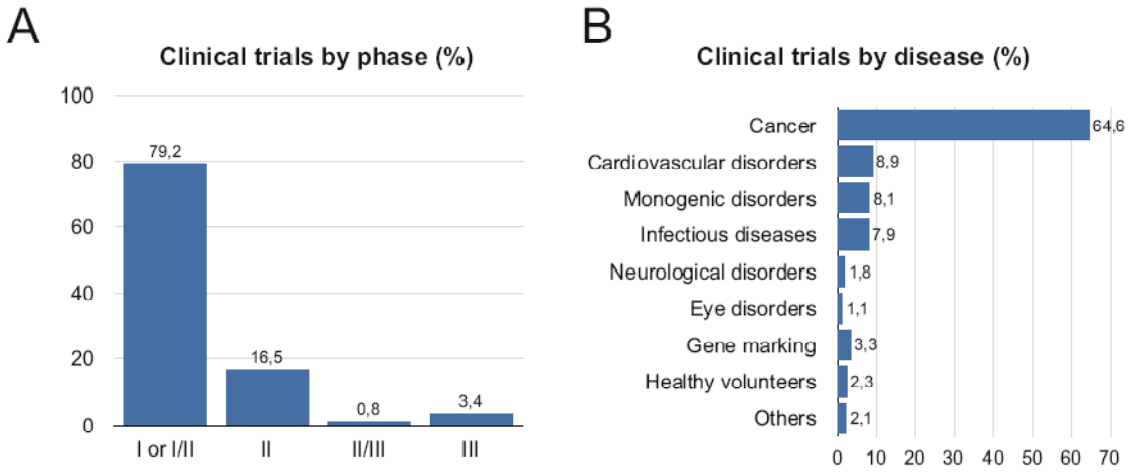

Figure 3. Distribution of the gene-based therapy clinical trials organized according the phase of experimentation (A) or type of disease (B) (Giacca, 2010).

Although the major part of DNA-based therapeutics is in early phases of clinical trials, this technology has emerged in recent years as a potential solution for a wide range of pathologies. Among which there are monogenic hereditary disorders (cystic fibrosis, hemophilia A and B, several combined immunodeficiency syndromes, familial hypercholesterolemia, ornithine transcarbamylase deficiency); cancer (breast cancer, ovary cancer, cervix cancer, colorectal cancer, pancreatic cancer, melanoma, leukemia, lymphoma, multiple myeloma); Infectious disorders (HIV, hepatitis B/C, influenza, adenovirus infection, cytomegalovirus infection, Epstein-Barr infection); Neurologic disorders (Alzheimer's disease, Parkinson's disease, multiple sclerosis, epilepsy); cardiovascular disorders (myocardial infection, peripheral artery disease, ischemic cardiomyopathy, pulmonary hypertension); eye diseases between other (anemia, rheumatoid arthritis, erectile dysfunction, type I diabetes) (Edelstein *et al.*, 2004; Giacca, 2010; *et al.*, 2005). Some examples of DNA-based therapeutics applied on these diseases, already commercialized and under development, are presented on Tables 2 and 3. Over 60% of the gene therapy clinical trials conducted so far have focused the cancer field, having as their target a vast series of neoplasias and exploiting various strategies, including anticancer vaccination (Figure 3 (B)). Interestingly, a growing number of trials are being conducted with the main objective of treating cardiovascular disorders. This field of gene therapy application has grown very rapidly in recent years (Edelstein *et al.*, 2004; Giacca, 2010).

Table 2. Approved products by regulatory agencies, under commercialization.

Therapeutic strategy	Product name	Vector	Gene	Target disease	References
Gene therapy	Gendicine	Adenoviral	p53 (tumor suppressor)	cancer	(Wilson, 2005)
	Vitravene	Antisense oligonucleotide	Inhibitor of immediate early region 2 (IE2) of human cytomegalovirus	Cytomegalovirus retinitis in HIV patients	(Patil *et al.*, 2005)
DNA vaccination	Gardasil	Virus-like particles	late protein 1 (L1) from HPV Capsid	Human Papillomavirus (HPV)	(Hung *et al.*, 2007)

Table 3. Examples of therapies under different phases of clinical development.

Therapeutic strategy	Vector	Gene	Target disease	Clinical development	Ref.
Gene therapy	Antisense oligonucleotide	Inhibitor of B cell leukemia/lymphoma 2 protein	Multiple myeloma	Phase III	(Patil *et al.*, 2005)
	Antisense oligonucleotide	Inhibitor of Intracellular Adhesion Molecule -1	Crohn's disease	Phase III	(Patil *et al.*, 2005)
	pDNA	Hepatocyte Growth Factor (HGF)	Critical limb ischemia	Phase III	(Sanada *et al.*, 2009)
	Adenovirus	Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)	cystic fibrosis	Phase I	(Lee *et al.*, 2005)
	Retrovirus	Chimeric T cell receptor (TCR)	HIV	Phase II	(Mountain, 2000)
DNA vaccination	pDNA	p24/p17 proteins and epitopes	HIV	Phase I	(Mwau *et al.*, 2004)
	pDNA	HPV-type 16 E7 protein	HPV	Phase I	(Sheets *et al.*, 2003)
	pDNA	hemagglutinin	Influenza	Phase I	(Drape *et al.*, 2006)
	pDNA	*Plasmodium falciparum* circumsporozoite Protein (PfCSP)	Malaria	Phase I	(Wang *et al.*, 1998)
	Heterologous prime-boost (pDNA + protein)	PfCSP	Malaria	Phase I	(Epstein *et al.*, 2004)
	pDNA	envelope glycoprotein	Ebola Virus	Phase I	(Martin *et al.*, 2006)
	pDNA	hepatitis B surface antigen (HBsAg)	Hepatitis B	Phase I	(Rottinghaus *et al.*, 2003)
	pDNA	Tyrosinase	Cancer	Phase I	(Liao *et al.*, 2006)

2. Methods for gene delivery

Success of any gene transfer procedure strictly depends upon the efficiency of nucleic acid internalization by the target cells. With very few exceptions, naked nucleic acids are, therefore, very poorly uptake into the cells due to their physicochemical properties (charge, size and poor stability). By this way, gene transfer must be facilitated using biological (viral and non-viral vectors), physical (for example, electroporation or high pressure injection) or chemical (cationic lipids or polymers) methods. A gene transfer system can be considered ideal if the following aspects have been satisfied: specificity and efficiency of gene transfer; magnitude and duration of expression; immunogenicity and manufacturing.

2.1. Biological methods

2.1.1. Viral vectors

Viruses were naturally evolved to infect cells having the benefit of to easily transfer their own genetic materials into the host cells (El-Aneed, 2004; Mountain, 2000), being for that reason the preferred vehicles for heterologous gene delivery (Brave *et al.*, 2007). In the most simplistic view, a viral particle is a small object composed of a nucleic acid and a few proteins that delay its degradation in the extracellular environment, mediate its internalization into the target cells and make use of the cellular machinery for production of viral encoded proteins (Brave *et al.*, 2007). Both RNA and DNA viruses have been evaluated as potential gene carriers, however, there is the need to modify them in the laboratory to eliminate their pathogenicity and retain their high efficiency in gene transfer (El-Aneed, 2004).

Five classes of viral vectors are currently in an advanced stage of clinical experimentation for human DNA-based therapy, such as adenovirus, retrovirus, lentivirus, herpes virus and adeno-associate virus. Initial research was focused on the implementation of both retroviral and adenoviral vectors, because these viruses exhibited high efficiency at delivering both DNA and RNA to numerous cell lines (Mintzer and Simanek, 2009; Lee *et al.*, 2005). Adenovirus contains double-stranded DNA with capacity to infect both dividing and non-dividing cells under lower risk of insertional mutagenesis (Anderson *et al.*, 2009), being important to remove the whole coding sequence of the viral genome to enhance the vectors capacity for therapeutic genes (El-Aneed, 2004). Nevertheless, adenovirus must be repeatedly administered because its expression is short lived, and its prior immune exposure can cause a severe, even lethal, inflammatory response (Anderson *et al.*, 2009; El-Aneed, 2004). On the other hand, retrovirus is a small RNA virus with DNA intermediate (with limited encapsulation capacity) that integrates into the host genome producing the viral proteins, which are removed when developing the gene delivery vector. Retroviral vectors already have the facility to integrate into the host genome for long-term expression, but it is unable to infect non-dividing cells

(Anderson *et al.*, 2009). Lentivirus can infect non-proliferating cells and the transfection efficiency in ovarian cancer cells was 10 times higher than retroviral vectors (El-Aneed, 2004). Lentiviral vectors offer the possibility of providing sustained expression of the therapeutic gene, as they integrate their genome into the host DNA (Lee *et al.*, 2005), being the most efficient method to transfect dendritic cells *in vitro* and *in vivo* (Anderson *et al.*, 2009). Overall, a detailed summary of the advantages and disadvantages of viral vectors are exposed on Table 4.

Table 4. Major advantages and disadvantages of viral vectors for DNA-based therapy (Mountain, 2000; El-Aneed, 2004; Brave *et al.*, 2007).

Viral vector	Advantages	Disadvantages
Adenovirus	Very high transfection efficiency *ex vivo* and *in vivo* Effective cellular uptake and proteins expression Stable constructions with low insertional mutagenesis Substantial clinical experience acquired in humans Rapid induction of immunity	Short duration of expression Difficult manufacture and storage Insert-size limited to 7.5 kb Repeat dosing ineffective owing to strong immune responses
Retrovirus	Fairly prolonged expression High transfection efficiency and substantial clinical experience *ex vivo* Low immunogenicity	Low transfection efficiency *in vivo* Transfects only proliferating cells Extremely difficult manufacture and storage Insert-size limited to 8 kb Safety concern of insertional mutagenesis
Lentivirus	Transfect a wide variety of cells (proliferating, non-proliferating and haematopoietic stem cells)	Safety concerns from immunodeficiency virus origins Extremely difficult manufacture and storage Insert-size limited to 8 kb
Adeno-associated virus	Very prolonged expression *in vivo* Efficiently transfects a wide variety of cells *in vivo* Can be modified to not carry any original viral genes Low immunogenicity and toxicity, proven to be safe and well-tolerated in gene therapy trials	Safety concern of insertion mutagenesis Very difficult manufacture and storage Insert-size limited to 4.5 kb Repeat dosing affected by neutralizing antibody responses Need for helper viruses to own production
Herpes virus	Top of viral vectors' capacity with insert-size of 40 kb Capacity to simultaneously deliver multiple genes	Limited therapeutic applications by both original pathologic and latent infectious nature

After this enthusiastic approach, the unfortunate death in 1999 of a patient with ornithine transcarbamylase deficiency following the intrahepatic infusion of an adenoviral vector (Giacca, 2010), the recent cases of T-cell leukemia in X-linked severe combined immunodeficiency infants following administration of a retroviral vector (Manthorpe *et al.*, 2005), and more generally, the growing perception of the general inefficacy of the protocols so far developed determined a decline in the number of clinical trial, having left the public disappointed and wondering about the future of gene therapy, in particular when using viral vectors (Giacca, 2010; Manthorpe *et al.*, 2005). In addition, fundamental problems associated with viral vector systems, including toxicity, immunogenicity, and limitations with respect to manufacturing and scale-up procedures, as reduced insert-size and difficulties on quality control, encouraged the investigation of other potential scaffolds to deliver exogenous DNA into targeted tissue (El-Aneed, 2004; Mountain, 2000; Mintzer and Simanek, 2009).

2.1.2. Non-viral vectors

Non-viral DNA-based therapy is a revolutionary nucleic acid-based therapy that relies on the delivery of multiple copies of genetic material to correct specific cellular disorders. Endowed with broaden application in numerous incurable pathologies with high rates of mortality and morbidity worldwide, non-viral DNA-based therapy encourages both scientific and medical communities towards the outcome of a cost-effective and exceptionally efficient alternative to conventional treatments (Rolland, 2005).

Gene medicines or nucleic acid-based biopharmaceuticals can be categorized on the basis of their potential therapeutic function as genes inhibitors or genes inductors (Patil *et al.*, 2005). Gene inhibitors (i.e. oligonucleotides, ribozymes, DNAzymes, aptamers, and small interfering RNAs (siRNAs)) are potent non-coding nucleic acids that silence defective genes at the mRNA level (Leonetti *et al.*, 1991). Gene inductors (cDNAs or plasmids containing transgenes) are protein-coding genes (Patil *et al.*, 2005) that can be divided into two groups, the gene vaccines and the gene substitutes. Gene vaccines are antigens of specific pathogens encoding either the genes or RNA that activate cell-mediated and humoral immune response and production of antibodies (Srivastava and Liu, 2003). Gene substitutes are transcriptionally fully competent genes introduced into cells to reimburse deficiency of a specific protein or its insufficient production, mostly applied on gene therapy (Giacca, 2010).

Non-viral vectors continue to be attractive alternatives to viruses and may overcome several problems encountered in viral vector-mediated therapy, including immune responses, limited DNA carrying capacity, recombination and high cost (Tros de Ilarduya *et al.*, 2010). In spite of their relative delivery inefficiency and need for increased doses due to the more-transient expression (Anderson *et al.*, 2009), non-viral vectors have greater potential for delivery of larger genetic units without insert-size limitation. They are less immunogenic and toxic without pathogenicity, being safer and easier to manufacture than viral vectors (Mountain,

2000; Medina-Kauwe *et al.*, 2005; Tros de Ilarduya *et al.*, 2010; Tangney *et al.*, 2006). According to the advantages of non-viral vectors and knowing that plasmid DNA (pDNA) is a double-stranded biomolecule with high molecular weight, easily constructed with any trangenes of interest, obtained in large scale under satisfactory purity degree by a simple manufacturing process with low cost, and able to transfect eukaryotic cells with satisfactory expression levels, this non-viral vector has been largely studied and applied on DNA-based therapies over the last years. A more detailed description of pDNA technology is presented in section 3.

Despite the advantages provided by non-viral vectors for gene-based therapy, the difficulty of introducing naked DNA into diseased cells is a serious bottleneck in the development of molecular medicine for therapeutic use. With the exception of antisense therapy, to execute effective pharmacological action the nucleic acids vectors must reach the cell nucleus (the target site of action), otherwise it looses its therapeutic value and may even cause side effects through non-intended interactions. Since many DNA-based compounds are new pharmaceutical drug candidates the effects of their human exposure have already been completely investigated, but the feasibility of their long-term use remains to be determined. In addition, very little is known about their cellular uptake, distribution, and metabolism (Patil *et al.*, 2005). Many studies on vector–cell interactions have reported that non-viral vectors bind and enter cells efficiently, but yield low gene expression, thus directing the attention to the intracellular trafficking of these vectors to understand where the hurdles occur (Medina-Kauwe *et al.*, 2005).

The success of the non-viral DNA-based therapy, namely by using plasmids, has been shown to be very much dependent on the various extra- and intracellular barriers that dramatically affect efficacy of the gene delivery systems (Figure 4) (Pathak *et al.*, 2009; Al-Dosari and Gao, 2009). After administration, the successful tissue distribution of plasmids depends on the extracellular barriers found, such as limited stability in blood, degradation or inactivation of pDNA by serum proteins or tissue and blood nucleases, phagocytosis by macrophages and adhesion to the tissue and cell types of interest avoiding off-target distribution (Anderson *et al.*, 2009; Pathak *et al.*, 2009). Once the pDNA reach the target cell, additional intracellular barriers are met, which needs to be crossed before the pDNA arrives at its intracellular target region, the nucleus. Figure 5 represents some possible intracellular barriers that are following described.

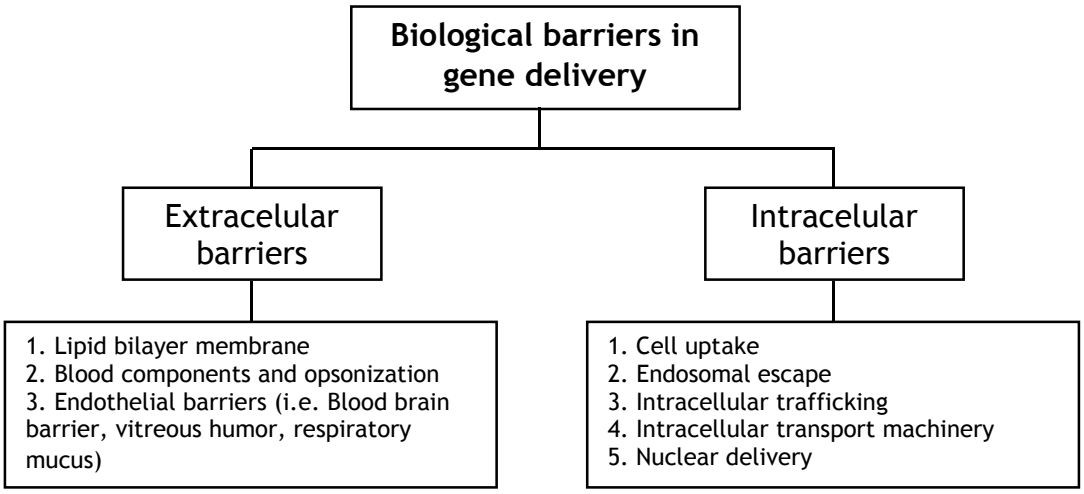

Figure 4. Schematic representation of several biological barriers in gene delivery (adapted from (Pathak *et al.*, 2009)).

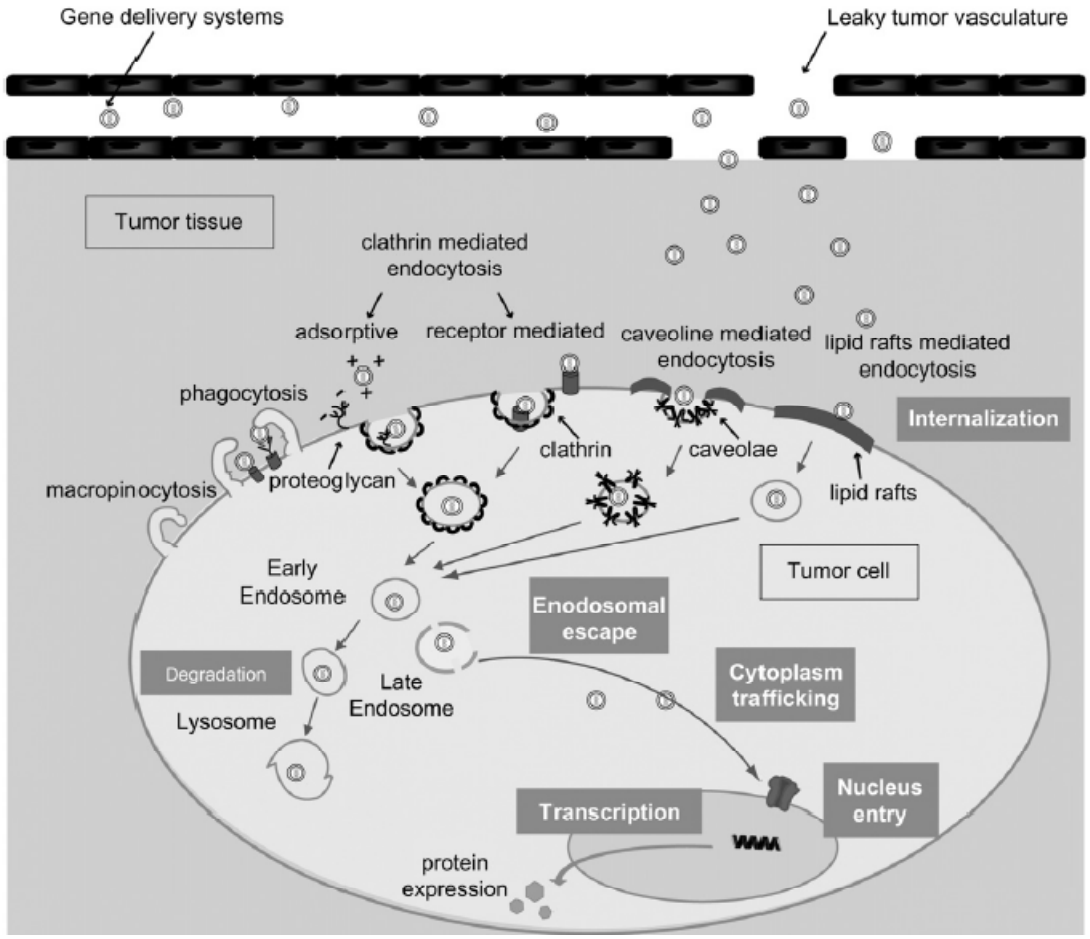

Figure 5. Schematic representation of the different hurdles encountered by a gene delivery system to enter and traffic into a tumor cell (Morille *et al.*, 2008).

The plasma membrane of living cells, a dynamic structure that is relatively lipophilic in nature, restricts the entry of very large, hydrophilic and negatively charged macromolecules as pDNA (Mintzer and Simanek, 2009; Rolland, 2005). By this way, internalization step in the target cell constitutes the first intracellular barrier for gene transfection process (Shin *et al.*, 2008). Electrostatic repulsions between the cell surface and pDNA are responsible for endocytosis of a small amount of pDNA by cells. This association is essentially mediated by heparin sulfate proteoglycans that are present on the cell surface. This binding process leads to endocytosis of particles with diameter lower than 200 nm (Medina-Kauwe *et al.*, 2005; Pathak *et al.*, 2009). The uptake levels can be improved by using chemical carriers that neutralize the anionic charges of pDNA backbone (described in section 2.3), in association with specific ligands conjugated with the backbone of the delivery system that promote preferential affinity by a particular type of receptors on the cell surface (Shin *et al.*, 2008; Hart, 2010). For this particular case there are a multitude of endocytic pathways that can be processed by the carrier systems: clathrin-mediated endocytosis via coated pits (adsorptive or receptor mediated), lipid-raft mediated endocytosis (caveoline-mediated or not), phagocytosis, macropinocytosis (Figure 5) (Anderson *et al.*, 2009; Morille *et al.*, 2008; Medina-Kauwe *et al.*, 2005; Mintzer and Simanek, 2009). After gene delivery systems surpass the cellular uptake by endocytosis they will be directed toward the endolysosomal compartment.

Naked pDNA must escape the endosomal/phagosomal compartment into the cytoplasm that represents a major barrier to pDNA efficient transfection (Medina-Kauwe *et al.*, 2005; Haisma *et al.*, 2011). In order to be effective, the vector has to be released from this compartment, preferably at an early stage. Given that cell internalization of pDNA occurs by endocytosis, and endosomal membranes possess an ATPase enzyme that actively transports protons from the cytosol into the vesicle, pDNA can be released by the proton-sponge phenomenon, which consists on accumulation of protons in the vesicle resulting in an influx of counter ions which causes osmotic swelling and rupture of the endosomal membrane (Haisma *et al.*, 2011; Morille *et al.*, 2008). Otherwise, endosomes will fuse with lysosomes, the main degradative compartment of the cell, that also contain a low pH environment and a high concentration of nucleases resulting in pDNA degradation (Pathak *et al.*, 2009). However, the application of specific carriers systems for pDNA can lead to different release processes according the carriers properties.

Once released from the endosomes, pDNA must finally displace through the cytoplasm to the nucleus to be transcribed into mRNA and subsequently translated into protein antigen (Anderson *et al.*, 2009). The unfavorable factor in this step is the presence of cytosolic nucleases, which degrade DNA with a half-life of 50–90 min (Pathak *et al.*, 2009). This is another reason for the importance of associating a carrier system that protects pDNA from degradation and improves its transport through the cell. At last, pDNA can diffuse into the nuclear region during the cell division stage because the nuclear membrane is temporarily

ruptured, unless the plasmids with size less than 10 nm can passively diffuse through nuclear pores or size less than 25 nm can be actively transported through nuclear pore complexes (Hart, 2010; Pathak *et al*., 2009).

2.2. Physical methods

Aiming the improvement of gene transfection using naked pDNA, several approaches of physical delivery methods have been employed, including needle injection (microinjection), needle-free injection (by high-pressure jets - jet injection; by bombardment with pDNA-coated microparticles - gene gun), electroporation, hydrodynamic gene transfer, sonoporation, laser assisted delivery and magnetofection (Anderson *et al*., 2009; Hart, 2010).

Microinjection using a needle is the most efficient and direct method to transfer pDNA into the cell cytoplasm or nucleus (Al-Dosari and Gao, 2009), despite to be a laborious technique, difficult to apply and inappropriate for the studies that require a large number of transfected cells. Jet injection methodology apply a liquid solution containing the naked pDNA that is applied to the skin as a jet of high velocity with the force to penetrate the skin and the underlying tissues, thus determining spread transfection of the areas of interest, being useful for gene therapy of skin metastasis in patients with breast cancer and melanoma (Mountain, 2000; Al-Dosari and Gao, 2009). Among the physical methods for gene transfer, one interesting approach is the possibility of delivering DNA into the cells by bombarding them with micron-sized beads carrying pDNA adsorbed onto their surface. Gene-gun delivery or also named ballistic consists on shooting gold, tungsten or silver particles at very high velocity into the tissues (Al-Dosari and Gao, 2009). These particles can easily cross the cell and nuclear membranes and release the DNA adsorbed on their surface into the nucleus, becoming more suitable than jet injection for pDNA vaccination and transfection of skin cells, since gene-gun requires much smaller pDNA doses (Mountain, 2000).

Electroporation was first reported in 1982 for gene transfer studies and is often used for cells that require milder methods of gene transfer. This process involves the exposure of cell membrane to high-intensity electrical pulses that can cause transient and localized pores, becoming the cell highly permeable to exogenous pDNA present in the surrounding medium (Mountain, 2000). Although at the end of the stimulus the membrane acquires again its normal properties, the application of this technology *in vivo* has limited accessibility of the electrodes to the internal organs and can induce tissue damage (Al-Dosari and Gao, 2009).

Hydrodynamic gene transfer or hydroporation method employs the high pressure as a driving force for gene transfer of large pDNA volume. Firstly, this process allows that pDNA to cross endothelial cell junctions, by inducing their separation, and afterward determines the transient formation of pores or microdefects in the plasma membranes of the target cells underlying the endothelium (Al-Dosari and Gao, 2009).

Both electroporation and hydrodynamic gene transfer are quite invasive, and thus difficult to apply for gene transfer to most organs. In contrast, sonoporation is used clinically for a variety of diagnostic and therapeutic applications. The ultrasound waves produced by sonoporation generate acoustic cavitation, which ultimately determines formation of micropores in the plasma membrane (Pathak *et al.*, 2009; Al-Dosari and Gao, 2009). Cavitation is increased by agents causing nucleation, such as echographic contrast agents based on microbubbles. In this case, rupture of the microbubbles caused by ultrasound increases permeability of the membrane and thus facilitates gene transfer (Al-Dosari and Gao, 2009).

In summary, these physical methods avoid the problems associated with endocytosis and facilitate the introduction of pDNA into the intracellular environment either directly (injection) or through the disrupted cell membrane, although the intracellular hurdles remain to be solved. Biodistribution of plasmids after intravenous administration can be markedly altered by formulating plasmids with a variety of chemical delivery systems (Rolland, 2005).

2.3. Chemical methods

Several non-viral vehicles of chemical nature have been proposed for safe delivery of therapeutic nucleic acids into the target cells as an alternative strategy to clinically dangerous virus-mediated delivery agents (Morille *et al.*, 2008). As it is summarized in Table 5, the chemical approaches can use a variety of materials including lipids (liposomes), polymers (polymeric nanoparticles, micelles, or dendrimers) and even organometallic compounds (some nanoparticles) as carriers to deliver nucleic acids into the target cells (Shin *et al.*, 2008). Ideally, a gene delivery system should be stable, biocompatible, non-toxic, cost effective and improve the solubility, protection and transfection of exogenous highly anionic genetic material, as pDNA, into the tissue-specific site (Wang *et al.*, 2010; Pathak *et al.*, 2009). Although the chemical carriers allow to overcome some problems related with the intracellular hurdles they also present some limitations such as poor oral bioavailability, instability in circulation, inadequate tissue distribution, and toxicity (Shin *et al.*, 2008).

Table 5. Examples of different chemical carriers systems for DNA delivery (Mintzer and Simanek, 2009; Shin *et al.*, 2008).

Nature of carriers systems	Examples of compounds
Cationic lipids	Lipofectin TM (DOTMA) Lipofectamine TM (DOSPA)
Polymers	Poly(L-lysine) (PLL) Polyethylenimine (PEI)- homopolymeric - structure variations Polymethacrylate Carbohydrate-Based Polymers - ß-Cyclodextrin - Chitosan - Poly(glycoamidoamine) - Schizophyllan - Dextran) Linear Poly(amido-amine) (PAA) Biodegradable Polymers - Poly(4-hydroxy-L-proline ester) - Poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) - Poly(amino-ester) - Phosphorus-Containing Polymers
Dendrimers	Polyamidoamine Dendrimers (PAMAM) Poly(propylenimine) Dendrimers (PPI) Poly(L-lysine) Dendrimers Phosphorus-Containing Dendrimers Carbosilane Dendrimers
Polypeptides	Tat-Based Peptides Antennapedia Homeodomain Peptide MPG Peptide Transportan Peptide
Nanoparticles	Quantum Dots Gold Nanoparticles Silica Nanoparticles Carbon Nanotubes Lipid-Based Nanoparticles - Solid Lipid Nanoparticles (SLNs) - Cerasomes Polymeric Hydrogels

Numerous studies over the past decade have focused on designing cationic compounds (lipids or polymers) that can form complexes with DNA and can avoid both extra- and intracellular barriers for gene delivery (Mintzer and Simanek, 2009). Interestingly, the complexation of pDNA mediated by electrostatic interactions between the negatively charged phosphate backbone of pDNA and cationic molecules leads to charge neutralization and a compaction of the nucleic acid. It has been shown that the size of the complex formed varies significantly depending on the type of cationic structure used, although the preparation conditions

including concentration of pDNA, pH, type of buffer, and N/P ratio also affect the complex size. Therefore, the physicochemical properties of the resulting complex are determined by the relative cation/anion amounts, preparation parameters (mixing speed, time, temperature of mixing, concentration of reactants, etc.) and material properties such as hydrophobicity, molecular weight, and charge density. The morphology of pDNA complexes formed with cationic polymers is independent of the polymer used (Mintzer and Simanek, 2009). For the particular case of cationic lipids as Lipofectamine TM, initially it was suggested that lipoplexes were delivered into the cytoplasm by direct plasma membrane fusion, but it is now agreed upon that liposome-mediated gene transfer proceeds primarily through endocytosis. Following cellular uptake by endocytosis, the cationic lipids of the carrier destabilize the anionic lipids of endosomal membrane, resulting in a flip-flop phenomenon, causing the pDNA release into the cytoplasm (Morille *et al*., 2008).

Overall, to find the ideal carrier for pDNA delivery is necessary the establishment of an equilibrium between the physicochemical properties of the pDNA/carrier complexes and the restrictions imposed by the extra- and intracellular barriers.

3. Plasmid DNA technology

The demand for efficient production and purification methods of pDNA has largely increased in response to rapid advances of pDNA application in both prophylactic (DNA vaccines) and therapeutic levels (gene therapy), taking into account the advantages of non-viral over viral vectors, previously referred. It is well known that the success of gene transfer to eukaryotic cells and subsequent expression of the target gene encoded on pDNA is strictly affected by the vector manufacturing process (Ferreira *et al*., 2000a). Hence, the need to perform an efficient transfection emphasized the development of novel platforms that allow the easy recovery of gene vectors under a high copy number and a satisfactory purity degree. However, first is essential to understand the structural properties and the stability of the plasmid that will influence the implementation of adequate production and purification processes.

3.1. Plasmid design

In general, the therapeutic plasmid vector can be divided in two distinct regions that have different roles. One part of the pDNA is important for the manufacturing machinery used by bacteria, which encodes the bacterial origin of replication that controls the plasmid copy number per bacterial cell and a selectable marker (Figure 6) that normally provides antibiotic resistance for the bacteria transformed with the plasmid encoding this gene (Liu, 2003; Williams *et al*., 2009).The other part of the plasmid includes a complete eukaryotic

expression cassette (Figure 6), composed by the transcribed region domain (including the sequence encoding the protein(s) of interest limited by two domains, the promoter/enhancer and terminator (Kaslow, 2004). Plasmids formulations give the possibility of genetically manipulate the plasmid composition, adding genes that for example encode cytokines or co-stimulatory molecules (unmethylated CpG motifs) increasing the protein production or enhancing immune responses. Also some structural conformations of plasmids, may contribute significantly to increase the biological activity of the expressed protein(s), being thus considered the preferred vectors for clinical applications (Manthorpe *et al.*, 2005; Liu, 2003).

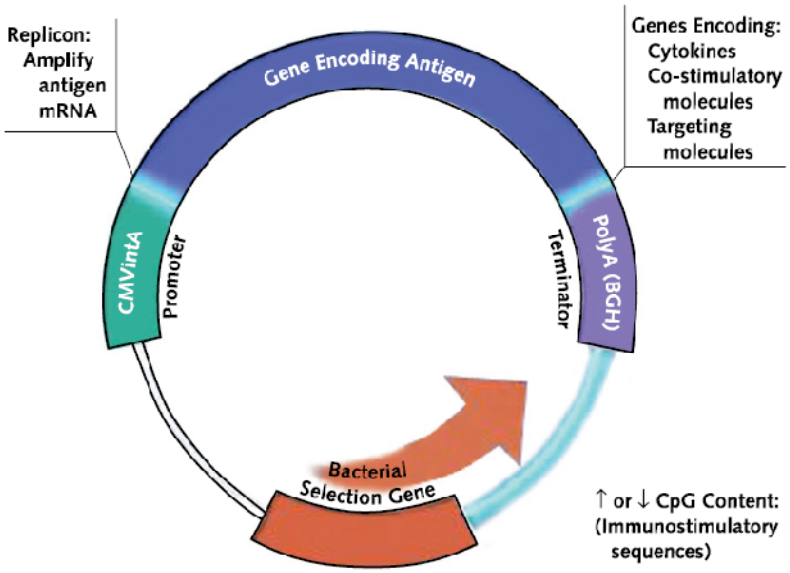

Figure 6. Schematic representation of a pDNA vector (Srivastava and Liu, 2003).

3.2. Plasmid structure, stability and storage for clinical applications

Plasmids are covalently closed, circular double-stranded DNA molecules, with a size from 200 up to more than 100.000 base pairs, that naturally exist in both, eukaryotic and prokaryotic cells and having the capacity of auto-replication independently from the chromosome of the host (Schleef, 2001). Each strand of a pDNA molecule is composed by a linear polymer of deoxyribonucleotides linked through phosphodiester bonds. The inner of the double helix is highly hydrophobic due to the close packing of the aromatic bases, while the outside is limited by phosphate groups (negatively charged for pH > 4) bounded to the sugars resulting in the hydrophilic backbone (Sinden, 1994). In the case of genomic DNA (gDNA), it is organized in an extremely compact structure due to the space restrictions inside the cellular nucleus, and the right-handed DNA double helix is further twisted in the opposite sense, resulting in a negatively under-winding DNA. Similarly, the helix axis of pDNA is naturally presented in the supercoiled (sc) conformation into the cytoplasmic space of the host cell. Biologically, negative supercoiling is advantageous because it promotes the necessary

unwinding and strand separation during replication, recombination and transcription (Sinden, 1994; Schleef, 2001).

Therefore, plasmid vector under sc topology is considered the most active and efficient isoform on transfection and gene expression on the eukaryotic cells, because it is the only naturally intact and undamaged pDNA conformation (Schleef and Schmidt, 2004; Cupillard *et al.*, 2005; Cherng *et al.*, 1999). However, other ineffective topologies of pDNA (Figure 7 (A)) can arise as consequence from the damage of the sc isoform. The breakage of one sc DNA strand leads to loss of the molecular coiling, resulting in the open circular (oc) isoform. Due to the total relaxed structure of oc isoform, the breakage of both strands at the same position or the accessibility to the restriction enzymes strongly favors the appearance of linear pDNA conformation (Schleef and Schmidt, 2004). In addition, the described topological plasmid isoforms can exist as equivalent dimmers by doubling in size (Figure 7 (A)), which normally occurs during the replication or by homologous recombination processes of the host cell (Schleef, 2001). Both oc and linear isoforms are considered less efficient in inducing gene expression because they can be randomly damaged at important gene locations, especially the promoter or gene coding regions (Schleef and Schmidt, 2004; Cherng *et al.*, 1999). Curiously, the different plasmid topologies occupy different sizes according their own conformation. In Figure 7 (B) is perceptible that sc isoform is the most compact isoform, while oc dimer the most expanded molecule. These size properties are useful for the identification of the different plasmid isoforms contained in the same sample by agarose gel electrophoresis. Beyond the size, they also present different bases exposition between plasmid topologies, being the bases more exposed on sc isoform due to the supercoiling phenomenon (Strick *et al.*, 1998). However, all these topologies represent the same plasmid and this can be proved because when they are treated with restriction enzymes, they are all disintegrated into linear isoform of the same size (Schleef, 2001).

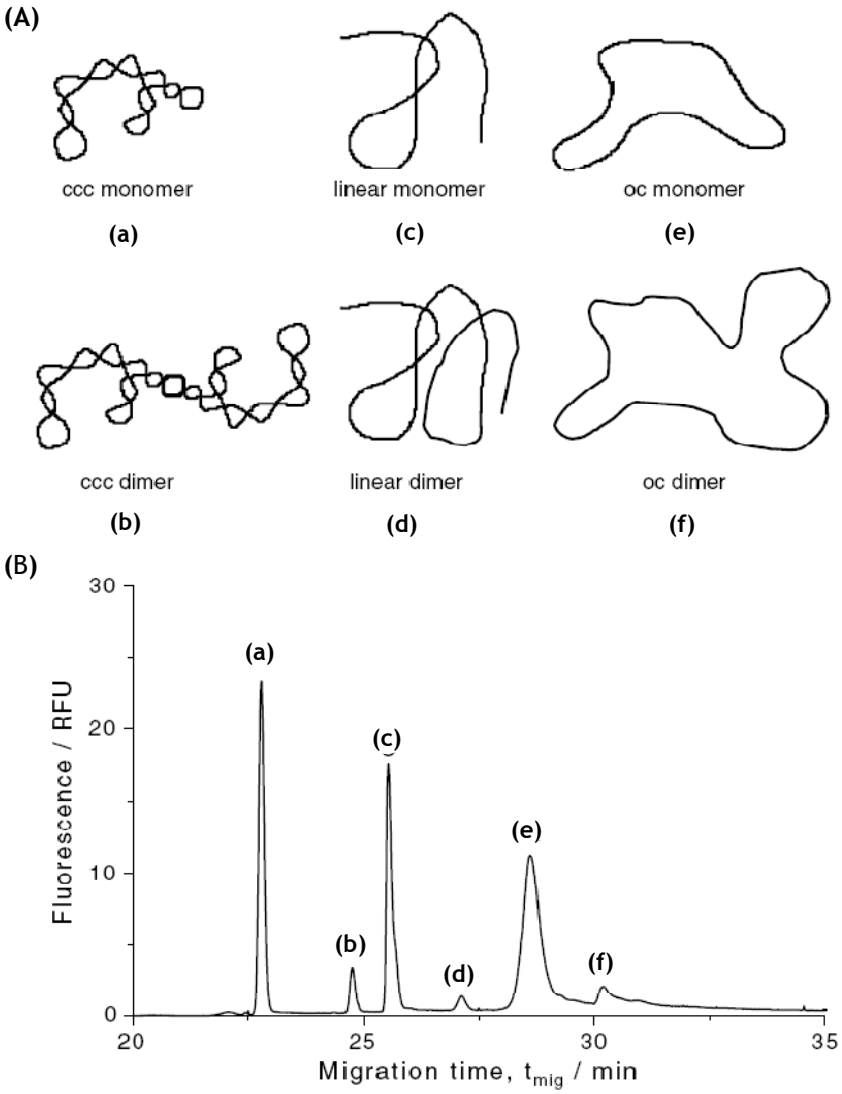

Figure 7. Different topologies of pDNA. (A) Examples of the possible pDNA conformations. (B) Topologic assessment of a plasmid DNA sample with different conformational variants by capillary gel electrophoresis (Schleef and Schmidt, 2004).

With regard to stability, pDNA has some very distinct advantages over protein-based pharmaceuticals. Proteins can be unfolded irreversibly by small changes in their tertiary or quaternary structure under relatively mild conditions (precipitation or aggregation), while the plasmids only loss the biological activity with a chemical modification of the phosphodiester backbone (Middaugh *et al.*, 1998). Thus, it is important to monitor the sc conformation stability that can be affected by processes within the plasmid-producing host or while processing the biomass (enzymatic or mechanic degradation) (Schleef, 2001; Schleef and Schmidt, 2004).

During the sc pDNA replication within an appropriate host, the cell machinery promote the appearance of topoisomerases (nuclease-active enzymes) that catalyze supercoiling and

relaxation of circular DNA, mainly on polyA sequences (Schleef, 2001; Oliveira *et al.*, 2009). The strategies for sc pDNA extraction and purification must be careful applied in order to avoid the use of enzymes or extreme alkaline conditions. If the pH is maintained below 12.5 during alkaline lysis, the sc pDNA isoforms start unwind as a consequence of the alkali-promoted hydrogen bond disruption (Diogo *et al.*, 1999). However, the anchor base pairs remain to prevent the complete separation of complementary strands, which serve as nuclei for the complete renaturation of pDNA molecules during a subsequent neutralization step (Diogo *et al.*, 1999). If cell lysis is carried out at a pH higher than 12.5, the plasmid anchor base pairs may be lost resulting in irreversibly denatured sc pDNA conformation appearing oc or even linear isoforms (Schleef, 2001; Diogo *et al.*, 1999).

Temperature is another critical factor that affects the structural stability of sc pDNA, either during the plasmid production or storage. The temperature increase results in extended thermal motion, which promotes a gradual unwinding of the DNA helix (Sousa *et al.*, 2007; Cherng *et al.*, 1999). Contrary to what happens for 24 and 4 ºC, storage of plasmid DNA at −80 and −20 ºC has shown no alteration concerning the plasmid conformations (Schleef and Schmidt, 2004). Therefore, for sc pDNA to be useful in clinical applications must be carefully isolated and purified, protected from enzymes and alkaline conditions and kept stable for extended periods of time, preferably at a convenient storage temperature. However, several studies already revealed good alternatives to store pDNA for some months, without losing the transfection efficiency and stability by freeze-drying and also adding some stabilizers such as sugars (Talsma *et al.*, 1997; Maitani *et al.*, 2008). These facts strengthen the theory that the use of non-viral pDNA vectors is advantageous over the viral ones, being easily stored and distributed in large amounts to undeveloped countries.

3.3. Plasmid manufacturing

The simplicity of the production methods is also one of the advantages of non-viral pDNA vectors for clinical applications instead of viral vectors. Over the years, several effective techniques for pDNA manufacturing have been developed, although the major challenge remains to achieve scalable and economical means of producing large quantities of sc pDNA fulfilling the requirements of regulatory agencies such as the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medical Products (EMEA) (Stadler *et al.*, 2004), presented in Table 6. The importance of to strict purify pDNA and remove the host impurities is related with the possibility of these foreign components originate adverse effects and inflammatory responses to the patients, if presents in quantities higher than those recommended.

Table 6. Characterization of bacterial cell lysates and accepted levels of impurities present in final sc pDNA preparation for clinical applications, recommended by regulatory agencies (adapted from (Stadler *et al.*, 2004; Ferreira *et al.*, 2000a)).

Bacterial cell lysates		**Final pDNA product**	
Component in focus	**Content (%)**	**Range of aceptance**	**Recommended assay**
RNA	21 %	Undetectable	Agarose gel electrophoresis
Proteins	55%	Undetectable	Micro-BCA protein assay
gDNA	3%	< 2 µg/mg pDNA	Real-time PCR
Endotoxins	3%	< 0.1 EU/µg pDNA	LAL assay
pDNA (all isoforms)	< 3%	> 97% sc pDNA	Analytical HPLC
Other components	15%	——	——

The pDNA manufacturing comprises several steps being started with the preparation of a bench scale, with the construction and selection of appropriate expression vectors and production microorganisms. After the establishment of these steps it is possible to follow to the selection and optimization of the host growth conditions to the plasmid production by fermentation. Finally, the isolation and recovery of pDNA can be performed with the integration of strategies to harvest and disrupt the host cells, to isolate and clarify the cellular lysate and to remove the impurities and preferentially purify the sc pDNA isoform (Ferreira *et al.*, 2000a). Figure 8 schematizes the most important steps to be considered during the sc pDNA manufacturing. Cell lysis and plasmid isolation and purification procedures are dependent of the inherent requirements of the Table 6.

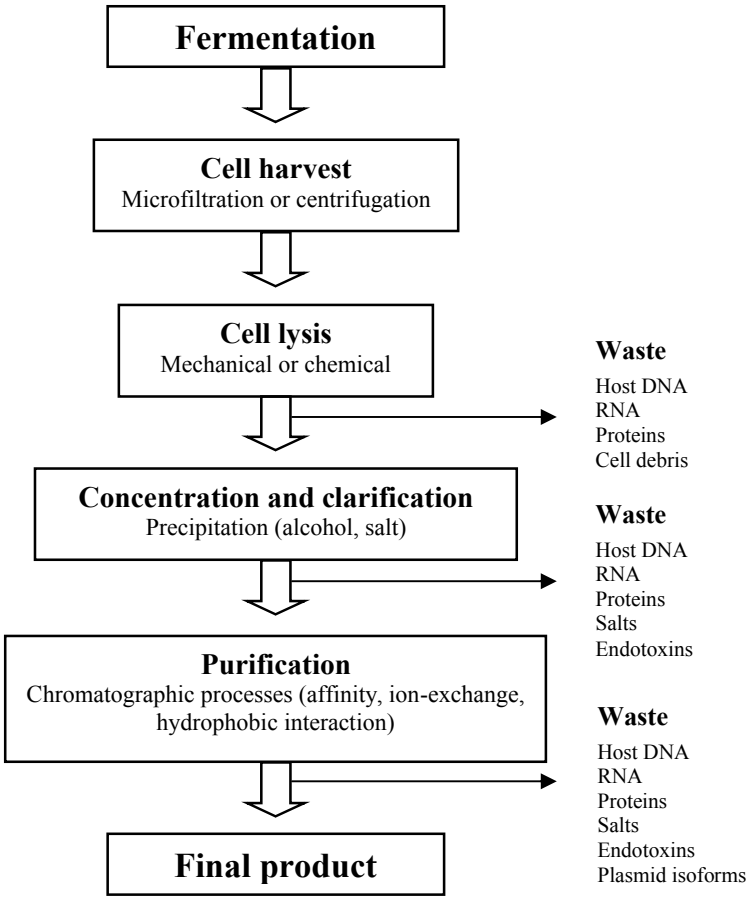

Figure 8. Schematic representation of the main unit operations to be considered during manufacturing process of sc pDNA together with the eliminated impurities in each step (adapted from (Ferreira *et al.*, 2000a)).

3.3.1. Fermentation

Plasmids are biosynthesized by autonomous replication in *Escherichia coli* (*E. coli*), an aerobic bacterium with a history of safe use in the bio-industry as a producer of many recombinant proteins. Following the preparation of a master cell composed by competent *E. coli* cells transformed with the target plasmid previously constructed, it is important to develop the *E. coli* fermentation step, to maximize plasmid copy number per cell.

Plasmids encode a variety of phenotypic functions which are not essential for the host organisms, but give them properties for a higher adaptability to distinct environments (Schleef, 2001). For example, the antibiotic resistance gene (Figure 6) allows the selective bacterial fermentation in the presence of a specific antibiotic, in order to promote the selective growth of bacteria containing plasmids encoding the antibiotic resistance gene (Manthorpe *et al.*, 2005). Studies of the effects of bacterial fermentation conditions on the production of plasmid shown that plasmid yield can be improved by supplementing media

with amino acids and temperatures between 37 and 40 ºC (Silva *et al.*, 2009). Indeed, manipulating the *E. coli* growth conditions, among which oxygen dissolved, temperature, selectivity with a specific antibiotic, nutrients concentration and pH, it is possible to improve the cell growth which is reflected on plasmid copy number (Silva *et al.*, 2011).

3.3.2. Cell harvest and lysis steps

Following the fermentation, the total amount of cells is recovered by centrifugation or microfiltration. Knowing that the centrifugation is the most used method, microfiltration offer the advantage of to wash away spent media components and extracellular impurities prior to beginning purification, although the number of times that a membrane can be used is limited (Manthorpe *et al.*, 2005).

Since plasmids are replicated in cytoplasmic space, the *E. coli* host cells must be lysed to release the molecule of interest. One of the limitations to the recovery of desired molecule is that beyond pDNA, all the host intracellular components (RNA, gDNA, endotoxins and proteins) are also released, and at this stage pDNA represents less than 3% (w/w) of the lysate content (Stadler *et al.*, 2004). Given that the host impurities present physicochemical properties similar to pDNA, the lysis step and the subsequent processes of isolation and purification must be carefully designed in order to reduce or eliminate the host impurities without loss or damage of the sc pDNA desired conformation.

Among the three possible methods to disrupt *E. coli* cells, chemical lysis is the most widely used, because the mechanical method employs ultrasound, which increases the temperature, being aggressive for sc pDNA structure (Chamsart *et al.*, 2001), while the enzymatic strategy is not accepted by the regulatory agencies due to the adverse effects of pharmaceuticals administration with residues of animal-derived materials, such as RNases (Stadler *et al.*, 2004; Ferreira *et al.*, 2000a). The most widely used chemical method is based on alkaline lysis, relying on the disruption of cells at high pH with NaOH in the presence of SDS, followed by the release and denaturation of gDNA, cell wall material and most of the cellular proteins (Diogo *et al.*, 2005). As it was previously referred, the application of pH higher than 12.5 causes intra-strand association of pDNA, appearing denatured pDNA (Schleef, 2001; Diogo *et al.*, 1999). By this reason, it is important to control the pH during the alkaline lysis, being considered a critical unit operation. After cell disruption, denatured gDNA and cellular debris are precipitated with a potassium acetate-based neutralizing solution. Different operations can remove this insoluble material, whereas the majority of pDNA remains in the supernatant. During these manipulations care should be taken to avoid shear-induced cleavage of gDNA, which has not the same capacity of refolding in a DNA double strand again as pDNA, resulting in small fragments that will not aggregate (Schleef and Schmidt, 2004). Throughout the lysis process, several impurities are removed, such as cell debris, most of

gDNA, endotoxins, part of RNA and some proteins, but it still remaining a large amount of impurities mixed with pDNA isoforms (Table 6).

3.3.3. Isolation (concentration and clarification)

For the preparation of plasmid extracts to be applied in the subsequent purification steps it is recommended the use of concentration and clarification techniques, designed to reduce the proteins and host nucleic acids content and to increase the plasmid mass fraction, named as primary isolation (Ferreira *et al.*, 2000a). The concentration of pDNA extracts can be achieved by precipitation with an alcohol, removing most of endotoxins, and some proteins (Manthorpe *et al.*, 2005). The proteins remaining in the lysate are usually removed by salting out phenomenon using a high concentration of chaotropic salts, which have the additional advantage of precipitating high molecular weight RNA together with the proteins (Ferreira *et al.*, 2000a).

3.3.4. Purification

Along the last two steps, the amount of *E.coli* host impurities is considerably reduced, whilst being present on pDNA-containing extract under non-acceptable quantities. At this stage, the chosen purification strategy needs to eliminate all the host impurities and non-effective plasmid isoforms, leaving the homogeneity of the final plasmid sample higher than 97% of sc pDNA isoform (Table 6). In addition, most of the critical impurities share common characteristics of negative charge [RNA, genomic DNA (gDNA) and endotoxins] molecular mass (gDNA, endotoxins) and hydrophobicity (endotoxins) (Stadler *et al.*, 2004) with pDNA, as well as pDNA isoforms which only differ on structural conformation. These similarities make difficult the purification procedure and this issue will be explored in detailed on section 5. Liquid chromatography, a high-resolution analysis method that is well characterized and well established in the biopharmaceutical industry, is central in process-scale manufacturing of therapeutic sc pDNA. According to the chromatographic principles, such as different size, charge, hydrophobicity, accessibility of nucleotide bases, the topological constrains imposed by supercoiling and affinity; different chromatographic strategies, such as size-exclusion, ion-exchange, hydrophobic interaction, reversed-phase, thiophilic adsorption and affinity can be applied either as an isolated or integrated steps (Diogo *et al.*, 2005; Sousa *et al.*, 2008a). Table 7 summarizes the advantages and drawbacks associated to the different chromatographic methods already used for pDNA purification.

Table 7. Chromatographic modes applied for pDNA separation, purification and quantification (adapted from (Diogo *et al.*, 2005; Sousa *et al.*, 2008a)).

Chromatography type	Advantages	Drawbacks	Major applications	References
Size-exclusion	Isolation of pDNA and gDNA from RNA, small molecules and endotoxins; Powerful as a finishing step.	Long chromatographic runs; Low chromatographic resolution with high samples dilution; Co-elution of pDNA isoforms and gDNA.	Final polishing step Group separation Endotoxin removal	(Whisenant *et al.*, 1988); (Ferreira *et al.*, 1997); (Li *et al.*, 2007)
Ion-exchange	Separation of pDNA isoforms in some cases; Rapid and simple removal of low charge density impurities Widely applied in preparative and analytical scales.	Low resolution between sc pDNA and other isoforms or gDNA; Co-elution of pDNA and RNA or endotoxins; Non-specific chromatographic phases; Low capacity.	pDNA capture and concentration pDNA quantification	(Prazeres *et al.*, 1998); (Tiainen *et al.*, 2007); (Eon-Duval and Burke, 2004); (Ferreira *et al.*, 2000b)
Hydrophobic interaction	Efficient separation of pDNA from endotoxins and single-strand nucleic acids; Rapid chromatographic runs, useful for analytical, preparative and industrial scales.	Non-binding of pDNA or difficult isoforms separation; Elution with high salt concentration.	pDNA quantification Impurities capture	(Diogo *et al.*, 2000); (Diogo *et al.*, 2001); (Diogo *et al.*, 2003); (Iuliano *et al.*, 2002); (Smrekar *et al.*, 2010)
Reversed-phase	Separation of pDNA from crude lysates; Possibility of isolating sc pDNA.	Requirement of organic solvents; Loss of pDNA integrity.	pDNA capture pDNA quantification	(Huber, 1998)
Thiophilic adsorption	Isolation of pDNA from impurities; Separation of pDNA isoforms.	Elution of pDNA in a high salt concentration.	pDNA capture pDNA concentration	(Lemmens *et al.*, 2003); (Sandberg *et al.*, 2004)
Affinity (in particular the strategy based on amino acids ligands)	Selective isolation and specific purification of sc pDNA isoform in a single operation; Efficient elimination of RNA, gDNA, proteins and endotoxins.	For Histidine-affinity chromatography, the elution of sc pDNA occurs with high salt concentration and relatively low yields.	sc pDNA capture pDNA quantification Impurities elimination	(Sousa *et al.*, 2006); Sousa *et al.*, 2008b); (Sousa and Queiroz, 2011)

Amongst the several chromatographic strategies implemented for pDNA purification, some of them present more advantages than other due to the specificity and selectivity that allow the purification of the sc pDNA isoform, and the use of mild salt conditions without organic compounds, avoiding the loss of pDNA integrity. Therefore, affinity chromatography seems to be the most adequate technique to properly purify sc pDNA isoform, eliminating the remain plasmid isoforms and host impurities in one single unit operation (Sousa *et al.*, 2008a).

Affinity chromatography is a methodology extensively described for proteins that allows the elimination of additional purification steps, enhancing the product quality, increasing yields and improving process economics (Lowe *et al.*, 2001). In fact, affinity chromatography has the singular ability of analyzing or purifying biomolecules on the basis of their biological function or individual chemical structure due to the use of a specific binding agent (Lowe *et al.*, 2001; Kanoun *et al.*, 1986). This particularity together with the evolution of ligand design, focused on the association of the selectivity of natural ligands and the high capacity and durability of synthetic systems (Lowe *et al.*, 2001; Mondal and Gupta, 2006), has permitted the application of several affinity chromatography strategies for nucleic acids purification (Sousa *et al.*, 2008a).

Immobilized metal ion-affinity chromatographic matrices are composed by chelating agents, such as nitrilotriacetic or iminodiacetic acids, facilitating the coordinate transition of metal ions, mainly Ni(II) and Cu(II) (Nastasijevic *et al.*, 2008). These stationary phases are able to selectively adsorb single stranded nucleic acids, such as RNA and denatured gDNA, through metal ion interactions with aromatic base nitrogens, especially those present on purine bases (adenine and guanine) (Cano *et al.*, 2005), whereas oligonucleotides duplexes, pDNA and gDNA, showed low IMAC binding affinity (Murphy *et al.*, 2003). Triple-helix affinity chromatography is based on recognition of homopurine specific sequences introduced on pDNA by a complementary oligonucleotide covalently linked to a chromatographic matrix through the formation of Hoogsteen hydrogen bonds resulting on a triple-helix (Wils *et al.*, 1997; Ito *et al.*, 1992). The association and dissociation of triple-helixes occurs at acidic and alkaline conditions respectively (Ito *et al.*, 1992). Although this method allows to specifically bind sc pDNA reducing the level of contaminating RNA, endotoxins and gDNA in a single step, it also occurs the loss of pDNA during the wash step leading to low yields (Wils *et al.*, 1997). Protein-DNA affinity chromatography exploits the natural interaction between a pDNA motif, such as lac operon sequence, and a recognizer protein, for this specific case the lac repressor protein, immobilized on the matrix (Hasche and Voss, 2005; Forde *et al.*, 2006). In this method, RNA and proteins can be eliminated, but gDNA also showed a specific interaction with the repressor being co-eluted with pDNA (Hasche and Voss, 2005). Amino acid-DNA affinity chromatography has revealed to be a promissory approach that selectively recognizes the sc pDNA isoform through specific amino acids bound to the agarose matrix, such as histidine and arginine, which allow eliminating the remaining pDNA isoforms and all the

impurities present on sc pDNA-containing extract (Sousa *et al.*, 2006; Sousa *et al.*, 2008b). The application of these amino acids as affinity ligands takes advantage of several interactions that can be developed with the different components present on the initial impure extract facilitating its selective separation. Among these different interactions it was described the presence of the hydrophobic, electrostatic, cation-π, van der Waals forces and/or hydrogen bond interactions (Sousa *et al.*, 2010). Moreover, these interactions can be favored or disfavored by manipulating the elution conditions, such as the temperature, flow rate and buffer composition (pH, ionic strength or presence of competitive agents). More details about this specific affinity chromatography are referred in section 5.

Regarding the sc pDNA purification, not only the chromatographic principles have been explored but also the chromatographic supports have been developed and optimized at chemical and structural levels in order to exceed the limitations of traditional stationary phases. In section 5 it will be extensively discussed the structural evolution of several chromatographic supports applied on plasmid purification field and highlighted the advantages of combining speed, resolution and capacity of some modern supports with the selectivity and specificity of some affinity ligands.

3.4. Plasmid quality control

Plasmid DNA quality depends of the manufacturing process, mainly the lysis, isolation and purification steps, being necessary a strict control and assessment of the remaining impurities on the final sample by several methods. Host RNA detection by agarose gel electrophoresis or analytical High-Performance Liquid Chromatography (HPLC) must be performed since pDNA should be obtained without using RNase. Proteins can be quantified by colorimetric assays, like the Bradford or the BCA assays (bicinchoninic acid) (Schleef and Schmidt, 2004). Although southern blot or hybridization can be used for detention of gDNA fragments, the most sensitive assay is the real-time Polymerase Chain Reaction (PCR), a kinetic method that uses a TaqMan probe to quantify gDNA contaminations (Smith *et al.*, 1999). Lipopolysaccharides, commonly designated by endotoxins, can be determined by kinetic measurement of Limulus Amoebocyte Lysate (LAL) assay (Stadler *et al.*, 2004). Finally, topological structure of pDNA can be characterized and monitored by agarose gel electrophoresis, or analytically by capillary gel electrophoresis (Schleef and Schmidt, 2004). Recently, a new analytical method based on arginine-affinity chromatography was developed to specifically quantify the amount of sc pDNA isoform present in a purified pDNA sample (Sousa and Queiroz, 2011).

No guideline exists, indicating a certain value or specification for clinical material (Schleef and Schmidt, 2004). However, several regulatory identities recommended that the final pDNA sample resultant from manufacturing process must be free from host constituents, such as RNA and proteins (undetectable), gDNA (< 2 μg/mg pDNA) and endotoxins (< 0.1 EU/μg pDNA),

with a sc isoform content higher than 97% (Stadler *et al.*, 2004; Ferreira *et al.*, 2000a). For example, the removal of gDNA to the lowest possible level is required due to the concerns associated to the risk of insertion on genetic material on eukaryotic cells (Schleef and Schmidt, 2004). Likewise, lipopolysaccharides constituents of outer *E. coli* cell wall may dramatically reduce *in vitro* transfection efficiencies in various cell lines and *in vivo* promote strong biological effects after entering in the blood stream, even at low concentration because of its toxicity (Wei *et al.*, 2007; Schleef and Schmidt, 2004). If the final, pDNA therapeutic product should respect the requirements imposed by regulatory agencies, hence, *in vitro* transfection studies must be performed to test the purity degree and efficiency of sc isoform. The optimization of delivery systems, as for example by using the natural polymer of chitosan (Gaspar *et al.*, 2011b; Gaspar *et al.*, 2011a), (described on section 2.3), should also be considered before testing in animal models or humans to avoid the hurdles that decrease the transfection efficiency.

4. References

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Paper I

5. Advances in chromatographic supports for pharmaceutical-grade plasmid DNA purification

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(*Submitted*)

Advances in chromatographic supports for pharmaceutical-grade plasmid DNA purification

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Abstract

Chromatographic technology is undoubtedly one of the most diverse and powerful purification methods for downstream processes applications, affording high resolution for bioseparation of molecules from complex mixtures. The diversity and quantity of biomolecules present in the crude extracts as well as the similarities between contaminants and the target biomolecule are considered the critical challenges in extraction and purification steps. Thus, it is important to optimize the purification protocol to achieve maximum recovery of the target sample. The difficulty of combining speed, resolution, capacity and selectivity when using the classical bead stationary phases has even been called the "dilemma of therapeutic molecules chromatography". By this way, the structure of chromatographic supports has been continuously developed to provide a rapid and efficient separation. In addition, the application of specific ligands to improve the selectivity for the target molecule has also been investigated.

The present review discusses the structural progress and evolution of the chromatographic supports that have been largely used for proteins and recently adapted for plasmid DNA (pDNA) purification. Nowadays, the most desirable form of pDNA for gene therapy and DNA vaccination is the supercoiled (sc) isoform, due to its stability and higher transfection efficiency over other plasmid topologies. The successful clinical applications of sc pDNA in several human diseases impose an improvement of productivity. However, the main challenge is not only to produce high quantities of sc pDNA but also to preserve its quality, fulfilling the strict requirements recommended by the regulatory agencies. In order to obtain satisfactory amounts of homogeneous sc pDNA preparations, the continuous research and development of specific and suitable stationary phases, including new ligands, has been essential for the efficient capture of this pharmaceutical biomolecule. Thus, this review will focus the chemical and structural classification of the different media, some of the specific ligands used for pDNA bioseparation and the immobilization techniques. Many successful advances have been achieved on plasmids purification field, with the application of supermacroporous monolithic matrices and amino acids-based ligands, being a promissory association.

Keywords: Chromatographic supports, ligands, monoliths, plasmid DNA, selectivity.

Outline

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Acknowledgments

References

1. Introduction

Chromatography is one of the most used methods in biotechnology, either at analytical and preparative level. Due to its simplicity, robustness and high reproducibility it can be applied in achievement of a variety of biomolecules, such as plasmid DNA (pDNA). Actually, for therapeutic areas under development, such as gene therapy and DNA vaccination, the major interest is not only to produce high quantities of plasmids as non-viral vectors but also to obtain and conserve its quality, fulfilling the purity requirements of regulatory agencies [1,2]. Therefore, the success of gene transfer by pDNA into the cells and subsequent expression is strictly affected by the plasmid manufacturing process (Fig. 1) [3]. In fact, one of the most important concerns when working on pDNA purification is the adjustment of all operational conditions to enhance the performance of the biotechnological strategy, guaranteeing the structural and functional stability of the most biologically active conformation of pDNA, the supercoiled (sc) isoform [4].

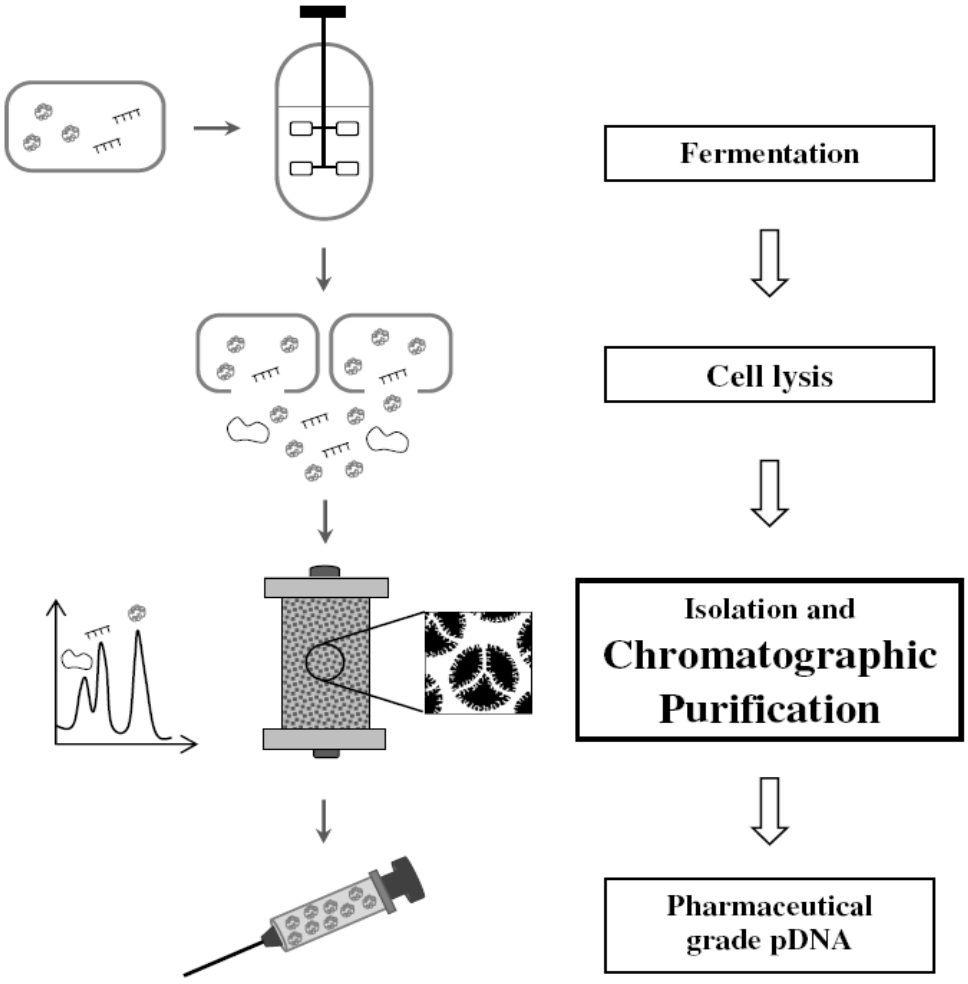

Figure 1. Chromatographic purification step as part of the downstream processing for pharmaceutical-grade sc pDNA preparation. sc pDNA; oc pDNA; RNA.

The separation and purification process commonly starts with alkaline lysis of the transformed host, for example *Escherichia coli* (*E. coli*), removing part of the cell walls, proteins and genomic DNA (gDNA), but leaving RNA as the main impurity of the target sc plasmid conformation together with other plasmid topologies, some proteins, endotoxins and gDNA fragments [5]. Most of these impurities share analogous chemical, physical and structural properties with pDNA that are summarized in Table 1, among which negative charge (RNA, gDNA and endotoxins), molecular mass (gDNA and endotoxins) and hydrophobicity (endotoxins) [6].

Table 1. Physical, chemical and structural similarities between pDNA and *E. coli* lysate molecules.

Parameter	oc pDNA	sc pDNA	gDNA	RNA	Endotoxins
Size	✓	✓	✓		✓
Negative charge	✓	✓	✓	✓	✓
hydrophobicity	✓	✓			✓
Bases exposition		✓		✓	
Nucleic acids conformation	relaxed circular; double-stranded	compact supercoiled; double-stranded	denatured single/double-stranded	single-stranded	

Different chromatographic techniques including size-exclusion, anion exchange, hydrophobic interaction, reversed phase or affinity chromatography, have been continuously developed and implemented to isolate the sc pDNA from the remaining host impurities either single or combined, with more or less success [6]. In fact, the similarity between sc pDNA and other ineffective plasmid topologies and host impurities aforementioned (Table 1) represent the main challenge when designing pDNA purification processes, and can only be surpassed by making use of high resolution, selectivity and high dynamic binding capacity chromatographic supports. By this way, the need of improving the chromatographic supports and ligands arose in order to achieve higher resolution and capacity, as well as to develop a specific biorecognition with the desired plasmid conformation, allowing an efficient purification in a simple unit operation. The present review focuses on the structural evolution of the chromatographic supports required in pDNA purification for therapeutic applications. The ligands immobilization on matrices with different formats to increase the accessibility and specificity to the target molecule will also be reported.

2. Chromatographic support requirements for pDNA purification

Nowadays, the collection of techniques known as chromatography may be the most prevalent and useful analytical and preparative methodologies. Due to their versatility and resolution, chromatographic separations of complex mixtures of biological products are used for many

purposes in research and industrial biotechnology [7]. In liquid chromatography, the sample components are distributed between stationary and mobile phase, owing to the dynamic separation of the mixture in a flow system [8]. The nature of the stationary phase determines the nature of the mobile phase. The optimization of both stationary and mobile phase is required to get optimal performance, because they are associated [7,8]. For instance, the mobile phase should be proper according to the nature of the stationary phase, notwithstanding the characteristics and stability of the target molecule that can be a limitation. In pDNA purification for pharmaceutical applications, the main challenge for the different biotechnological strategies developed is to eliminate the *E. coli* host impurities, maintaining the highest supercoiling degree as possible [4].

Successful plasmid purification largely depends on the selection of a suitable solid support. Indeed, the support format should ideally be macroporous with high chemical and physical stability [8] and should selectively capture plasmids, while at the same time should exhibit low nonspecific adsorption, high binding capacity and mass transfer, maintaining good flow properties throughout processing (See Section 3.2). Likewise, the stationary matrix must preferably be incompressible, inexpensive, simple to use, reusable in several chromatographic runs and sanitized by alkaline conditions [9]. The matrix may be commercially available or can be made by attaching a suitable ligand to a solid support via the appropriate chemical reactions (see Section 4).

Pore dimension is correlated with the exclusion limit, which defines the size range of molecules that cannot enter in the pore [8,10]. Hence, for an ideal chromatographic support, the size of the pores should be at least five times larger than the average size of the target biomolecule for its easy access to immobilized ligands, increasing the binding capacity. Otherwise, pores excessively large allow unrestricted access of large molecules to the immobilized ligands, but imply surface area reduction and consequently lower ligand density and lower capacity. Pores extremely small hinder the molecules access to the pores staying the purification only dependent of the specificity of surface area [8]. In reality, the pore size of a matrix is inversely correlated to its surface area, which in turn, directly affects the amount of immobilized ligand and thus the binding capacity [9,11].

Overall, for plasmids separation, the solid supports should be adapted to the physicochemical aspects of the target plasmids. The large size of plasmids implies the use of matrices with a defined pore geometry, whereas the chemical characteristics of these biomolecules require a suitable surface properties of the solid support [8]. Undoubtedly that the chemical composition of the chromatographic supports determines the preferential interactions established with the target molecule, allowing its retention whereas the undesirable molecules are eluted. By this way, the chemical nature of the functional groups will determine the chromatographic action principles that will reflect in the success and efficiency of the purification step [4,6]. The possibility to perform chemical modifications or

to immobilize specific ligands in the surface of the chromatographic supports should result in increased stability of the matrix and in the improvement of the established interactions. On the other hand, the physical and structural characteristics of the solid supports will decide the availability and accessibility of the functional groups to the target molecules [8]. As it was previously refereed, an equilibrium between the structural shape and the number of available binding sites is required to take advantage of a high binding capacity and productivity. Therefore, the combination of the ideal chemical and structural characteristics of the chromatographic matrices can result in a suitable purification process to obtain biological samples under a pharmaceutical-grade for therapeutic applications.

3. Classification of different supports

The classification of different chromatographic supports is not a simple process, because they can be organized by different categories. The present review will mainly center in the classification according the physical and structural properties of the constituent material, being also briefly described the different supports according to the chemical properties.

3.1. Chemical properties of the constituent material

The constituent material of chromatographic supports can be classified according to the chemical properties in natural polymers, synthetic polymers, inorganic material and composite material (summarized in Table 2). Natural polymers, such as cellulose, agarose, dextran and chitosan are often used because they present low unspecific adsorption [9,12]. However, cellulose is less advantageous over the other natural polymers because its fibrous structure limits the flow rate. In some cases, the material has to be crosslinked due to the soft constitution of these natural polymers, in order to improve the surface area properties of the solid support. The substitution with hydroxyl groups makes these materials more hydrophilic, remaining enough groups available to introduce ligands that will also modify the surface in accordance with the intended purpose [9]. The synthetic polymers, like polyacrylamide derivatives, polymethacrylate and polystyrene polymers, are more hydrophobic than the natural polymers, being necessary to coat the surface area to avoid recovery loss. On the other hand, they are characterized as chemically stable because of their resistance to extreme conditions of pH and oxidation environmental. The most common inorganic material is hydroxyapatite, silica and glass [13,14]. All these materials present advantages and disadvantages in chromatography application, for example hydroxyapatite develops good selectivity with target molecules but presents poor flow properties, unlike glass that it is not so selective but has excellent flow and mass transfer properties due to its rigid and porous structure [15]. Silica is the principal inorganic material that can be coated with natural or synthetic polymers obtaining also a good selectivity. Nevertheless, the support constituted by a hydrogel polymerized into a macroporous shell of silica, for example, is

considered a composite material, where it is possible to work at extremely high flow rate without loosing binding capacity [16]. In some studies mentioned in Table 2, the basic material is adapted in order to coat or graft an additional layer onto the surface (cellulose, dextrane, agarose or glass) with other materials to attain the intended purpose.

Table 2. Examples of different basic material constituent of chromatographic supports mainly applied in pDNA purification.

Category	Basic material	Physical shape	Trade name	References
Natural polymer	Cellulose	Fibrous crosslinked	CELBEADS	[17]
	Nitrocellulose	Fibrous		[18,19]
	Dextran	Particles Particles crosslinked	Sephacryl S Sephadex G	[20] [21]
	Agarose	Particles Particles with dextran modification	Sepharose 4B Superdex	[4,22,23] [24]
Synthetic polymer	Polyacrylamide derivate	Cryogel	Trisacryl or Hyper	[25]
	Polymethacrylate	Porous continuous bead	CIM	[26-30]
	Polystyrene	Microparticles		[31]
Inorganic material	Hydroxyapatite	Porous crystal		[13]
	Silica	Fibers		[14,32]
	Glass	Coated with polystyrene		[33]
Composite material	Silica+hydrogel	Gigapores		[16]
	Methacrylate+cryogel	Supermacroporous	PHEMAH	[34]

3.2. Physical and structural properties of the constituent material

The enormous variety of biological molecules that need to be efficiently purified from a mixture to apply in pharmaceutical industry forces the continuous development of new chromatographic supports, more appropriate to obtain the target molecule with a suitable purity degree. The great development on chromatography has been achieved mainly for proteins purification [12,35-37]. Nevertheless, some of these advances are applied on purification of other biomolecules, obtaining enthusiastic results. The chromatographic research has improved the format of the material in order to satisfy the purity requirement for more complexes molecules, maintaining satisfactory recovery yields. Several

chromatographic supports with different structural and physical properties (Fig. 2) have been used for pDNA purification [25,34,38,39-41]. Nowadays, the advances in this field allow the purification of the desired plasmid conformation, rapidly, with an adequate removal of impurities and a reduced sample loss. Therefore, an overview of the evolution of the most common supports, among which dispersed particles non-porous or porous, membranes and monoliths including cryogels, used in pDNA purification will be presented below. The advantages and disadvantages of these chromatographic supports are summarized in Table 3.

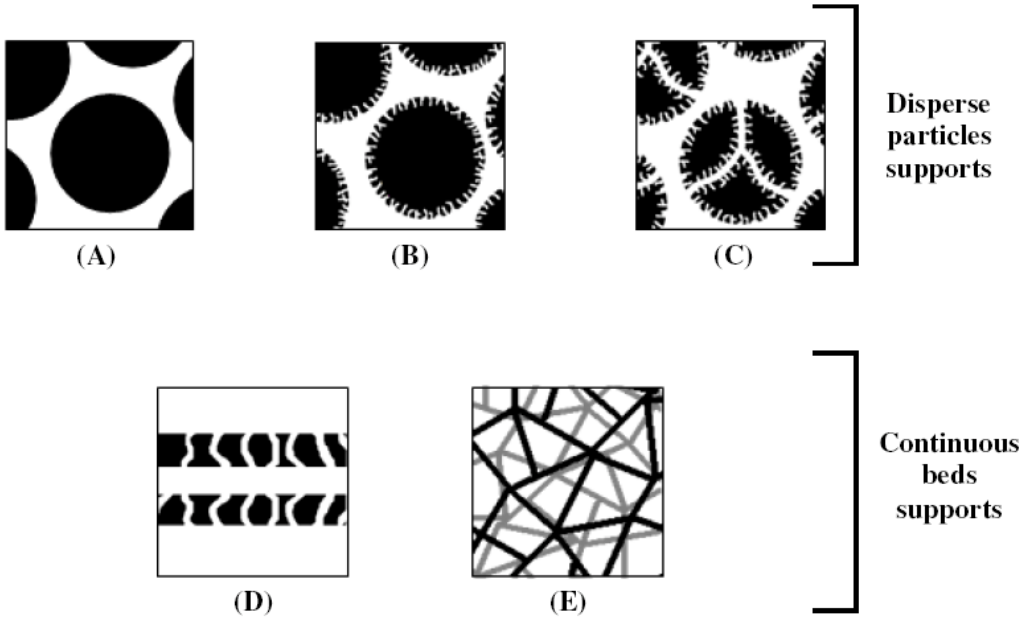

Figure 2. Schematic representation of the physical and structural properties of the constituent materials of the different chromatographic supports. (A) Diffusive non-porous particles; (B) Diffusive porous particles; (C) Perfusion particles; (D) Two sheets membranes stacked; (E) Monoliths.

Table 3. Summary of advantages and drawbacks related with structural advance in chromatographic stationary phases used for pDNA bioseparation.

Structural properties	Advances	Drawbacks
Dispersed particles		
- Non-porous	Rapid separation and good resolution.	Low specificity, capacity and efficiency.
- Porous	Better binding capacity and selectivity.	High flow resistance that leads to high back pressure.
- Perfusion	Improvement of mass transfer resistance and increase surface area.	Efficiency dependent of pore exclusion and void volume between the particles.
Membranes	Better chromatographic resolution, efficiency and productivity. Mass transport by convection instead diffusion.	High cost per low surface area and lack of radial connectivity between the different pores resulting in low binding capacity.
Monoliths	Material polymerization directly on column avoiding scale-up and scale-down problems, without dead volume. Convective channels interconnected providing more binding sites and fast mass transfer under low back pressure.	Not suitable for size exclusion chromatography, depending of the channel size.

3.2.1. Dispersed particles

The solid supports used for plasmids achievement should be highly porous, with good chemical and physical stability, while at the same time should exhibit a selective capture of the target, with low nonspecific adsorption and high capacity, maintaining the flow properties throughout process. As it was previously referred, the chromatographic supports must be inexpensive, readily available, and simple to use. However, several studies have been performed with matrices under different structural forms, like dispersed particles or continuous beds, porous or non-porous, which contribute by different ways for plasmid purification.

3.2.1.1. Non-porous

The non-porous dispersed particles are more useful for quality control, on-line monitoring and purity evaluation of biomolecules than for preparative purposes. Due to a total absence of pores (Fig. 2(A)), the mass transfer resistance and diffusion effects are eliminated, being possible to obtain a rapid separation and analysis with high resolution [42]. Likewise, the lack of internal pore structure allows good recovery, avoiding the conformational changes of

biomolecules that could lead to a loss of their biological activity. However, the loading capacity of the columns packed with non-porous sorbents is relative low and unspecific due to the simple and small surface area of these particles. Thus, the use of small non-porous particles on preparative chromatography is not recommended because they cause extremely high-pressure drop. To overcome the problem of low specific surface and to obtain high chromatographic efficiency, the non-porous packing materials are often coated with some polymers like dextran [24,42] or grafted with specific ligands that facilitate the access to the target molecules and increase the adsorptive surface area. For example, a particular study applying polystyrene based microparticles coated with quaternary amines revealed an increased dynamic binding capacity for pDNA, being possible to employ this matrix in the extraction of pDNA from *E. coli* lysate in a lab-scale capture step [31]. Therefore, non-porous particles have been mainly used for the implementation of analytical strategies to quantify pDNA, taking advantage of hydrophobic [38,43] or affinity ligands [44].

3.2.1.2. Porous

Aiming to overcome some limitations of non-porous particles, arises the implementation of solid materials with defined porosity only in the surface of the particle, as it is represented in Fig. 2(B). The chromatographic matrices should be highly porous to increase the contact surface area for the biomolecules, enabling increase the binding capacity and selectivity due to the exclusion limit imposed by the porous size [10], as mentioned above in section 2. Theoretically, smaller molecules are preferred because they allow faster mass transfer between outer flow and interior of the porous particle, making possible to use higher flow rates and maintaining an efficient capture. However, these molecules also lead to higher flow resistance, greater potential to form aggregates and collapse the particles [24], increasing the accumulation of contaminants such as cellular debris and denatured proteins of the sample which can also contribute to high back pressure.

The traditional porous particles are composed by a porous shell and a solid core [25]. For this reason, the mobile phase is stagnant inside the pore and the molecules only penetrate by molecular diffusion implying the increase of mass transfer resistance [45]. This behavior is harmful for therapeutic proteins and plasmids that are susceptible molecules at long procedures, can undergo degradation and consequently lose the biological activity [16,46,47]. For the successful implementation of porous stationary phases in chromatography, a compromise between pore size of the solid support and the diameter of the target molecules should be taken into account. For example, in a particular study it was prepared a specific anion exchanger matrix that allowed an efficient discrimination between RNA and pDNA present in a complex *E. coli* lysate [20]. Large plasmid molecules did not bind to such beads since they are too large to enter the pores and therefore cannot come into contact with the positively charged matrix in the inner parts of the beads. Otherwise, RNA molecules present in a clarified lysate, readily enter the pores and become adsorbed [20]. Thus, taking

advantage of the different size of these molecules, and preparing particles with an uncharged layer and a core containing cationic groups, it was possible to combine size exclusion and anion exchange principles to efficiently separate two negatively charged molecules.

To improve the role of conventional porous particles discussed above, perfusion particles (Fig. 2(C)) containing a network of large through-pores accessible by diffusion were introduced. This kind of superporous particles allow an intraparticle mass transport by molecular diffusion [48], improving the effect of mass transfer resistance resultant of the mobile phase stagnated inside the pore. The performance (e. g. resolution and capacity) of these superporous particles considerably increased due to the efficient mass exchange between the mobile and stationary phases under increased flow rates [12] and the additional surface of porous walls to bind large molecules. This superporous technology was firstly applied in agarose beads for proteins purification by ion-exchange, affinity and hydrophobic chromatography [49-51]. A comparative study between superporous and non-porous agarose beads revealed that the superporous beads gave satisfactory separation with a twice-increase on the purification factor and on the concentration of the desired product, at flow velocities five times higher than was possible for homogeneous beads [51]. In a more recent study, the superporous agarose anion exchangers were applied for plasmid isolation from a *E. coli* lysate, and showed to have four to five times higher plasmid binding capacity than the corresponding non-porous agarose beads [39]. The tendency to have greater surface area for plasmid binding using superporous particles was also observed and confirmed in the same work by confocal microscopy of the beads with adsorbed, fluorescently labeled plasmids under binding conditions.

Diffusion and convection are the main modes of mass transport to be considered in superporous dispersed (Fig. 2(C)) and non-dispersed particles (Fig. 2(D) and (E)). The movement by diffusion can be explained as the migration of solutes from an area of high concentration to an area of low concentration. Traditional porous particles rely almost exclusively on diffusive mass transport [45,48]. Nevertheless, the diffusion movement is injured by using large molecules that directly decrease the dynamic binding capacity, being difficult to work at higher flow rates [9,52,53] and consequently affecting the resolution. Most of these limitations are dependent of pore exclusion [10], as previously referred. Otherwise, convective mass transport is only imposed by the flow rate, and is independent of diffusion or molecular size. For this case, the flow rates normally used are 10-20 times higher than the velocities applied in diffusive particles, and under these flow conditions the binding capacity and resolution are almost unaffected [52,53]. In fact, the interconnected throughpores bring intraparticle convective transport into the bead and differs from diffusive pores that are characterized mainly by providing a substantial surface area.

Therefore, the perfusion technology based on superporous particles described above brought the advantage of using the high flow rates to the chromatographic media, without sacrificing

resolution. It was described that the application of this technology to dispersed particles allows the formation of convective channels on the interior of the particle (Fig. 2(C)), but the transport of the sample through these supports is still dominated by diffusion. On the other hand, despite a significant improvement of the mass transport inside the channels of the dispersed particles, they still present a disadvantage related with the void volume between the particles packed in a column, which implies that the mobile phase still flows around rather than through the particles. However, the implementation of this methodology in continuous beds material as membranes or monoliths (Fig. 2(D) and (E)) already implies that the mass transport becomes governed mainly by convective movement.

The continuous beds supports with convective channels, instead of superporous, are often designed as macroporous [54] or gigaporous [55,56] perfusion material, depending of the pore size or the intended aim. For example, a comparative study of the effect of intraparticle mass transfer parameters on the surface and pore diffusion in macroporous and gel-filled gigaporous stationary phases were performed for protein chromatography [48]. The obtained results revealed that the diffusion is a significant mechanism of mass transfer in gel-filled gigaporous stationary phase, since the intraparticle diffusivity increase with the retention factor, what was less evident for the macroporous support. Gigaporous chromatographic material was developed to overcome the problems associated with slow mass transfer in conventional porous microspheres and to produce porous with a controlled diameter [57]. Excessive hydrophobicity of these perfusion chromatographic polystyrene-supports was controlled by coating with poly(vinyl alcohol) and agarose [56,57], avoiding non-specific binding and biomolecules degradation. Thus, these gigaporous structures were developed to increase the capacity of stationary phases, although this parameter should be controlled because it could decrease when the pores become too large. Normally, such materials consist of a rigid skeleton containing the large pores, which are filled with the soft gel designed as hydrogel that increases the surface charge density [16]. In this case, the capacity is significantly increased while the flow rate dependency of the chromatographic separation lies between that of the perfusion and the conventional porous particles.

However, when the foremost objective is to purify pDNA, some problems arise due to its high molecular weight that is responsible for the increase viscosity of pDNA extracts resulting in lower diffusion coefficients [58]. This trouble gains significance when the conventional chromatographic supports with dispersed particles are used. The pores number and the convective channels introduced through the pores are not sufficient to overcome the diffusion limitation and the void volume between the particles, or to improve the binding capacity of the support for the target molecules, which is also considered to be a limiting factor in pDNA chromatography [59-61]. Also the small size pores are not engineered to handle large molecules as pDNA [6]. From this stand point, the continuous chromatographic supports with large pores and the absence of transport limitations make membrane or monolith-technology as an attractive option to porous particles for pDNA bioseparation [40,62].

3.2.2. Membranes

Membranes technology is often used for filtration or ultrafiltration, and some times as alternative to perform polishing purification [19,63]. They are mainly used to separate large molecules from small ones in desalting processes, and also in downstream processing to remove cell debris [18], colloidal or suspended solids and viruses. Membrane adsorbers arise as potential support for better chromatographic resolution, efficiency, as well as productivity [64]. Meanwhile, they present a real limitation, mainly for industrial applications, associated to the combination of higher cost per volume of adsorbent and lower throughput due to lower unit surface areas [63].

Membrane chromatography differs from column chromatography based on dispersed particles, in the geometry and the structure of the stationary phase (Fig. 2(D)). The typical stationary phase in membrane chromatography is a flat disk (diameter in the centimeter range, thickness in the micro- to millimeter range) bearing a large number of throughpores with similar and optimized diameter [65]. Membranes can be considered as monoliths due to the extreme geometry, but the dimension in the axial direction is very short, since they are used as a single sheet [66]. Membranes are prepared as very thin layers and therefore scale up is not possible while preserving this monolithic structure. However, they can be stacked to provide an additional volume and consequently to improve capacity (Fig. 2(D)). Furthermore, the immobilization of specific ligands with affinity, ion exchange, hydrophobic or reversed phase characteristics to the membranes surface can increase the selectivity of these materials [36]. By this way, membranes devices present different structures and can be classified not only as porous sheets loaded with specific binding particles and stack of sheet membranes, but also under hollow fiber and radial flow membranes [67]. These chromatographic supports can have origin in different constituent materials such as nitrocellulose [18,19], silica derivatives, polypropylene, nylon, chitin and chitosan [36], among other.

The target molecule hydrodynamic movement is considered the main difference between membranes and porous particulate material. The mass transport in membrane supports take place by convention through the membrane pores [64], becoming a faster process than through the dead-ended pores in conventional particles by diffusion flow [67]. Also in membranes structure, it is important to have attention to the pores size. Microporous membranes adsorbers are lately employed as alternative technology to Protein A resins for capture and polishing steps in the purification of monoclonal antibodies [68,69]. Notwithstanding, the adoption of this technology has been slow because membrane chromatography has been limited by a lower binding capacity than the achieved for the conventional columns. This disadvantage together with the possibility of the microporous membranes collapse with large molecules, makes this support inadequate to be applied at pDNA purification, being preferred to use the macroporous membranes [36].

Membrane technology has been widely investigated and improved in the last years, being introduced either for ultrafiltration or purification steps on downstream process of the pDNA. Morao and co-workers recently proposed a mass transfer model for predicting sieving coefficients of sc pDNA by ultrafiltration with narrow pore membranes [70]. On the other hand, membrane chromatography has also gained impact on purification of pDNA from a complex *E. coli* lysate. For example the nitrocellulose membranes develop a negative chromatography behavior, retaining contaminants like proteins, endotoxins, RNA and chromosomal DNA, while pDNA has the capacity to cross the membranes becoming more clarified [18,19]. Likewise, combining the hydrophobicity and macroporosity of the rigid cross-linked cellulose beads, namely CELBEADS, with the molecular exclusion of sc pDNA, it was possible to obtain this molecule free from the smaller proteins, RNA, and linear fragments of gDNA that preferentially adsorbed to this matrix, in a single purification step after alkaline cell lysis [17].

In another study, the application of anion exchange membrane chromatography on pDNA purification from *E. coli* lysate solution, confirmed that the elution curve was independent of the flow rate [71]. Nevertheless, some pDNA molecules bound irreversibly to the membrane surface and were not removed completely during elution. Recently, a study including three modification procedures to functionalize membrane supports with a linear alkyl chain ligand, allowed the separation of the model plasmid pVAX1-LacZ (6050 bp) from RNA in clarified *E. coli* cell lysates, with good resolution, based on hydrophobic chromatography principle [72].

3.2.3. Monoliths

Over the time increasingly appear strict requirements to the purity degree required in biomolecules for therapeutic applications. These challenges make necessary the continuous development of new chromatographic materials and strategies. In accordance, monoliths emerged as an excellent solution and have been extensively investigated and improved by some research groups. However, Strancar and co-workers are the primarily responsible for the major breakthrough in the development of short monolithic layers [67,73,74]. Nowadays, the balanced combination between the research, development, and marketing of BIA Separation company, Slovenia, has greatly increased the success of the wide set of monolithic products under the commercial name Convective Interaction Media (CIM) [66].

In reality, monoliths are considered the fourth-generation of the chromatographic stationary phases [11]. Its structural appearance seems with several membranes sheets stacked [75], but instead that they are polymerized directly in a column as a single unit [9,67]. Taking into account this fact, monoliths media do not require packing, avoiding all the process development and validation. Likewise, some problems related to the scale-up and scale-down variations in packing quality that can also be affected by the different operators, and the need to repack a column due to the inadvertent introduction of air are also eliminated. This

continuous bed can be prepared in a rigid disk or cylindrical tube [65], by polymerization of different materials, resulting in a chromatographic support highly porous. The pore size is dependent of the polymerization reaction, being possible adjust this parameter to the intended goal [76,77]. The monolith can be polymerized and kept into the column using a flexible inflatable wall with ring-shaped, which facilitate the target molecule access at entire monolithic structure [67]. Nevertheless, this construction can be unfavorable when extreme flow rates and pressure drop are exerted. Probably this is the reason for the silica monoliths are not yet available for preparative and industrial scale. On the other hand, the monoliths constituted by polymethacrylate material are more suitable and easier to fit into a housing comparing with silica rods since they present a lower stiffness [11].

One important characteristic that distinguishes the conventional supports with packed particle beds and the innovative monolithic technology is the absence of wasted space in the case of monoliths [9], as it is depicted in Fig 1(E). The void/dead volume between dispersed particles causes diffusion restrictions that lead to additional peak broadening and decreases the separation efficiency [75]. This disadvantageous parameter is also responsible by the lost of capacity and resolution, since turbulent mixing in the void space erodes the separation achieved by the surface chemistry. In the case of the monoliths, the entire continuous monolithic bed is functional, except the circulating-ring that supports the polymerized matrix. In this respect, monoliths have an advantage over membrane chromatography as well. Although the mass transport on membranes also occurs by convective movement, the flow disorder between layers and dead volumes within the housings contribute to turbulent mixing and sacrifice some of the resolution achieved by the surface chemistry.

Another important distinction is related with the convective channels in monoliths that are highly interconnected [41,66,78]. Monolithic columns are characterized by specific permeability resultant from their morphological and structural/physical properties [28]. This fact creates high surface availability and accessibility, as well as uniform frontal migration throughout the support [75]. Besides the monolithic structure is based on large throughpores that facilitate the flow of the liquid through the column at comparatively low back-pressure, in some cases the own pore skeleton has small pores to enhance the binding capacity [75,77]. The arrangement between convective flow and the high interconnectivity is considered the main advantage of monoliths that allows rapid separations to be carried out with extremely short beds [25]. Owing to these remarkable characteristics, monolithic chromatography supports have the ability to maintain both resolution and capacity independently from flow rate.

In parallel, research advances into new polymers, configurations, and surface chemistries promises to bring the benefits of monoliths to an increasingly broad range of applications. Whereas the conventional columns normally apply the small high-resolution particles for laboratorial scale and larger particles for process scale in order to keep pressure drops on

columns low enough, the same monolithic column can be used on both small and industrial scales [11,78]. Even for analytical scale in-process and final control of new therapeutics, the monolithic columns have been recently applied with great efficiency due to the fast and enhanced mass transfer between the stationary and mobile phases provided by these methodology [28]. In general, the monoliths nature applied for preparative separation is polyacrylamide or polymethacrylate [11,77], and for analytical separation or capillary electrochromatography is polystyrene or quinidine [79]. The performance of a chromatographic support mainly depends of the geometrical shape and size of the external porous [8]. The porosity of polymethacrylate monoliths is dependent of all parameters that affect the solubility of polymethacrylate chains, among which the solvent, additives, pressure and mainly temperature [77]. Knowing that the reaction between glycidyl methacrylate and ethylene dimethacrylate monomers to obtain the polymethacrylate is a strong exothermal process, the temperature increase should be controlled to generate a monolith with homogeneous porosity [77].

Comparing the proteins versus pDNA monolithic chromatography, the number of theoretical binding sites is much higher for plasmids, although these molecules exhibit lower diffusivity (1/10) and larger hydrodynamic radii (10-50 times) than proteins [80]. Monoliths are therefore considered the material of choice for the purification and analysis of pDNA [11,28,80], due to their proper structure, that allows to overcome the limitations associated to the conventional matrices, such as low capacity and plasmid degradation resultant from the extended retention time. In addition, the small diffusivity of large proteins, pDNA and viruses observed into pores of conventional media reinforces that the monolithic media are the best solution for separation and efficient purification of these molecules [9,28,78].

Because this continuous bed is constituted by a single piece of macroporous material, characterized by a highly interconnected network with large diameter (Fig. 2(E)), the binding capacity for large biomolecules as pDNA is widely improved [41,67]. By this way, all the mobile phase is forced to flow through the channels via convection, resulting in a very fast mass transfer between mobile and stationary phases, having a positive effect on the separation. In fact, in conventional supports, several parameters such as unspecific binding, plasmid purity and structural integrity, recovery and peak resolution are mainly affected by the extended chromatographic residence time and back-pressure [9]. These effects can be surpassed by monolithic technology, reducing the column length [67], increasing the flow rates guarantying resolution. Figure 3 confirms the independence of separation and peaks resolution of pDNA isoforms (open circular (oc) and sc) under different flow rates [41]. Sousa and co-workers also demonstrated that the non-grafted polymethacrylate monolith has the ability to selectively separate plasmid isoforms, with good binding capacity for the sc plasmid conformation [41], becoming a potential support to be applied on direct capture of sc pDNA from a impure and complex extract of *E. coli* lysate.

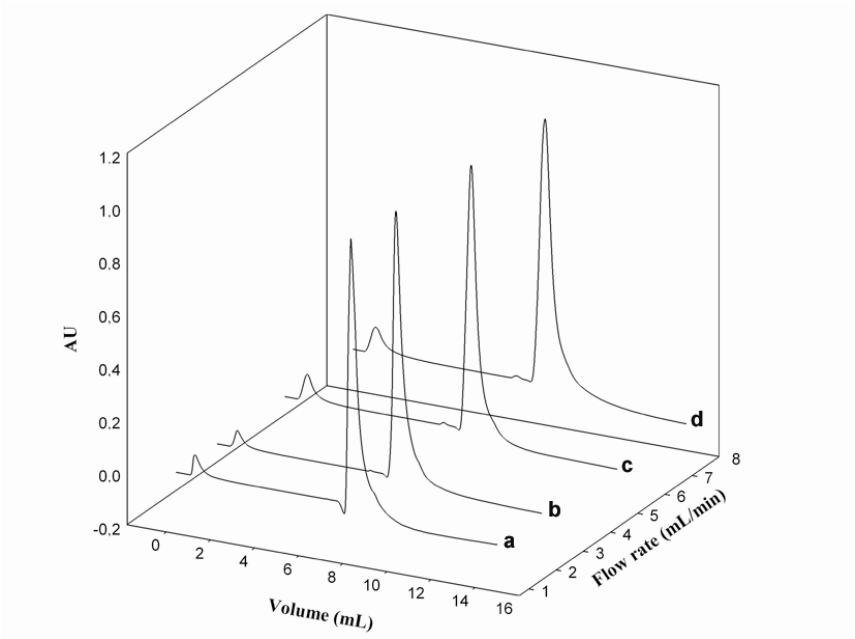

Figure 3. Effect of flow rate on resolution of pDNA isoforms (1 mL/min a); 2.5 mL/min b); 5 mL/min c) and 7.5 mL/min d)). The peaks obtained in each chromatographic run correspond to oc and sc pDNA isoforms efficiently purified (data not shown).

Monolithic technology can be applied for almost all kinds of operation mode and chromatographic principles. Although for size exclusion chromatography has not been much explored, a monolith equipped with mesopores was already developed by Urban *et al*. (2007), which seems to be suitable to separate molecules according to the different sizes [81]. The most common chromatographic principle applied for pDNA purification is the ion exchange strategy [29,82-86], however making use of different ligands there are other studies describing the application mainly of hydrophobic and affinity chromatographic principles. Furthermore, adjusting the monolithic channel diameter at the range of the target biomolecules, it is also useful to employ monoliths to extract large molecules because of their higher surface area and productivity [11]. Accordingly, a recent study showed that some monolithic supports present good abilities to bind plasmids with large size, reaching binding capacities of 12.0 mg/mL for a plasmid with 39.4 kbp [87].

Curiously, similar to hydrogels preparation, also cryogels can be obtained by polymerization of specific materials but in combination with the advantageous mass transfer properties and permeability of rigid supports, as it is the monoliths case. Cryogels are prepared at temperatures below − 10ºC by frozen of monomers dissolved in water, forming ice crystals. The shape and size of the final pores are dependent of the freezing process, designed of cryopolymerization [25]. The major advantage of cryogel chromatography is the ability to process crude solutions that contain non-clarified cell homogenates as well as entire cell suspensions (bacterial and mammalian) [11,88-90], properly extracting the target molecule

[91,92]. They are mostly used for affinity applications, such as proteins [93], pDNA [62] and viruses [94] purification and for particle separation and cells immobilization because of its high selectivity. The transport of solutes inside the cryogel column is achieved mainly due to convection rather than diffusion, using flow rates higher than conventional particles supports with low flow resistance [25]. However, this gel grafted support presents low binding capacity for proteins (does not exceed 1 mg/mL for proteins) [25], being a disadvantage to apply at industrial scale.

Interestingly, when cryogel material is combined with the permeability of supermacroporous monoliths, the resultant chromatographic support can significantly increase the capacity, as well as selectivity and affinity properties to specific molecules [25], as pDNA. Normally, the monoliths highly macroporous associated with cryogel material are constituted by polyacrylamide and other organic solvents [95]. A particular study, using the advantageous monolithic structure coated with a cryogel substance composed by the polymer poly (hydroxyethyl methacrylate) [PHEMA] associated to the histidine affinity ligand, revealed high binding capacity values for pDNA, within the range obtained with monoliths, and was also demonstrated the possibility to purify pDNA from a bacterial cell lysate [34].

4. Ligands immobilization for supercoiled pDNA purification

4.1. The importance of ligands

The physical and structural nature of the different supports aforementioned allows a partial separation of some impurities present in *E. coli* lysate and pDNA molecules. However, the similarities of sc pDNA with other non-functional plasmid isoforms and also with host impurities (Table 1) can difficult the establishment of an adequate chromatographic strategy to isolate the sc pDNA in a single unit operation [4,6,96]. For example, the application of non-porous particles matrix can be useful if the particles surface are coated with charged polymers taking advantage of anion exchange chromatography [24]. This technique is quite used in plasmids purification field [97], being verified that nucleic acids elution occurs according the increase of the overall net charge that is dependent on the chain length [98]. But in same cases, the supports present low resolution for pDNA and endotoxins, gDNA or RNA [96,98] due to their identical structure and charge. The application of porous particles can provide a partial separation of the large molecules that are eluted directly through the particles and the small ones that are retarded passing through the channels of the pores, based on size exclusion chromatography. In reality, the retention of low molecular weight impurities, among which RNA, proteins and endotoxins, was obtained with the Superose support, while gDNA and pDNA were excluded together because both present high molecular weight [99].

Knowing that the application of the supports under their basic structure is not enough for pDNA purification, the need to employ other chromatographic principles emerges as an alternative to overcome these limitations. The preparation of appropriate supports for specific chromatography requires the immobilization of ligands with restrict and desired characteristics, either to porous or non-porous material. The association of adequate ligands to the solid support takes advantage of a higher selectivity and specificity for the target biomolecule. The advances on chromatographic technology by coupling of specific ligands allow that this strategy achieves an efficient and directed purification. The benefits from the selectivity of chromatographic supports, applied in particular for sc pDNA molecule, are described below.

4.2. Ligands applied in pDNA purification

The immobilization of a specific ligand conjugated with the coating of defined polymers and the structural shape of the support provides a variety of matrices that can be used on different chromatographic principles, respecting the requirements imposed by the target molecules. As it was previously referred, the difficulties on sc pDNA efficient purification from the other plasmid isoforms and host impurities can be solved by the conjugation of these parameters. Table 4 presents a brief summary of some examples of ligands that have been used for pDNA purification.

Table 4. Examples of different ligands immobilized onto different solid supports and involved on several chromatographic principles for pDNA purification.

Ligands	Chromatographic principle	References
Polycations (based on amino groups)	Anion exchange	[29,31,62,64, 86,98]
Alkyl chains (phenyl-, epoxy-, butyl-)	Hydrophobic interaction	[38,43,51,72,100]
Aromatic thioethers	Thiophilic adsorption	[101]
Metal-ion (Cu^{2+}, Zn^{2+}, Ni^{2+})	Immobilized metal affinity	[102,103]
Oligonucleotides	Triple-helix affinity	[104]
His-tagged lac repressor	Protein-DNA affinity	[105]
Amino acids (histidine, arginine, lysine)	Amino acid-DNA affinity	[44,106,107]
Coated with cryogel + histidine amino acid	Affinity	[34]

The most common and simple immobilization on different structural supports is based on crosslinked reaction with specific charged polymers, namely positive amines that allow employing the chromatographic principle of anion exchange [31,64,98]. This support modification is useful for pDNA purification from the *E. coli* complex lysate due to the polyanionic character of the nucleic acids molecules. The pDNA double strand molecules are easily captured by these cationic supports, because a preferential interaction between negatively charged phosphate groups in the pDNA backbone is promoted with the positively charged functional groups on the stationary phases [97]. In this particular ion-exchange chromatography, the electrostatic interactions are prevalent due to the attraction between the nucleic acids and the matrices that present opposite charges. The elution of retained nucleic acids is established by a slight increase of salt concentration, which firstly favors the displacement of the molecules with smaller charge density, depending of the chain length and conformation [98].

To take advantage of hydrophobic interaction chromatography most of the matrices contain long alkyl chains and aromatic rings with hydrophobic properties as ligands attached to the solid support. The principle of hydrophobic chromatography is based on the requirement of high salt concentrations because in this case the ionic strength is responsible for the removal of water molecules around the hydrophobic groups of the target biomolecules and the ligands favoring its attraction [6]. This strategy has been used at preparative, analytical and industrial scales, although the high salt concentration is associated with environmental and economic impacts for industrial scale. Elution process is attained by decreasing the salt concentration of the mobile phase, which weakens the hydrophobic interactions previously established [108]. The elution order of the bound molecules is dependent on the hydrophobicity degree of each molecule. For the particular case of nucleic acids retention, pDNA was efficiently eluted and isolated from the endotoxins and single-stranded nucleic acids by decreasing ionic strength [109]. The retention of nucleic acids to the hydrophobic matrices is mainly affected by their size, structure and hydrophobic bases composition and exposure [5]. Normally, the higher bases exposure in the small RNA molecules and in the more compact sc pDNA isoform allows a stronger retention than for the larger gDNA or oc pDNA molecules, being possible to isolate all these components [110]. Nevertheless, in some cases it has been difficult to achieve an efficient separation of the pDNA conformations. For example, the pDNA isoforms in the phenyl-sepharose hydrophobic matrix at 1.5 M of ammonium sulfate are eluted together in the flowthrough without interacting with the matrix, whereas the fragmented gDNA and single strand RNA promote some hydrophobic interactions with the matrix being eluted later [43]. In this study it has been showed that the bases exposition of sc pDNA molecules was not sufficient to develop hydrophobic interactions with phenyl-sepharose matrix.

The pursuit of a suitable ligand that promotes a specific biorecognition with sc plasmid conformation encouraged the application of affinity chromatography. Initially, this

methodology applying specific ligands immobilized in stationary phases was not extensively used for pDNA. Furthermore, the biological origin of the first ligands used was the biggest disadvantage because they had trend to be fragile or toxics for the biological samples and are associated with low binding capacities [111]. Nowadays, this technique has been developed and improved with new synthetic ligands with increased capacity and durability, which are used as specific binding agents to analyze, explore or purify biomolecules taking advantage of their biological function or individual chemical structure [4,111]. The ideal ligand must be highly safe (non-toxic), stable to avoid physical or chemical damages during the immobilization process, specific and selective for the molecule of interest and inert to all other compounds present in the complex sample. Undoubtedly, for the successful implementation of affinity chromatography on sc pDNA purification, the attachment chemistry, the ligands and the spacers between the ligands and the matrix should be carefully designed, screened and optimized for stability, selectivity, low nonspecific binding and high capacity for the target biomolecule [14,112]. In some cases the use of specific spacers can increase the accessibility and availability of the ligands or can also contributes with additional interactions for the specificity of the purification.

Additionally, the design of selective ligands for the purification of biomolecules can be complex, time consuming and expensive. However, their implementation into sc pDNA purification by affinity chromatography processes allows the elimination of some downstream steps, increasing yields and improving the product quality [111], which can justify the initial investments. On the other hand, the knowledge and understanding of nature of interactions involved between the appropriate ligand and sc pDNA is also required, whereas this biorecognition of target molecule by the ligand must be stable under different binding and elution conditions. Finally, in this chromatography methodology, elution steps can be performed either specifically, using a competitive ligand, or non-specifically, by changing the pH, temperature, ionic strength or polarity. These parameters should be adjusted depending on the solid support used, for the manipulation of the specific interactions that can be involved between the ligands and the sc pDNA, among which electrostatic, cation-π, hydrophobic, van der Waals forces and/or hydrogen bond interactions [113-115].

Several ligands associated to anion exchange, hydrophobic, thiophilic adsorption, immobilized metal affinity, triple-helix affinity and protein-DNA affinity chromatography have been used for pDNA purification (Table 4), being recently implemented the amino acid-DNA affinity technology [4]. The selection of the amino acids as ligands was based on natural occurrence of many different interactions between proteins and nucleic acids in biological organisms, which mainly involve basic amino acids such as histidine or arginine [115]. In addition, several atomic studies have described preferential interactions occurring between particular positively charged amino acids and nucleic acid bases [113,114]. Although histidine, arginine and lysine belong to the positively charged amino acids group, the agarose matrices with these immobilized ligands applied on pDNA chromatography have showed different elution

behavior [106,107,116]. Curiously, for all these three matrices it was described a specific biorecognition by the sc pDNA conformation, allowing the elimination of other plasmid isoforms [107] and host impurities[106,116]. These interesting findings are mostly related with the different bases exposition degree of the pDNA isoforms and also the remaining nucleic acids. The difference between plasmid isoforms is associated with the deformation induced by torsional strain in the sc conformation, which becomes the bases of this nucleic acid more turned to outside being more available to be involved on different interactions with the amino acids ligands than the bases of the oc isoform.

As it was referred, amino acids ligands develop preferential and specific interactions with the nucleic acid bases. The main interactions of histidine with the DNA bases can be ring stacking hydrophobic interactions, among other, due to the presence of its aromatic ring, being necessary to use high salt concentration (ammonium sulfate) to promote the total retention of pDNA, and thereafter by decreasing the ionic strength, all the species are gradually eluted from this matrix [106]. Likewise, histidine-agarose support was also applied to specifically purify 6S RNA from other *E. coli* sRNA species [117]. Additional studies with model synthetic oligonucleotides were performed to better understand the complexity of the interactions involved between histidine and nucleic acids [118]. This study confirmed that besides ring stacking hydrophobic interactions, also water mediated and bifurcated hydrogen bonds are established between the histidine amino acid and the nucleotide bases, preferentially with guanine base, and van der Waals contacts with phosphate groups of nucleotides [119], depending of the conditions imposed during the elution steps.

The implementation of arginine and lysine affinity chromatography arise as good alternative in relation with histidine matrix because the total retention is established with low salt concentration (sodium chloride) and the species are eluted with a slight increase of ionic strength [116,107]. The similar elution conditions can be related with the constitution of both amino acids, and particularly with the presence of amino groups. Nevertheless, arginine revealed a preferential interaction with sc pDNA isoform that was the last nucleic acid to be eluted from this matrix [116]. The studies using model synthetic oligonucleotides also revealed that arginine participates in multiple hydrogen bonds mainly with guanine, but other interactions (electrostatic, hydrophobic interactions, dipole-dipole forces, cation-π interactions) may also be present and become dominant depending on the conditions used [120]. Meanwhile, a specific study of the chromatographic behavior of each *E. coli* nucleic acid and pDNA isoforms on lysine matrix showed that the most retained nucleic acid was RNA [121]. Therefore, according with the reported results it is suggested that lysine chromatography can be a potential technology mainly on RNA purification and arginine chromatography seems to be more suitable for sc pDNA purification, using both low salt concentrations.

Indeed, affinity chromatography has been the purification strategy more adequate to specifically achieve the sc active conformation of pDNA isolated from the other pDNA isoforms and also the host impurities. The possibility to choose the ideal ligand facilitates the establishment of certain interactions with the nucleic acids. These interactions can be manipulated and favored by modification of the elution conditions such as temperature and buffer composition (pH, ionic strength or presence of competitive agents), taking advantage from the ligands properties. Hence, the multiplicity of the chromatographic ligands and the elution factors that can be used in order to promote the suitable interactions with the molecule of interest shows the versatility of the affinity chromatography.

4.3. Immobilization techniques

The immobilization techniques should be adequate to the nature of the ligand and also the constituent material and structure shape of the chromatographic support. For example, when both the support and ligand are stable to high pH, the immobilization procedure of ligands must be conducted under basic conditions. Several approaches have been reported for different attaching techniques of specific ligands that can be grouped in covalent and non-covalent immobilization. Some of the most important immobilization strategies are described below, especially those that are used for ligands with amino groups.

The most common strategies for the non-covalent immobilization can be performed via adsorption or entrapment [122]. Adsorption process is very attractive and easily achieved without significant structural or functional damages to the ligand. However, a slight variation of the elution conditions can result on ligand desorption from the support surface. On the other hand, the entrapment process is normally used on sol-gel materials, as cryogels, by incorporation of the ligand on the polymerization mixture that will cover all the support [122].

Besides the covalent immobilization can affect the biological activity of the ligand, because this strategy involves a chemical reaction between the ligand and the solid support, it is preferred when reactive residues of the amino acids are used, among which are described the guanidyl group of arginine, the imidazolyl group of histidine and ε-amino group of lysine [75]. In reality the most suitable groups for immobilization are the carbohydrates, especially in oxidized form, the nucleophilic primary or secondary amines, the sulfhydryl groups or the hydroxyl groups. Several reactions can be used to form the stable covalent bond, avoiding unspecific interactions. Cyanogen bromide (CNBr) method is the most used immobilization method in traditional polysaccharide or agarose supports used for affinity chromatography. The procedure is relatively simple and easy to perform combining an ice cold, basic solution of CNBr with the solid support [123]. In general, for continuous beds it is used the epoxy method that involves nucleophilic attack by the amine groups of the ligand to the epoxy

groups present on the supports, resulting on a stable secondary amine linkage. This can result in low amounts of immobilized ligand or long immobilization times [124-126].

Other amino-based coupling method consists on formulation of Schiff base through first the conversion of epoxy groups into diols that are afterward oxidized with periodic acid to give aldehyde groups, which can react with primary amines on ligands [124]. Glutaraldehyde method is closely related approach with the Schiff base technique but involves more steps for support preparation and results in a longer spacer between the ligand and the support [125,127]. The hydrazide method is also similar to the Schiff base method with more additional steps, resulting in a higher activity for glycoproteins and carbohydrate-containing ligands [128].

Carbonyldiimidazole (CDI) method requires a first activation of the support, usually monoliths, beginning by conversion of epoxy groups into diols, which are then reacted with 1,19-carbonyldiimidazole to produce imidazolyl carbamate active groups [129]. At this step, the supports are ready to be rapidly immobilized by means of a nucleophilic substitution between the activated sites and primary amines of the ligand, resulting in a stable amide linkage. Curiously, the CDI method was already used with GMA/EDMA monoliths to immobilize L-histidine [54]. CDI technology provides advantages over the antecedent methods because the immobilization spends less time than the epoxy method and less steps that Schiff base or glutaraldehyde methods. However, there is a possibility of the ligands activities to be lower than the Schiff base technique [124]. The disuccinimidyl carbonate (DSC) method is identical to the CDI process also in low stability of the activated support, requiring a proper care be taken to avoid side reactions due to hydrolysis [54].

The coordination method is widely used to prepare the immobilized metal-ion affinity chromatographic matrices. This process consists on the application of the epoxy method to covalently couple the chelating agent like iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA) to the solid supports, followed by the addition of metal ions to this material to form coordination complexes [122].

5. Conclusions and future trends

Over the last years, several efforts and improvements have been established to prepare an ideal chromatographic support for the separation and purification of large biomolecules as pDNA under sc active conformation for therapeutic applications, taking into account the guidelines of regulatory agencies that are increasingly strict. Besides improving the mechanical stability of the conventional particle matrices as well as more mass transfer and use of higher volumetric flow rates, with the progress of pore design and pore size in membrane and monolithic continuous beds, it is still missing something to achieve the optimal chromatographic media. The difficulty on final attainment of sc pDNA sample suitably

purified is related with the complexity of the *E. coli* lysate extract and the physical, chemical and structural similarities between the sc pDNA and the other non-functional plasmid isoforms and host nucleic acids proteins and endotoxins.

The methacrylate monolithic columns have been considered as an enabling tool for developing fast and efficient separation processes using low back pressure, capacity, stability and productivity for plasmids. Affinity chromatography is a unique separation method, which allows the purification of biomolecules on the basis of biological functions rather than individual physical or chemical properties. Due to its high specific interaction between the ligand and biomolecules of interest, affinity chromatography has been widely adapted in the downstream processing of plasmids. For example, although using the conventional non-porous particles, amino acids-DNA affinity chromatography has showed a biorecognition by the sc plasmid isoform and the possibility to isolate this molecule under required purity degree, by manipulating the elution conditions. However, combining the selectivity achieved using amino acids affinity ligands with monolithic supports, we could expect better results with no limited flow rates and higher binding capacity and productivity, resulting in a final sc pDNA product suitably purified under a pharmaceutical-grade for therapeutic applications.

According to the advantageous characteristics provided by the monolithic supports previously described and the specificity and selectivity obtained with the amino acid ligands, the adequate immobilization of these ligands on the monoliths becomes a promising strategy to find the support that can recognize and purify the sc pDNA isoform from the remaining lysate constituents with high productivity.

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Chapter 3

Paper II

Histidine affinity chromatography of homo-oligonucleotides. Role of multiple interactions on retention

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Histidine affinity chromatography of homo-oligonucleotides. Role of multiple interactions on retention

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ABSTRACT: The recent application of histidine–agarose affinity supports in plasmid purification takes advantage of the biorecognition of nucleic acid bases by the histidine ligand. This consideration prompted the need for better understanding the interactions involved in affinity chromatography of plasmid DNA with the histidine–agarose support. In this work, we used synthetic homo-deoxyoligonucleotides with different sizes (1–30 nucleotides long), to explore the effect of several conditions like hydrophobic character of the individual bases, presence of secondary structures, temperature, pH and salt concentration on the mechanism of retention of nucleic acids to histidine–agarose support. One of the most striking results shows that histidine interacts preferentially with guanine, and the presence of secondary structures on polyA and polyG oligonucleotides has a significant influence on retention. Otherwise, the temperature manipulation has not shown a direct influence on oligonucleotide retention, only inducing conformational changes on secondary structures. Overall, the results obtained provide valuable information for the future development and implementation of histidine and other amino acids as ligands in chromatography for the purification of plasmid DNA and other nucleic acids, by improving the knowledge of the interactions involved as well as of the parameters influencing the retention. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: affinity chromatography; histidine–agarose support; multiple interactions; oligonucleotides; secondary structure

Introduction

The development of efficient processes to achieve the production of large quantities of highly pure supercoiled plasmid DNA (pDNA) is impacting industry because of the expansion of new therapeutic strategies like gene therapy or DNA vaccination (Ferreira *et al*., 2000). However, the therapeutic use of pDNA requires it to be free of any other contaminant material (Prazeres *et al*., 1999). According to international regulations, a content of sc form higher than 97% is required (Stadler *et al*., 2004) to induce the most efficient transfection and expression rate in eukaryotic cells (Urthaler *et al*., 2005).

For the purification of pDNA, several chromatographic methods have been reported (Lemmens *et al*., 2003; Prazeres *et al*., 1998; Diogo *et al*., 1999, 2005; Tan *et al*., 2007), but not all strategies achieve efficient pDNA isoforms separation. Chromatographic operations based on affinity interactions between pDNA or impurities with specific ligands immobilized on stationary phases (Sousa *et al*., 2008a,b) have not been used extensively for pDNA purification. Despite the lack of high-capacity supports, the high specificity and efficiency of affinity interactions are probably optimum if a single chromatographic step is required (Diogo *et al*., 2005; Sousa *et al*., 2007a).

Chromatographic supports and membranes derivatized with histidine were first used for protein (Kanoun *et al*., 1986; Wu *et al*., 1992) and oligosaccharide (Delattre *et al*., 2005, 2008) purification. The fact that interactions between histidine and nucleic acid bases have been recognized at atomic level in several protein–DNA structures (Hoffman *et al*., 2004; Luscombe *et al*., 2001) fostered the study of plasmid purification with a histidine–agarose matrix. A recent study has proved that it is possible to use a single histidine–agarose chromatography step to purify sc pDNA from other isoforms and *E. coli* impurities present in a clarified lysate, with an optimized ammonium sulfate gradient (Sousa *et al*., 2006). The histidine–agarose support combines the mild hydrophobic characteristics of an epoxy spacer arm with an affinity histidine ligand, which can be responsible for biorecognition of nucleic acid bases (Sousa *et al*., 2005). These considerations prompted the need for better understanding of the interactions involved between nucleic acids and the histidine–agarose support.

Recognition of a specific nucleotide sequence by a DNA-binding protein is predicted at atomic level by studying possible interactions between the amino acids of the latter and the nucleotides of the former (Hoffman *et al*., 2004; Luscombe *et al*., 2001). To assess the interactions behind amino acid–base recognition, some studies investigate the presence of hydrogen bonds, van der Waals contacts, electrostatic interactions and water-mediated bonds (Luscombe *et al*., 2001). This study revealed that, for interactions

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Abbreviations used: pDNA, plasmid DNA.

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with histidine at high salt concentration, the apparent affinity is due to the ability to produce many bifurcated hydrogen interactions preferentially with guanine (Luscombe *et al*., 2001). The stacking interactions, which are defined as the π–π interactions may also occur between nucleosides and the aromatic side-chains of histidine (Allers and Shamoo, 2001).

The magnitude of hydrophobic retention is determined by the free energy change for the equilibrium of the biomolecule between the bulk mobile phase and the stationary phase domains. In general, for hydrophobic interaction the number of water molecules and salt ions released increases with the number of nucleotides and hydrophobicity of the individual bases (Diogo *et al*., 2003), but for low ionic strengths, however, electrostatic interactions between the biomolecules and the solvent are dominant over hydrophobic interaction and this behavior may be inverted (Diogo *et al*., 2002).

As it is described at atomic level, the histidine has preference for guanine, so it becomes important to evaluate how the oligonucleotide bases composition affects the interaction. Oligonucleotides with a G-rich sequence can form a higher-ordered structure consisting of G-quartets, called G-quadruplex (Nagatoishi *et al*., 2007), by planar association of four guanines in a cyclic Hoogsteen hydrogen bond (Tuntiwechapikul *et al*., 2006). This unique structure has recently been proposed to exist in living cells and has drawn attention as a target for gene expression control, cell division, and design of drugs for cancer (Nagatoishi *et al*., 2007).

In this work, we used synthetic homo-deoxyoligonucleotides of adenine (polyA), thymine (polyT), cytosine (polyC), guanine (polyG) and uracil (polyU) with different molecular masses, to explore the effect of several conditions like temperature, pH and ammonium sulfate concentration on the mechanism of retention of nucleic acid single strands to the agarose-based histidine support. The results suggest that multiple interactions between oligonucleotides and the histidine ligand play an important role in their selective retention in accordance with the molecular mass of the oligonucleotides, the hydrophobic character of the individual bases, the presence of secondary structures, the concentration of ammonium sulfate in the eluent and the influence of temperature.

Experimental

Materials

L-Histidine–agarose gel was purchased from Sigma (St Louis, MO, USA). Lyophilized homo-deoxyoligonucleotides with different molecular masses were purchased from Sigma. The homo-deoxyoligonucleotides were first ressuspended in 10 mM Tris pH 8.0 and used without further purification. For the modulation of chromatography experiments analytical-grade ammonium sulfate and tris(hydroxymethyl)aminomethane (Tris) from Merck (Darmstadt, Germany) were used.

Chromatography of the Oligonucleotides

Chromatography was performed in an ÄKTA Purifier system (GE Healthcare Biosciences, Uppsala, Sweden). A 10 mm diameter × 20 mm long (about 1.6 mL) column was packed with commercial L-histidine–agarose gel. This support is characterized by the manufacturer as a cross-linked 4% beaded agarose matrix with a 12-atom spacer and an extent of labelling between 1 and 2 μmol/mL. The column was equilibrated at 5, 12, 15, 20, 24 and 35°C with various concentrations of ammonium sulfate (1.5, 1, 0.5 and 0 M) in 10 mM Tris–Cl (pH 8.0) at a flow-rate of 1 mL/min. A 50 μL volume (about 2.5 μg) of the various oligonucleotides samples was loaded onto the column, and isocratic elution of each oligonucleotide was carried out at identical concentrations of ammonium sulfate. The absorbance of the eluate was continuously measured at 260 nm. The water jacketed column was connected to a circulating water bath MultiTemp III to maintain the appropriate temperature in each experiment. After the chromatographic runs, the column was washed with 3 column volumes of this buffer without salt, in order to remove any molecules that remained bound.

Results and Discussion

A 2.5 μg amount of each homo-deoxyoligonucleotide (polyA, polyT, polyG, polyC and polyU with 1, 3, 6, 15 and 30 bases) was injected into the histidine–agarose column and eluted with different concentrations of ammonium sulfate and at different temperatures.

In Fig. 1, we can see the retention pattern chromatograms achieved at 5°C for the different oligonucleotides studied. Each chromatogram represents the elution profiles performed with a particular oligonucleotide, testing different salt concentrations on the eluent. In general, the retention of oligonucleotides increases when the ammonium sulfate concentration increases. If a sufficiently high salt concentration is used, the oligonucleotides become strongly retained in the column, and can only be eluted by decreasing the salt concentration. The magnitude of the ionic strength necessary to promote this interaction depends on the hydrophobic character of the oligonucleotides (Diogo *et al*., 2002). The increase in retention time with salt concentration is controlled by the displacement of water molecules. Adsorption leads to the release of a much larger number of water molecules (119–418) than salt ions (1–18) (Esquibel-King *et al*., 1999). This situation is consistent with the fact that hydrophobic interactions are driven by an increase in entropy resultant from the release of water molecules from the surface of the stationary phase upon adsorption, as has been described for the hydrophobic interaction of several proteins/hydrophobic ligand systems (Esquibel-King *et al*., 1999). This trend is expected for adsorption processes dominated by hydrophobic interactions, because the number of water molecules released should be proportional to the hydrophobic contact area between the molecule and the ligand (Diogo *et al*., 2003). Comparing the retention only considering the hydrophobicity of the mononucleotides, it can be seen in Fig. 1 that the hydrophobicity of A is higher than the hydrophobicity of T, which in turn is higher than the hydrophobicity of U (the same can be seen in poly 15). The effect of the base composition on the hydrophobic interaction can be explained on the basis of the relative hydrophobicity of the individual bases, and of the primary structure of the oligonucleotides. The difference between A and T is probably the result of the existence of two aromatic rings in the case of the adenine and the difference between T and U is due to the presence of an additional hydrophobic methyl group in the thymine base, as indicated by Diogo *et al*. (2002).

As for the exact type of interactions between the histidine ligand and the DNA bases, these may include hydrogen-bonding between H-donor and H-acceptor N-atoms in the non-protonated histidine with base edges, ring stacking/hydrophobic interactions,

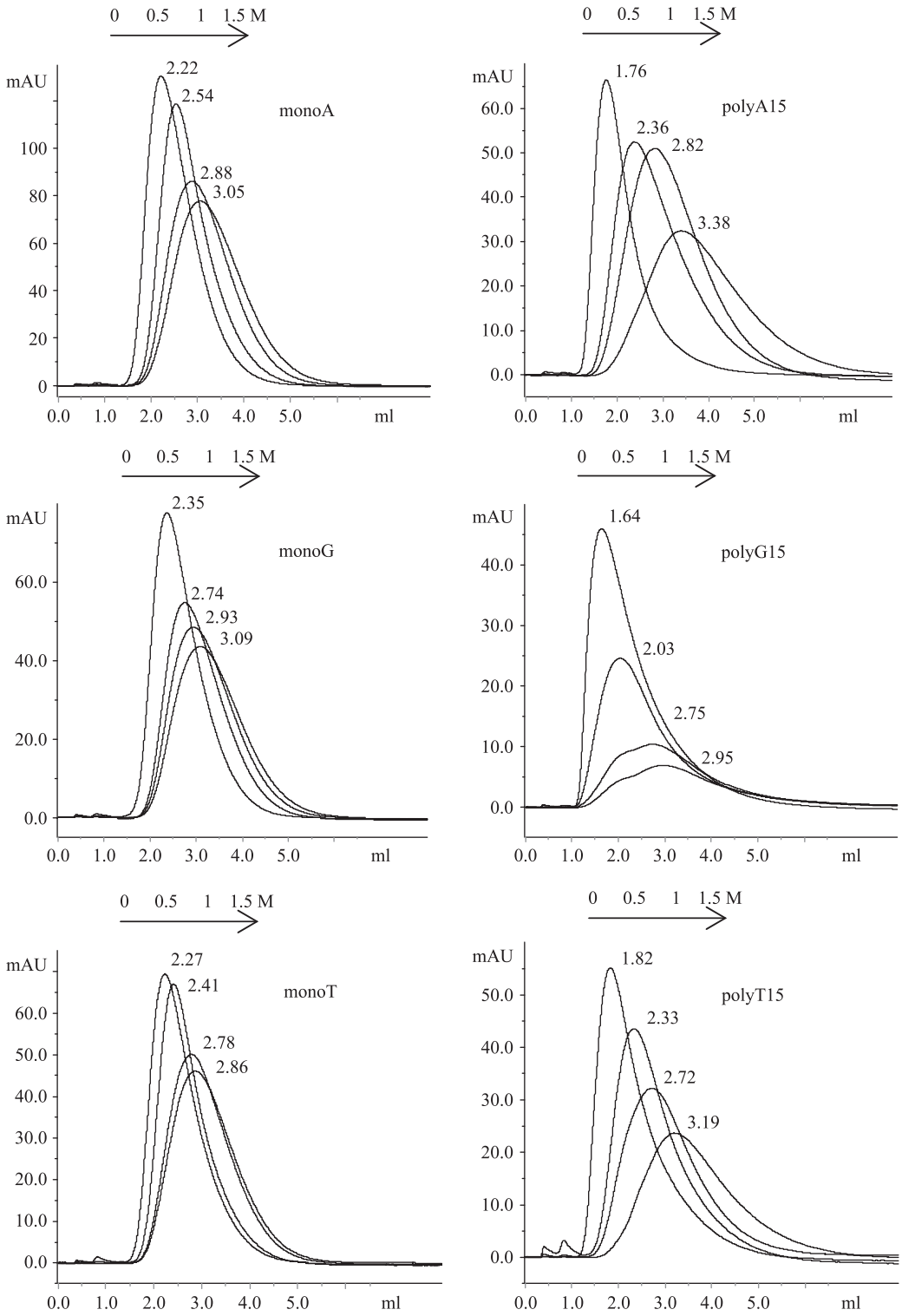

Figure 1. Affinity chromatography profiles of homo-deoxyoligonucleotides monoA, monoG, monoT, monoU, monoC, polyA15, polyG15, polyT15, polyU15 and polyC15 on L-histidine–agarose gel. The curves represent the interaction of nucleotides as ammonium sulfate concentration (0, 0.5, 1 and 1.5 M) is increased, all eluted at 5°C. The label on the top of each peak corresponds to the retention time.

water-mediated hydrogen bonds and van der Waals interactions (Hoffman *et al.*, 2004; Luscombe *et al.*, 2001). We can observe in Fig. 1 the retention time in mononucleotide chromatograms and it is evident that the monoG is more retained than the monoA. It can also be noted that these two bases are retained with greater intensity than monoT, monoC and monoU. These results are in accordance with statistical data for interactions between amino acids and DNA nucleotides, where histidine interacts preferentially

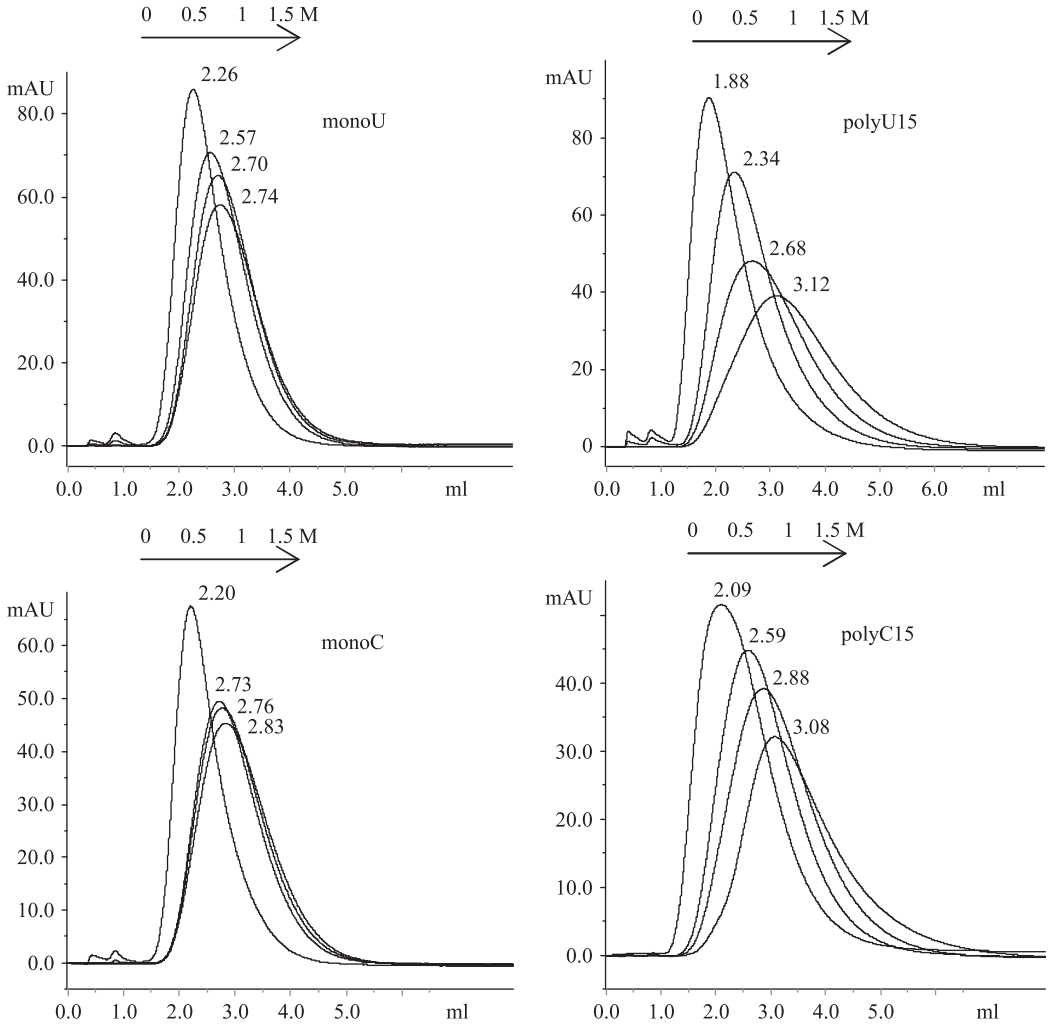

Figure 1. (*Continued*)

with monoG by hydrogen-bond, but it can also retain monoA and monoT with less intensity (Hoffman *et al*., 2004). The interaction of monoG with histidine was found to be more intense because of the bifurcate hydrogen-bond to N7 and O6 of guanine, which might confer a preference for this base. However, the affinity of histidine for many base types, especially for guanine and adenine, is also explained by their ability to produce extensive ring-stacking interactions in structures with suitably deformed DNA (Choo and Klug, 1997). If the nucleotide bases are less exposed, the histidine is positioned with the plane of the ring facing the DNA, therefore maximizing the contact surface area (Luscombe *et al*., 2001). The interaction histidine–guanine was found to be quite rare in biological protein–DNA complexes, suggesting that it plays a significant role in specific recognition (Mandel-Gutfreund *et al*., 1995).

Knowing that naturally the histidine interacts preferentially with monoG (Hoffman *et al*., 2004), it was found interesting to study if this concept could be reproduced in the chromatographic retention behavior of different guanine oligonucleotides on the histidine matrix. Figure 2 shows the representative chromatograms of monoG, polyG3, polyG6 and polyG30 (polyG15 is presented in Fig. 1), obtained for the temperature of 5°C, with different salt concentration on the eluent. The expected increased retention in monoG when increasing salt concentration was visible, because the increase in ammonium sulfate concentration promotes the increase in retention time and the decrease in peaks height. However, in the case of the other oligonucleotides of guanine (polyG3, 6, 15 and 30), the presence of the salt promoted a heterogeneity of the peaks. This behavior suggests that different isoforms of the oligonucleotide can co-exist. These isoforms may arise due to the possible interactions between molecules of guanine that can lead to inter- or intramolecular associations depending on the size of oligonucleotide (Campbell and Parkinson, 2007).

DNA can adopt conformations differing substantially from the Watson–Crick double helix. Some of them are favored by purine bases, which is interesting because purine bases probably stabilized the first precursors of the present-day nucleic acids. The best-known of the purine base-specific conformers of DNA is the guanine quadruplex (Kejnovska *et al*., 2003). DNA quadruplexes are formed from guanine-rich repeats that self-associate into higher-order four-stranded structures; nevertheless, the short G-rich sequences associate together as intermolecular quadruplexes (Campbell and Parkinson, 2007). The potential of guanine oligonucleotides to be organized in different structures induces an heterogeneity when they are in solution and consequently may be differently retained to the support. For example, for the polyG3 in Fig. 2 two overlapping peaks are visible in the

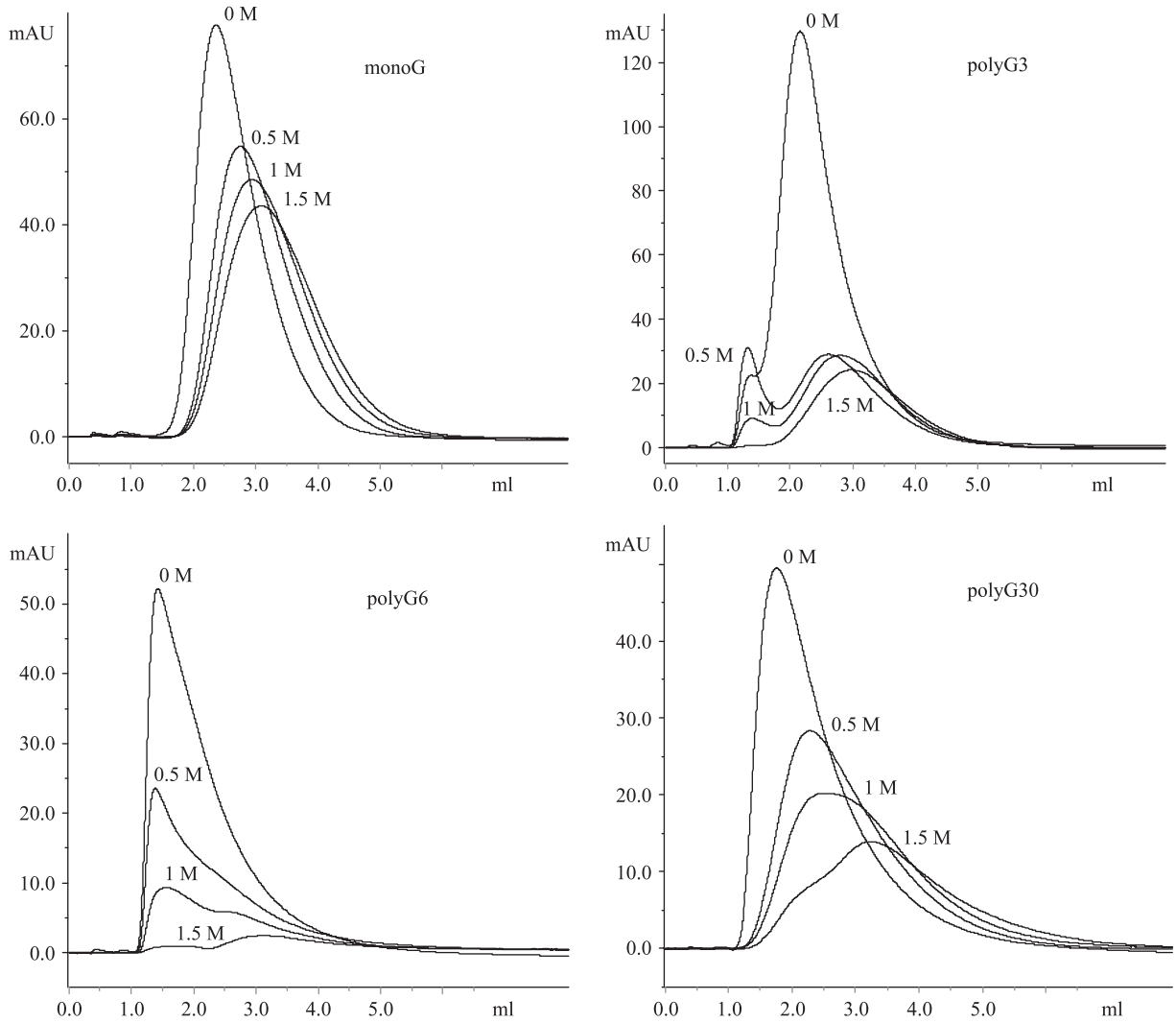

Figure 2. Affinity chromatography profiles of homo-deoxyoligonucleotides monoG, polyG3, polyG6 and polyG30 on L-histidine–agarose gel. The several curves of each chromatogram represent the interaction of nucleotide as ammonium sulfate concentration (0, 0.5, 1 and 1.5 M) is increased, all eluted at 5°C.

presence of the buffer Tris–Cl or even at low salt concentrations (for instance the ammonium sulfate) according to what was previously described (Kejnovska *et al*., 2003). This leads us to believe that in this case polyG3 molecules could associate together as intermolecular quadruplexes. Since the polyG 6, 15 and 30 have long guanine-rich repeats, they can self-associate into higher order four-stranded structures as intramolecular quadruplexes (secondary structures). These considerations are grounded in the heterogeneity of the peaks corresponding to these oligonucleotides, as we can see in Fig. 2.

Figure 3 shows the retention behavior of the various polyA nucleotides at extreme temperatures (5 and 35°C), for different salt concentrations in the eluent. We can observe that the retention factor increased with the salt concentration for all the molecules studied. With relation to the effect of molecular mass of oligonucleotides on retention, it was observed that for polyC, polyT and polyU the retention increased as the molecular mass also increased (results not shown). However, an exception to this behavior was found for polyG and polyA oligonucleotides. In general, in the polyA experiments, the retention of oligonucleotides increases with the increase in the number of bases, but there are some contradictory behaviors. At 0.5 M of ammonium sulfate concentration, the interaction of polyA with the histidine–agarose support is stronger for the lower-molecular-mass oligonucleotides (retention time 2.30 and 2.71 for polyA30 and polyA3, respectively), e.g. Fig. 3(A, B). This would not be the expected behavior when hydrophobic interactions are dominant because the global hydrophobic character should increase with the molecular mass. For this case, at relatively low salt concentrations, we suggest that electrostatic interactions between the charged surface of the oligonucleotides and the surface of the ligand are prevailing over hydrophobic interaction. These results are probably a consequence of the higher dipole moment of adenine, which is responsible for stronger ion–dipole interactions between the solute and the eluent (Diogo *et al*., 2002).

Previous studies based on nucleotides retention reported that the hydrophobicity of adenine is higher than that of thymine (Ikuta *et al*., 1984). This order of elution, however, is reversed for the case of the poly 30 molecules (Diogo *et al*., 2003). Accordingly, in our work, polyA30 was also less retained than polyT30, for the elution with 1.5 M of ammonium sulfate at 5°C (data not shown). Furthermore, by comparing the retention of polyA30 and polyA3,

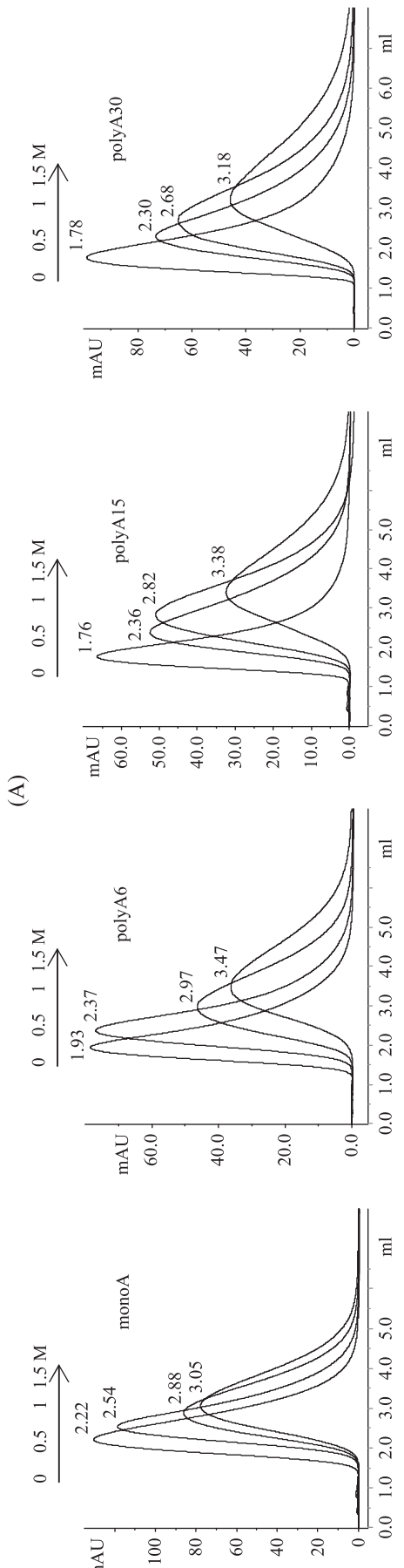

Figure 3. Affinity chromatography profiles of homo-deoxyoligonucleotides monoA, polyA6, polyA15 and polyA30 on L-histidine–agarose gel, (A) corresponding to elution of oligonucleotides at temperature of 5°C and (B) corresponding to elution of oligonucleotides at temperature of 35°C. The several curves of each chromatogram represent the interaction of nucleotide as ammonium sulfate concentration (0, 0.5, 1 and 1.5 M) is increased. The label on the top of each peak corresponds to the retention time.

a different behavior from the expected was also observed since a lower retention was achieved for polyA30 oligonucleotide (retention times 3.18 and 3.42 for polyA30 and polyA3, respectively), e.g. Fig. 3(A). These results are probably a consequence of the existence of secondary structures for the case of polyA30. In fact, secondary structures can shield the interaction sites by decreasing the exposure of the hydrophobic bases, giving retention times that are lower than expected, as previously described (Diogo *et al*., 2003).

Despite the nonsystematic effect of temperature on the retention of oligonucleotides studied, some curious results were achieved. Conversely to the findings at 5°C, for the experiments performed at 35°C in Fig. 3(B), an increased retention was obtained by increasing the molecular weight of adenine oligonucleotides. In this way, the results of Fig. 3(B) suggested that the increase in temperature could tend to disrupt the interactions that stabilize the secondary structures, and consequently, increase the interaction with the support. Owing to the presence of secondary structures in polyA, the interactions between the molecule and the stationary phase may not be proportional, as occurs with totally denatured molecules; this means that at 35ºC the molecule is more denatured than at lower temperatures (Diogo *et al*., 2003). These results are in agreement with previous studies, where it was reported that the temperature increase could affect pDNA conformation, by inducing conformational changes (predenaturation of secondary structures) and consequently affect pDNA retention, but not directly influence the interaction of the molecules to the histidine stationary phase (Sousa *et al*., 2007b).

The direct effect of temperature increase on conformational changes on molecules presenting secondary structures was also revealed in the experiments using polyA30 eluted with 1.5 M ammonium sulfate, at different temperatures, e.g. Fig. 4(A). It was possible to observe that the retention time increased with the temperature. In these circumstances, an increase in temperature decreases the degree of local base stacking, inducing the disruption of the polyA30 secondary structures, which increases the hydrophobic contact area of the oligonucleotides. In this particular case, the conformational change induced by the increase in temperature (Sousa *et al*., 2007b) seems to be the predominant phenomenon that governs the interaction with histidine-support. This phenomenon is also corroborated by the results found for all the other oligonucleotides studied at different temperatures, as exemplified in Fig. 4(B) for polyA30 eluted at 0.5 M of ammonium sulfate. In this case a systematic tendency of retention with increasing temperature was not verified, probably because at this low salt concentration the secondary structures are not favored and the effect of the temperature is not so relevant. Similarly in the experiments performed with other oligonucleotides not presenting secondary structures, there was not observed a systematic influence of temperature in retention, using either high or low salt concentrations (results not shown).

The effect of elution buffers pH (a range between 5 and 8) on the oligonucleotides retention in the histidine–agarose support was also studied. We have observed that, in general, the retention of the oligonucleotides increased when the pH value was decreased in the elution buffer without ammonium sulfate (retention time 2.71, 2.68, 2.60 and 2.36 for pH 5, 6, 7 and 8, respectively), as we can see in Fig. 5(A). However, the retention of oligonucleotides remained unchanged in the pH range studied, if the elution buffer contained 0.5 M of ammonium sulfate (retention times 2.81, 2.81, 2.81 and 2.74 for pH 5, 6, 7 and 8, respectively) as we can see in Fig. 5(B). These results suggested that with lower salt content the ionic interactions are predominant. For the lower pH values the electrostatic interactions occurring between the phosphate group of oligonucleotides and the histidine ligand are responsible for the higher retention (pK_a of histidine 6.5). In the presence of salt other interactions, such as hydrophobic interactions, could be favored and the effect of pH on oligonucleotides retention was not verified.

This behavior is also an evidence that, depending on the experimental conditions, different interactions could be predominantly involved and explain the different retention of oligonucleotides. In general, we suggest that the affinity interaction is responsible for the specific recognition of particular oligonucleotide bases but also involves multiple interactions occurring between the histidine ligand and the oligonucleotides.

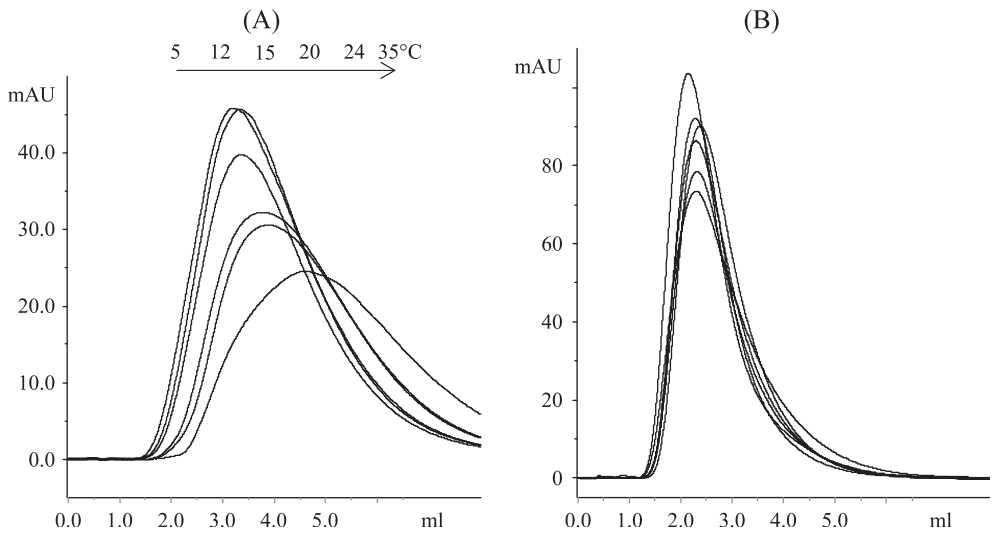

Figure 4. Affinity chromatography profiles of nucleotide polyA30 on L-histidine–agarose gel; the several curves of each chromatogram represent the interaction of nucleotide as temperature (5, 12, 15, 20, 24 and 35°C) is increased. (A) Elution of polyA30 at 1.5 M ammonium sulfate and (B) elution of polyA30 at 0.5 M ammonium sulfate.

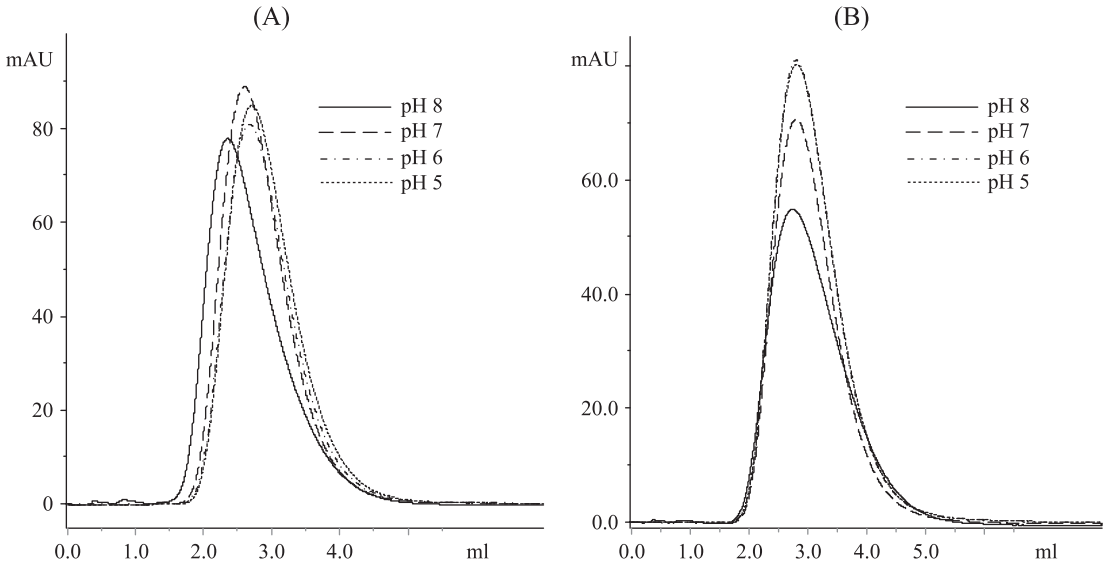

Figure 5. Affinity chromatography profiles of monoG nucleotide on L-histidine–agarose at 5°C. The curves of each chromatogram represent the retention of mononucleotide at different pH values (5–8). (A) Elution of monoG at 0 M ammonium sulfate and (B) elution of monoG at 0.5 M ammonium sulfate.

Conclusions

In general it can be concluded that multiple interactions between oligonucleotides and the histidine ligand play an important role in their selective retention. Several parameters such as the molecular mass of the oligonucleotides, the hydrophobic character of the individual bases, the presence of secondary structures, the pH and the concentration of ammonium sulfate in the eluent have been shown to have a significant influence on oligonucleotide retention to the histidine support. The retention behavior of the different bases investigated and the ability of salt to modulate the interaction strongly indicates that hydrophobic interactions and the biorecognition of particular oligonucleotide bases by histidine ligands play a major role in the retention. It was also concluded that the temperature has not a direct influence on oligonucleotide retention. In fact, temperature can promote conformational changes on molecules presenting secondary structures, thus affecting the retention of oligonucleotides on the histidine support.

This study provides a further understanding of the interactions involved in the selective recognition of supercoiled plasmid DNA, by histidine–agarose affinity support, bringing additional knowledge to overcome some problems and improve the histidine chromatography affinity technique.

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Paper III

Selectivity of arginine chromatography in promoting different interactions using synthetic oligonucleotides as model

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Original Paper

Selectivity of arginine chromatography in promoting different interactions using synthetic oligonucleotides as model

Arginine has been effectively used in several chromatography methodologies to improve recovery, resolution, and to suppress aggregation. Recently, arginine chromatography was used to fully separate supercoiled and open circular plasmid DNA isoforms. The specific recognition of supercoiled plasmid isoform by arginine was hypothesised to be due to the ability of arginine matrix to be involved in complex interactions that are partly dependent on the conformation of the DNA molecule. In light of these considerations a study was conducted to understand the several interactions that a DNA molecule can promote with the arginine support, in accordance with the chromatographic conditions established. Consequently, knowing the ideal conditions to promote the specific interactions, it could be possible to perform a more targeted and efficient purification. This work describes the chromatography of oligonucleotides with sizes up to 30 bases on the arginine-agarose gel. The effect of several conditions like hydrophobic character of the individual bases, molecular mass of the oligonucleotides, presence of secondary structures, temperature and elution buffer composition (salt and arginine supplemented buffer) was investigated. According to previous atomic data referent to possible interactions between amino acids and DNA nucleotides, arginine can preferentially interact with guanine by hydrogen bond, but other interactions (ionic interactions, van der Waals contacts, water mediated bonds) may also be present and become dominant depending on the conditions used. The results also revealed that the application of arginine in the elution buffer led to an effective elution of oligonucleotides from the arginine chromatographic support by a competition strategy. In general, it was suggested that the affinity interaction promoted by the arginine support is responsible for the specific recognition of particular oligonucleotide bases, involving multiple interactions.

Keywords: Affinity chromatography / Arginine-agarose support / Oligonucleotides / Selectivity

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1 Introduction

The pursuite of effective, robust and economical processes to conform with the strict quality assurance in production of therapeutic biomolecules has been accompanied by the development of highly selective and sophisticated strategies based on affinity chromatography (AC) [1]. Affinity methods present several advantages such as the reduction of the number of required steps, increased yields and improved process economics. However, some limitations are also associated with these methods, mainly in view of the biological origin of the ligands [1]. Because the ligands with biological origin tend to be fragile and associated with low binding capacities, a new approach is considered for designing synthetic ligands that would combine the selectivity of natural ligands with the high capacity and durability of synthetic systems [2, 3]. There are several studies that relate important advances in ligand design for specific proteins, but its development for plasmid DNA (pDNA) purification has not been extensively described [4, 5].

AC separates biomolecules on the basis of a reversible interaction between the target biomolecule and its bio-

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Abbreviations: AC, affinity chromatography; **pDNA**, plasmid DNA

specific ligand, which is coupled to a chromatography matrix. The specific interactions occurring between ligand and target molecule can be the result of electrostatic interactions, hydrophobic interactions, van der Waals forces and/or hydrogen bonding [6]. Overall, the specific nature of the underlying interactions is a major advantage of AC because, in a single step, affinity purification can offer immense advantages over other less-selective and time-consuming multi-step procedures [7–9]. The concept of using less-selective ligands has also been applied to pDNA purification, mainly by using amino acids as immobilized ligands, which, as predicted from atomic studies based on molecular modelling, might preferentially interact with specific nucleic acid bases [6, 10].

Amino acids ligands were used in AC of proteins [3], oligosaccharides [11] and endotoxins [12], and, very recently, for the first time, their application for pDNA purification was asserted [13, 14]. In general, the positively charged amino acid arginine mediates the largest number of contacts in protein-nucleic acid interactions [10]. The characteristics of arginine, namely (i) its ability to interact in different conformations, (ii) the length of its side chain, and (iii) its ability to produce good hydrogen bond geometries, also point to the possibility of specific recognition mechanisms [6]. Preliminary studies that employed arginine matrices to purify pDNA revealed the presence of specific interactions with plasmid molecules and, importantly, a significant recognition of the supercoiled isoform [9]. In light of these considerations a study was conducted to understand the several interactions that the DNA molecule can promote with the arginine chromatographic support, in accordance with the conditions established for its elution. Thus, knowing the type of preferential interaction between the arginine ligand and a nucleotide base or a specific bases sequence, and thus the preferential conditions of the interaction and the composition of the plasmid, it could be possible to perform a more targeted and efficient purification.

Synthetic oligonucleotides can also be utilised in many molecular biology applications inducing PCR, DNA sequencing, and genotyping [15]. Furthermore, short chain oligonucleotide molecules, with various specific sequences, represent particularly attractive models to study nucleic acid adsorption because they allow a clear interpretation of the experimental data [16]. This work describes the AC of synthetic homo- and hetero-oligonucleotides with sizes up to 30 bases on the arginine-agarose gel. Hence, the effect of several conditions like hydrophobic character of the individual bases, molecular mass of the oligonucleotides, presence of secondary structures, temperature and elution buffer composition (salt and arginine supplemented buffer) was investigated.

2 Experimental

2.1 Materials

The commercial support Arginine-Sepharose 4B gel was purchased from GE Healthcare Biosciences (Uppsala, Sweden). This support is characterised by the manufacturer as a cross-linked 4% beaded agarose matrix with a 12-carbon atom spacer, a ligand density between 14 and 20 μmol/mL, the mean particle size is 90 μm and the bead size range is between 45 and 165 μm. Lyophilised synthetic homo-oligonucleotides with different molecular mass were purchased from Sigma-Aldrich (St Louis, MO, USA). The hetero-oligonucleotides: GGG-GGG-CCC-GGG-GGG (H1), GGG-GGG-AAA-GGG-GGG (H2), GGG-AAA-GGG-AAA-GGG (H3) were synthesised by Eurogentec (Seraing, Belgium). Other chemicals used in the elution buffer were NaCl purchased from Panreac (Barcelona, Spain), L-arginine purchased from Sigma-Aldrich and Tris from Merck (Darmstadt, Germany). All solutions were freshly prepared using water ultra-pure grade, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and the elution buffers were filtered through a 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. The lyophilised homo- and hetero-oligonucleotides were first resuspended in 10 mM Tris-Cl pH 8.0 and used without further purification.

2.2 Procedure

Chromatography was performed in an ÄKTA Purifier system (GE Healthcare Biosciences, Uppsala, Sweden). A 10 mm diameter × 20 mm long (about 1.6 mL) column was packed with the commercial arginine-sepharose 4B gel. The column was equilibrated at 5, 12, 15, 20, 24 and 35°C with an appropriate loading buffer (NaCl or L-arginine) at a flow-rate of 1 mL/min. The water jacketed column was connected to a circulating water bath Multi-Temp III to maintain the appropriate temperature in each experiment. The column was initially equilibrated with 10 mM Tris-Cl buffer pH 8.0, subsequently, 50 μL (about 2.5 μg) of the oligonucleotides samples were loaded on the column and then eluted by using a linear gradient elution from 0 to 1 M NaCl (100% of buffer B) unless otherwise stated. The absorbance of the eluate was continuously measured at 260 nm. After the chromatographic runs, the column was washed with three column volumes of the 100% of buffer B, in order to remove any molecule that remained bound.

3 Results and discussion

Arginine-agarose support was recently used to effectively purify pDNA [17] and to isolate both plasmid isoforms

(supercoiled and open circular) [9], and it was suggested that the binding mechanism involves phenomenological interactions like biorecognition between amino acids and pDNA [14], including, electrostatic interactions, hydrophobic interactions, (multiple) hydrogen bond interactions, dipole-dipole forces, cation-π interactions, *etc*. Since plasmids are negatively charged due to the phosphate groups in the DNA backbone, it is easy to predict a favourable electrostatic interaction between the plasmid phosphate groups and arginine ligands. On the other hand, complex interactions extend the concept of simultaneous bonds further, since the arginine ligand can bind with more than one base step simultaneously [6]. Overall, in situations that double strands are present, such as the plasmid, a cross-linking may occur between the arginine ligand and the bases of both chains in different planes. These interactions enable amino acids to recognize short DNA [6] or RNA [18] sequences. By this way, an investigation was performed to understand the several interactions involved between the DNA molecule and the arginine support, in accordance with the conditions established for its elution.

For this study, a 2.5 μg amount of each homo-oligonucleotide (polyA, polyT, polyG and polyC with 1, 3, 6, 15 and 30 bases) was injected into the arginine-agarose column, previously equilibrated with 10 mM Tris-Cl buffer pH 8.0, and eluted with a linear gradient between 0 and 1 M NaCl using different temperatures.

3.1 Homo-oligonucleotides

In Fig. 1, we can observe the chromatographic profile achieved at 12°C for the different poly15 homo-oligonucleotides studied, eluted with an increased NaCl gradient. In this study the oligonucleotide presenting higher retention on arginine matrix was the polyG15 (Fig. 1). This retention behaviour can be tentatively explained through several arguments. The retention does not simply follow the hydrophobicity of the oligonucleotides, because although the guanine base is characterised as the most hydrophobic base [16], what is in accordance with its higher retention on the arginine matrix, the adenine base that is more hydrophobic than the pyrimidine bases presented a weaker interaction and was less retained (Fig. 1). Moreover, the elution pattern is also different from that of the mononucleotides which elute in the order G, C, T and A (results not shown). Hence it is concluded that more specific and more complex interactions are driving the retention of the oligonucleotides on the arginine phase. Thereby, interactions like electrostatic, hydrophobic, π-cation stacking, hydrogen bond and van der Waals interactions may occur between arginine and the nucleotide bases. In fact, previous studies based on molecular modelling [6, 10] described a favoured and stronger interaction of arginine with the guanine base by occurrence of a large number of hydrogen interactions. The fact that this amino acid can interact with guanine by two or more hydrogen bonds [4] (Fig. 2) could be another explanation for the longer retention of polyG15 in the arginine support.

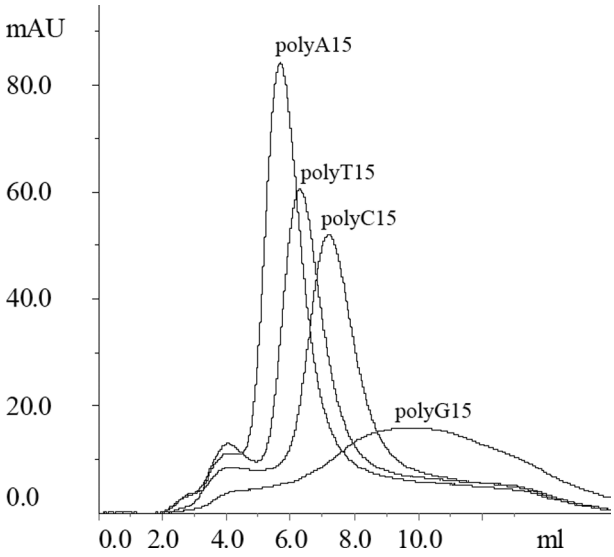

Figure 1. AC profiles of homo-oligonucleotides polyA15, polyT15, polyC15 and polyG15 on arginine-agarose gel, at 12°C. Elution was performed at 1 mL/min by increasing the NaCl concentration in the eluent from 0 to 1 M.

The arginine amino acid can also promote amino-π and cation-π interactions with aromatic rings of nucleic acid bases, and as both involve a positively charged group, they shall be designated as cation-π interactions [19]. These interactions may be present, since arginine is positively charged and the nucleotide bases are composed of aromatic rings, enabling them to a displacement of π-electrons. Furthermore, the positive character of arginine is also exploited in the electrostatic interactions promoted because of the presence of the phosphate group. For this case, the ionic interactions that are considered are the interactions involving the arginine molecule with O-1 and O-2 of the phosphodiester backbone, for the reason that these atoms are readily accessible and are frequently found in a good position for electrostatic interactions [20, 21]. Overall, the combined action of these interactions is reflected in a preferential binding of arginine amino acid with a guanine bases sequence, and if the plasmid has a sequence rich in guanine, we can purify it more adequately.

3.2 Oligonucleotide molecular mass

The analytical techniques for the separation of oligonucleotides are in principle based on the discrimination of the molecular size (length) or charge [15]. A different preferential interaction pattern was obtained when test-

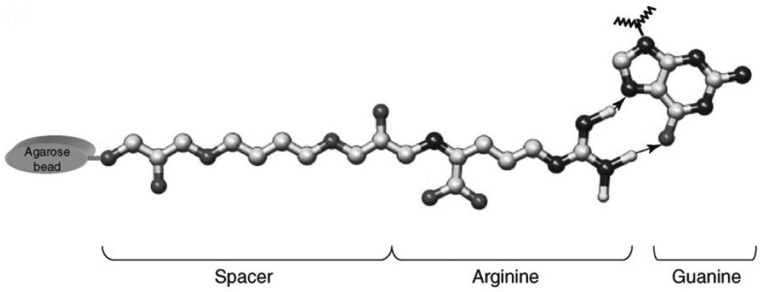

Figure 2. Schematic representation of one type of multiple hygrogen bond designated by bidentate interaction, involving two hydrogen bonds, between arginine immobilised ligand (donor atom) and guanine (acceptor atom) [4].

ing mononucleotides or poly15 oligonucleotides, adenine being more retained in the first case and the guanine base in the second, it was interesting to know the behaviour of these oligonucleotides with different sizes. Figure 3(a) shows the representative chromatograms of monoA, oligoA3, oligoA6, polyA15 and polyA30, obtained at a temperature of 12°C, using an increased NaCl gradient between 0 and 1 M.

In Fig. 3(a) it is possible to observe an expected increase on retention when the molecular mass of the adenine oligonucleotides was increased. Thus, at a constant temperature and for a fixed salt gradient, in the absence of additional effects (such as the presence of secondary structures), the retention time of the oligonucleotides was proportional to its number of hydrophobic bases, in accordance to what has been described by a previous work [22]. But in the case of guanine oligonucleotides (monoG, oligoG3, oligoG6, polyG15 and polyG30) in Fig. 3(b), the retention pattern was different from the expected one. Heterogeneity on the G6 oligonucleotide is evident as a minor peak at about 5.5 mL and a major peak at about 9 mL (Fig. 3(c)) is found. Moreover, the retention time of polyG15 is higher than that for polyG30. This behaviour suggests that different secondary structures of the oligonucleotide can coexist under these conditions. These secondary structures were already described as frequent and are usually designated by guanine quadruplexes that may occur due to the possible interactions between molecules of guanine leading to inter- or intramolecular associations, depending on the size of oligonucleotide [23, 24]. The quadruplexes can form arrays of four hydrogen bonds in which each base acts as both donor and acceptor of two hydrogen bonds with other guanines, and pairing between bases is of Hoogsteen type [25]. This unique structure has recently been proposed to exist in living cells and has drawn attention as a target for gene expression control, cell division [26], and selective anticancer agents (telomerase inhibitors and/or transcriptional repressors of c-MYC oncogene) [27]. Studies regarding G-quadruplex binding selectivity are essential to the development of G-quadruplex ligands for therapeutic use [27]. The potential of guanine oligonucleotides to be organised in different secondary structures induces a heterogeneity when they are in solution which is reflected in a two peaks profile (Fig.

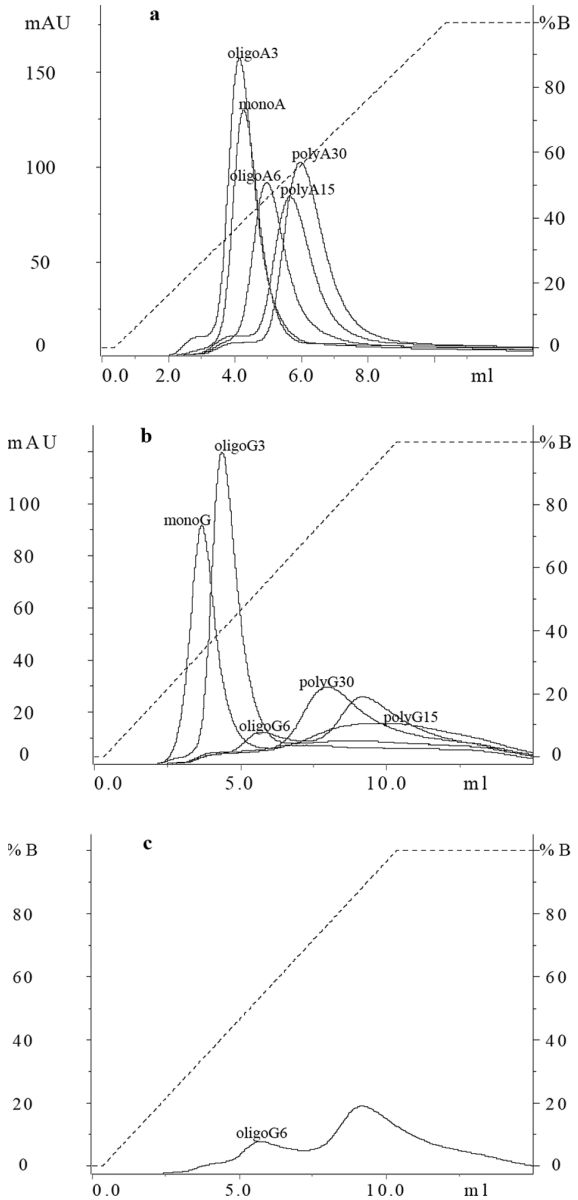

Figure 3. Affinity chromatographic profiles of homo-oligonucleotides monoA, oligoA3, oligoA6, polyA15 and polyA30 (a); monoG, oligoG3, oligoG6, polyG15 and polyG30 (b) and only oligoG6 (c) on arginine-agarose gel, at 12°C. Elution was performed at 1 mL/min by increasing the NaCl concentration in the eluent from 0 to 1 M, represented by %B in a Y secondary axis through the dashed line.

Table 1. Retention time[a)] (min) of oligonucleotides loaded on arginine-agarose support with an increased NaCl gradient between 0 and 1 M, at different temperatures (5, 12, 15, 20, 24, 35°C).

Oligonucleotide	Temperature (°C)					
	5	12	15	20	24	35
monoA	4.221	4.311	4.329	4.344	4.379	4.449
polyA30	5.902	5.977	6.165	6.310	6.431	6.777

a) For chromatographic conditions see Section 2.

3(b)). A temperature increase also destabilised the conformational structure of quadruplexes, leading to an increased exposure of the bases and consequently increased retention (data not shown).

3.3 Temperature

Temperature is a parameter that might significantly influence oligonucleotide interactions with arginine matrix and consequently affect the retention, or even the chromatographic selectivity [28]. The elution profiles obtained when loading an oligonucleotide sample (*e.g.* monoA or polyA30) onto the arginine-agarose column have shown that oligonucleotide retention increased when the temperature was increased (Table 1). This is the reverse mechanism previously found for histidine-AC of homo-oligonucleotides, where the interaction was mainly attributed to base exposition [24]. This work is in agreement with previous studies, where it was described that the temperature increase directly influenced the retention of the pDNA to the arginine stationary phase, being verified an increased retention [9]. Hydrophobic interactions involving both the aliphatic portion of the side chain of arginine and the 12-carbon spacer arm may also play an important role in the binding of oligonucleotides. Although the binding was observed at moderately low ionic strength (<1 M NaCl), unlike what is typical in hydrophobic interaction chromatography, the increased retention at higher temperatures [22] suggests that hydrophobic interactions may be involved, and was also verified in the representation of van't Hoff plots (ln*k′ versus* 1/T) (Fig. 4) [29, 30].

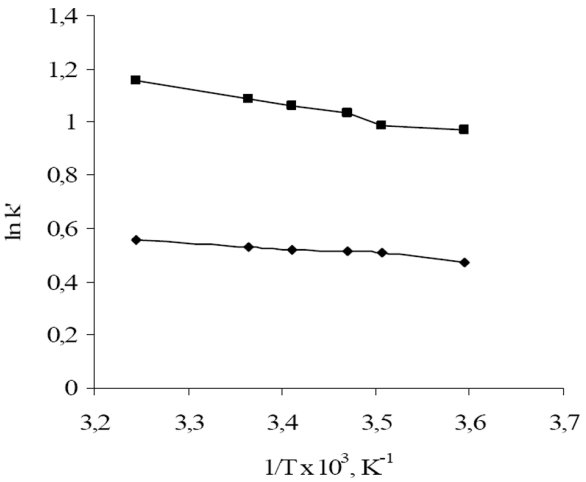

Figure 4. Van't Hoff plots of retention data obtained with monoA (♦) and polyA30 (■) (represented in Table 1) on arginine column.

3.4 Hetero-oligonucleotides

The analysis of hetero-oligonucleotides in ÄKTA purifier system was considered an important study to understand the effect of small changes in the oligonucleotides composition on the global retention, due to the different properties of the bases and interactions with the matrix. In fact, oligonucleotides of the same length but different sequence may exhibit different retention behaviour. This was demonstrated by the analysis of three different hetero-oligonucleotide ladders, as presented in Fig. 4. The separation of G homo-oligonucleotides is generally a problem because of the strong inter- and intramolecular interactions of G-rich DNA sequences [24]. Therefore, to study the impact of nucleotides contribution to the retention, we have used a sequence of guanines with an insertion of other nucleotides sequences.

Curiously, in hetero-oligonucleotides study (Fig. 5), the addition of three C nucleotides in the overall G oligomer (GGG-GGG-CCC-GGG-GGG) decreased the retention of this hetero-oligonucleotide (H1) compared with retention of the homo-oligonucleotide HomoG (GGG-GGG-GGG-GGG-GGG). Most interestingly, the retention of G oligomer with an insertion of three A nucleotides (GGG-GGG-AAA-GGG-GGG) slightly decreased the retention of this hetero-oligonucleotide (H2) by comparison with the previous H1. The retention of the third hetero-oligonucleotide (H3) with two insertions of three A nucleotides (GGG-AAA-GGG-AAA-GGG) was the least retained, confirming the evidence that the presence of adenine reduces the interaction with the arginine support. Some studies describe that higher-resolution IMAC HPLC can separate hetero-oligonucleotides with sporadic mismatches, presumably through interactions with bases in the disordered region [31]. Our above results have shown that the interaction of the hetero-oligonucleotides is

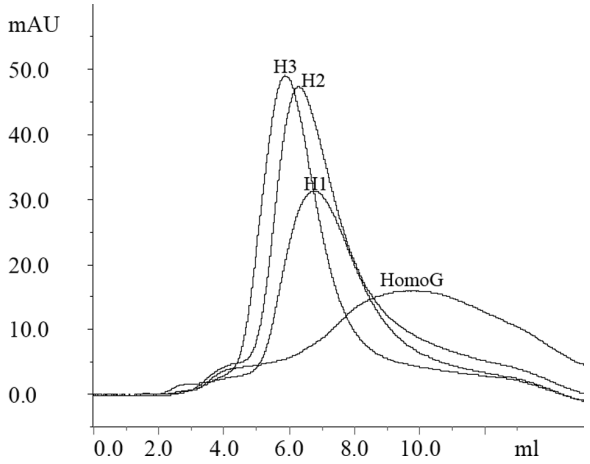

Figure 5. Comparison of chromatographic profile between homo-oligonucleotide polyG15 (HomoG (GGG-GGG-GGG-GGG-GGG)) and three different hetero-oligonucleotides; H1 (GGG-GGG-CCC-GGG-GGG), H2 (GGG-GGG-AAA-GGG-GGG) and H3 (GGG-AAA-GGG-AAA-GGG), from arginine-agarose, at 12°C. Elution was performed at 1 mL/min by increasing the NaCl concentration in the eluent from 0 to 1 M.

affected by the addition of new bases, such as A and C, in the sequence of G oligomers decreasing the retention, and if the addition is a sequence of the adenines the interaction is less favoured than with a cytosines sequence. However, the retention time correlates well with the binding affinities of the respective A and C homo-oligonucleotides in Fig. 1, which corroborates the hypothesis that different interactions may be involved in retention of hetero-oligonucleotides on the arginine-support. On the other hand, it is possible to suggest that the insertion of small sequences containing different bases on G oligomers can minimize the tendency of the formation of G-quadruplex, because the chromatographic peaks profile was different.

3.5 Complementary and non-complementary oligonucleotides

It is known that the nucleotides forming the DNA double chain bind through hydrogen bonds, and more specifically adenine forms two hydrogen bridges with thymine and guanine is involved in three hydrogen bridges with cytosine. Considering this, it was thought interesting to verify the retention behaviour of mixtures of two complementary and non-complementary oligonucleotides with the same size. Accordingly, the chromatographic experiments were performed by loading a sample constituted by the same quantity of polyG15 and polyC15 (complementary bases), and eluted by using a NaCl increased gradient as presented by the dashed line in Fig. 6. As it is possible to observe in the resulting chromatogram, the

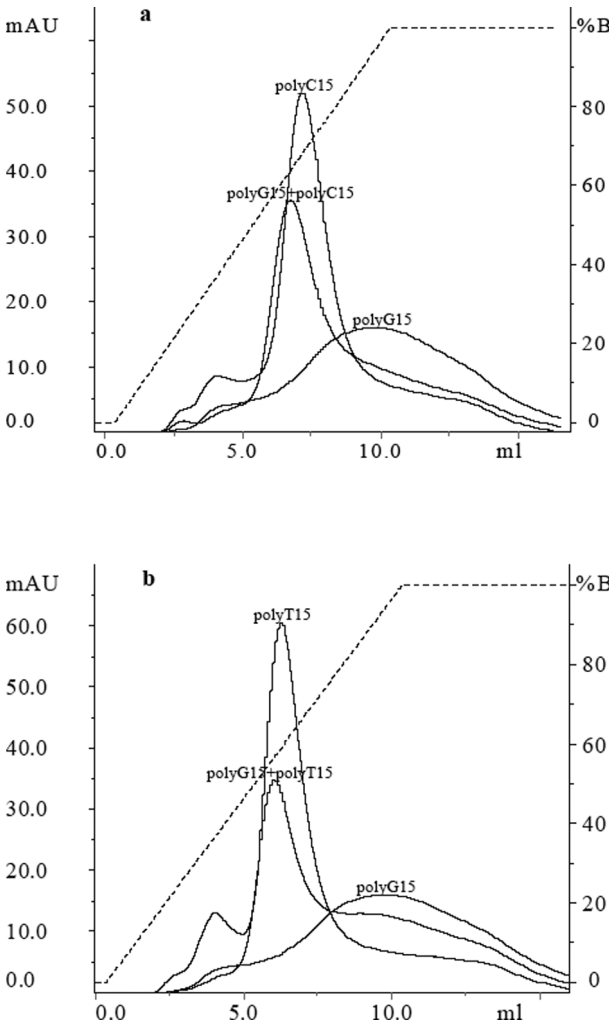

Figure 6. (a) Comparison of chromatographic profiles of polyG15, polyC15 and a sample constituted by an equivalent amount of polyG15 and polyC15 (complementary bases); (b) comparison of chromatographic profile between polyG15, polyT15 and a sample constituted by equivalent amount of polyG15 and polyT15 (non-complementary bases), from arginine-agarose, at 12°C. Elution was performed at 1 mL/min by increasing the NaCl concentration in the eluent from 0 to 1 M, represented by %B in a Y secondary axis through the dashed line.

elution of the mixture occurred in a single peak, and the retention time obtained was lower than the retention time of polyG15 and polyC15 when they were injected separately (Fig. 6(a)). Otherwise, when the sample injected is composed by the same quantity of polyG15 and polyT15, two peaks with distinct elution times were obtained (Fig. 6(b)). This behaviour leads us to suppose that in the second experiment both oligonucleotides present in the mixture were eluted according to the retention time of each isolated oligonucleotide, therefore, it is possible to observe a split peak that overlaps with the individual peaks. On the other hand, when the

Table 2. Retention data[a)] for adenine oligonucleotides using arginine and NaCl supplemented buffer mobile phases.

Eluent	Retention time (min)				
	monoA	polyA3	polyA6	polyA15	polyA30
Arginine[b)]	4.869	4.239	7.815	12.940	18.615
NaCl[b)]	5.061	4.714	8.843	14.961	32.701

a) For chromatographic conditions, see Section 2.
b) Concentration of elution buffer, 0.1 M.

bases are complementary (Fig. 6(a)), probably some additional interactions between oligonucleotides occurred leading to a double-stranded structure presenting less available sites to interact with the arginine support, resulting in the lower retention time.

3.6 Arginine in elution buffer

Arginine is extensively applied in purification strategies in a wide range of situations. This amino acid was accidentally discovered as a useful reagent able to suppress aggregation of the proteins during refolding, thereby increasing refolding efficiency [32]. For addition, arginine solubilises proteins from loose inclusion bodies, resulting in efficient production of active proteins [32]. Arginine suppresses protein–protein interactions in solution and also non-specific adsorption to gel permeation chromatography columns [33]. Lindner and coworkers performed a selective chemical modification of arginine residues in peptides by LC-MS and characterised arginine oligomers and a dendrimeric, arginine-containing peptide since this amino acid exerts a strong influence on both the mass spectrometric detection and the chromatographic retention [34]. This amino acid also facilitates elution of bound proteins from various column resins, including Protein-A or dye affinity [35] columns and hydrophobic interaction columns [33]. More recently it was demonstrated that this competition strategy with arginine in elution buffer is capable of the total elution of pDNA indicating that the interaction of pDNA with the matrix was weakened by competition [9, 14].

In this work, the elution of the oligonucleotides by addition of an arginine-supplemented buffer was tested. Table 2 schematically represents the retention time obtained in elution of the polyA oligonucleotides using a linear gradient with 0.1 M NaCl or with 0.1 M arginine added to the elution buffer without NaCl. Comparing the retention time of the oligonucleotides, it was verified that the elution with arginine was faster than the elution with NaCl. It was also observed that the peaks obtained with arginine in the elution buffer have a well defined chromatographic profile [33], indicating that the addition of arginine in the eluent can promote the preferential binding of oligonucleotides to the free arginine present in the buffer, weakening the interaction with the matrix. These results are in accordance with published data showing that this competition strategy is able to elute bound proteins from arginine-agarose columns [36]. In light of these results, we suppose that the arginine can be used as the elution buffer to lower concentrations as 50 and 20 mM (data not shown) relative to other salts. The lower concentration of arginine used suggests that its effectiveness is due to factors other than electrostatic effects [35], involving affinity interactions.

4 Concluding remarks

Amino acid-based AC represents a promising approach for the purification of pDNA and other nucleic acids, because it combines the selectivity of a naturally occurring biological interaction with the simplicity of a single small molecule used as a ligand. To achieve higher efficiency and selectivity in arginine-based AC, it has become essential to identify the interactions involved with the arginine support to purify nucleic acids.

In order to understand the mechanism of interaction of pDNA with arginine-agarose surface, the retention process of synthetic homo- and hetero-oligonucleotides molecules with sizes up to 30 bases was studied. Several factors like the hydrophobic character of the individual bases, the molecular mass of the oligonucleotides, the presence of secondary structure, temperature and elution buffer composition (salt and arginine supplemented) were analysed to achieve their influence on oligonucleotides retention on the agarose support. In general, this study has shown that arginine chromatography can specifically recognise the different bases of oligonucleotides. The underlying mechanism in this study involves phenomenological interactions like biorecognition which are themselves made up of elementary interaction forces such as (multiple) hydrogen bond, electrostatic, hydrophobic interactions, dipole-dipole forces, cation-π interactions, *etc*. The molecular mass and presence of secondary structures in oligonucleotides influence the hydrophobic character of the molecules and consequently its retention on the arginine-based matrix. The differences on retention achieved by using oligonucleotides with mixed base composition in different

orders, demonstrated that the type of bases present on the oligonucleotides sequence plays the most important role in the retention behaviour. In addition, the bases exposure is a crucial factor to determine the type of favoured interactions. The electrostatic interaction could be the principal phenomenon explaining the retention of single-stranded oligonucleotides, but it was proved that the interaction and retention of double-stranded oligonucleotides on arginine support significantly decreased, as a result of the diminished exposure degree of bases.

Overall, this work is a confirmation that depending on the experimental conditions, different interactions could be predominantly involved and explain the different retention of oligonucleotides. The results obtained provide valuable information for the future development and implementation of arginine and other amino acids as ligands in chromatography to perform a more targeted and efficient purification of several nucleic acids.

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Paper IV

Biorecognition of supercoiled plasmid DNA isoform in lysine-affinity chromatography

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Short communication

Biorecognition of supercoiled plasmid DNA isoform in lysine-affinity chromatography

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ABSTRACT

The use of plasmid DNA-based therapeutics relies on procedures that efficiently purify the supercoiled plasmid isoform. The present study describes a new strategy that uses a lysine ligand in affinity chromatography to efficiently separate supercoiled and open circular plasmid DNA isoforms. To achieve higher specificity in this chromatography it is essential to characterize the behaviour of binding/elution of supercoiled isoforms. The results show that the lysine support promotes complex interactions with supercoiled isoform, according to ionic strength and temperature manipulation.

1. Introduction

The progress of disease-related genes and the possibility to manipulate the gene set-up in some organisms has fostered the development of innovative human DNA therapeutics, such as DNA vaccination, gene therapy and recombinant biopharmaceuticals [1]. In 2007, around 30% of gene therapy clinical tests started to use plasmids as non-viral vectors in vaccines for the treatment of cancer [2]. For the successful implementation of clinical approaches using plasmid DNA (pDNA)-based therapies, increasingly developments of production and purification procedures are required [3].

The biosynthesis of pDNA by *Escherichia coli* (*E. coli*) results in a highly enriched supercoiled (sc) pDNA extract, which is advantageous since sc pDNA is considered the most efficient isoform at transferring gene expression [4,5]. In this way, a particular downstream processing is required for the elimination of cellular components of the *E. coli* host, as well as for the reduction the open circular (oc), linear and denatured pDNA isoforms that occur due to conformational changes of sc plasmid. The main objective is the recovery of sc isoform (higher than 97%) according to the standards established by regulatory agencies [6].

One of the major bottlenecks found in the chromatography application for pDNA purification is that many stationary phases display poor separation selectivity towards pDNA and impurities due to their similar binding affinities [7]. The affinity chromatography (AC) takes advantage of the undoubted specificity and efficiency of pDNA or impurities to specific immobilized ligands [3,8,9]. These affinity interactions are probably the most adequate to purify sc pDNA, even when a single chromatographic step is required.

Supercoiled pDNA purification strategies that use amino acids (histidine and arginine) as immobilized ligands have recently lead to interesting results [10,11]. Particularly, immobilised histidine was used to specifically recognize sc pDNA present in the complex *E. coli* lysate, allowing the elimination of host impurities and the required purification degree [11]. In addition, arginine–agarose support has also been efficiently applied to separate plasmid isoforms, revealing a recognition of sc isoform [10]. Similarly to what is described for arginine, some molecular modelling studies described that lysine is a positively charged amino acid that mediates the largest number of contacts in protein–nucleic acid interactions [12,13]. Lysine–agarose was already used to separate RNA species of different molecular weight with a linear gradient of NaCl [14]. Building on these considerations, it is interesting to verify if the lysine matrix may contribute to the separation of sc and oc pDNA isoforms and to study the lysine-base molecular recognition mechanism by affinity chromatography.

2. Material and methods

Lysine-Sepharose 4B gel was obtained from GE Healthcare Biosciences (Uppsala, Sweden) and the Qiagen Plasmid Purification Maxi Kit was purchased from Qiagen (Hilden, Germany). Water was ultra-pure grade, purified with a Milli-Q system from Millipore. All salts used were of analytical grade.

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2.1. Bacterial production

The 6.05 kpb plasmid pVAX1-*LacZ* (Invitrogen, Carlsband, CA, USA) used in the experiments was produced by a cell culture of *Escherichia coli* DH5α. Growth was carried out at 37 °C using Terrific broth medium (20 g/l tryptone, 24 g/l yeast extract, 4 ml/l glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4) supplemented with 30 μg/ml kanamycin. Growth was suspended at the late log phase [*OD*600 ~13] and the cells were recovered by centrifugation and were stored at −20 °C.

2.2. Lysis and isolation of plasmid DNA

pDNA was purified using the Qiagen (Hilden, Germany) plasmid maxi kit according to the manufacturer's instructions. The protocol is based on a modified alkaline lysis procedure. Following lysis, binding of pDNA to the Qiagen anion exchange resin is promoted under appropriate low-salt and pH conditions. Impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then is concentrated by isopropanol precipitation.

2.3. Preparative chromatography

Chromatography was performed in a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden). A 16 mm × 100 mm (~20-ml) column was packed with the commercial support Lysine-Sepharose 4B gel (GE Healthcare Biosciences, Uppsala, Sweden). This support is characterized by the manufacturer as a cross-linked 4% beaded agarose matrix with a 12-carbon atom spacer and a ligand density between 4 and 7 μmol/ml. Typically, the experiments were performed at 12 °C, through a circulating water bath to maintain the appropriate temperature. The column was equilibrated with 165 mM NaCl in 10 mM Tris buffer (pH 8.0) and the system was run at a flow rate of 1 ml/min. Plasmid sample (200 μl) was then injected at the same flow rate and the absorbance was monitored at 280 nm. After elution of unbound species with 165 mM NaCl in 10 mM Tris buffer (pH 8.0), the ionic strength of the buffer was increased stepwise to 250 mM NaCl in 10 mM Tris buffer (pH 8.0). Fractions were pooled according to the chromatograms obtained and kept for further analysis as described bellow. After chromatographic runs, it was necessary to wash the lysine medium with at least 5 bed volumes of 2 M NaCl in 10 mM Tris buffer, pH 8.0.

2.4. Gel electrophoresis

The fractions recovered from the chromatographic experiments were analysed by horizontal electrophoresis using 15-cm-long 1% agarose gels (Hoefer, San Francisco, CA, USA), which were stained with ethidium bromide (0.5 μg/ml). Electrophoresis was carried out at 100 V, for 1 h, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0).

3. Results and discussion

Initial experiments were performed to choose the best strategy for pDNA binding/elution, being observed that total (oc + sc) pDNA retention was obtained at 150 mM NaCl in 10 mM Tris buffer (pH 8.0), and total elution was verified at 250 mM with the same salt. During these experimental studies it is observed a need for strict control in order to maintain the reproducibility since a slight variation of the conductivity (salt concentration or temperature) affected the plasmid retention. The column was equilibrated with 165 mM NaCl in 10 mM Tris buffer (pH 8.0) at 12 °C, using a flow rate of 1 ml/min. Fig. 1 represents the pDNA chromatographic profile obtained when 200 μl (200 μg/ml) were injected. After plasmid injection, unbounded species were eluted due to lower affinity to the matrix. The ionic strength of the buffer was then increased to 250 mM NaCl in 10 mM Tris buffer (pH 8.0) and the highly bound species were eluted. Fractions were pooled according to the chromatograms obtained and were analysed by an agarose gel electrophoresis.

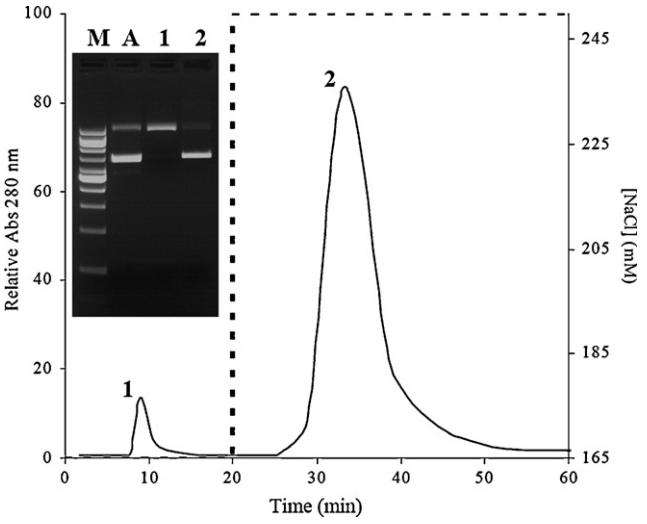

Fig. 1. Chromatographic separation of pDNA (200 μg/ml) isoforms with a lysine–agarose matrix. Elution was performed by stepwise increasing NaCl concentration in the eluent from 165 to 250 mM, as represented by dashed line. Agarose gel electrophoresis analysis of each peak is represented in the respective figure. Lane M: molecular weight marker; lane A: pDNA sample injected onto the column (oc + sc); lane 1: oc; lane 2: sc.

In Fig. 1, the analysis of electrophoresis gel revealed that the first peak of unbounded species corresponds to the oc isoform (lane 1), and the second peak was attributed to the sc isoform (lane 2). As judged by the gel, both isoforms were totally isolated. These results suggest that the recognition of lysine matrix by sc isoform can be related with supercoiling phenomenon that is a consequence of deformations induced by the torsional strain leading to a higher exposition of the bases of sc isoform compared with the oc isoform. This phenomenon can be considered as an explanation for the fact of lysine ligand to distinguish and differentially interact with both isoforms (Fig. 1) further suggesting a specific recognition of the sc pDNA isoform.

Although lysine can only use a single side chain atom for binding, and the hydrogen bonds are less likely to resemble the ideal geometry than for arginine [3], there are clear evidences that lysine is a multiple donor amino acid and promotes hydrogen complex interactions (bidentate) preferentially with guanine base, according to what was found in molecular modelling studies [12]. Even though the salt concentrations (250 mM NaCl) needed to elute bound plasmid isoforms from lysine matrix (Fig. 1) are lower than those typically used in other anion-change chromatographic supports (higher than 500 mM), the presence of electrostatic interactions between the plasmid phosphate groups and lysine ligand (positively charged) should be considered, similarly to what happens in studies with arginine [5].

To better understand the mechanism for specific recognition of sc pDNA with lysine–agarose, some experiments of competitive elution with elution buffers containing different kinds of amino acids were performed. A 40 min linear gradient between 10 mM Tris buffer and 250 mM of each amino acid was used. Arginine was chosen as a positively amino acid in the elution buffer and pDNA was eluted during the linear gradient. This behaviour is due to the

positively character of this amino acid that promote a preferential binding of pDNA with free arginine present in the buffer by electrostatic interactions, inducing its elution together with arginine. When it was used glutamic acid as negative amino acid in the elution buffer, pDNA was also eluted because glutamic acid promoted the displacement of the bound pDNA. So, it was observed that the use of a negative amino acid can also promote a competitive elution, competing with pDNA, particularly with the phosphate groups, for the lysine ligands surface. Valine was chosen as amino acid with aliphatic side chain, and for this case, the elution gradient did not induce pDNA elution. This fact shows that the preferential interaction between pDNA and lysine support is ionic and not hydrophobic, because despite this amino acid have a carbon lateral chain, pDNA remained bound at the matrix (results not shown). These data allowed the knowledge of the underlying mechanism and the responsible groups for the recognition of pDNA by lysine, which can be extremely helpful to the further efforts in development of novel materials for purification of nucleic acids.

To further confirm the matrix selectivity at the same elution conditions, one sample with sc isoform (200 μg/ml) obtained after lysis and purification with the Qiagen kit, and another sample with oc isoform (200 μg/ml) achieved by incubation of a sc pDNA sample at room temperature for 5 days (following by electrophoresis gel), were used (results not shown).

The effect of temperature on selectivity was already described in other studies and it has been proved that the temperature play an important role in pDNA affinity chromatography with amino acids ligands. In the histidine matrix, the temperature increase only affected the nucleic acids molecules conformation. Some studies described that the temperature increase induces plasmids torsional strain changes leading to relaxation and consequent decrease in the interaction of sc pDNA with the histidine ligands. However, the study of single-strand molecules revealed that the increase in temperature induced a greater exposure of hydrophobic contact area of these molecules leading to an increase in their retention [15]. On the other hand, in arginine chromatography, the pDNA molecules retention is directly influenced by temperature, suggesting that hydrophobic interactions may be involved [10,16]. In the study of separation of ribonucleic acids on lysine–agarose, the decrease of temperature promotes a greater retention of the tRNA species to the column [14]. Therefore, several chromatographic analysis were triggered to demonstrate how temperature influences the retention of pDNA isoforms on the lysine support. A linear gradient (3 column volumes) between 160 and 250 mM NaCl Tris buffer (pH 8.0) was used, and to stabilize the required temperature (5, 12, 15 and 24 °C) for each experiment, a circular water bath was connected to the column. Fig. 2 represents the elution profiles obtained when loading a pDNA sample containing oc and sc pDNA isoforms into the lysine matrix. It was evidenced that, while the temperature of the column decreases, also the sc isoform retention decreases, being partially eluted in the first peak together with the oc isoform. This tendency can be monitored by the different height of peaks and

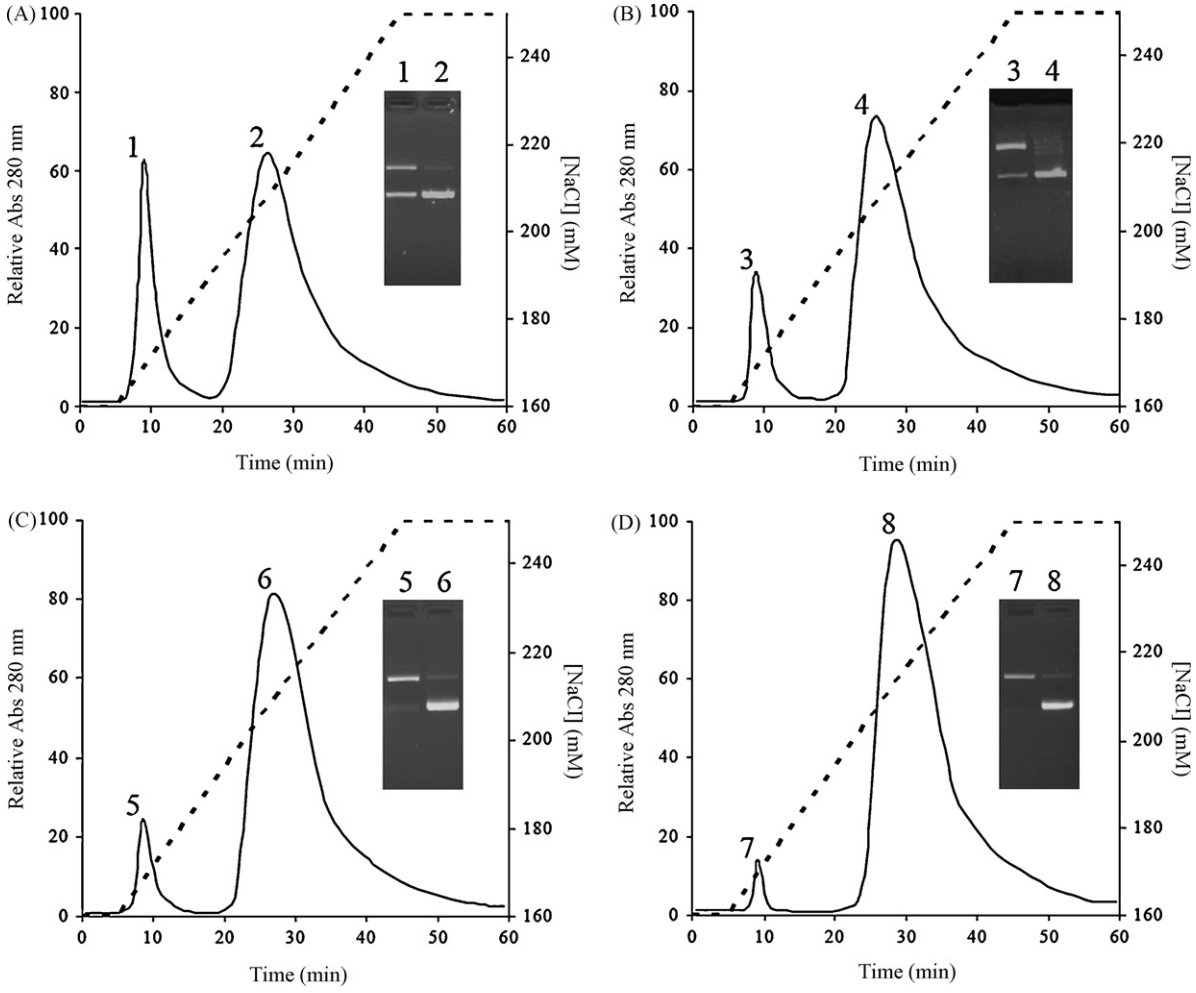

Fig. 2. Affinity chromatographic profiles of oc and sc pDNA isoforms retention on lysine–agarose support, at different temperatures: (A) 5 °C; (B) 12 °C; (C) 15 °C and (D) 24 °C. Elution was performed at 1 ml/min by linearly increasing the NaCl concentration in the eluent from 160 to 250 mM (3 column volumes). Agarose gel electrophoresis analysis of each peak is represented in each chromatogram.

by electrophoresis gel presented in each chromatogram (Fig. 2). The fact of the retention of sc isoform increased with increasing temperature may be related to the presence of hydrophobic interactions with the aliphatic portion of side chain or the 12-carbon epoxy spacer arm of lysine ligand [7].

Curiously, in this work it is visible that the first peak of each chromatogram was eluted approximately at same retention time, as well as happens with the second peak. However, in arginine study [10], the two peaks tend to approach and the two isoforms are more retained due to the temperature increase. These different behaviours suggest that, in case of lysine chromatography, the temperature increase allows stronger sc isoform retention, and this interaction at low temperatures is not so favoured. Thus, it is evident that temperatures above 12 °C promote a significant effect in sc isoform distribution, increasing the specificity of the retention and favouring the total plasmid isoforms separation. As previously demonstrated by the competitive elution studies, the hydrophobic interactions are not the preferential forces involved in sc plasmid retention, and as it is known a temperature increase significantly weaken nonspecific interactions like hydrogen bonds. Hence, the behaviour found with the temperature increase suggests that the ionic interaction between lysine and pDNA is strengthened favouring the recognition of sc isoform.

In general, some positively charged amino acids were already used as affinity ligands to efficiently separate sc and oc pDNA isoforms, but each matrix can specifically recognize and differently interact with the sc isoform. In the case of histidine-based affinity matrix it was achieved the possibility to separate the pDNA isoforms using a high ammonium sulphate concentration. This fact can be considered a disadvantage indicating the need for further improvements. It also verified that the temperature had only a direct influence on molecules conformational structure [15]. Otherwise, the arginine matrix revealed an extreme sensitivity of the binding/elution behaviour of the biomolecules with respect to the ionic strength of the buffers used. The type of interactions established between the arginine ligand and the nucleic acid molecules were different. Thus, the molecules retention was performed at low sodium chloride concentrations and their elution was obtained with a slight increase of salt concentration [10]. In the arginine support studies, the temperature effect was more evident on the retention [16]. In the present study, plasmid isoforms were also separated using mild salt conditions and it is suspected the involvement of different interactions. As it was previously described, in lysine chromatography, the temperature increase developed an important role allowing a specific interaction with the sc isoform. Interestingly, unlike arginine, the retention time of oc and sc isoforms in lysine matrix was not changed with the temperature increase, but an increased specificity was observed favouring the total plasmid isoforms separation.

For the first time, it is presented a new affinity chromatographic process based on naturally occurring interactions, between the lysine amino acid and plasmid molecules, promoting an efficient separation of sc and oc isoforms. We suggest that the underlying mechanism involves phenomenological interactions like biorecognition between the lysine matrix and pDNA isoforms, including hydrogen, electrostatic, van der Waals and hydrophobic interactions. Lysine-affinity chromatography can be another potential purification technique to obtain the sc pDNA directly from a clarified *E. coli* lysate in the required conditions for therapeutic applications, since in this work it was proved that this matrix recognize specifically the sc isoform. The use of amino acid matrices and mild salt conditions become this technique more appropriate and economic to apply at biotechnological process and industrial scale.

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Paper V

Impact of lysine-affinity chromatography on supercoiled plasmid DNA purification

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Impact of lysine-affinity chromatography on supercoiled plasmid DNA purification

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ABSTRACT

Gene therapy and DNA vaccination cover a variety of applications using viral and non-viral vectors as vehicles of choice for treatment of genetic or acquired diseases. Recently, most therapeutic applications have been performed with non-viral biological agents preparations highly enriched in supercoiled plasmid molecules and it has been concluded that this isoform is more efficient at gene transfection than open circular isoform. This work describes for the first time a new strategy that uses lysine-chromatography to efficiently eliminate *Escherichia coli* impurities as well as other ineffective plasmid isoforms present in a complex clarified lysate to purify and obtain pharmaceutical-grade supercoiled plasmid DNA. The quality control tests indicated that the levels of impurities in the final plasmid product were below the generally accepted specifications. Furthermore, the delivery of the purified product to eukaryotic cells, the cell uptake and transfection efficiency were also analyzed. The results showed that the transfection efficiency reached with the application of the supercoiled plasmid conformation, purified with lysine-agarose, was higher than the values achieved for other plasmid topologies. Therefore, this study presents a new enabling technology to obtain the completely purified non-viral vector, able to act with good efficiency as gene therapy delivery vehicle in several diseases like cancer.

1. Introduction

In the last decades, a technological advance in medical and pharmaceutical areas allowed the accomplishment of several human clinical trials, testing the ability to inject plasmids providing therapeutic benefits in the untreatable pathologies [1–4]. Effective DNA vaccination and gene therapy require the control of both the location and function of therapeutic genes at specific target sites within the patient's body [5]. The main challenge in the development of molecular medicine is to introduce naked DNA into diseased cells overcoming the extra- and intracellular cell barriers. Most gene administration methods are based on viral vectors [6] but this delivery system has raised safety and regulatory concerns because of their toxicity and immunogenicity [7]. Thus, the non-viral vectors arise as a good alternative, overcoming the problems associated to the viral vector-mediated therapy [6], becoming the most attractive gene-transfer system to be used as biopharmaceutical product [5,8,9]. Therefore, it is important to continue the development of non-viral vectors, as plasmid DNA (pDNA), for efficient transfection and gene expression.

Almost all processes, for the manufacture of pDNA in sufficient quantities to clinical applications, needed to improve the productivity [10], mainly in the following operations: fermentation, lysis, isolation, purification and transfection (Fig. 1). Plasmids are mostly biosynthesized by *Escherichia coli* (*E. coli*) fermentation, intending to maintain high plasmid copy number per bacterium [1]. After the bacterial cell harvest, normally by centrifugation or microfiltration techniques, it is performed the cell lysis, which is considered the critical operation of the pDNA manufacturing process [11]. This step can affect the ratio of supercoiled (sc) plasmid to other forms (Fig. 1) and the plasmid amount, as well as the physicochemical characteristics of cellular impurities that must be removed during the downstream process. The isolation of crude plasmid can be done by precipitation with salt in order to reduce the presence of RNA and other host impurities [12], like proteins and endotoxins. The final step to obtain pDNA as a highly pure product for therapeutic applications is the plasmid purification, through of liquid chromatography [13].

Several common approaches exploit one or more of the following purification processes: size exclusion, anion exchange, hydrophobic interaction and affinity chromatography [14]. Nevertheless, the affinity concept is gaining impact with the development of new supports with specific ligands that improve the binding capacity for pDNA [15] and combine different interactions promoting a biorecognition of sc conformation [13].

Supercoiled pDNA, due to its structure extremely compact and functional, is considered the most efficient isoform at inducing gene expression comparing with other conformational variants

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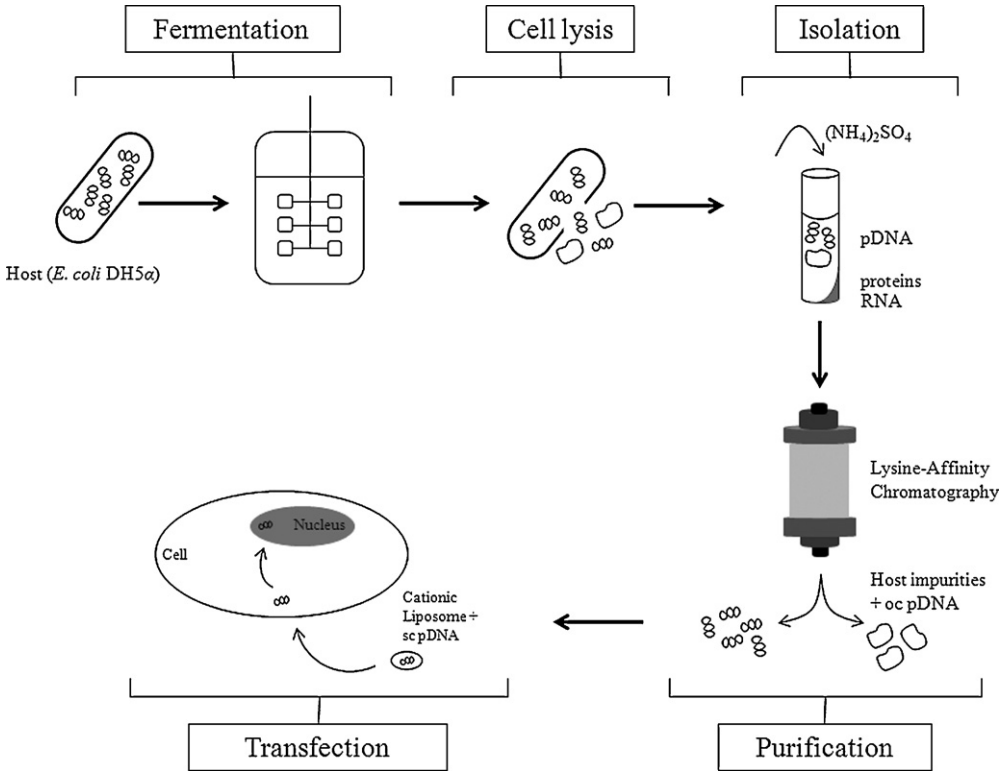

Fig. 1. Schematic representation of downstream processing of sc pDNA preparation, according to quantity and purity degree required for clinical applications, using lysine-affinity chromatography as a major purification step. *E. coli*, *Escherichia coli*; $(NH_4)_2SO_4$, ammonium sulfate; oc, open circular; sc pDNA, supercoiled plasmid DNA.

[16] (open circular (oc) and linear isoforms, obtained from the damage of the sc form) [1,17]. According to regulatory agencies, such as Food and Drug Administration (FDA), a content of sc form higher than 97% is required to apply in gene therapy and DNA vaccination [10]. This sc plasmid isoform should be relatively free from impurities, such as bacterial genomic DNA (gDNA) (<2 ng/μg of pDNA), endotoxins (<0.1 EU/μg of pDNA), RNA and host proteins (undetectable) [11]. Knowing that the total amount of pDNA present in the *E. coli* extract represents less than 3% of the global content, it becomes essential developing adequate purification processes to isolate the sc isoform, as it is represented in Fig. 1.

The selection of affinity matrices with amino acids ligands was mainly supported by the natural occurrence of protein–DNA complexes in biological systems [18] and because some atomic evidences suggested the existence of favored interactions between particular amino acids and nucleic acids bases [19–21]. Recently, several amino acids, such as histidine [22,23] and arginine [24,25] have been tested as affinity ligands in agarose chromatographic supports to specifically purify sc pDNA from a clarified *E. coli* lysate. A new agarose support with lysine ligand was first experimented with a pre-purified native (oc + sc) pDNA sample and the separation of both plasmid isoforms was achieved [26]. With this study, it was also possible to understand the interaction mechanism underlying the specificity of the support that allowed a biorecognition of the sc isoform. The applicability of this matrix to efficiently purify and isolate this isoform from the other plasmid topologies and *E. coli* host impurities in a single chromatographic step, considering the requirements of the regulatory agencies, is evaluated, for the first time, in the present study. These findings will strengthen the possibility of using lysine-affinity chromatography as a potential enabling technology in the downstream process of sc pDNA for therapeutic applications.

2. Materials and methods

2.1. Materials

Lysine-Sepharose 4B gel was obtained from GE Healthcare Biosciences (Uppsala, Sweden). Sodium chloride and ammonium sulfate were purchased from Panreac (Barcelona, Spain) and tris(hydroxymethyl) aminomethane (Tris) from Merck (Darmstadt, Germany). All solutions used in chromatographic experiments were freshly prepared using deionized water ultra-pure grade, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and analytical grade reagents. The elution buffers were filtered through a 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. The iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used for genomic DNA quantification. Unless otherwise stated, the reagents used for Hela and COS-7 culture were obtained from Sigma (St. Louis, MO, USA) and the reagents for the transfection experiments were obtained from Invitrogen (Carlsbad, CA, USA). The 6.05-kbp plasmid pVAX1-*LacZ* (Invitrogen, Carlsband, CA, USA), designed for the development of DNA vaccines, was used as a model plasmid. This vector contains the Human CytoMegalovirus (CMV) immediately early promoter/enhancer, the Bovine Growth Hormone (BGH) polyadenylation signal, a T7 promoter/printing site, a multiple cloning site, a kanamycin resistance gene, a pUC origin and a reporter (β-galactosidase) gene used as a positive control for transfection and expression in the cell line of choice. The host strain used to obtain the several nucleic acids was *E. coli* DH5α.

2.2. Plasmid production

The pVAX1-*LacZ* amplification was obtained by *E. coli* DH5α fermentation as described by Sousa *et al.* 2010 [27], carried out at 37 °C using the Terrific Broth medium (20 g/L tryptone, 24 g/L yeast

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extract, 4 mL/L glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 30 μg kanamycin/mL. The cells were harvested at the late log phase by centrifugation and the pellets were stored at −20 °C. Plasmid-free *E. coli* DH5α cells were also grown under the same conditions as described before, but with no antibiotic present.

2.3. Alkaline lysis and clarification of E. coli lysate

Cells were lysed through a modification on the alkaline method [28] as described by Diogo et al. [29]. Bacterial pellets (obtained from centrifugation of 250 mL cell broth at 5445 × *g* (30 min, 4 °C)) were thawed and dissolved in 20 mL resuspension buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM ethylene-diamine tetraacetic acid (EDTA), pH 8.0). Alkaline lysis was performed by adding 20 mL of a 200 mM NaOH, 1% (w/v) sodium dodecylsulfate (SDS) solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated by adding and mixing 16 mL of prechilled 3 M potassium acetate, pH 5.0 (20 min on ice). The precipitate was removed by centrifuging twice at 20,000 × *g* (30 min, 4 °C) with a Sigma 3-18K centrifuge. The concentration and reduction of impurity content was performed according to a previously published method [29], since the main objective of this work is to explore the purification strategy. In a short description, the pDNA in the supernatant was precipitated by adding 0.7 volumes of isopropanol and incubating on ice for 30 min. The pDNA was recovered by centrifugation at 16,000 × *g* (30 min, 4 °C). The pellets were then redissolved in 2 mL of 10 mM Tris-HCl buffer, pH 8.0. Next, ammonium sulfate was dissolved in the pDNA solution up to a final concentration of 2.5 M, followed by a 15 min incubation on ice. Precipitated proteins and RNA were removed by centrifugation at 16,000 × *g* (20 min, 4 °C). The supernatant was desalted by passing through PD-10 desalting columns (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions, using 10 mM Tris-HCl buffer (pH 8.0) as the elution buffer.

2.4. Isolation of E. coli nucleic acids

Nucleic acids (gDNA and RNA) were isolated from pDNA-free DH5α *E. coli* cells. gDNA was isolated using the Wizard genomic DNA purification kit from Promega (Madison, WI, USA), according to the manufacturer's instructions. For RNA isolation, the cells were lysed by a modification of the alkaline method [28], the resultant lysate was clarified by ammonium acetate precipitation and nucleic acids were concentrated by polyethylene glycol 6000 precipitation as previously described [12].

2.5. Affinity chromatography

Chromatography was performed in a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Uppsala, Sweden). A 16 mm diameter × 100 mm long (≈20 mL) column was individually packed with the commercial lysine-agarose gel. The chromatographic runs were performed at 5 °C, by using a specific column containing a water jacket tube connected to a circulating water bath to maintain the suitable temperature. The column was first equilibrated with 29% of buffer B (250 mM NaCl in 10 mM Tris-HCl buffer, pH 8.0) and the remaining 71% corresponds to buffer A (10 mM Tris-HCl buffer, pH 8.0) at a flow rate of 1 mL/min. After elution of unbound species the ionic strength of the buffer was increased stepwise to 37% of buffer B and then to 100% of the same buffer. The clarified lysate obtained after desalinization was loaded onto the column using a 200 μL loop at the same flow rate. In all separations and throughout the entire chromatographic run the optical density was monitored at 280 nm, while 2 mL fractions were pooled according to the chromatograms obtained, and used for further electrophoresis analysis. For the evaluation of the matrix efficiency on multiple isolations of the sc pDNA isoform and to assess the final content of impurities (proteins, gDNA and endotoxins) present in the sc pDNA purified product, they were used 5 different chromatographic runs. After chromatographic runs, it was necessary to wash the lysine matrix with at least 5 bed volumes of 2 M NaCl in 10 mM Tris-HCl buffer, pH 8.0.

2.6. Agarose gel electrophoresis

Pooled fractions were analyzed by horizontal electrophoresis using 15 cm-long 1% agarose gels (Hoefer, San Francisco, CA, USA), stained with ethidium bromide (0.5 μg/mL). Electrophoresis was carried out at 100 V, for 40 min, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). The gel was visualized in a Vilber Lourmat system (ILC Lda, Lisbon, Portugal). In order to confirm the homogeneity of isoforms recovered from the first and second chromatographic peaks, the band density of each sample pooled and analyzed by electrophoresis was evaluated through of Bio-Rad Quantity One® software (Hercules, CA, USA) using the band analysis toolbox.

2.7. Plasmid quantification

The pDNA concentration and purity was evaluated after each separation process, in the fractions pooled from lysine-chromatography, according to an adaptation of the analytical method previously described [30]. Briefly, the HPLC method based on hydrophobic interaction chromatography was developed for the assessment of pDNA concentration and purity in crude *E. coli* lysate or other extracts. The Phenyl Sepharose Source column (Amersham Biosciences) was used to separate the double-stranded pDNA molecules that elute in the flow through, from the more hydrophobic impurities that are delayed (gDNA and proteins) or retained (RNA and proteins) in the analytical column [30]. Therefore, the 4.6/100 mm HIC Source 15 PHE PE column (Amersham Biosciences) was connected to an ÄKTA Purifier system (GE Healthcare Biosciences). Initially, the column was equilibrated with 1.5 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer, pH 8.0. Afterward, 20 μL of a sample suitably diluted in the equilibration buffer were injected and eluted at a flow rate of 1 mL/min. After the injection of the sample, all unbound material was eluted with 1.5 M $(NH_4)_2SO_4$ in 10 mM Tris-HCl buffer, pH 8.0, for 4 min, and then the elution buffer was instantaneously changed to 10 mM Tris-HCl buffer pH 8.0 without ammonium sulfate. The last elution condition was maintained for 6 min to elute bound species. Next, the column was re-equilibrated for 10 min with the equilibration buffer to prepare the column for the next run. The absorbance of the eluate was recorded at 260 nm. The concentration of pDNA in each sample was calculated using a calibration curve constructed with pDNA standards (1–400 μg/mL) purified with a commercial Qiagen kit (Hilden, Germany). The purity degree was defined as the percentage of the pDNA peak area related with the total area (area of all peaks on the chromatogram). Isolated gDNA and RNA samples were also injected, in order to observe the retention pattern of these nucleic acids in the analytical column.

2.8. Protein quantification

Total protein was quantified using the micro-BCA (bicinchoninic acid) protein assay kit form Pierce (Rockford, USA), according to the manufacturer's instructions. A calibration curve was constructed with Bovine Serum Albumin (BSA) as the standard protein (0.01–0.1 mg/mL), using 10 mM Tris-HCl pH 8.0 to dilute the samples. Samples with high salt concentration were desalted against 10 mM Tris-HCl pH 8.0 before analysis.

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2.9. Genomic DNA quantification

Genomic DNA was quantified through of real-time polymerase chain reaction (PCR) in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad), as described previously [31]. Sample collected after lysis was diluted 100 fold beforehand. Sense (5′-ACACGGTCCAGAACTCCTACG-3′) and antisense (5′-CCGGTGCTTCTTCTGCGGGTAACGTCA-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 I. The gDNA concentration was determined using a calibration curve generated by serial dilution of *E. coli* DH5α gDNA (purified with the Wizard gDNA purification kit; Promega) in the 5 pg to 50 ng/μL range. Negative controls (no template) were run at the same time of each analysis.

2.10. Endotoxin quantification

Endotoxin contamination was assessed using the ToxinSensor™ Chromogenic *Limulus* Amoebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript, USA, Inc.) according to the manufacturer's instructions. The calibration curve (from 0.005 to 0.1 EU/mL) was performed from a 10 EU/mL stock solution provided with the kit. To avoid the external endotoxins interference, samples to analyze and samples from the kit were diluted, or dissolved respectively, with non-pyrogenic water, which was also used as the blank. All tubes and tips used to perform this quantification were endotoxins-free.

2.11. Cell culture

Hela and COS-7 cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) at pH 7.4 containing 100 units/mL antibiotic-antimycotic (penicillin/streptomycin), supplemented with 10% Fetal Bovine Serum (FBS) (Biochrom AG, Berlin, Germany), at 37 °C in a 5% CO_2 atmosphere incubator.

2.12. FITC fluorescent labeling of pDNA

The preparation of fluorescently labeled pDNA was performed according to the method previously described [32] with some modifications. Briefly FITC (10 mg) was activated overnight with 2-(4-aminophenyl)-ethylamine in 150 μL of dimethyl-formamide (DMF) under stirring, at room temperature (≈24 °C). Afterwards, FITC-aniline conjugate was reacted with $NaNO_2$ (110 μmol) in 0.5 M HCl at 0 °C under stirring, for 5 min. The reaction was stopped by adding 100 μL of 1 M NaOH solution. FITC-diazonium salt was further mixed with the pVAX1-*LacZ* plasmid samples in Tris-HCl buffer (pH 8.0) at room temperature (≈24 °C), under stirring, for 25 min. The FITC-labeled plasmid was recovered by centrifugation at 16,000 × *g* after precipitation using 0.7 volumes of ice cold isopropanol.

2.13. In vitro transfection

To perform *in vitro* transfection experiments for fluorescence assay, Hela cells were seeded on a 24-well plate at a density of 2.5×10^4 cells/well (with a lamella per well coated with poly-D-lysine) and incubated during 20 h. Immediately before transfection, cells were rinsed and supplemented with 500 μL of fresh culture medium without antibiotic at pH 7.4, whereas FITC-pDNA/polycation complexes were prepared as briefly described below. FITC-labeled pDNA solutions and appropriate volumes of Lipofectamine™ 2000 were separately diluted in 50 μL (for each transfection assay) of Opti-MEM® I medium, according to the manufacturer's instructions. After a 5-min incubation of lipofectamine at room temperature (≈24 °C), the respective FITC-labeled pDNA solution was added and the mixture was incubated for 20 min at room temperature (≈24 °C) to allow the formulation of FITC-pDNA/lipofectamine complexes. A fraction of the mixture (100 μL) was then added to each well containing cells and medium. The cells were incubated at 37 °C under a 5% CO_2 atmosphere. After 6 h of incubation, the medium and complexes were removed, and cells were rinsed once with PBS and cultured with antibiotic and 10% FBS supplemented medium (DMEM).

2.14. Fluorescence

The fluorescence experiments were performed after Hela cells *in vitro* transfection. Following the change of the medium, transfected cells were fixed with 4% paraformaldehyde in PBS and incubated for 20 min. The cells were then permeabilized with 1% Triton X-100 and blocked with 10% FBS and 0.1% Tween 20 in PBS for 3 h. Subsequently the cells were washed 10 times with PBST. Hoechst 33342 dye (2 μM) (catalogue number: H1399; Molecular Probes, Invitrogen, Carlsbad, CA, USA) was then added and incubation proceeded for 15 min at room temperature (≈24 °C), followed by 10 washing steps with PBST. Afterwards the samples were then mounted with DAKO mounting medium and the fluorescence was visualized by confocal microscopy in a Zeiss LSM 510 Meta system (Zeiss Imaging/Systems), using a ×63 objective with an image zoom scan of 1.0.

2.15. Transfection efficiency quantification

The transfection efficiency was determined after 24 h of transfection by using the β-Gal Staining Kit (Invitrogen). In this case, the transfection experiments were performed as previously described, but with COS-7 cell line and without conjugation of FITC with pDNA/lipofectamine complexes. This method enables the determination of the efficiency of cell transfection with pVAX1-*LacZ*, because the *lacZ* gene product, β-galactosidase, catalyses the hydrolysis of β-galactosides such as X-gal, producing a blue color that can be visualized under the microscope using a ×10 objective with a image zoom scan of 1.0. The transfection efficiency is determined as the percentage of blue cells compared to the total cell number (5 randomly acquired fields in each well).

3. Results

3.1. Lysine affinity chromatography

In the present work, it is exploited that the applicability of lysine-affinity chromatography in the pDNA downstream process, to isolate sc plasmid conformation from a complex *E. coli* lysate. Therefore, several retention/elution experiments were performed in order to obtain different elution patterns for gDNA, pDNA isoforms and RNA present in the clarified *E. coli* lysate. In a first step, lysine-agarose column was equilibrated with 29% of buffer B (constituted by 250 mM NaCl in 10 mM Tris-HCl buffer, pH 8.0), while the remaining 71% corresponds to buffer A (10 mM Tris-HCl buffer, pH 8.0) at 5 °C. After injection of the pDNA-containing clarified lysate (200 μL) using an isocratic elution mode of 1 mL/min, it was obtained a first peak resultant from the elution of unbound material (Fig. 2A). The ionic strength of buffer B was then increased to 37% for 20 min in order to elute specific molecules in a second peak. The chromatographic run was concluded with a final elution step, using 100% of buffer B for 30 min, to recover the strongly bound species.

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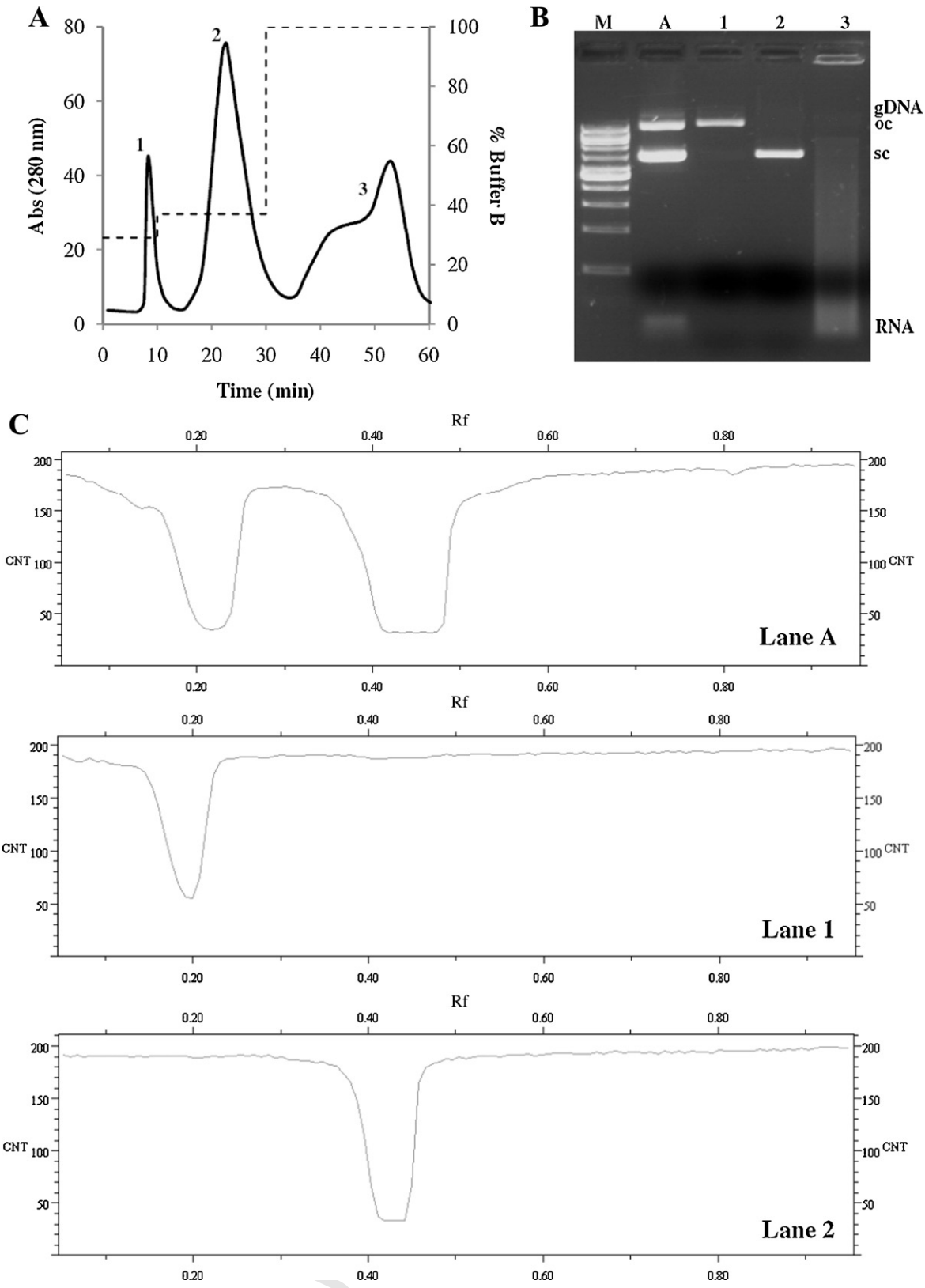

Fig. 2. (A) Chromatographic purification of sc plasmid isoform from a clarified *E. coli* lysate using lysine-affinity chromatography. Mobile phases – buffer A: 10 mM Tris-HCl buffer pH 8.0; buffer B: 250 mM NaCl in 10 mM Tris-HCl buffer pH 8.0. Elution was performed at 1 mL/min by stepwise gradient increasing the buffer B percentage in the eluent from 29% to 37% of buffer B, and finally to 100% of buffer B, as represented by the dashed line. (B) Agarose gel electrophoresis analysis of the samples recovered from each peak of the chromatogram. Lane M: molecular weight marker; Lane A: feed sample injected onto the column. (C) Lane densities of particular samples analyzed by electrophoresis.

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With this study it was also verified that the chromatographic performance was dependent of a suitable conductivity control. Therefore, the salt concentration and environmental temperature should be monitored to attain the ideal plasmid separation conditions.

The presence of several peaks in the chromatogram (Fig. 2A) suggests a differential recognition of the plasmid isoforms and host biomolecules by the lysine matrix. The fractions pooled on each peak were analyzed by agarose electrophoresis. Fig. 2B confirms that the first peak mostly corresponds to the oc isoform that did not interact with the support (lane 1, peak 1), while sc isoform remained bound under the same conditions, being identified in the second peak (lane 2). The electrophoretic evaluation of the fractions recovered from the last peak revealed the presence of RNA (lane 3), by comparing with the bands of lane A (feed sample injected onto the column). The oc and sc isoforms homogeneity, analyzed on lanes 1 and 2 of electrophoresis, was also confirmed by the determination of the lanes density presented in Fig. 2C.

3.2. Analytic parameters quantification

The electrophoresis of the fractions pooled from the chromatographic experiment indicated that the sc pDNA eluted in the second peak is 100% pure, with no detection of RNA. However, to certify if the lysine-agarose chromatography can be applied in a single step to isolate sc pDNA from host biomolecules and other plasmid isoforms for therapeutic applications, further characterization of quality parameters is required.

3.2.1. Plasmid concentration and purity degree

The determination of pDNA concentration and the evaluation of residual impurities present in sc plasmid sample isolated by lysine preparative chromatography aforementioned, was performed according to an adaptation of the analytical method previously published [30], as briefly described in Section 2.7. Fig. 3 presents the analytical chromatographic profiles of several samples recovered throughout the manufacturing process of sc pDNA, as well as of the isolated gDNA and RNA samples to facilitate the identification of the impurities peaks. The analysis of the *E. coli* lysate sample showed a large quantity and diversity of biomolecules, as revealed by the multi-peak chromatogram (Fig. 3A). Knowing that the first peak eluting at 0.7 min corresponds to pDNA molecule [30], and by comparison with retention time of isolated nucleic acids (Fig. 3B and C), it was determined that the second and fourth peaks are due to the presence of gDNA and RNA respectively, while the remaining peaks (third and fifth) are other impurities. The isopropanol and ammonium sulfate precipitations, performed to clarify the lysate sample, contribute significantly to reduce the impurity content (Fig. 3D). When sc pDNA sample from second peak (Fig. 2A) of lysine-agarose chromatography was analyzed (Fig. 3E), only a single plasmid peak appeared in the chromatogram. The interference present after the elution of sc pDNA is due to the change of elution buffer as it is shown in Fig. 3F representing the negative control with the injection of binding buffer.

Table 1 presents the values of concentration and purity of pDNA, as well as the purification factor and yield achieved throughout the several steps of the process. The sc pDNA concentration obtained in the second peak of lysine chromatography was 7.34 ± 0.17 μg/mL, whereas the purity estimated from the relative peak area was 100%, what is in agreement with previous electrophoretic analysis and support the theory that the lysine matrix can be used as a final purification step. The total recovery of native plasmid (oc + sc) accomplished in the purification step corresponds to 55% yield, where 46% corresponds to the sc isoform recovery (Table 1). The relatively low yield can be related with the composition of the

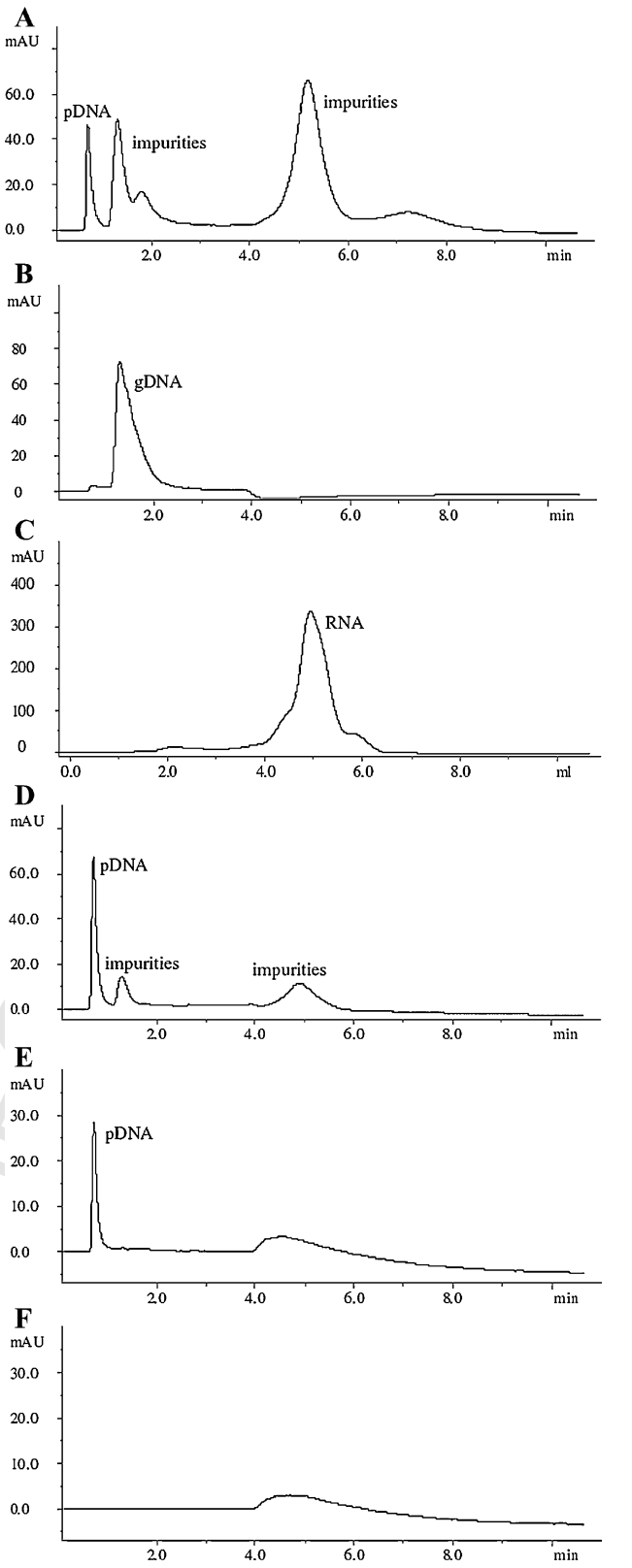

Fig. 3. Analytical chromatographic profiles of several nucleic acids and samples recovered throughout the purification process. (A) *E. coli* lysate; (B) genomic DNA; (C) RNA; (D) feed sample injected onto the column; (E) sc isoform fraction (peak 2); and (F) zero (injection of binding buffer).

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Table 1
Analysis of purity and recovery yield of the sc pDNA isolated from *E. coli lysate* through of lysine-affinity chromatography.

Process step	Volume (mL)	pDNA		Purity (%)	Purification factor	Process yield (%)
		(μg/mL ± SD, $n = 5$)	(μg)			
Primary isolation						
Alkaline lysis	15	12.91 ± 0.43	193.58	6.76	–	–
$(NH_4)_2SO_4$ precipitation	10	18.07 ± 0.65	180.69	37.50	5.55	93.30
Lysine chromatography						
Peak 1 (oc)	4	4.49 ± 0.15	18.00	80.30	11.88	9.30
Peak 2 (sc)	12	7.34 ± 0.17	88.10	100	14.79	45.50

impure extract. Most of critical impurities share common characteristics of negative charge (gDNA, RNA and endotoxins), molecular mass (gDNA and endotoxins) and hydrophobicity (endotoxins) with pDNA [10]. Therefore, the clarification and purification are the two main steps that can affect the yield of sc pDNA manufacturing. The clarification step involves high ammonium sulfate concentration to promote the salting out phenomenon, favoring the precipitation of molecules containing hydrophobic areas. Thus, proteins, endotoxins, RNA and denatured gDNA are eliminated in this step, but a small amount of sc pDNA can also be precipitated. Furthermore, the characteristics of the contaminants significantly constrain the purification of target molecule, since the affinity ligands can also bind other biomolecules [33], leading to a decrease on the purification process yield. As it is shown in Fig. 2A, lysine matrix mainly interacts with the nucleic acids presenting higher bases exposition, such as RNA and sc pDNA. By this reason, RNA can be considered a key competitor for sc pDNA purification from *E. coli* lysate with lysine chromatography, decreasing the binding capacity of this support, or even the global yield of the sc plasmid recovery.

Nevertheless, the plasmid yield of 55% resultant in this work can be considered a reasonable value when compared with similar purification strategies. Previous studies of plasmids purification based on affinity chromatography strategies, such as triple-helix affinity chromatography [34] or histidine-affinity chromatography [23] achieved 42% and 45% of yield, respectively.

3.2.2. Proteins, gDNA and endotoxins contamination

The proteins were not detected on the purified oc and sc plasmid pools, by using the micro-BCA assay, while the RNA fraction had a residual concentration of 10.8 ± 0.54 μg/mL and the sample resulting from ammonium sulfate precipitation had, 12 ± 0.32 μg/mL. The analysis of gDNA contamination was performed by a suitable technique of real-time PCR. The results suggest a significant reduction on gDNA content throughout the clarification process and also in the lysine purification step, because the initial sample had 272 ± 1.35 ng gDNA/μg pDNA and the sc plasmid fraction had 2 ± 0.02 ng gDNA/μg pDNA (136-fold reduction). The methodology used to purify the sc pDNA was also efficient in removing endotoxins, which decreased from 9.5 ± 0.55 EU/μg of pDNA to 0.1 ± 0.01 EU/μg of pDNA (a 99% reduction).

These results were satisfactory considering the specifications recommended by the regulatory agencies [10,11]. However, it is known that the presence of these impurities in sc pDNA sample are undesirable due to their toxicity for the cells transfected with this sample [14]. Bearing this in mind, the transfection efficiency evaluation is required to investigate the biological effects of residual impurities, such as RNA, gDNA, proteins and endotoxins, in sc plasmid purified by this new lysine-affinity chromatographic strategy. In addition, the transfection level obtained with the plasmid sample purified with this matrix should be compared with the transfection level obtained in previous works using other chromatographic techniques, to analyze the efficiency of this new affinity strategy in the removal of host impurities.

3.3. Transfection experiments

3.3.1. Cellular localization of plasmid/lipofectamine complexes

The mechanism of cell uptake and localization of plasmid/lipofectamine complexes was observed with a confocal laser scanning microscope using FITC-pDNA and Hela cell line as it was established and optimized in previous work [32]. After the *in vitro* transfection experiments and fluorescence procedure, it was ascertained that the FITC-pDNA/lipofectamine complexes were condensed to form aggregates, which cross the cell membrane and can internalize into the cell. As it is observed in Fig. 4A, the FITC-pDNA/lipofectamine complexes correspond to the green areas that are near the nucleus blue-stained with Hoechst. Fig. 4B confirms that the green aggregates of stained plasmid are located inside the cell, particularly in the cytoplasm.

3.3.2. Transfection efficiency

To improve the characterization of sc pDNA sample resultant from *E. coli* lysate purification with lysine-affinity chromatography, the transfection efficiency experiments of isolated plasmid isoforms were performed and compared with the same plasmid purified with a commercial kit based on anion-exchange chromatography (Fig. 5). The COS-7 cell line is normally used for this procedure. Earlier studies have reported that some factors can lead to lower transfection efficiency, such as stoichiometry of pDNA/lipofectamine complex and serum concentration/pH of the transfection medium [35], as well as the type of cell line used [36]. However, in our study, the transfection efficiency (reflected in the expression level of β-galactosidase) obtained with sc plasmid fraction (43.96% ± 1.29) was practically three times higher than the obtained with the oc isoform fraction (17.06% ± 2.25), and it was also significantly higher when compared with the efficiency (22.12% ± 2.88) achieved with pDNA control sample (kit), as it is summarized in Table 2.

4. Discussion

The applicability of lysine-affinity matrix to purify the biological active sc pDNA from an impure *E. coli* lysate extract was evaluated. The characterization techniques allow the confirmation of the purity and activity of this plasmid. In the results of Fig. 2A and B, the separation of nucleic acids constituents from *E. coli* clarified lysate and the differential recognition by the lysine ligands is a consequence of several interactions. Particularly, electrostatic interactions can occur between the nucleic acids phosphate groups

Table 2
Summary of transfection efficiency achieved through of *in vitro* experiments using COS-7 cell line and applying different plasmid samples, with the same concentration (143 μg/mL).

Plasmid DNA	Transfection efficiency (% ± SD, $n = 3$)
Control (kit)	22.12 ± 2.88
oc	17.06 ± 2.25
sc	43.96 ± 1.29

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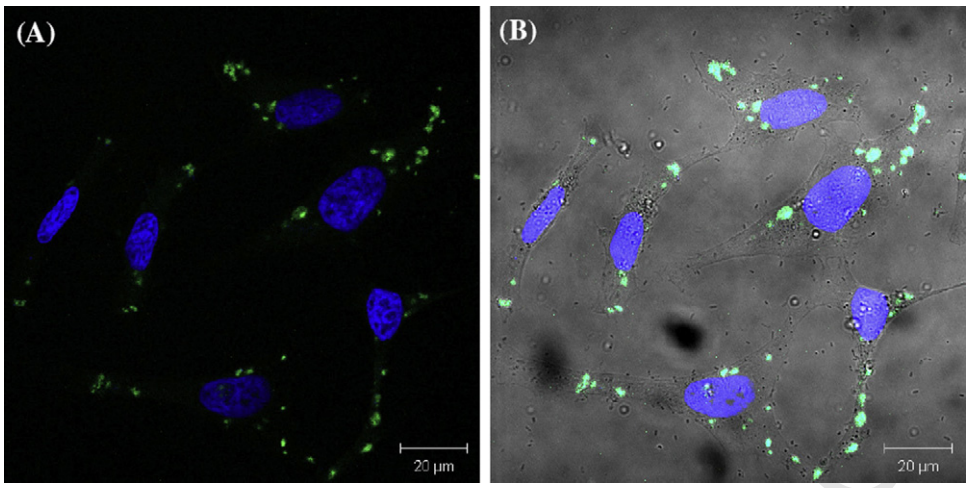

Fig. 4. Confocal microscopy of cellular localization of FITC-pDNA/lipofectamine complexes inside Hela cells (630×). (A) Co-localization of FITC-pDNA corresponds to the green areas, and the nuclei of cells were stained with Hoechst 33342 dye (blue) (image zoom scan, 1.0×). (B) The same image with phase contrast to observe the cellular delimitation.

and the lysine amino acid positively charged. Likewise, the lysine amino acid has some characteristics, such as the lateral side chain with significant length and capacity to interact in different conformations producing good hydrogen bond geometries, which reinforce the specific recognition mechanism. Molecular modeling studies evidenced that in protein–DNA interactions lysine promotes complex hydrogen-bonds preferentially with guanine base edges [21]. This positive amino acid performs an analogous interaction to the end-on conformation by placing N atom between more than one acceptor atom, being possible the same ligand to be acceptor and donor hydrogen atoms [20]. These multiple interactions can be responsible for the different retention of nucleic acids and lysine matrix, depending of the bases exposure degree. In this way, the oc plasmid isoform was first eluted because the bases are less exposed than in the sc isoform, which remain bound to lysine matrix. Indeed, the sc pDNA conformation results from the supercoiling phenomenon, which is a consequence of deformations induced by the torsional strain, leading to a more compact molecule with higher bases density than oc isoform [37]. The single-strand RNA molecule has the bases more exposed, being more retained than the other nucleic acids, in a similar way as obtained in our previous work [27].

Moreover, the fact that lysine amino acid can promote multiple interactions with the nucleic acids, also suggests that this support can be applied in the purification of different plasmids, even presenting different sizes, bases composition or exposure degree. In accordance to previous results [38], a slight adjustment of the binding and elution conditions will allow the target plasmid purification. In fact, this previous work shows that the retention on the histidine-agarose matrix is not directly affected by the plasmid size, because the smaller pUC19 (2.7 kbp) and the larger pVAX1-*LacZ*gag (7.4 kbp) plasmids were more retained than pVAX1-*LacZ* (6.05 kbp) [38]. The preferential retention of the pVAX1-*LacZ*gag to the histidine matrix was related with the 1.3 kbp adenine-enriched insert [38]. Taking into account that the lysine amino acid recognizes preferentially the guanine base [21], it will be expected an increased retention of plasmids with inserts mainly constituted by guanine.

In general, the differential interaction that was verified with both plasmid isoforms is in accordance with a previous study [26] that reported the biorecognition of pDNA isoforms in lysine-affinity chromatography. Fig. 2C confirms that the plasmid pool recovered in the peak 2 contains 100% of the sc isoform, without other nucleic acid impurities, in agreement with the recommendation of the regulatory agencies [10]. The temperature used on the chromatographic runs was 5 °C, because in a previous work [27], we have verified an increased RNA retention time using low temperature conditions what led to an improvement on separation between this impurity and sc pDNA. The analytical chromatograms in Fig. 3 clearly show a gradual reduction of impurities throughout the process until to obtain the sc plasmid isoform totally purified, confirming the result of agarose electrophoresis. Likewise, the content of residual proteins, gDNA and endotoxins present in sc plasmid sample is below to the level recommended by the regulatory agencies [10,11]. The global quantification of proteins

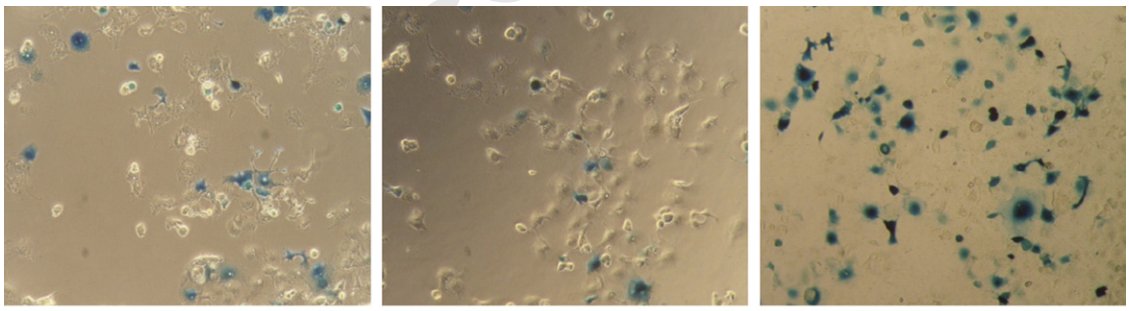

Fig. 5. Typical results of *in vitro* transfection experiments with COS-7 cells (100×) using different plasmid samples, with the same concentration (143 μg/mL). (A) Native pDNA (oc + sc) control sample obtained with a Qiagen kit; (B) oc plasmid and (C) sc plasmid obtained from chromatography with lysine-agarose support.

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and endotoxins revealed that the ammonium sulfate precipitation step was very efficient [39], and the residual amount of these impurities was further eliminated during the purification procedure. With relation to gDNA contaminant, a recent study reports that the presence of bacterial gDNA in a purified plasmid sample for therapeutic benefits causes significant necrosis of skeletal muscle cells [40]. By this way, the gDNA must be reduced to the lowest value as possible, in plasmid preparation for vaccines because of the risk of insertion [1]. In fact, the gDNA molecule was mostly eluted in the first peak together with oc isoform (Fig. 2A) because the bases exposure degree is lower than in sc pDNA or RNA molecules.

Similar to the study of Ishii and collaborators, about the mechanism of cell transfection with plasmid/chitosan complexes [35], in our work it was also verified that the preparation of fluorescently labeled pDNA with FITC was efficient, as well as the cell uptake and the beginning of accumulation near the nucleus (Fig. 4). As illustrated in Fig. 5 and confirmed by the values present in Table 2, the transfection efficiency obtained with sc plasmid topology was almost three times higher than the transfection rate achieved with oc isoform (both purified with lysine-affinity chromatography) and the double comparing with the native pDNA purified with a commercial kit (based on ionic chromatography). Although different cell lines have been used, the transfection efficiency of COS-7 cells (44%) obtained with sc pDNA conformation purified with the process developed in the present work was identical to the transfection efficiency of CHO cells (50%) achieved with sc pDNA purified with histidine-agarose matrix [23]. Likewise, other processes developed to achieve a clinical pDNA product revealed that the transfection efficiency is enhanced when using the sc pDNA conformation [17,41]. A particular study using arginine-agarose to purify a therapeutic plasmid encoding the tumor suppressor gene p53 showed that sc pDNA isoform is also responsible for a significant enhancement in the expression of the target protein [41]. Thus, the results obtained confirmed that the process developed with lysine-agarose for purification of clinical grade pDNA present a good performance. Overall, the novelty presented in this study is the application of the lysine-affinity chromatography, for the first time, to specifically and efficiently purify the most biologically active sc plasmid conformation from a much more complex and impure extract, resultant from the recombinant production of the plasmid in *E. coli*. Considering the relevance of obtaining pharmaceutical-grade pDNA, the quality and purity of the plasmid product obtained was evaluated by using the methodologies required by FDA. Also the biological activity of the supercoiled plasmid was verified by measuring the transfection efficiency, in *in vitro* tests. The levels of impurities present in isolated sc pDNA sample are in accordance with the specifications of regulatory agencies in terms of RNA and proteins (undetectable), gDNA (2 ± 0.02 ng/μg of pDNA), endotoxins (0.1 ± 0.01 EU/μg of pDNA) and sc pDNA homogeneity (100% sc). Thus, the lysine ligands provided high specificity and ability to purify the sc pDNA under a pharmaceutical-grade. Furthermore, the knowledge of the ideal conditions for sc pDNA purification and the selectivity provided by the lysine matrix for the different nucleic acids can be useful in a future association of the lysine ligands with a specific chromatographic support of higher capacity than traditional agarose supports. With this future approach it is also expected to improve the scalability and the performance of this downstream operation integrated in a global process.

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Paper VI

Differential interactions of plasmid DNA, RNA and genomic DNA with amino acid-based affinity matrices

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Research Article

Differential interactions of plasmid DNA, RNA and genomic DNA with amino acid-based affinity matrices

The development of a strategy to plasmid DNA (pDNA) purification has become necessary for the development of gene therapy and DNA vaccine production processes in recent years, since this nucleic acid and most of contaminants, such as RNA, genomic DNA and endotoxins, are negatively charged. An ideal separation methodology may be achieved with the use of affinity interactions between immobilized amino acids and nucleic acids. In this study, the binding behaviour of nucleic acids under the influence of different environmental conditions, such as the composition and ionic strength of elution buffer, and the temperature, is compared with various amino acids immobilized on chromatography resins. Supercoiled (sc) plasmid isoform was isolated with all matrices used, but in some cases preferential interactions with other nucleic acids were found. Particularly, lysine chromatography showed to be an ideal technology mainly on RNA purification using low salt concentration. On the other hand, arginine ligands have shown a greater ability to retain the sc isoform comparatively to the other nucleic acids retention, becoming this support more adequate to sc pDNA purification. The temperature variation, competitive elution and oligonucleotides affinity studies also allowed to recognize the dominant interactions inherent to biorecognition of pDNA molecule and the affinity matrices.

Keywords: Affinity chromatography / Amino acid matrices / Biorecognition / Nucleic acids
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1 Introduction

The demand for efficient production and purification methods of plasmid DNA (pDNA) has vastly increased in response to rapid advances of pDNA application as a non-viral vector for gene therapy or particularly for vaccination [1, 2]. Plasmids are usually produced in a recombinant *Escherichia coli* (*E. coli*) host by fermentation and represent around 3% w/w of the *E. coli* extract. Furthermore, most of the critical contaminants share similar characteristics of negative charge (RNA, genomic DNA (gDNA), endotoxins), identical in size (gDNA, endotoxins) and hydrophobicity (endotoxins) [3] with pDNA. However, the number and complexity of the processing steps in the extraction, isolation, purification and formulation of pDNA induce structural stress resulting in damage to plasmid molecules [4], namely supercoiled (sc) isoform. Given that, the specifications of pDNA for therapeutic applications are as follows: appearance (clear, colourless solution), plasmid homogeneity (>97% sc) [3], proteins (not detectable, BCA (<3 μg/mg pDNA)), RNA (not detectable, 0.8% agarose gel), gDNA (<2 μg/mg plasmid, PCR) and endotoxin (<10 EU/mg plasmid, LAL assay). Indeed, a special downstream processing is required for the elimination or reduction of cellular components of the *E. coli* host to acceptable levels according to the requirements established by regulatory agencies.

The diversity of biomolecules present in pDNA-containing extracts and the structural and chemical similarities between pDNA and impurities are some of the main challenges of improving or establishing novel purification procedures. Various types of chromatography, based on differences in size, charge, hydrophobicity and affinity of different molecules in a mixture, have been employed to purify pDNA [5]. Size exclusion chromatography has limited capacity and selectivity for pDNA [6] and hence is ideally used as a finishing step for sc pDNA purification from residual contaminants [7]. For anion-exchange chromatography, selectivity towards pDNA or impurities (*e.g.* RNA, gDNA and endotoxin) is poor due to their non-specific binding to the anion-exchange resin [5]. On the other hand, hydrophobic interaction chromatography allows an efficient separation of pDNA from endotoxins and single-stranded

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Abbreviations: gDNA, genomic DNA; **oc,** open circular; **pDNA,** plasmid DNA; **sc,** supercoiled

nucleic acids, owing to non-binding of pDNA [8]. However, pDNA elution occurs at high salt concentrations [9] and cannot obtain an efficient isoforms separation. Reverse-phase chromatography also exploits the hydrophobic interaction between target molecules and resin, getting the isolation of sc pDNA, but the use of organic solvents which are often toxic is a major disadvantage for this technique [10]. Several affinity approaches have already been successfully developed for a variety of applications, but we will focus here on a specific affinity chromatographic method and its possible contributions to purify and selectively separate the nucleic acids present in *E. coli* lysate.

Affinity chromatography simulates and exploits natural biological processes such as molecular recognition for the selective purification of the target molecule. However, despite the affinity methods have the purification power to eliminate additional steps, increasing yields and improving process economics, they do suffer other problems, particularly in regard to the biological origin of the ligands [11, 12]. By this way, synthetic ligands were developed to combine the selectivity of natural ligands with the high capacity, durability and cost effectiveness of synthetic systems [11, 13]. In the last decades, affinity interactions between target proteins and specific immobilized ligands have been more exploited than with target nucleic acids, but a few relevant applications have been described [12]. Among which, we have immobilized metal affinity chromatography [14], triple-helix affinity chromatography [15], protein-DNA affinity chromatography [16] and amino acid-DNA affinity chromatography [17–19].

Recently, an effort was proposed to adjust natural occurrence phenomena, such as the association between nucleic acids and proteins in biological organisms, to practical and biotechnological procedures in affinity chromatography. Amino acids have been widely used in biotechnology applications [20]. Since amino acids are natural compounds, they can be safely used in pharmaceutical applications, *e.g.* as a solvent additive for protein purification and as an excipient for protein formulations. Moreover, some particular amino acids are recognized as suppressors of protein aggregation during protein refolding [21], efficient agents to solubilize proteins from loose inclusion bodies and good competition agents when included in elution buffer [22]. Furthermore, based on the atomic studies demonstrating that some positively charged amino acids can preferentially promote specific interactions with nucleic acid bases [23, 24], such as electrostatic interactions, hydrophobic interactions, van der Waals forces and/or hydrogen bonding, we have developed a new approach of affinity chromatography designed for pDNA purification [12]. Through amino acid-DNA affinity chromatography, it became possible to obtain a specific binding with sc pDNA isoform in a single chromatographic step, whereas RNA, gDNA, proteins and endotoxins are efficiently eliminated [12].

According to these considerations, a comparative study was performed to explore and analyze the feasibility of using amino acid-DNA affinity chromatography for purification and isolation of several nucleic acids. Short-chain oligonucleotides molecules, with specific sequences, also represent attractive models to understand the nucleic acid adsorption, since they allow a clear interpretation of the experimental data [25]. For this purpose, the binding behaviour of pDNA isoforms, RNA, gDNA and oligonucleotides under the influence of different environmental conditions, such as the composition and ionic strength of elution buffer, and the temperature, was investigated with various amino acids immobilized on chromatographic resins.

2 Materials and methods

2.1 Materials

L-Histidine-agarose gel was obtained from Sigma-Aldrich (St. Louis, MO, USA), arginine-sepharose 4B and lysine-sepharose 4B gels were obtained from GE Healthcare Biosciences (Uppsala, Sweden). Qiagen Plasmid Purification Maxi Kit was from Qiagen (Hilden, Germany). Lyophilized synthetic homo-oligonucleotides with different molecular mass were purchased from Sigma-Aldrich. Other chemicals used in the elution buffer were sodium chloride and ammonium sulphate purchased from Panreac (Barcelona, Spain). L-Arginine, L-glutamic acid, L-valine, L-histidine and imidazole were acquired from Sigma-Aldrich and Tris from Merck (Darmstadt, Germany). All solutions were freshly prepared using water ultra-pure grade, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and the elution buffers were filtered through a 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. The lyophilized homo-oligonucleotides were first hydrated with 10 mM Tris-Cl, pH 8.0, and used without further purification.

2.2 Fermentation

The 6.05-kbp plasmid pVAX1-*LacZ* (Invitrogen, Carlsbad, CA, USA) used in the experiments was produced by a cell culture of *E. coli* DH5α. Growth was carried out at 37°C using Terrific Broth medium (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4) supplemented with 30 μg/mL kanamycin. Growth was suspended at the late log phase and cells were recovered by centrifugation and the pellets were stored at −20°C. Plasmid-free *E. coli* DH5α cells were also grown under the same conditions as described before, but with no antibiotic present.

2.3 Alkaline cell lysis and pDNA isolation

pDNA was purified using the Qiagen Plasmid Maxi Kit according to the manufacturer's instructions. The protocol is based on a modified alkaline lysis procedure. Following

lysis, binding of pDNA to the Qiagen anion exchange resin is promoted under appropriate low-salt and pH conditions. Impurities are removed by a medium-salt wash, and pDNA is eluted in a high salt buffer, being then concentrated through an isopropanol precipitation.

2.4 Isolation of *E. coli* nucleic acids

gDNA and RNA were isolated from pDNA-free DH5α *E. coli* cells. gDNA was isolated using the Wizard genomic DNA purification kit from Promega (Madison, WI, USA), according to the manufacturer's instructions. For RNA isolation, the cells were lysed by a modification of the alkaline method [26], the resultant lysate was clarified by ammonium acetate precipitation and nucleic acids were concentrated by polyethylene glycol 6000 precipitation as described previously [27].

2.5 Interactions of pDNA isoforms, RNA, gDNA and oligonucleotides with immobilized amino acid affinity matrices

Chromatography was performed in a fast protein liquid chromatography system (Amersham Biosciences). A 16 mm diameter × 100 mm long (approximately, 20 mL) column was individually packed with the commercial histidine-, arginine- and lysine-agarose gels. The column was equilibrated with appropriate loading buffer, as described below, at a flow rate of 1 mL/min. Plasmid samples (200 μL) were injected at the same flow rate and the absorbance of the eluate was continuously monitored at 280 nm. Following plasmid isoforms separation with an appropriate elution gradient, as described below, the fractions were pooled according to the chromatograms obtained, and used for further electrophoresis analyses after concentration and desalting with Vivaspin concentrators (Vivascience). Other chromatographic runs were also performed by injecting *E. coli* nucleic acids (gDNA and RNA) isolated as described above. The elution scheme and conditions were identical to those used for pDNA purification. Some experiments of pDNA competitive elution with elution buffers containing different kinds of amino acids were performed, through a linear elution gradient as described below. All experiments were conducted at room temperature unless otherwise stated.

Oligonucleotides chromatography was performed in an ÄKTA Purifier system (GE Healthcare Biosciences). A 10 mm diameter × 20 mm long (about 1.6 mL) column was packed with each commercial amino acids matrix. The column was equilibrated at 5, 12, 15, 20, 24 and 35°C with an appropriate elution buffer (ammonium sulphate or sodium chloride) at a flow rate of 1 mL/min. The water-jacketed column was connected to a circulating water bath Multi-Temp III to maintain the appropriate temperature in each experiment. In total, 50 μL (about 2.5 μg) of the oligonucleotides samples were injected on the column and the absorbance of the eluate was continuously measured at 260 nm. After the chromatographic runs, the column was washed with three column volumes of elution buffer, in order to remove any molecule that remained bound.

2.6 Agarose gel electrophoresis

The fractions recovered from each chromatographic experiment were analyzed by horizontal electrophoresis using 15-cm-long 1% agarose gels (Hoefer, San Francisco, CA, USA), stained with ethidium bromide (0.5 μg/mL). Electrophoresis was carried out at 100 V, for 40 min, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0).

3 Results and discussion

Affinity chromatography is a liquid chromatographic technique that separates biomolecules on the basis of a specific, reversible interaction between the target biomolecule and its specific ligand, which is coupled to a chromatography matrix. The choice of matrix and elution conditions to be used will depend on the molecular properties of biomolecules and the physicochemical and thermodynamic nature of their molecular interactions [28]. If the biomolecule is retained with only a weak or moderate affinity, it is possible to elute the target biomolecule applying an elution buffer under isocratic conditions, being thus designated as weak affinity chromatography or dynamic affinity chromatography [29]. More strongly retained substances can be eluted by changing the mobile phase or column conditions. For this case, the elution steps can be performed either specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity depending on the matrix used and the chemical characteristics of the biomolecules [28].

In this study, the interactions between nucleic acids and amino acid–ligands in affinity chromatography were explored, and it is shown that, although the three immobilized amino acids belong to the same amino acids group (polar, positively charged), they promote different interactions, depending on the conditions used, such as, the composition and ionic strength of elution buffer or the temperature.

3.1 Effect of ionic strength and elution buffer composition on pDNA isoforms binding

Recent studies have demonstrated that histidine [30] and arginine [31] can be used as ligands to isolate and purify sc pDNA using appropriated elution strategies. For histidine-chromatography, a stepwise decreasing ammonium sulphate concentration between 2.3 and 2 M was applied,

allowing plasmid isoforms separation, being sc pDNA stronger retained on the histidine support than open circular (oc) [30]. In the arginine-chromatography studies, pDNA isoforms' isolation was achieved with the application of a stepwise increasing sodium chloride gradient in the eluent between 200, 225 and 300 mM. In this example, arginine ligands distinguished and differentially interacted with both pDNA isoforms, suggesting a specific recognition for the sc isoform [31].

Similarly to these amino acid matrices, lysine–agarose was also studied by our research group for the capacity to isolate pDNA isoforms. In this case, a successful isoforms separation was obtained with an elution strategy identical to that used on arginine study, but with sodium chloride concentrations between 165 and 250 mM [19]. An example of chromatographic profile obtained in lysine-chromatography is shown in Fig. 1, and through electrophoresis analysis it was possible to verify that the first peak corresponded to the oc and the second peak was attributed to the sc isoform [19]. Hence, it was demonstrated that it is possible to obtain the plasmid isoforms totally isolated through an adequate elution strategy in function of amino acid immobilized on the support.

Comparing the three chromatographic strategies used to purify the isoforms of pDNA, it is evident that in all cases there is a specific interaction and a biorecognition involving the sc isoform. The ability of these amino acid matrices to distinguish and differentially interact with both isoforms is explained by the different degree of bases exposition. In all cases, it is possible to establish a specific retention of sc isoform on the matrix, probably because the bases of sc isoform are more exposed than the bases of the oc isoform due to the deformations induced by the torsional strain associated with supercoiling [19, 30, 31]. However, the nature of the interactions involved in each case is not the same. For example, in the study of histidine ligands, it was demonstrated the involvement of several

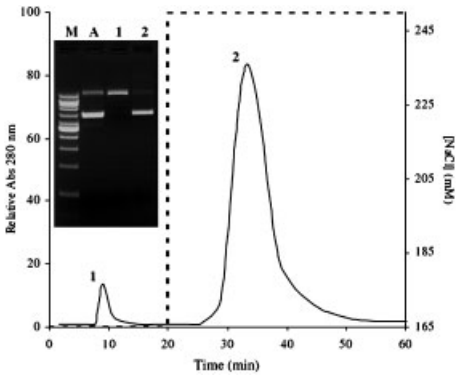

Figure 1. Effect of ionic strength on separation of oc and sc pDNA isoforms by lysine-chromatography. Elution was performed by stepwise increasing NaCl concentration in the eluent. Agarose gel electrophoresis analysis of each peak is represented in respective chromatogram. Lane M, molecular weight marker; lane A, pDNA sample injected onto the column (oc+sc).

interactions on pDNA retention with high salt concentration [30]. However, as to promote the pDNA elution it was necessary to decrease the salt concentration, it was suggested that ring staking is the dominant interaction, once that hydrogen bonds do not weaken with lower salt concentrations [30].

For arginine- and lysine-chromatography, the elution strategy was quite different. These matrices are highly effective in binding pDNA at low ionic strength, indicating the presence of strong interactions with the charged phosphate groups in the pDNA backbone, although in the case of lysine the interactions are weaker than in arginine [19, 31]. In these cases, to elute pDNA isoforms, a slight increase of salt concentration was necessary, confirming the predominance of electrostatic interactions. The biorecognition of sc isoform by these matrices is also related with bidentate and complex hydrogen bonds, van der Waals contacts or water-mediated hydrogen bonds, that promote an additive effect in the specificity for this plasmid isoform.

Therefore, the arginine- and lysine-based affinity matrices revealed an extreme sensitivity of the binding/elution behaviour of the plasmid isoforms with respect to the ionic strength. Although that, in the case of histidine-based affinity matrix, it was necessary high salt concentration of ammonium sulphate to separate the pDNA isoforms, becoming this matrix less advantageous to be applied in pDNA purification, comparing with the other two amino acid matrices.

3.2 Effect of temperature on pDNA, RNA and gDNA retention

After to know the pDNA retention behaviour on these matrices, it was also important to study how the other nucleic acids behave. The effect of temperature on pDNA stability is already known [32], and this parameter may also significantly influence pDNA interactions with the immobilized amino acids. Thus, several chromatographic experiments were designed to analyse how temperature affects the adsorption of pDNA isoforms, as well as the other *E. coli* nucleic acids, to the amino acid matrices. The water-jacketed column was connected to a circulating water bath to maintain the appropriate temperature (temperature extremes, 5 and 24°C) in each experiment.

In histidine-chromatography, the column was initially equilibrated with 2.3 M $(NH_4)_2SO_4$ in 10 mM Tris buffer (pH 8.0), after pDNA sample injection (200 μL), a linear gradient (three column volumes) up to 10 mM Tris buffer (pH 8.0) was used. The elution of other isolated nucleic acids was carried out under the same conditions described previously. For the arginine experiments, a linear gradient (three column volumes) between 200 and 300 mM NaCl in 10 mM Tris buffer (pH 8.0) was used for each nucleic acid and for the lysine studies, the gradient was performed between 160 and 250 mM NaCl 10 mM Tris buffer (pH 8.0).

The retention time of RNA, gDNA and oc plasmid isoform in the histidine matrix was not affected with the temperature increase, as shown in Fig. 2A. However, sc isoform retention decreased as the temperature increased. This behaviour suggests that the temperature increase may affect the sc isoform conformation inducing sc plasmid torsional strain changes, leading to relaxation and to a consequent decrease in the interaction of this molecule with the histidine ligands. These results are supported by another study, about the retention of single-stranded molecules (oligonucleotides) with histidine, where also occurred structural changes of the molecules due to the temperature increase [33]. An increase retention time of nucleic acids could be expected if the involved interactions were only of hydrophobic nature. Nevertheless, according to these studies, this behaviour was not verified suggesting, that the temperature increase has a predominant effect on the molecules structure.

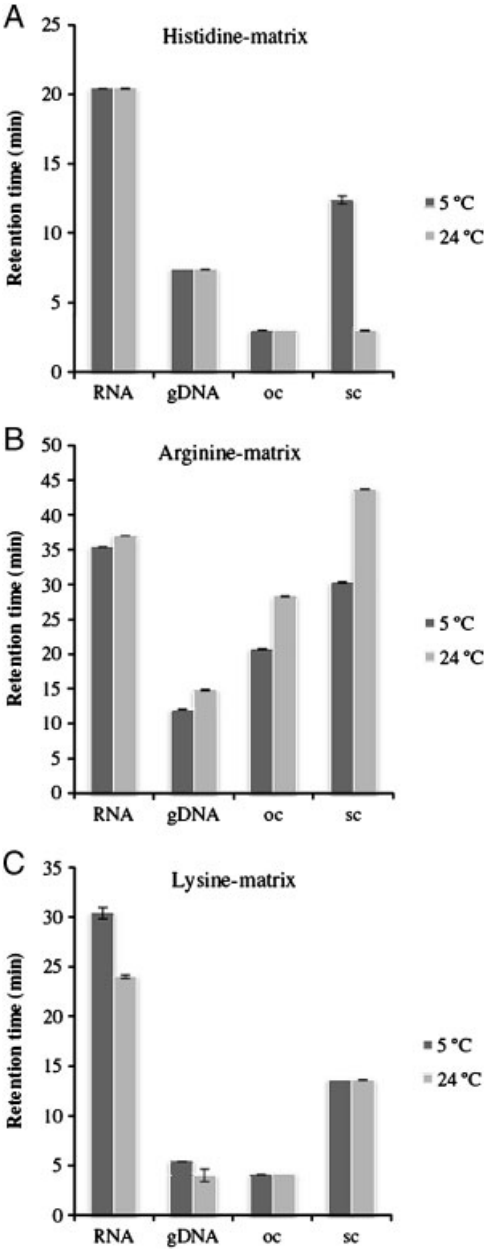

Figure 2. Effect of temperature on the retention time of nucleic acids on affinity matrices with immobilized amino acids (5 and 24°C). Elution was performed at 1 mL/min by an appropriate elution gradient (three column volumes).

On the other hand, in arginine-chromatography (Fig. 2B), it was verified an increase on retention of all nucleic acids studied with the temperature increase. In this study, the nucleic acids molecules retention to the arginine ligands is directly influenced by temperature, suggesting that the temperature increase favours the involvement of the hydrophobic interactions [34]. The changes on conformational structure can also be involved in this case, but the temperature effect on interaction between nucleic acids and the ligands seems to prevail. Curiously, in Fig. 2B, it is observed that the retention time of isolated RNA (35.4 min) is similar to the isolated sc plasmid isoform (30.3 min) for the lowest temperatures studied (5°C). On other hand, the retention time of RNA molecule in a complex sample was significantly lower. The stronger interaction of isolated RNA sample with the arginine ligands might be due to the fact that bases are more available to interact with immobilized ligands, because of the single-stranded structure of RNA. The different interaction behaviour of double- and single-stranded nucleic acids during the purification process was already described for immobilized metal affinity chromatography studies, where plasmid and gDNA showed low binding affinity, whereas RNA and single-stranded oligonucleotides bind strongly to matrices [35]. The authors revealed that when RNA is single injected it can bind to several matrices, whereas in the presence of endotoxins, it was not retained. This change in RNA elution behaviour showed that some compounds can appear as competitive agents by the available binding sites on the matrices. Also in arginine-chromatography was verified that the isolated RNA bind preferentially with arginine ligands, comparatively with the RNA incorporated in a complex-clarified lysate sample [18].

Figure 2C shows the retention time of each nucleic acid in lysine-chromatography when submitted to 5 and 24°C. For RNA and gDNA experiments, it is evident a decrease on retention time with the temperature increase. This behaviour, opposite to what happens in arginine-chromatography, is not completely understood. But an identical tendency was observed in a study of separation of ribonucleic acids on lysine–agarose, where the decrease of temperature promoted a greater retention of the tRNA species on the column [36]. In plasmid isoforms study, the retention time was not changed with the temperature increase (Fig. 2C). In fact, it was already shown that for low temperatures, the lysine selectivity for sc isoform is decreased, and a fraction of this isoform eluted with oc isoform [19].

The amino acid-based chromatography can overcome some limitations of other kinds of chromatography, among

which, limited capacity to purify pDNA (size exclusion chromatography), poor selectivity towards pDNA or the other nucleic acids (anion-exchange chromatography), non-specificity to obtain an efficient pDNA isoforms separation (hydrophobic interaction chromatography) and to avoid the use of organic solvents (reverse-phase chromatography). In addition, it gives the possibility to choose the most suitable conditions to purify a particular nucleic acid molecule, depending on the objective of the study. sc plasmid isoform was isolated with all matrices used, but in same cases preferential interactions with other nucleic acids were found. For example, the histidine- and lysine-chromatography can be applied on purification or isolation of RNA because the interaction is stronger. Associating the lysine support with low temperatures, it is possible to promote an increased retention of RNA and, at the same time, to decrease the pDNA retention. The lysine-chromatography can be a good strategy when the sample is to be applied in therapeutic treatments, because of the low sodium chloride concentration used. The use of low temperatures can avoid the sample degradation during the time that it is retained on the column. On the other hand, arginine-chromatography can be used, with temperatures near to 24°C, to purify the sc pDNA isoform from *E. coli* lysate for therapeutic applications. Under these conditions, it is possible to maintain the sc isoform retained, being the contaminants eluted before sc isoform.

3.3 Competitive elution studies

In affinity chromatography, the elution of a target solute that is bound to the affinity ligands can be achieved through changing the ionic strength, pH or polarity of elution buffer [28]. However, it is also possible to add a competing agent in the elution buffer becoming the elution strategy more selective and biospecific. The competing agent can bind to the retained target or to the immobilized ligand, depending of their characteristics, thus allowing to predict the interactions that may be involved.

Some competitive elution experiments were performed, using pDNA as the target molecule and elution buffers containing different amino acids. The elution gradients applied were adapted to ensure that the pDNA sample is on ideal conditions to bind to the matrix ligands, and will be eluted only due to the competing agent addition. Several amino acids with different characteristics (positive, negative and hydrophobic amino acids) were chosen to add in the elution buffer in order to evaluate the kind of competition that may be developed.

Due to the solubility limitation of each chosen amino acid, only a concentration of 250 mM was possible to prepare for these experiments. Histidine-chromatography was first equilibrated with 2.5 M $(NH_4)_2SO_4$ in 10 mM Tris buffer (pH 8.0), after pDNA injection, a linear 40 min gradient to 2.5 M $(NH_4)_2SO_4$ supplemented with 250 mM arginine (positively charged) in 10 mM Tris buffer (pH 8.0) was used. The same elution conditions were employed for glutamic acid (negatively charged), valine (hydrophobic character) and histidine (aromatic ring) amino acids. The results obtained are summarized in Table 1. In these experiments, it was not observed the pDNA elution, suggesting that under these conditions the amino acids have not promoted any competition mechanism. By this way, another competing agent, with higher solubility than the previous amino acids, was tested. Ammonium sulphate solution was supplemented with 500 mM of imidazole (with identical structure of histidine ligand) and with the same elution gradient it was observed a partial elution of pDNA. These results suggest that the predominant interaction between pDNA and histidine ligands is the ring staking/hydrophobic interaction due to high salt concentration used, and because this interaction is weakened when the imidazole is present to compete.

In the case of arginine, it was also tested this elution strategy, thus arginine-column was equilibrated with 10 mM Tris buffer (pH 8.0) to promote the total retention of pDNA, and a 40 min linear gradient to 250 mM of some amino acid mentioned above was applied. pDNA sample elution was only achieved when the elution buffer contained the arginine amino acid (Table 1). This behaviour may be due to the positive character of this amino acid that promotes a preferential binding of pDNA with free arginine present in the buffer by electrostatic interactions, inducing its elution.

Competitive elution studies were already performed to lysine-chromatography [19] through an elution gradient similar to that used in arginine matrix. Comparing the results obtained with the other two matrices studied (Table 1), it was verified that the use of arginine on elution buffer also promoted the plasmid elution, as it was also verified with arginine-column. Curiously, in these previous lysine experiments, when glutamic acid was used as negative amino acid in the elution buffer, pDNA was

Table 1. Summary of retention and elution behaviour of pDNA sample in function of competitive elution strategy applied to affinity matrices with immobilized amino acids[a)]

Matrix	Competing agent	pDNA behaviour	Competition behaviour
Histidine	Arginine	Retention	–
	Glutamic acid	Retention	–
	Valine	Retention	–
	Histidine	Retention	–
	Imidazole	Partial elution	+ (Ligand)
	Arginine	Elution	+ (Ligand)
Arginine	Glutamic acid	Retention	–
	Valine	Retention	–
	Arginine	Elution	+ (Ligand)
Lysine	Glutamic acid	Elution	+ (pDNA)
	Valine	Retention	–

a) +, with competition; –, without competition.

also eluted for the reason that glutamic acid promoted the displacement of the bound pDNA [19]. Hence, it was observed that the use of a negatively charged amino acid can also promote a competitive elution, competing with pDNA, particularly with the phosphate groups, for the lysine ligands surface.

The elution experiments with valine competing agent (hydrophobic amino acid) did not reveal any plasmid elution for the last two matrices. This fact shows that the preferential interaction between pDNA and arginine and lysine supports is ionic and not hydrophobic, because despite the valine amino acid has a carbon lateral chain, pDNA remained bound to the matrix (results not shown).

In a general way, the competition study has shown that free arginine present in the elution buffer could link to the plasmid, promoting its biospecific elution on arginine and lysine columns, due to the competition with the ligand [28], as also happened with imidazole on histidine column. Glutamic acid was linked to the ligand, only on lysine column, also promoting the biospecific elution of plasmid, but in this case due to the competition with the plasmid [28]. These data gave further information to understanding the underlying mechanism, as well as the interactions involved and the responsible groups for the recognition of pDNA by amino acid matrices studied in this study, which can be extremely helpful to the further efforts in development of novel strategies for purification of nucleic acids.

3.4 Affinity of oligonucleotides

The application of amino acids ligands to purify pDNA has demonstrated a biorecognition by sc plasmid isoform, suggesting that these matrices can develop complex interactions that are partly dependent on the conformation of the DNA molecule [12]. Synthetic oligonucleotides have been applied in several areas as molecular biology, because these molecules are associated with some techniques namely PCR, DNA sequencing and genotyping [37]. Furthermore, short-chain oligonucleotide molecules, with various specific sequences, represent particularly attractive models to study nucleic acid adsorption because they allow a clear interpretation of the experimental data [25]. In light of these considerations, a study was conducted using the oligonucleotides as model to understand the several interactions that a DNA molecule can promote with the amino acids supports, in accordance with the chromatographic conditions established. This knowledge can be useful to perform a more targeted and efficient purification as it is described in our previous studies [33, 38].

Histidine–agarose support was equilibrated with different concentrations of ammonium sulphate (0, 0.5, 1 and 1.5 M in 10 mM Tris buffer (pH 8.0)) at several temperatures (5, 12, 15, 20, 24 and 35°C), with a flow rate of 1 mL/min. A 2.5 μg amount of each oligonucleotide (polyA, polyT, polyG, polyC and polyU with 1, 3, 6, 15 and 30 bases) was loaded onto the column and an isocratic elution gradient was carried out at identical salt concentrations. The same experiments were performed with arginine and lysine–agarose supports but with an appropriate elution gradient. These columns were previously equilibrated with 10 mM Tris buffer (pH 8.0), and the oligonucleotides were eluted with a linear gradient between 0 and 1 M NaCl in 10 mM Tris buffer (pH 8.0) also using different temperatures.

In Table 2, it is possible to analyse some representative results of the retention time of poly30 oligonucleotides, for each matrix to the temperature of 5 and 35°C. For histidine-chromatography case, the experiments performed at 1.5 M of ammonium sulphate are presented due to the higher retention obtained with this salt concentration. In these circumstances, the oligonucleotide that presented higher retention with the three amino acids ligands was the polyG30. This retention behaviour does not simply follow the hydrophobicity of the oligonucleotides, because although the guanine base is characterized as the most hydrophobic base [25] (what is in accordance with its higher retention on the amino acid matrices (Table 2)), the adenine base, also being a purine base, is less retained that thymidine base (pyrimidine). Previous studies based on the molecular modelling [23, 24] described a favoured and stronger interaction of histidine, arginine and lysine with the guanine base by occurrence of a variety of hydrogen interactions. In a general way, the same preference by guanine base was also verified in our previous studies [33, 38]. Hence, other interactions can be involved in retention mechanisms, such as electrostatic interactions between phosphate groups of oligonucleotides and the positive charge of amino acids ligands, as well as cation–π or van der Waals interactions. In summary, it becomes evident that the binding mechanism involves phenomenological interactions, such as biorecognition between amino acid ligands and oligonucleotides.

Temperature has been described as a parameter that influences significantly the analytes' interaction with amino acid matrices, consequently affecting the retention, or even the chromatographic selectivity [39]. Analyzing

Table 2. Retention time[a)] of oligonucleotides loaded on histidine, arginine and lysine–agarose supports with appropriate elution gradients, at different temperatures (5 and 35°C)

Matrix	Temperature (°C)	Retention time of oligonucleotides ± SD (min)		
		polyA30	polyG30	polyT30
Histidine	5	3.182 ± 0.020	3.235 ± 0.005	2.534 ± 0.027
	35	4.593 ± 0.031	2.991 ± 0.014	2.947 ± 0.005
Arginine	5	5.902 ± 0.052	7.175 ± 0.022	6.807 ± 0.031
	35	6.777 ± 0.047	8.125 ± 0.040	7.201 ± 0.033
Lysine	5	4.851 ± 0.035	6.095 ± 0.024	5.657 ± 0.040
	35	5.919 ± 0.016	6.676 ± 0.028	6.413 ± 0.021

a) For chromatographic conditions, see Section 2.

the results of Table 2, it is shown that, in a general way, oligonucleotides retention increased when the temperature was increased, mainly with arginine and lysine supports. This tendency is in accordance with the previous studies, where it was described that the temperature increase directly influenced the retention of pDNA to the amino acids stationary phase, being verified an increased retention [31]. A contradictory behaviour on histidine-chromatography was observed for polyG30 retention with temperature increase. In this particular case, the temperature increase led to a decrease of polyG30 retention, because this oligonicleotide can present secondary structures or G-quadruplexes under these salt conditions that are affected by the temperature increase. It was already described that an increase in temperature can disrupt the interactions that stabilize these molecular structures affecting retention [33].

4 Concluding remarks

The successful implementation of new therapeutic strategies is partially dependent on the progress of pDNA manufacturing technology. Amino acid-based chromatography represents a particularly promising approach because it combines the selectivity of a naturally occurring biological interaction with the simplicity of small molecule. This article has explored the three types of matrices with immobilized amino acids recently employed in pDNA purification, and has examined the applications and selectivity that these methods can have in function of different environmental conditions.

In a general way, the lysine-chromatography showed to be an ideal technology for RNA purification using a low salt concentration, whereas histidine ligands immobilized need high salt concentrations to purify sc pDNA. On the other hand, arginine ligands exhibited a greater ability to efficiently retain the sc isoforms. On the other hand, the temperature manipulation has also demonstrated the possibility of combining the preferential elution of a particular target nucleic acid, exploring the nucleic acids retention with the amino acid matrices depending on the selected column. Competitive elution studies suggested that depending on the competing agent applied on the elution buffer, different interactions, such as hydrophobic or ionic interactions promoted due to the competition with the ligand or with the plasmid, are prevalent relatively to the other interactions involved on the pDNA biorecognition. Generally, a favoured and stronger interaction between amino acid ligands and guanine base of oligonucleotides was verified on the affinity studies, probably by occurrence of a variety of hydrogen interactions, although other interactions are present.

Overall, this comparative study brings more evidences that the binding mechanism inherent on biorecognition between amino acids and nucleic acids, namely pDNA, can result from the combination of several interactions such as electrostatic interactions, hydrophobic interactions, (multiple) hydrogen bond interactions, van der Waals forces and cation–π interactions. However, depending on the environmental conditions established and the amino acids ligands used, some interactions can be more favoured than others, becoming more evident under these conditions. The high resolution obtained with these three supports and the low salt concentrations used with arginine and lysine supports indicate that these could be promising and economic methods to apply at biotechnological process and industrial scale.

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Paper VII

Performance of a non-grafted monolithic support for purification of supercoiled plasmid DNA

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Performance of a non-grafted monolithic support for purification of supercoiled plasmid DNA☆

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ABSTRACT

The use of therapeutics based on plasmid DNA (pDNA) relies on procedures that efficiently produce and purify the supercoiled (sc) plasmid isoform. Several chromatographic methods have been applied for the sc plasmid purification, but with most of them it is not possible to obtain the required purity degree and the majority of the supports used present low capacity to bind the plasmid molecules. However, the chromatographic monolithic supports are an interesting alternative to conventional supports due to their excellent mass transfer properties and their high binding capacity for pDNA. The separation of pDNA isoforms, using short non-grafted monolithic column with CarbonylDiImidazole (CDI) functional groups, is described in the current work. The effect of different flow rates on plasmid isoforms separation was also verified. Several breakthrough experiments were designed to study the effect of different parameters such as pDNA topology and concentration as well as flow rate on the monolithic support binding capacity. One of the most striking results is related to the specific recognition of the sc isoform by this CDI monolith, without flow rate dependence. Additionally, the binding capacity has been found to be significantly higher for sc plasmid, probably because of its compact structure, being also improved when using feedstock with increased plasmid concentrations and decreased linear velocity. In fact, this new monolithic support arises as a powerful instrument on the sc pDNA purification for further clinical applications.

1. Introduction

The requirements in medicine and molecular biology science, demand large quantities of highly pure and homogenous plasmid DNA (pDNA) for different applications such as cloning, large scale protein production, DNA vaccination or gene therapy [1]. The impact of plasmid structure and conformation on the transfection process efficacy has received some attention in the past. Thus, the supercoiled (sc) pDNA isoform is the desired topological form since it induces the most efficient access to the nucleus of the cell and consequently improves gene expression in eukaryotic cells [2]. According to international regulations, the product-quality is defined as a percentage of the sc isoform compared to the total pDNA, being that the purity of the sc pDNA must be 100% with the homogeneity degree higher than 97% of sc isoform from *Escherichia coli* (*E. coli*) host [3]. This foreseeable necessity led to amplified efforts within both research and industry to improve the effectiveness of sc pDNA production and purification methodologies [4].

For the purification of pDNA several chromatographic methods based on particulate supports have been reported. Besides conventional techniques such as anion exchange, hydrophobic interaction and size exclusion chromatography [5], other methods were tested with more or less success. The affinity concept has gained impact with the development of new supports combining different and more specific interactions with pDNA. Based on the natural occurrence of protein–DNA complexes in biological systems, and the atomic studies demonstrating the existence of favored interactions between particular amino acids and nucleic acid bases [6,7], it was recently developed by our research group a new affinity chromatographic methodology using some amino acids immobilized on agarose matrix for the isolation and purification of several nucleic acids [8]. Particularly, histidine [9], arginine [4] and lysine [10] were already used as ligands to efficiently purify sc pDNA, and the recognition of this isoform proved the presence of specific interactions between pDNA molecule and the amino acid-based matrices studied. Despite these results obtained with the affinity matrices, some problems remained to be solved, such as the low capacity of available supports for pDNA and the low diffusivity of pDNA samples due to their high molecular weight. Considering these facts, it is necessary to study and develop more suitable supports to overcome these problems.

Monolithic sorbents represent one of the newest developments of chromatographic stationary phases for biomolecules separation

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and purification. A typical monolith is a continuous bed constituted by a single piece of highly porous solid material, whose pore size depends on the polymerization process [11,12]. The ligands immobilized on these chromatographic supports can be several biological agents as antibodies, enzymes, lectins or amino acids [13] and peptides.

The chromatographic support named as Convective Interaction Media (CIM) was developed with the aim of obtaining a short chromatographic layer, ensuring well-defined, narrow pore-size distributions, excellent separation power and exceptional chemical stability [11]. These innovative CIM disks present numerous advantages comparing to conventional supports applied on sc pDNA purification, among which it is important to refer: high binding capacity due to excellent mass transfer properties and a huge quantity of accessible binding sites for large biomolecules as pDNA [14]; capacity to achieve very fast separation and purification with high reproducibility both at small and large scale [15]; simple handling, flow independent resolution [16]; the target molecule can be eluted in a concentrated form with a reduced biomolecule degradation due to short contact times with the chromatographic matrix [17].

This novel chromatographic material has been largely employed to separate immunoglobulins [18], oligonucleotides [19], proteins [19,20] and recently to purify pDNA [14,21–23]. Knowing that an agarose based matrix with immobilized histidine amino acid allows an efficient separation of pDNA isoforms [9], it becomes interesting to study a new non-grafted glycidyl methacrylate monolith (BIA Separations, Ljubljana, Slovenia) since this support is constituted by CarbonylDiImidazole (CDI) chemical groups. Pointing to the possibility of specific recognition mechanisms between imidazole ring and pDNA molecules, the applicability of CDI monolithic support to purify sc isoform seems to present great potential and is exploited in the present work. Additional chromatographic characterization based on breakthrough experiments was also designed to study the effect of parameters such as, pDNA topology and concentration, as well as the different flow-rate on the monolith dynamic binding capacity.

2. Experimental

2.1. Materials

All experiments were carried out in a 0.34 mL (3 mm thick and 12 mm diameter) non-grafted CDI poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith packed into a CIM disk housing, provided by BIA Separations (Ljubljana, Slovenia). This CIM epoxy monolith was initially synthesized from glycidyl methacrylate and ethylene dimethacrylate monomers in presence of porogens dodecanol and cyclohexanol by BIA Separations Company. The next step consisted in hydrolyzing the epoxy monolith with 0.5 M H_2SO_4 at 60 °C for 2–3 h to obtain hydroxyl groups. Finally, the hydroxyl groups were treated with 1,1′-carbonyldiimidazole to originate the imidazole carbamate reactive groups, as it was described by Bencina et al. [24]. Qiagen Plasmid Purification Maxi Kit was from Qiagen (Hilden, Germany). The ammonium sulfate used in the elution buffers was purchased from Panreac (Barcelona, Spain). All solutions were freshly prepared using deionized water ultra-pure grade, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and analytical grade reagents. The elution buffers were filtered through a 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically.

2.2. Methods

2.2.1. Bacterial cell culture

The 6.05-kbp plasmid pVAX1-*LacZ* (Invitrogen, Carlsband, CA, USA) used in the experiments was produced by a cell culture of *E. coli* DH5α, being obtained a high plasmid copy numbers enriched in sc isoform. Growth was carried out at 37 °C using Terrific broth medium (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4) supplemented with 30 μg/mL kanamycin. Growth was suspended at the late log phase ($OD_{600} \approx 13$) and the cells were recovered by centrifugation and were stored at −20 °C.

2.2.2. Alkaline cell lysis and pDNA isolation

Plasmid DNA was isolated from *E. coli* bacteria after alkaline lysis using the Qiagen (Hilden, Germany) plasmid maxi kit according to the manufacturer's instructions. The protocol is based on a modified alkaline lysis procedure, being considered a critical unit operation, because this step can influence the final ratio of sc plasmid. Unfavorable environmental conditions, such as extreme pH and temperature, affect the helical repeat of DNA promoting its unwinding, being generated other forms such as open circular (oc) isoform. Following lysis, binding of pDNA to the Qiagen anion exchange resin is promoted under appropriate low-salt and pH conditions. Impurities are removed by a medium-salt wash, and pDNA is eluted in a high-salt buffer, being then concentrated through an isopropanol precipitation. The pDNA obtained at the end of alkaline lysis contained around 90% of sc isoform, while the remaining 10% are due to the presence of oc isoform, as revealed by agarose electrophoresis. This pDNA isoform was applied for preparative chromatography studies and dynamic binding capacity measurements. Open circular (oc) pDNA, also used for dynamic binding capacity experiments, was prepared by incubating a sc pDNA sample at room temperature (24 °C). The sample was monitored over the time by electrophoresis analysis until the total conversion of sc plasmid to oc isoform was observed (about 3 days).

2.2.3. Preparative chromatography

All experiments were performed using 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. The monolithic column was equilibrated with 2.3 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0) and the system was run at a flow rate of 132.5 cm/h. Plasmid sample, resultant from alkaline lysis and pre-purification with the Qiagen Kit, was homogenized in 50 mM phosphate buffer (pH 8.0) and the ionic strength was corrected, dissolving the ammonium sulfate quantity required for the sample volume that will be injected (loop with 20 μL). The absorbance was monitored at 260 nm. After elution of unbound species with 2.3 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0), the ionic strength of the buffer was decreased by shifting the eluting buffer to 0 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0) to elute bound species. Following plasmid isoforms separation with an appropriate elution gradient, as described above, the fractions were pooled according to the chromatograms obtained, and used for further electrophoresis analysis after concentration and desalting with Vivaspin concentrators (Vivascience). A posterior study was conducted to analyze the effect of different flow rates (53, 132.5, 265 and 397.5 cm/h) on the resolution of both plasmid isoforms. All experiments were performed at room temperature.

2.2.4. Dynamic binding capacity (DBC) measurement for pDNA

The monolithic column, with non-grafted CDI-disk previously applied in preparative chromatography, was used for the determination of the dynamic binding capacity for pDNA. These experiments were conducted using different pDNA topologies (sc and oc) and concentrations (0.05, 0.1, 0.15 and 0.2 mg/mL), as well as different flow rates (26.5 and 53 cm/h). The column was equilibrated with 2.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0), and it was necessary to prepare the feedstock with high plasmid quantities to test the parameters referred above. After several alka-

line lysis processes using the Qiagen Kit, the final plasmid product was homogenized in 50 mM phosphate buffer (around 100 mL) and the ionic strength was corrected, dissolving the ammonium sulfate necessary, but in this case for a concentration of 2.5 M. The binding capacity can be measured by different methods [25]. In this work, it was applied the open-loop frontal analysis that is similar to closed-loop frontal analysis, but the feedstock solution is collected after crossing the whole system instead of being transported back into the closed circuit. At the end of each experiment, the binding capacity was determined by the breakthrough area integration method [26]. Briefly, each breakthrough experiment was derived from a 100% of saturated column. Then the sample volume corresponding to the adsorbed amount of plasmid was calculated by numerical integration of the detector response. The area obtained from the filled column was subtracted from that for the empty column. In this step, the void volume was correctly discounted from the DBC determination. This area is equivalent to the sample volume, which was required to saturate the column, and can be related with sample concentration that remained bound per millilitre of the support, reflecting the support capacity. Normally, the capacity values are represented at 10% of the breakthrough that corresponds to 10% of the column saturation, being calculated in the same way. Finally, the elution of the bound plasmid was achieved by decreasing the ammonium sulfate concentration in the mobile phase to 0 M in a stepwise manner.

2.2.5. Gel electrophoresis

The fractions recovered from each chromatographic experiment, after being efficiently desalinized, were analyzed by horizontal electrophoresis using 15-cm-long 1% agarose gels (Hoefer, San Francisco, CA, USA), stained with ethidium bromide (0.5 μg/mL). Electrophoresis was carried out at 100 V, for 40 min, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0).

3. Results and discussion

CIM supports represent the fourth generation of monoliths chromatographic supports. These glycidyl methacrylate-based monolithic columns are characterized by a single piece that contains pores highly interconnected, forming a network of channels [15]. Thus, the whole mobile phase is forced to run through these pores duo to the pressure difference, therefore the mass transfer between stationary and mobile phase is based on convection rather than on diffusion, increasing their mobility by several orders of magnitude. This transport mechanism enables very fast separations and purification of components as well as flow-unaffected resolution and dynamic binding capacity [15]. The latter is especially important for the purification of molecules on preparative level where the productivity is essential. Besides their attractive hydrodynamic characteristics, the non-grafted CDI monolith, provided by BIA Separations (Ljubljana, Slovenia) becomes a potential chromatographic support for pDNA purification.

3.1. Supercoiled pDNA purification

Initial experiments were performed to choose the best strategy to achieve the binding and elution of the pDNA isoforms, being observed that the total (oc + sc) pDNA elution was obtained at 1 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0). However, the partial retention of sc isoform was established at 2 M and total retention of both isoforms was verified at 2.5 M with the same salt (data not shown). These results suggest that it is necessary to apply high ammonium sulfate concentrations on the binding buffer and to optimize the concentration range between 2.5 and 2 M to obtain the ideal plasmid isoforms separation.

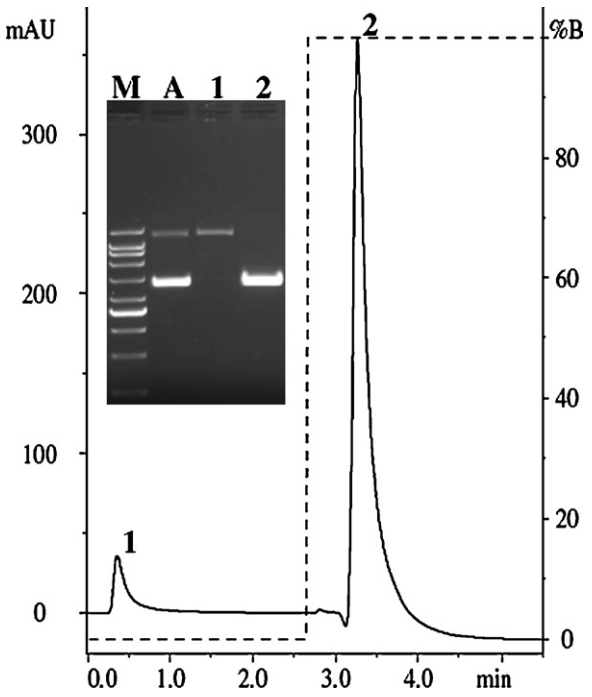

Fig. 1. Chromatographic purification of sc isoform pDNA using a non-grafted CDI monolith. Mobile phase – buffer A: 2.3 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 8.0; buffer B: 50 mM phosphate buffer pH 8.0. Step elution was performed at 132.5 cm/h (2.5 mL/min) by increasing the buffer B percentage in the eluent from 0% to 100% of buffer B, as represented by the dashed line. UV detection at 260 nm. Injection volume was 20 μL. Agarose gel electrophoresis analysis of each peak is represented in respective chromatogram. Lane M: molecular weight marker; lane A: pDNA sample injected onto the column (oc + sc); lane 1: oc; lane 2: sc.

The monolithic column was equilibrated with 2.3 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0) using a flow rate of 132.5 cm/h. After native plasmid DNA (oc and sc isoforms) sample injection and binding to the column, a first elution step designed to elute the species with lower affinity to the matrix was carried out with the same salt concentration of the equilibrium buffer. The elution of highly bound species was then performed with a second gradient step by decreasing to 0 M $(NH_4)_2SO_4$ (in 50 mM phosphate buffer, pH 8.0). The agarose gel electrophoresis analysis of the fractions eluting from the column (Fig. 1) proved that the first peak of unbound material corresponds to the oc isoform (lane 1), whereas the second peak was attributed to the sc isoform (lane 2). As judged by the gel, both isoforms were totally isolated and sc isoform appears completely purified. The fact of carbonyldiimidazole groups of monolith differentially interact with both plasmid isoforms at high concentration of ammonium sulfate (Fig. 1), evidenced by a stronger retention of sc isoform, can be tentatively explained by the supercoiling phenomenon. Hypothetical interactions of the CDI monolith with the pDNA backbone can be ruled out because phosphate and sugar groups are equally exposed in both isoforms. Under these ionic strength conditions the isoforms retention is mostly due to the hydrophobic interactions. Nevertheless, the observed selectivity between these different isoforms must be due to interactions of the imidazol ring with the bases of sc pDNA but not with the bases of oc pDNA. The reason for this behavior is the deformation induced by torsional strain in the sc isoform that becomes the bases of this nucleic acid more exposed than the bases of the oc isoform. Previous works related with affinity chromatography have revealed the same preference by this isoform [9,10]. Curiously, when the histidine–agarose matrix was applied to purify the sc isoform [9], the salt and ionic strength conditions used were the same to those used in the present work. Consid-

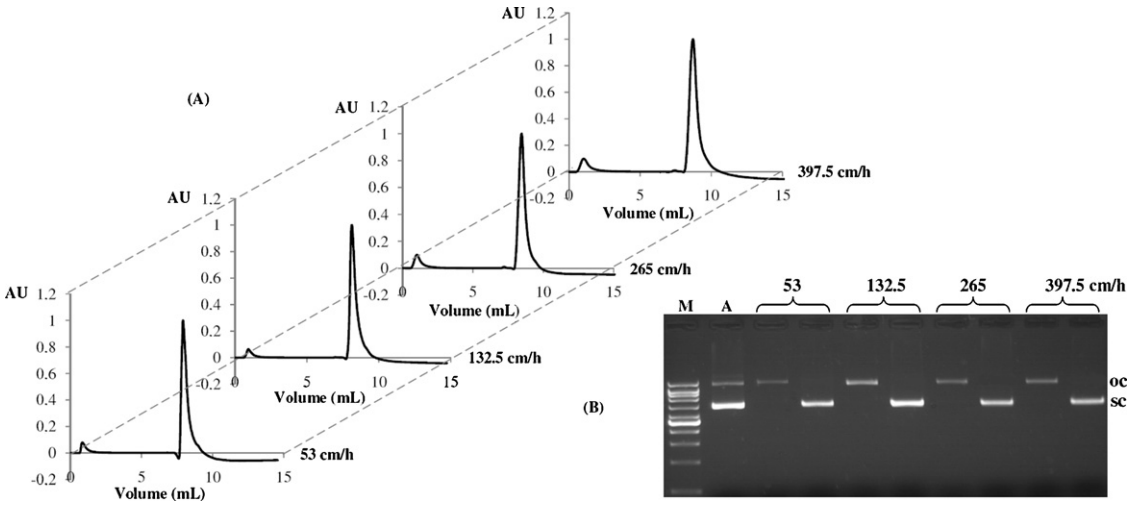

Fig. 2. (A) Effect of flow rate on resolution of pDNA isoforms. Experiments were performed in the same salt and gradient conditions applied for the sc isoform purification (presented in Fig. 1) at different flow rates (53, 132.5, 265 and 397.5 cm/h). (B) The peaks obtained in the chromatographic runs at different flow rates were identified by agarose gel electrophoresis. Lane M: molecular weight marker; lane A: pDNA sample injected onto the column (oc + sc).

ering that histidine matrix is composed by an epoxy spacer arm connected to a histidine amino acid, which is also constituted by an imidazole ring, it can be suggested that the recognition of sc isoform by both matrices, histidine–agarose and CDI–monolith, is mainly related with imidazole ring interactions. As it was described on histidine study [9], the interactions responsible for this specific recognition are mostly hydrophobic since high ammonium sulfate concentrations were used. Nevertheless, other elementary interaction forces that constitute the affinity interactions are also involved, such as (bidentate) H-bond interactions between the H donor and the H acceptor atoms in the nonprotonated imidazol ring with base edges, ring stacking/hydrophobic interactions, and water-mediated H bonds, that allow a stronger binding with sc plasmid bases.

3.2. Effect of flow rate on plasmid isoforms separation

Considering the physical and chemical constitution of methacrylate monolithic columns, high flow rates are expected to be used for semi-preparative or preparative purification of biomolecules as pDNA [16], and no change is expected on the separation selectivity. In fact, some studies proved that the molecules separation is flow-independent due to the monoliths characteristics [15,16]. Therefore, to verify the impact of flow rate on resolution of isoforms, the same elution gradient previously established for the ideal separation was used. Fig. 2(A) shows the resulting chromatograms for the separation of pDNA isoforms at the different flow rates; 53, 132.5, 265, and 397.5 cm/h. All chromatograms were normalized in function of the elution volume that was the same for all experiments performed, but a significant reduction on the chromatographic run time was verified when higher flow rates were used. The purity of each plasmid isoform separated with different flow rates was followed by electrophoresis as shown in Fig. 2(B). Evaluating the chromatograms and the respective electrophoresis, it is clearly evident that no changes have occurred in the separation efficiency of plasmid isoforms for the different flow rates under study. It is also possible to observe that the chromatograms overlap each other even at the highest applied flow rate of 397.5 cm/h. In this case the separation was completed in 2 min. This result is in accordance with other studies employing anion-exchange monoliths for pDNA purification where it was also verified good peaks resolution even at increased linear velocities [14].

3.3. Dynamic binding capacity determination

3.3.1. Effect of pDNA topology

The determination of column binding capacity is a critical component of the purification process development. Monoliths are known as versatile matrices that present good structural characteristics responsible for their high dynamic binding capacities and flow-unaffected resolution, when compared for instance with alternative agarose-based supports. In the present study, the influence of pDNA topology (oc and sc isoforms) on the binding capacity was evaluated. Breakthrough experiments were performed using feedstock solutions with the respective pDNA isoform at 0.05 mg/mL (Fig. 3). The feedstock used to study the effect of sc plasmid topology on capacity was obtained immediately after alkaline lysis and pre-purification with the Qiagen Kit. Although most of plasmid obtained presents sc conformation, a residual quantity of this isoform is converted into oc form, being obtained a solution with around 90% of sc isoform and 10% of oc isoform. On the other hand, when it

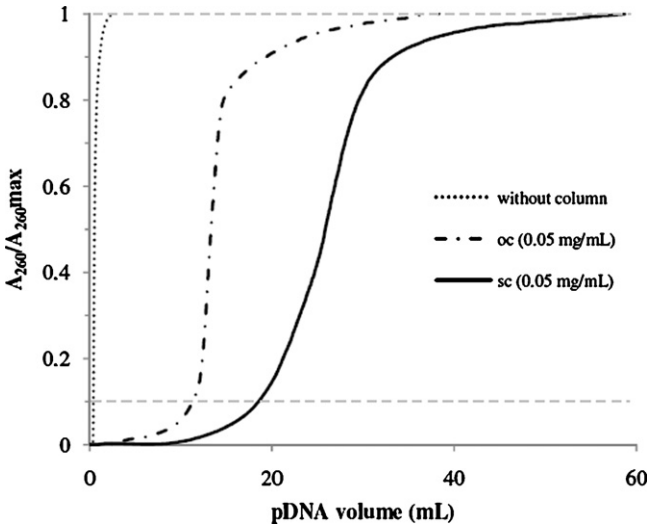

Fig. 3. Influence of plasmid topology on normalized breakthrough curves of CDI monolith. Representation of breakthrough experiments without column, open circular and supercoiled conformations. Flow rate: 53 cm/h (1 mL/min); feedstock: sc or oc plasmid solutions, at 0.05 mg/mL, were prepared in 2.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 8.0.

Table 1
Effect of sc plasmid concentration on total and 10% dynamic binding capacity of non-grafted CDI monolith. The breakthrough experiments were performed on a single monolithic disk with 0.34 mL, at flow rate of 53 cm/h (1 mL/min). The loading was constituted by sc isoform dissolved in 2.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer, pH 8.0.

Plasmid concentration (mg/mL)	DBC ± SD, $n = 2$	
	10%[a]	Total[b]
0.05	2.193 ± 0.003	3.640 ± 0.005
0.10	2.206 ± 0.004	3.900 ± 0.011
0.15	2.215 ± 0.003	4.412 ± 0.006
0.20	3.380 ± 0.006	6.359 ± 0.008

[a] Dynamic binding capacity at 10% breakthrough (mg/mL).
[b] Total dynamic binding capacity (mg/mL).

was studied the effect of oc conformation, sc form was converted to oc isoform by incubation at room temperature (24 °C). For this case, it was considered that the feedstock had almost 100% of oc isoform because after 3 days only this isoform appeared on the electrophoresis analysis. A significant difference in the binding capacity curves was observed for the two pDNA conformations, and the lower capacity was obtained with oc pDNA solutions. This behavior can be explained by the high supercoiled degree of sc isoform that enables the reduction of the superficial contact area, facilitating the binding of other biomolecules and improving the capacity. Thus, in this work, the breakthrough curve of oc isoform presents less capacity than sc isoform, probably because oc molecule has larger apparent size. These results strengthen the theory that the apparent size or conformation of the pDNA plays an important role in binding to chromatographic supports [27,28].

Analyzing the profile of the curves obtained in Fig. 3 it is observed that instead of the curves sharp increase, they present a typical S shape and a gradual creep up until the column was saturated. The profile obtained for both breakthrough curves of pDNA isoforms is also in agreement with the work of Mihelic and collaborators, who found out that some factors can lead to appearance of S shape curves and to the extension of the time required for equilibration [25]. These factors that affect and can decrease the dynamic binding capacity of monolithic matrices can be the increasing flow rates, solute size and high viscosity of mobile phases. Moreover, particular studies revealed that the increased capacity can be presumably associated to biomolecules agglomeration, also having a pronounced influence on the tailing of breakthrough curves, when it is applied the open-loop frontal analysis [29]. Thus, the higher dynamic binding capacity found for the sc pDNA isoform can also be related with the higher compactness of this biomolecule.

3.3.2. Effect of pDNA concentration

Since it was demonstrated that the feedstock of sc pDNA isoform allowed a higher capacity value, it is also interesting to verify whether different concentrations of the feedstock affect the CDI monolith capacity. For this purpose, several breakthrough experiments were performed using different pDNA concentrations (0.05, 0.1, 0.15 and 0.2 mg/mL), with the flow rate of 53 cm/h. The results presented in Table 1 show that dynamic binding capacity can be improved by increasing the sc pDNA concentration in the feedstock. For a concentration value of 0.05 mg/mL the monolith capacity was 3.640 mg/mL, whereas at 0.20 mg/mL the capacity increased to 6.359 mg sc pDNA/mL gel. These results are satisfactory when compared with other values described for the pDNA binding capacity (≈8 g/L [21]) obtained with a non-grafted dimethylaminoethyl (DEAE) anion-exchange methacrylate monolith. These intermediate capacity values are associated to the fact of being used non-grafted monoliths. Nevertheless, a comparative study between non-grafted and grafted DEAE weak anion-exchange methacrylate monoliths revealed an improvement on capacity, around 17 mg/mL, when it was used the grafted monolith [17]. These outcomes suggest that the CDI monolith capacity could be increased after grafting this monolith. On the other hand, the capacity obtained for this non-grafted CDI monolith is 6 or 12 times higher than the capacity achieved on histidine agarose-based supports with proteins (1 mg/mL) [30] or pDNA (0.5 mg/mL) [27], respectively. Therefore, the CDI monolith emerges as an interesting support to apply on affinity chromatography that is normally associated with low dynamic binding capacity.

The improvement on monolith capacity with the increase of sc pDNA concentration in the feedstock can be explained by the compaction degree that plasmid molecules suffer, leading to a significant reduction on the molecular apparent size, consequently increasing the surface area on the support for molecules adsorption. The compaction phenomenon can be related with the fact that plasmid molecules in more concentrated solutions suffer a significant compression and have a smaller radius of gyration [28]. On the other hand, it was also described that using high salt concentrations, the intermolecular repulsion between DNA molecules can be reduced, allowing DNA to pack more closely on the surface and leading to higher binding capacity [21]. These results are in accordance with previous plasmid-based works using histidine–agarose chromatography [27] and membrane chromatography or filtration [28,31].

At the end of each breakthrough experiments, the pDNA that remained bound to the monolith was recovered by decreasing the ionic strength of the elution buffer. Besides the target molecule was eluted in a small volume and in a concentrate form, also the contact time with the matrix was short. These conditions become an important criterion to be considered for the choice of the support to apply on pDNA purification, given that they avoid the pDNA degradation [14].

In order to quantitatively evaluate the column loading and the dissociation constant (K_d) from adsorption isotherm between the sc pDNA and CDI monolith support, frontal analysis chromatography was used, according to what is described in a recent publication [32]. This method assumes a Langmuirian behavior, and the K_d value was quantified with the equation $1/[C(V_R - V_M)] = (K_d/m_L)(1/C) + (1/m_L)$; where m_L is the total number of available binding sites in the column, V_M is the column void volume and V_R is the volume required to elute a continuously applied concentration of pDNA (C) from the column. The data were analyzed by linear regression. Through of equation we can plot $1/[C(V_R - V_M)]$ vs $1/C$ to obtain the ordinate intercept value corresponding to $1/m_L$ and the slope corresponding to K_d/m_L.

Using previous equation and the breakthrough experiments obtained with CDI monolith at different sc plasmid concentrations, the m_L value was found to be $2.50 \pm 0.23 \times 10^{-9}$ mol and K_d $4.81 \pm 0.21 \times 10^{-8}$ M. Previous works also developed a quantitative affinity chromatography technique for determination of the binding constants characterizing the biospecific adsorption and desorption phenomena [33]. In fact, the risks of irreversible biomolecules adsorption and denaturation are minimized when the value of K_d is between 10^{-4} and 10^{-8} M. By this way, the dissociation constant value obtained was near to 10^{-8} M, revealing a good affinity interaction between the ligand and pDNA, which indicates that CDI monolith is a good affinity support.

3.3.3. Effect of flow rate

To verify if a reduction in the flow rate utilized can result in an improvement of the binding capacity, some breakthrough experiments with a 0.1 mg/mL solution of sc pDNA were conducted at 26.5 and 53 cm/h (Fig. 4). Through analysis of normalized curves obtained for both flow rates, it is clear in Fig. 4 that to the experiment of lower flow rate (26.5 cm/h) is associated a slightly increase on the support capacity for pDNA binding. This result is in agree-

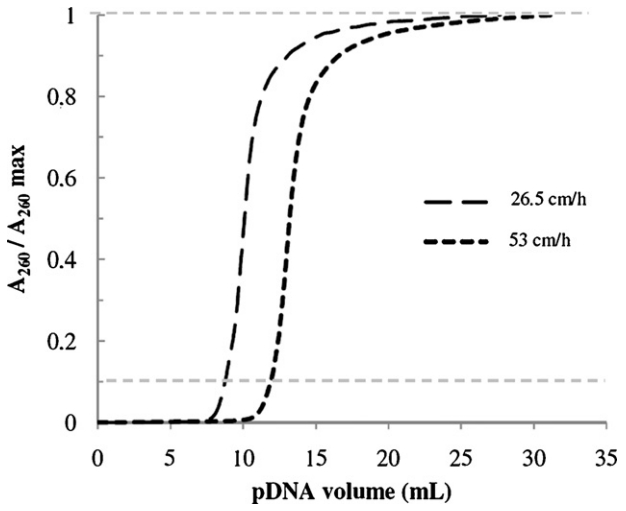

Fig. 4. Breakthrough curves of sc plasmid DNA in CDI monolithic support at different flow rates (26.5 and 53 cm/h). Feedstock: sc plasmid solution, at 0.1 mg/mL, was prepared in 2.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 8.0.

ment with a recent publication [27] since the effect of lower flow rate on the retention behavior of pDNA can be related with the increasing contact time between the matrix and the solutes, favoring the attainment of equilibrium and inducing a more efficient binding. Haber and collaborators also found out that sc pDNA becomes less extended at lower flow rates, thus occupying a smaller area and increasing the binding capacity [31].

Curiously, there are some disagreements in the literature about the effect on the flow rate. For example, Bencina et al. [21] demonstrated that flow velocity up to 700 cm/h has no effect on plasmid DBC. Also Krajnc et al. [34] recently reported flow unaffected capacity for a 39.4 kbp plasmid at linear velocity up to 300 cm/h. Otherwise, further extension of this range up to 1000 cm/h performed by Urthaler et al. [14] and Zochling et al. [35] indicated a small decrease of binding capacity, as well as happens in our study but only with a linear velocity of 53 cm/h.

4. Conclusions

The successful implementation of new pDNA-based therapeutic strategies is partially affected by the vector manufacturing process. Due to the singularity of plasmid molecules, its efficient purification is still challenging. The novel non-grafted monolithic support, employed in the present study for the pDNA purification, offers several potential advantages over traditional supports, including higher selectivity and productivity and good capacity. The separation process of plasmid isoforms through a CDI monolith has shown to be flow-independent, involving a specific recognition of the sc isoform. A maximum capacity was obtained when increased concentrations of sc plasmid conformation and decreased revealed linear velocities were used. The obtained K_d value, $4.81 \pm 0.21 \times 10^{-8}$, confirmed that CDI monolith support develops a good affinity interaction with pDNA, showing satisfactory affinity properties as a chromatographic support. Therefore, this glycidyl methacrylate monolith represents a robust purification matrix which enables efficient purification of sc isoform with a high purity degree. This fact opens new possibilities for further application of this chromatographic technology to efficiently isolate sc isoform from other pDNA isoforms and *E. coli* host impurities present in the clarified lysate.

Acknowledgements

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Paper VIII

Successful application of monolithic innovative technology using a carbonyldiimidazole disk to purify supercoiled plasmid DNA suitable for pharmaceutical applications

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Successful application of monolithic innovative technology using a carbonyldiimidazole disk to purify supercoiled plasmid DNA suitable for pharmaceutical applications

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ABSTRACT

The growing demand on plasmid DNA (pDNA) manufacture for therapeutic applications requires a final product with higher quality and quantity, spending the least time. Most of the current processes for pDNA production use at least one chromatographic step, which often constitutes a key-step in the purification sequence. Monolithic stationary phases are new alternatives to the conventional matrices, which offer fast separation of pDNA due to their excellent mass transfer properties and their high binding capacity for large molecules, as pDNA. However, the efficient recovery of pure pDNA focuses on a suitable balance of the feedstock, adsorbent and mobile phase properties. To satisfy the increasing demand for pharmaceutical grade plasmids, we developed a novel downstream process which overcomes the bottlenecks of common lab-scale techniques while complying with all regulatory requirements. This work reports an integrative approach using the carbonyldiimidazole monolith to efficiently purify the supercoiled (sc) pDNA active conformation from other plasmid topologies and *Escherichia coli* impurities present in a clarified lysate. The monolith specificity and selectivity was also assessed by performing experiments with plasmids of several sizes of 2.7, 6.05 and 7.4 kilo base pairs (kbp), verifying the applicability to purify different plasmids. Hence, the process yield of the pDNA purification step using the CDI monolith was 89%, with an extremely reduced level of impurities (endotoxins and gDNA), which was reflected in good transfection experiments of the sc plasmid DNA sample. Overall, the analytical results and transfection studies performed with the pDNA sample purified with this monolithic enabling technology, confirmed the suitability of this pDNA to be used in pharmaceutical applications.

1. Introduction

The rapid evolution of gene therapy and particularly of DNA vaccination promises to revolutionize the treatment of inherited and acquired diseases, by the development of specific DNA vectors in large quantities under pharmaceutical grade. Despite poor immunogenicity, advantages like safety, production simplicity and higher stability render non-viral vectors, such as plasmid DNA (pDNA), the most suitable alternative in relation to viral vectors [1,2]. However, an effective application of pDNA for therapeutic benefits has been hampered so far by issues associated with intracellular delivery, transfection efficiency and purity degree required for pDNA expression vectors to avoid adverse immune responses [3]. With regard to the implementation of pDNA vaccines, some investigations generated very encouraging results in the treatment of diseases like malaria and HIV [4,5].

Plasmid molecules are usually produced in *Escherichia coli* (*E. coli*) by fermentation, followed by alkaline cell lysis, primary isolation and purification steps [6]. Despite pDNA being a very stable biomolecule, during its manufacturing and recovery processes, it can undergo several types of stress that may disrupt its structural stability [7]. In this way, plasmid molecules that are essentially produced under the supercoiled (sc) isoform can also exist in a relaxed or open circular (oc) form, or other variants such as linear, denatured or dimeric conformations. Because the diversity of pDNA forms results from the damage of the sc isoform, and also because this is the only naturally intact form adapted to play an active role in eukaryotic cell physiology [8–10], the attainment of the isolated and purified sc plasmid isoform becomes essential. The challenges throughout the entire process are essentially aimed at eliminating host impurities that share common characteristics with pDNA, like negative charge (RNA, genomic DNA (gDNA), and endotoxins), molecular mass (gDNA and endotoxins) and hydrophobicity (endotoxins) [11], as well as at isolating the sc pDNA from the other plasmid conformational variants. These facts render more evident the need to develop and implement efficient

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methods to quickly capture, concentrate and purify the sc plasmid isoform.

Liquid chromatography is essential to the large-scale purification of plasmids, both as a process step and as an analytical tool. Over the last years several efforts have been made to develop adsorbents based on different chromatographic principles that would allow fast and efficient separation of pDNA [12]. However, some of these strategies apply stationary phases with poor selectivity towards pDNA and impurities, as well as to distinguish between the plasmid isoforms, due to the existing similarities. Recently, a new affinity chromatography approach, named amino acid–DNA affinity chromatography [6] was implemented to efficiently purify sc pDNA. This powerful technique is based on the application of amino acids as specific binding agents to analyze or purify biomolecules on the basis of their biological function or individual chemical structure [6,13,14]. The choice of amino acid ligands was also based on the computational models that explain the atomic and molecular forces that are predictable to occur between different amino acids and the different regions of nucleotides [15,16]. Although these ligands allowed for enhanced specificity and efficiency in the purification of plasmid DNA in a single unit operation [17,18], the conventional stationary phases still present some limitations.

Therefore, an innovative monolithic chromatography was recently implemented and characterized by our group, using the carbonyldiimidazole (CDI) disk that was already described to promote the separation of the plasmid isoforms [19]. Monoliths are now being considered the material of choice for the purification and analysis of proteins, pDNA and viruses [20–22] due to their appropriate structure. This continuous bed consists in a single piece of porous material, characterized by a highly interconnected network with large diameter [23]. In this way, all the mobile phase is forced to flow through to the channels via convection [24], resulting in a very fast transfer between mobile and stationary phases [25]. As large channels can easily accommodate large pDNA biomolecules, the monolithic supports offer a very high binding capacity for pDNA [19,23]. In general, the plasmid purity, recovery and peak resolution are coherently influenced by chromatographic residence time, which consequently can be changed by manipulating buffer flow rate and column length [26]. In accordance, for the optimal purification of larger biomolecules, the chromatographic column needs to be short, supporting higher flow rates [24,27] in order to reduce backpressure [28], unspecific binding, product degradation and minor changes in the structure of the biomolecule [29], without sacrificing resolution.

Knowing that the CDI monolithic disk is involved in a specific recognition mechanism with pDNA isoforms [19], it becomes interesting to test this support in an integrated process for the purification of sc pDNA isoform directly from a clarified *E. coli* lysate. The sc plasmid sample obtained should be free from the host contents in compliance with all regulatory requirements and capable of inducing gene expression with high efficiency [11,30]. The monolithic application also intends to overcome production bottlenecks, namely, low capacity for large molecules, sc form degradation resulting from extended retention time, and low process robustness due to flow dependent properties associated with conventional matrices. Finally, the applicability of the CDI monolith to purify pDNA molecules with different sizes was also investigated.

2. Experimental

2.1. General

2.1.1. Materials

All experiments were carried out in a 0.34 mL (3 mm thick and 12 mm diameter) non-grafted CDI poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith packed into a CIM disk housing, kindly provided by BIA Separations (Ljubljana, Slovenia). The Qiagen Plasmid Purification Maxi Kit was from Qiagen (Hilden, Germany). The ammonium sulfate used in the elution buffers was purchased from Panreac (Barcelona, Spain). All solutions were freshly prepared using ultra-pure grade deionized water, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and analytical grade reagents. The elution buffers were filtered through a 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany), and degassed ultrasonically. The iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used for genomic DNA quantification. Unless otherwise stated, the reagents used for A549, Hela and COS-7 cultures were obtained from Sigma (St Louis, MO, USA), and the reagents for the transfection experiments were obtained from Invitrogen (Carlsbad, CA, USA). The 2.7-kbp plasmid pUC19 and the 6.05-kbp plasmid pVAX1-*LacZ* were from Invitrogen (Carlsband, CA, USA). The 7.4-kbp plasmid pVAX1-*LacZ*gag was obtained by modification of pVAX1-*LacZ* with a 1.3-kbp insert [31]. The host strain used for obtaining the different plasmids and the several nucleic acids was *E. coli* DH5α.

2.1.2. Equipment

All preparative and analytical chromatographic experiments were performed using the ÄKTA purifier system (GE Healthcare Biosciences, Uppsala, Sweden) controlled by UNICORN software, Version 5.11. Agarose gels for electrophoresis analysis were revealed under UV light in a transilluminator system (ILC Lda, Lisbon, Portugal). Genomic DNA was quantified through real-time polymerase chain reaction (PCR) in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad).

2.2. Methods

2.2.1. Plasmids and bacterial culture

The plasmids amplification was obtained by *E. coli* DH5α fermentation, using a 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 kanamycin/mL for the cells transformed with pVAX1-*LacZ* and pVAX1-*LacZ*gag and 100 μg ampicillin/mL for the cells with pUC19. The bacterial growth was run overnight, at 37 °C under 250 rpm shaking, and the cells were harvested at the late log phase by centrifugation. Plasmid-free *E. coli* DH5α cells were also grown under the same conditions as described before, but with no antibiotic present.

2.2.2. Alkaline lysis and primary isolation of pVAX1-LacZ

E. coli cells were lysed using a modification of the alkaline method [32], as described by Diogo et al., 2000 [33]. Bacterial pellets (obtained from centrifugation of 250 mL cell broth at 5445 × *g* (30 min, 4 °C)) were thawed and resuspended in 20 mL of 50 mM glucose, 25 mM Tris–Cl, 10 mM ethylene-diamine tetraacetic acid (EDTA), pH 8.0. Alkaline lysis was performed by adding 20 mL of a 200 mM NaOH, 1% (w/v) sodium dodecylsulfate (SDS) solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated by adding and mixing 16 mL of prechilled 3 M potassium acetate, pH 5.0, (20-min incubation on ice). The precipitate was removed by centrifuging twice at 20,000 × *g* (30 min, 4 °C) with a Sigma 3–18 K centrifuge. The concentration and reduction of impurity content was performed according to a previously published method [33], given that the main objective of this work is to explore the purification strategy. Briefly, the pDNA in the supernatant was precipitated by adding 0.7 volumes of isopropanol and incubating on ice for 30 min. The pDNA was recovered by centrifugation at 16,000 × *g* (30 min, 4 °C). The pellets were then redissolved in 2 mL of 10 mM Tris–Cl buffer, pH 8.0. Next, ammonium sulfate was dissolved in the pDNA solution up

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to a final concentration of 2.5 M, followed by a 15-min incubation on ice. Precipitated proteins and RNA were removed by centrifugation at 16,000 × *g* (20 min, 4 °C). The supernatant was finally loaded directly on the chromatographic column.

2.2.3. Pre-purification of the plasmid species

To compare the applicability of the CDI disk to the isoforms separation of different plasmid samples less complex than the previously prepared, three plasmid species (pUC19, pVAX1-*LacZ* and pVAX1-*LacZ*gag) were purified using the Qiagen (Hilden, Germany) plasmid maxi kit, according to the manufacturer's instructions. The protocol is based on a modified alkaline lysis procedure. Following lysis, binding of pDNA to the Qiagen anion exchange resin is promoted under appropriate low-salt and pH conditions. Impurities are removed by a medium-salt wash. The plasmid DNA is eluted in a high-salt buffer and then concentrated by isopropanol precipitation.

2.2.4. Isolation of host nucleic acids and preparation of the linear pVAX1-LacZ sample

Nucleic acids (gDNA and RNA) were isolated from pDNA-free DH5α *E. coli* cells. Genomic DNA was isolated using the Wizard genomic DNA purification kit from Promega (Madison, WI, USA), according to the manufacturer's instructions. For RNA isolation, the cells were lysed by a modification of the alkaline method [32]. The resulting lysate was clarified by ammonium acetate precipitation and the nucleic acids were concentrated by polyethylene glycol 6000 precipitation, as previously described [34]. The linear pVAX1-*LacZ* sample was prepared by enzymatic digestion with the Hind III enzyme through a 1-h incubation at 37 °C.

2.2.5. Preparative chromatography

The previously characterized CDI monolith [19], was equilibrated with 2.2 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0) and the system was run at a flow rate of 1 mL/min. The lysate sample containing pVAX1-*LacZ* resulting from the ammonium sulfate precipitation was then injected (loop with 20 μL) in the monolith at the same flow rate. The absorbance of the eluate was continuously monitored at 260 nm. After elution of unbound species, the ionic strength of the buffer was decreased stepwise to 1.84 M of ammonium sulfate and then to 1.27 M of the same buffer. Finally, the monolithic column was washed with 50 mM of phosphate buffer, pH 8.0. The fractions were pooled according to the chromatogram obtained, and used for further electrophoresis analyzes after concentration and desalting with Vivaspin concentrators (Vivascience). Some studies were performed by loading increased amounts of the lysate sample (20, 50, 100, 200, 500 and 1000 μL) in the CDI monolith aimed at observing the influence of loaded sample in the purification efficiency. Another additional study was conducted to observe the retention behavior of the main impurities present in the *E. coli* lysate, namely, linear pDNA and host nucleic acids (gDNA and RNA), in the CDI monolith using the same elution gradient described above. All experiments were performed at room temperature.

2.2.6. Agarose gel electrophoresis

Pooled fractions were analyzed by horizontal electrophoresis using 15 cm, 1% agarose (Hoefer, San Francisco, CA, USA) gels, stained with ethidium bromide (0.5 μg/mL). Electrophoresis was carried out at 100 V, for 40 min, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). Lane density measurements were performed with Bio-Rad Quantity One® software (Hercules, CA, USA) using the lane analysis toolbox.

2.2.7. Plasmid DNA quantitative analysis

The pDNA concentration and purity were evaluated after each separation experiment for the fractions pooled following chromatography, according to an adaptation of the previously described method [35]. A 4.6/100 mm HIC Source 15 PHE PE column (Amersham Biosciences) was connected to an ÄKTA Purifier system. A gradient elution with decreasing salt concentration was used. Buffer A was 1.5 M $(NH_4)_2SO_4$ in 10 mM Tris buffer, pH 8.0 and buffer B was 10 mM Tris buffer, pH 8.0. Initially, the column was equilibrated with 0% of buffer B during 10 min. Subsequently, 20 μL of a sample suitably diluted in the equilibration buffer were injected and eluted at a flow rate of 1 ml/min. After the injection of the sample, all unbound material was eluted with 0% of buffer B for 2 min, and then the elution buffer was instantaneously changed to 100% buffer B. The last elution condition was maintained for 8 min to elute bound species. Next, the column was re-equilibrated for 10 min with the equilibration buffer to prepare the column for the next run. The absorbance of the eluate was recorded at 260 nm. The concentration of pDNA in each sample was calculated using a calibration curve constructed with appropriate pDNA standards (1–400 μg/mL) purified with a Qiagen commercial kit (Hilden, Germany). The purity degree was defined as the percentage of the pDNA peak area related to the total area (area of all peaks on the chromatogram).

2.2.8. Plasmid DNA qualitative analysis

The plasmid DNA qualitative analysis consists in the quantification of the levels of impurities, such as proteins, endotoxins and gDNA, present in the final sc pDNA sample purified with the CDI monolith. Protein concentration was measured with the micro-BCA (bicinchoninic acid) protein assay kit from Pierce (Rockford, USA), according to the manufacturer's instructions. A fraction of each sample (50 μL) was added to 200 μL of BCA reagent in a microplate and incubated for 30 min at 60 °C. A calibration curve was constructed with Bovine Serum Albumin (BSA) as the protein standard (0.01–0.1 mg/mL), and using 50 mM phosphate buffer pH 8.0 to dilute the samples. Samples with high salt concentration were desalted against 50 mM phosphate buffer pH 8.0 before the analysis.

Endotoxin contamination was assessed using the ToxinSensor™ Chromogenic *Limulus* Amoebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript, USA, Inc.) according to the manufacturer's instructions. The calibration curve (from 0.005 to 0.1 EU/mL) was performed with a 6 EU/mL stock solution provided in the kit. To avoid external endotoxin interference, samples to be analyzed and samples from the kit were diluted or dissolved, respectively, with non-pyrogenic water, which was also used as the blank. All tubes and tips used to perform this quantification were endotoxin-free and the entire procedure was performed inside of a laminar flow cabinet.

Genomic DNA was quantified through real-time polymerase chain reaction (PCR), as described previously [36]. The sample collected after lysis was diluted 100-fold beforehand. Sense (5′-ACACGGTCCAGAACTCCTACG-3′) and antisense (5′-CCGGTGCTTCTTCTGCGGGTAACGTCA-3′) primers were used to amplify a 181-bp fragment of the 16S rRNA gene. PCR amplicons were quantified by following changes in fluorescence of the DNA binding dye Syber Green I. The gDNA concentration was determined using a calibration curve generated by serial dilution of *E. coli* DH5α gDNA (purified with the Wizard gDNA purification kit; Promega) in the range of 5 pg to 50 ng/μL. Negative controls (no template) were run at the same time of each analysis.

2.2.9. In vitro transfection experiments

Hela and COS-7 cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's

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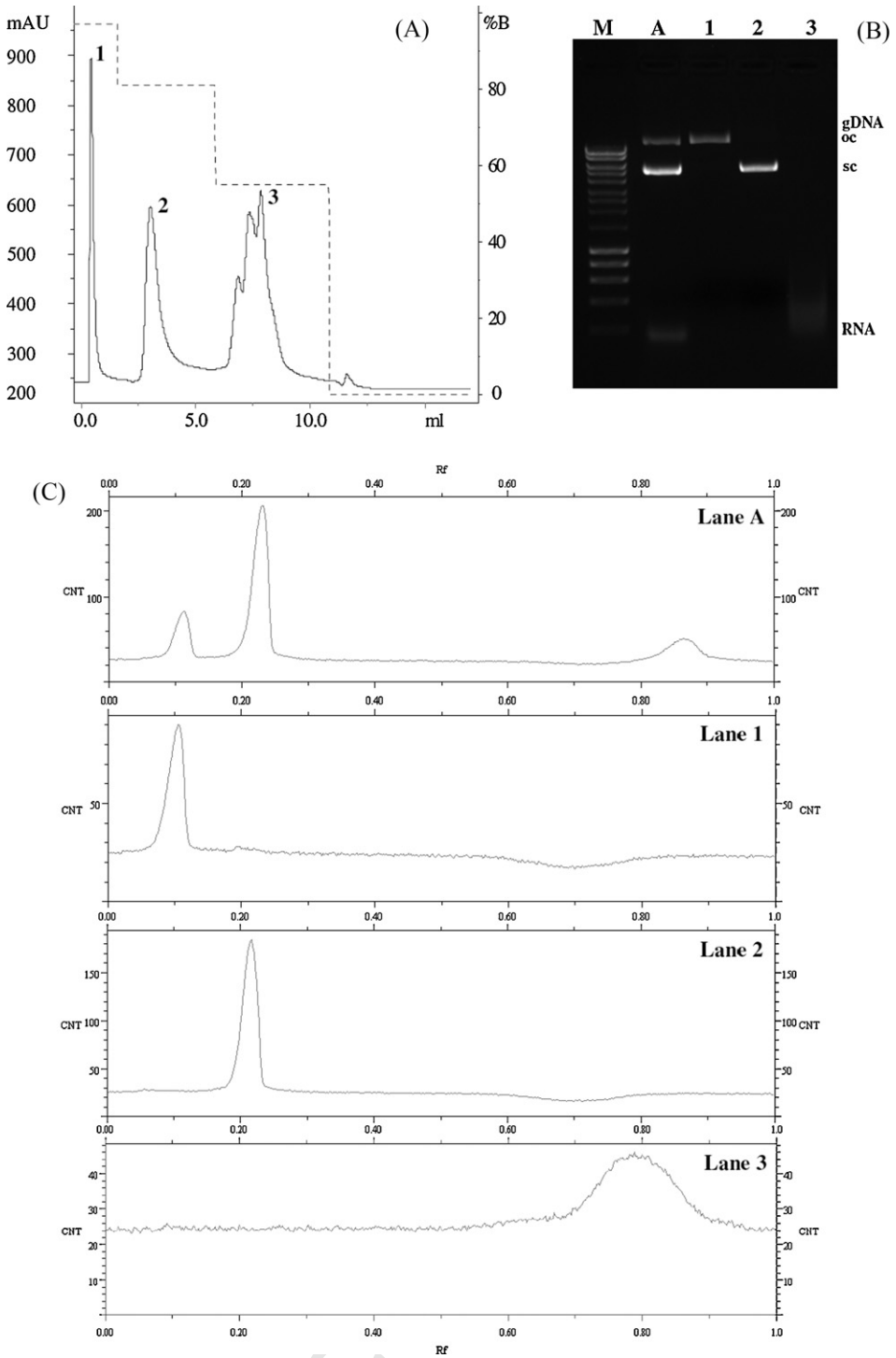

Fig. 1. (A) Chromatogram showing the selective purification of sc pDNA isoform from a clarified *E. coli* lysate using CDI monolith-chromatography. Mobile phase—buffer A: 50 mM phosphate buffer pH 8.0; buffer B: 2.3 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 8.0. Sample elution was performed at 1 mL/min by stepwise gradient decreasing the ammonium sulfate concentration, as represented by the dashed line. UV detection at 260 nm. Injection volume was 20 μL. (B) Agarose gel electrophoresis analysis of the samples recovered from each peak of the chromatogram. Lane M: molecular weight marker; lane A: feed sample injected onto the column. Fractions corresponding to peaks 1, 2 and 3 are shown in lanes 1, 2 and 3, respectively. (C) Density analysis of the corresponding lanes of each sample analyzed by agarose gel electrophoresis.

Modified Eagle's Medium (DMEM), whereas the A549 cell line was cultured in Ham's F12K medium. All media had the pH controlled at 7.4, containing 100 units/mL antibiotic/antimycotic (penicillin/streptomycin), supplemented with 10% heat activated Fetal Bovine Serum (FBS) (Biochrom AG, Berlin, Germany), at 37 °C in a 5% CO_2 humidified atmosphere.

One day prior to transfection, the cells were seeded on 24-well plates at a density of 2.5×10^4 cells/well in 500 μL of the respective medium without antibiotic (to promote transfection) and incubated during 20 h. Immediately before transfection, cells were rinsed and supplemented with 500 μL of fresh culture medium without antibiotic at pH 7.4, whereas pDNA/lipofectamine complexes were prepared as briefly described below. Plasmid

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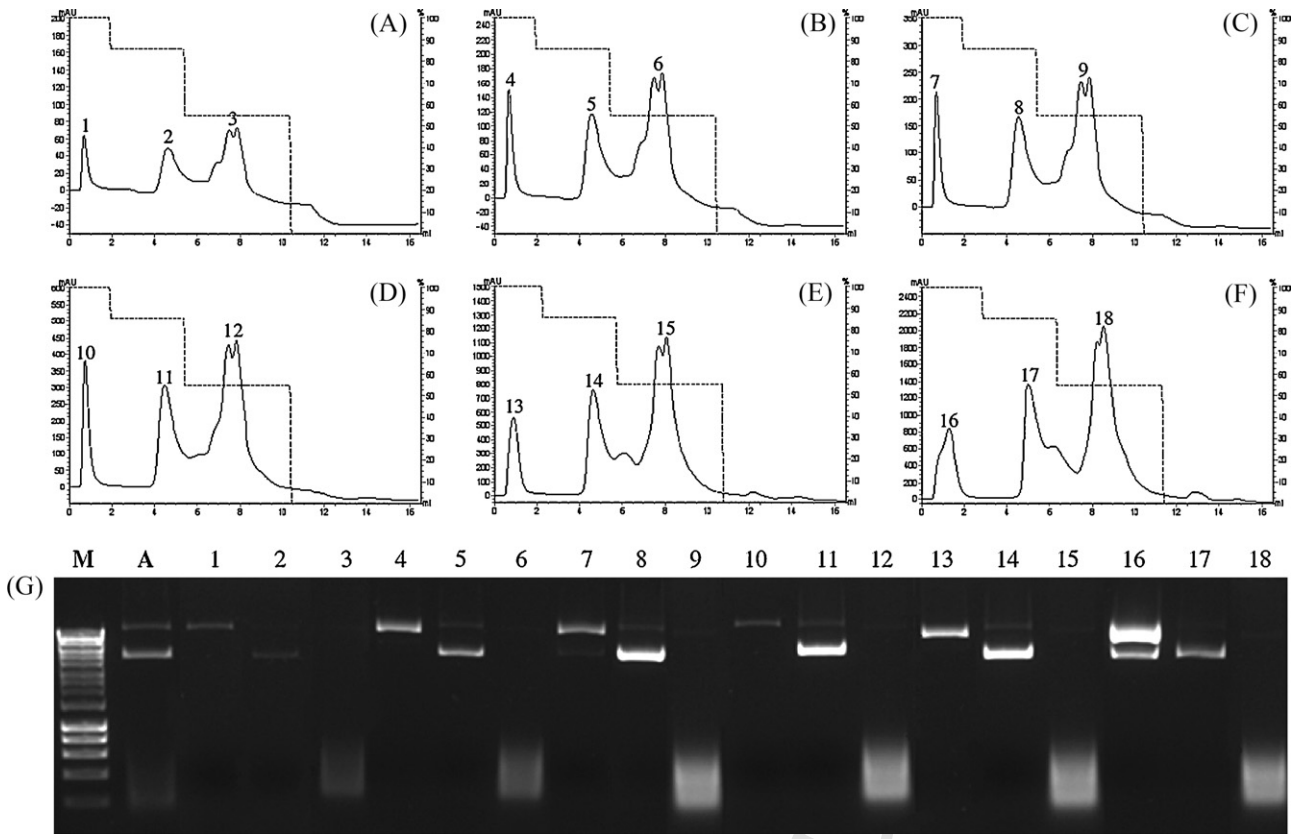

Fig. 2. Chromatographic profiles obtained by injection of increased *E. coli* lysate volumes onto the CDI monolith: (A) 20 μL; (B) 50 μL; (C) 100 μL; (D) 200 μL; (E) 500 μL and (F) 1000 μL. (G) Agarose gel electrophoresis analysis of the all peaks recovered from the chromatographic runs. Lane M: molecular weight marker; lane A: feed sample injected onto the column. Lanes 1–18: Fractions recovered from peaks 1–18, respectively.

solutions (pVAX1-*LacZ*) and appropriate volumes of Lipofectamine™ 2000 were separately diluted in 50 μL (for each transfection assay) of Opti-MEM® I medium, according to the manufacturer's instructions. After a 5-min incubation of lipofectamine at room temperature (≈24 °C), the respective pDNA solution was added and the mixture was incubated for 20 min at room temperature (≈24 °C) to allow the formulation of pDNA/lipofectamine complexes. A fraction of the mixture (100 μL) was then added to each well containing cells and medium. The cells were incubated at 37 °C under a 5% CO_2 atmosphere. After a 6-h incubation, the medium and complexes were removed, and the cells were rinsed once with PBS and cultured with antibiotic and 10% FBS supplemented medium (DMEM).

The transfection efficiency was determined after 24 h of transfection by using the β-Gal Staining Kit. This method enables the determination of the efficiency of cell transfection with pVAX1-*LacZ*, because the *lacZ* gene product, β-galactosidase, catalyses the hydrolysis of β-galactosides such as X-gal, producing a blue color that can be visualized under the microscope. The transfection efficiency is determined as the percentage of blue cells compared to the total cell number.

3. Results and discussion

3.1. Preparative chromatography of sc pDNA using a CDI monolith

Beforehand, a preliminary screen was performed to choose the best salt concentration range for a differential retention/elution pattern of the several nucleic acids present in a clarified *E. coli* lysate sample (data not shown). Linear and stepwise gradients were tested, and it was determined that the stepwise elution is more suitable to obtain the nucleic acids separation, using lower salt concentrations [37]. The chromatographic profile of the sc pDNA purification from a clarified *E. coli* lysate, using a CDI monolithic column, is presented in Fig. 1(A). Initially, the column was equilibrated with 2.2 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 8.0. After injection of the pDNA-containing clarified lysate sample (20 μL), a first peak was obtained with the same salt concentration of the equilibrium buffer, resulting from the elution of unbound material. After, the ionic strength of elution buffer was decreased to 1.84 M of ammonium sulfate in order to partially elute specific molecules in a second peak. The chromatographic run was concluded through a final elution step using 1.27 M of ammonium sulfate to elute the remaining bound nucleic acids, and also with an additional washing step with 50 mM of phosphate buffer to verify if all the species were eluted, as represented by the dashed line. As shown in Fig. 1(A), all the nucleic acids are isolated after 10 min. This result is in accordance with the advantages presented by the monolithic supports [23,24], given that the chromatographic runs are completed in less time than the used in a conventional matrix to the same purpose, where the chromatographic run usually takes about 120 min (12-fold higher) [17].

In order to establish a correlation between the several nucleic acids present in the clarified *E. coli* sample, and the peaks in the chromatogram, an agarose gel electrophoresis was performed (Fig. 1(B)). The analysis of the results revealed that the elution of the oc isoform occurs in the first peak (Fig. 1(B), lane 1), at high ionic strength. On the other hand, the elution of the sc isoform and RNA species only occurs with the decrease of ionic strength, being eluted in the second and third peaks, respectively (Fig. 1(B),

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lanes 2 and 3), corresponding to an increased retention at the CDI disk. Additionally, the results presented in Fig. 1(B) also suggest that the sc pDNA isoform was recovered with 100% homogeneity. Hence, to further support this hypothesis, lane density was also evaluated, and as Fig. 1(C) shows, the density peak of lane 2 indeed corresponds only to the sc isoform, that is therefore recovered with maximum purity, preserving the structural characteristics. These results are in accordance with a previous study [19] and show that the plasmid pool recovered in the peak 2 contains the sc isoform with high purity degree. Furthermore, the structural characteristics of short monolithic columns also explain these results, since the reduced contact time of the target molecule with the chromatographic matrix avoids the structural changes and pDNA degradation [29].

The global aim of the present work was to isolate and purify the sc pDNA isoform from a complex *E. coli* lysate under a suitable pharmaceutical-grade for further therapeutic applications. In this way, the knowledge of the maximum amount of sample that can be loaded onto the monolith using the established elution conditions and its influence in the plasmid purification efficiency arise as important parameters to be considered for the successful application of the CDI monolithic column on preparative chromatography of sc pDNA. To study this effect, increased amounts of the clarified lysate sample were loaded onto the CDI disk, using the same elution strategy described above (Fig. 2). The injection of increased sample volumes up to 500 μL (Fig. 2(E)), allowed the separation of the several nucleic acids, as it is possible to observe in the electrophoresis (Fig. 2(G), lanes 1–15). On the other hand, the injection of 1000 μL of sample seems to exceed the monolith capacity, there being observed a slight change in the chromatographic profile of the first peak (Fig. 2(F)). By the agarose electrophoresis analysis, it was revealed a partial elution of the sc pDNA isoform along with the oc isoform in the first peak (lane 16 of Fig. 2(G)). Although a small part of the loaded sample was eluted in the flowthrough without interacting with the matrix, the sc pDNA and RNA molecules that occupied the binding sites of the support were separated afterwards. The monolithic supports are described to present high dynamic binding capacity for pDNA molecules [19,38]. However, it is also important to adjust the elution conditions, when increased sample volumes are loaded, in order to optimize the monolith binding capacity for complex lysate samples.

The injection of the linear pDNA sample previously prepared by enzymatic digestion of pVAX1-*LacZ* purified with the Qiagen commercial kit, and pure gDNA and RNA samples isolated from pDNA-free *E. coli* DH5α cells was accomplished to confirm the elution tendency of these impurities and to compare with the three elution peaks present in the chromatogram of Fig. 1(A). Elution was carried out under the same conditions described above for the sc pDNA isolation (Fig. 3). The results obtained indicate that both linear pDNA and host gDNA impurities are eluted at high salt concentration, in the same elution circumstances of the oc isoform (Fig. 3(B)). Under these conditions, it was not verified an effective interaction of these impurities with the CDI monolith, it being eluted in the flowthrough. On the other hand, the RNA molecules remain retained at high salt concentrations, being mostly eluted at 1.27 M of ammonium sulfate (Fig. 3(C)), as it happens with the RNA from the *E. coli* extract in Fig. 1(A).

The data show that, under the established elution conditions, the CDI monolithic matrix interacts differently with the several nucleic acids present in the *E. coli* lysate sample. Thus, the monolith retains preferentially the RNA, as well as the sc pDNA isoform, but with less intensity, and there are no evidenced interactions with the oc isoform and gDNA molecules. The preference of the CDI monolith for the sc plasmid isoform, in comparison to the oc isoform, was already explained in the previous work [19]. Although the oc and sc isoforms are essentially double-stranded, the sc

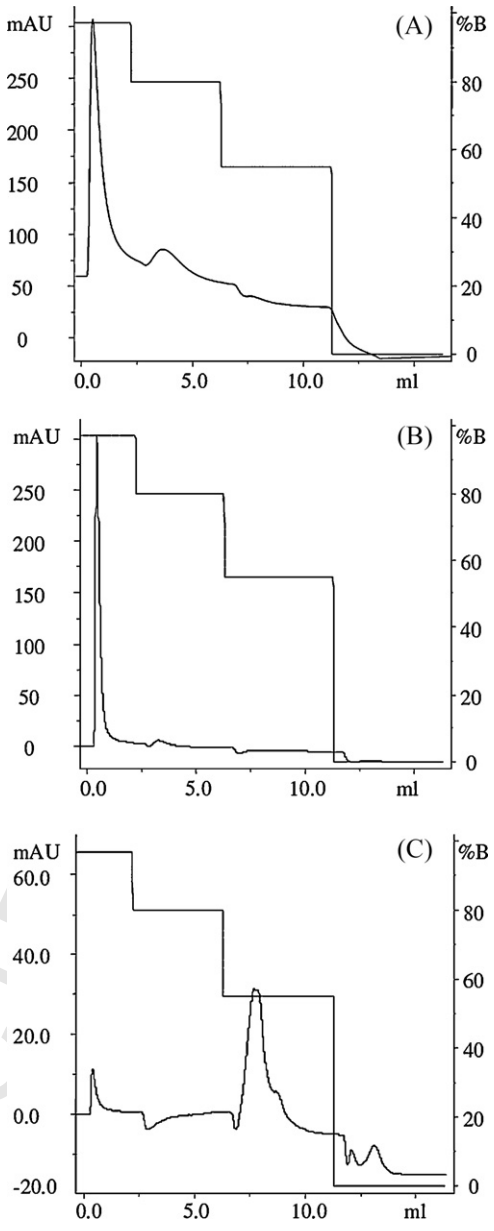

Fig. 3. Chromatographic representations of the elution behavior of different impurities, such as linear pDNA (A), *E. coli* gDNA (B) and RNA (C) using the CDI-monolithic column. The elution strategy used was the same of the previously established for the purification of sc pDNA isoform from a clarified *E. coli* lysate presented in Fig. 1.

pDNA can be specifically retained to the imidazole rings present in the CDI monolith due to a higher degree of base exposure promoted by the torsion and supercoiling phenomenon suffered in this plasmid conformational structure [19]. Therefore, the CDI ligands can develop different interactions responsible for the biorecognition of the sc plasmid isoform, namely hydrophobic interactions, but also hydrogen bonds and water mediated H bonds with the more exposed bases, as well as the electrostatic interactions with DNA backbone. The similar retention behavior found between gDNA and oc pDNA molecules can be related to the inadequate exposure of the nucleic acid bases of the oc isoform and the double-stranded fragments of the gDNA molecule denatured during its alkaline lysis [39]. Likewise for RNA, the stronger interaction of these molecules to the CDI monolith can also be related to bases that are more exposed to interact with the groups available, given the single-strand nature of RNA molecules. Other affinity-based methodologies, like immobilized metal affinity chromatography

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(IMAC) studies are in accordance with this tendency [40,41]. The double-stranded nucleic acids such as plasmid, gDNA and oligonucleotides duplexes showed low IMAC binding affinity, whereas RNA and single-stranded oligonucleotides bind strongly to IMAC matrices.

The chemistry of the CDI monolith used in the present study was prepared by BIA Separations with the purpose to be used for further immobilization procedures. However, the application of this non-grafted monolith directly on purification strategies has revealed that the CDI disk is also a suitable chromatographic matrix which presents high capacity, selectivity to separate several nucleic acids, and efficiency to purify the sc plasmid isoform. Furthermore, the alternative to perform a posterior immobilization of specific ligands to the CDI monolith gives the possibility to explore new conditions that can lead to increased capacity, selectivity or improvement of some present limitations such as the higher ammonium sulfate concentration used.

3.2. Analytic parameters for plasmid quality assessment

According to regulatory agencies, such as the Food and Drug Administration (FDA), the sc pDNA sample for pharmaceutical applications should be free from host impurities, with regard to the following criteria: appearance (clear, colorless solution), plasmid homogeneity (>97% supercoiled), proteins (not detectable, by micro-BCA method), RNA (not detectable by 0.8% agarose gel), gDNA (<2 μg/mg plasmid, PCR) and endotoxin (<0.1 EU/ng plasmid, LAL assay) [11,30,42].

Initially, the concentration and quality of the sc pDNA sample isolated by the CDI monolith were evaluated by high-performance hydrophobic interaction chromatography (Fig. 4) according to the method previously described [35]. The analytical chromatogram in Fig. 4(A) representative of the profile of the *E. coli* lysate, showed the elution of pDNA in the first peak, at 0.7 min [35], and a large amount and variety of impurities that are eluted later in a series of peaks. Comparing these results with Fig. 4(B) from the feed sample that was injected onto the monolithic column, it is evident that the isopropanol concentration and the ammonium sulfate-based clarification, performed throughout the process to obtain the pDNA sample, contribute significantly to reduce the impurity content [43]. When the sc pDNA sample recovered from the second peak (Fig. 1(A)) of the CDI monolith chromatography was analyzed (Fig. 4(C)), only a single plasmid peak appeared in the chromatogram. The interferences present after the elution of the sc pDNA are due to the injection effect and the change of elution buffer, as it is proved in the negative control, obtained with the injection of binding buffer (Fig. 4(D)). Therefore, the analytical chromatograms clearly show a gradual reduction of impurities throughout the isolation process until the sc plasmid isoform is obtained totally purified by the CDI monolith, confirming the result obtained by agarose electrophoresis, where neither RNA nor other host impurities were observed.

The analytical data allow us to assess the pDNA purity, the purification factor and the process yield (Table 1) achieved for the pDNA purification step using the CDI monolith. The plasmid purity of the feed sample injected in the monolithic column was determined from the ratio between the pDNA peak area and the sum of all the peak areas that appear in the HPLC chromatogram, there being achieved a value of 37%. On the other hand, the HPLC analysis of the sc pDNA recovered in the second peak (Fig. 1) only revealed the presence of the pDNA peak corresponding to approximately 100% of purity (Table 1). This result is in agreement with previous results and supports the hypothesis that the CDI monolithic matrix has a capacity and selectivity to purify the sc pDNA from an impure lysate sample and can be used as a final purification step. Exploiting the analytical data, it is also possible to determine the yield of the purification process, through the ratio between the pDNA mass present in the recovered sample and in the feed sample. As shown in Table 1, from 3.94 μg of pDNA contained in the feed sample, 2.93 μg of the sc plasmid were recovered and totally isolated from 0.56 μg of the oc isoform. Consequently, the total amount of pDNA (oc and sc isoforms) recovered in the monolithic purification step was 3.49 μg corresponding to a global yield of 89%, of which 74% resulted from the sc isoform purification step (Table 1). The partial loss of pDNA can be related to the concentration and desalting step performed with Vivaspin concentrators. Thus, the 74% sc pDNA yield achieved with the CDI monolith is significantly higher than the 45% of sc pDNA achieved with the conventional histidine-affinity matrix [17], or the 62% if using a macroporous material [44], and is similar to the 75% yield when anion exchange monolithic disks are used [23,45].

The protein quantification in the purified oc and sc plasmid pools was performed by using the micro-BCA method and it was verified that the proteins were not detected, as required by regulatory agencies [42]. The same was achieved for the third peak of RNA (Table 2). However, the significant reduction that occurred with the ammonium precipitation suggests that this step was quite efficient, and the residual amount of proteins was eliminated during the purification procedure.

The results shown in Table 2 indicate that a significant reduction of endotoxins content was also obtained throughout the alkaline lysis process and the chromatographic step with the CDI monolith, given that the feed sample had 202.21 EU/μg of pDNA, the clarified sample injected on the monolithic column had 18.87 EU/μg of pDNA, and the sc plasmid fraction had 0.1 EU/μg of pDNA. The endotoxins level in the final sample of sc pDNA is in compliance with the regulatory agencies specifications [11]. The first endotoxins reduction event can be related to endotoxins precipitation along with proteins during the ammonium sulfate precipitation step [43]. However, endotoxins were essentially cleared during the subsequent CDI monolithic purification step, with a purification factor of sc pDNA relatively to LPS of 189 times. This significant reduction is much more effective in comparison with other studies that described endotoxins removal by conventional matrices with immobilized amino acids [17,18,46].

The gDNA quantification was performed by real-time PCR that is a robust and more sensitive method when compared to the established Southern slot-blot method. The reduction of gDNA throughout the process is also shown in Table 2. The efficiency of the CDI monolith to eliminate gDNA was really significant because the residual amount of this molecule in the sc plasmid fraction was 0.57 ng gDNA/μg pDNA, which is lower than the 80 ng gDNA/μg pDNA obtained with anion exchange disk [23], and also inferior to the 2 ng gDNA/μg pDNA recommended by regulatory authorities [11,42].

3.3. Comparison of pDNA transfection efficiency

The presence of endotoxins in the sc pDNA sample for therapeutic applications is undesirable because the transfection efficiency can be drastically decreased in different cell lines [12]. Additionally, other concerns are associated with the presence of gDNA in plasmid preparations because of the risk of insertion, further stressing the need to reduce this impurity to the lowest possible level [8]. Therefore, *in vitro* additional studies of transfection efficiency were conducted to investigate the biological effects of residual impurities present in the sc plasmid sample. Three different cell lines were used to certify the tendency of transfection efficiency of the several plasmid samples used.

In Fig. 5 it is presented the transfection efficiency, reflected in the expression level of the β-galactosidase, oc and sc plasmid pVAX1-*LacZ* samples resulting from the purification step of the

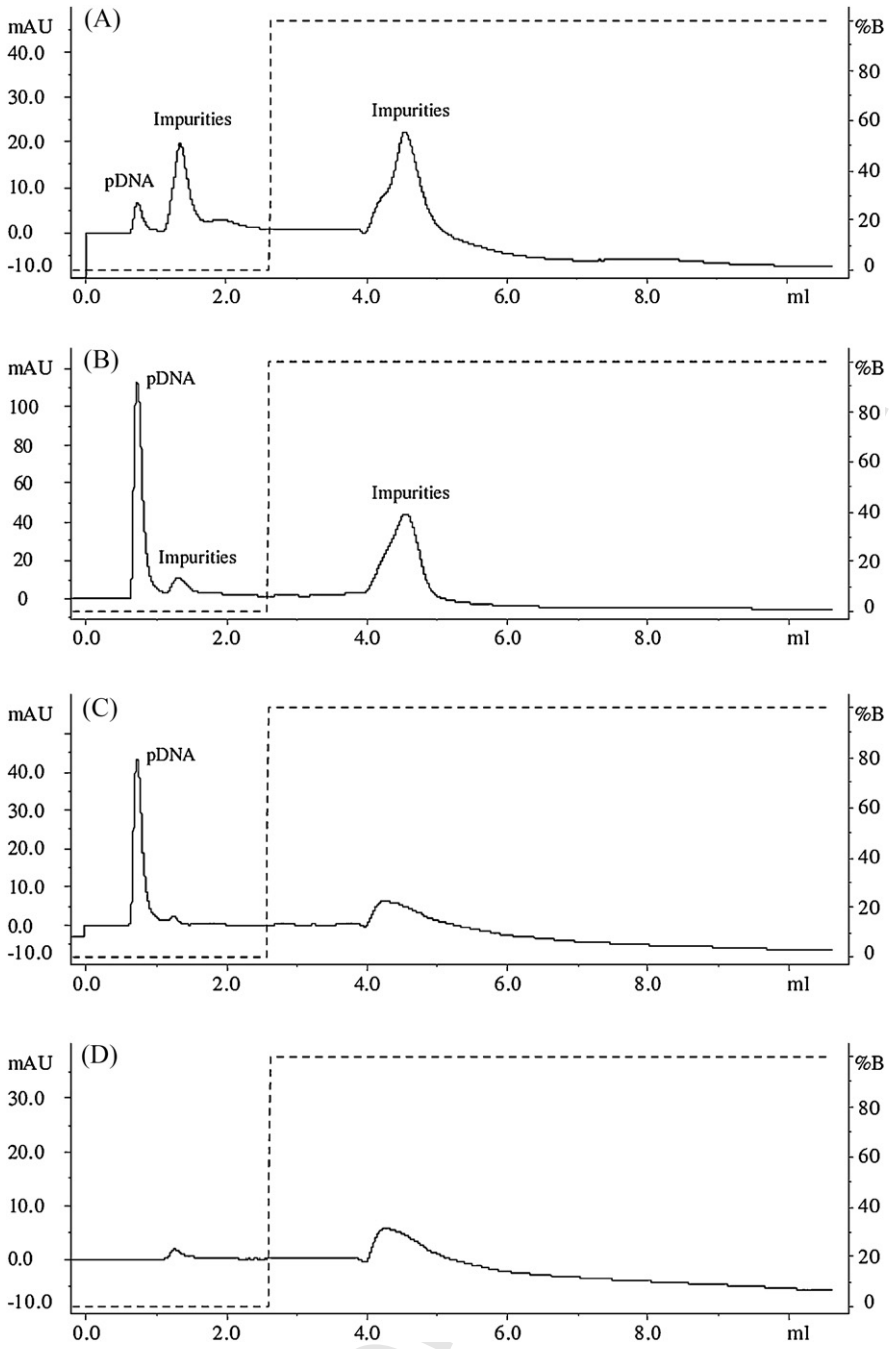

Fig. 4. Analytical chromatographic profiles of several pDNA-containing samples recovered at different steps of the purification process. (A) *E. coli* lysate; (B) feed sample injected onto the column; (C) sc isoform fraction (peak 2 in Fig. 1); and (D) zero (injection of binding buffer).

Table 1
Performance of CDI monolithic column on the purification of sc pDNA from *E. coli* lysate samples.

Process step	Volume (mL)	pDNA		HPLC purity (%)	Purification factor	Step yield (%)
		(μg/mL)	(μg)			
Feed sample	0.02	196.79	3.94	36.82	–	–
CDI monolith chromatography						
Peak 1 (oc)	0.08	6.97	0.56	82.70	2.25	14.20
Peak 2 (sc)	0.08	36.64	2.93	100	2.72	74.40

These values were obtained by HPLC analysis of plasmid solutions previous and after the purification step.

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Table 2
Quantitative analysis of the proteins, gDNA and endotoxins in the sc pDNA sample collected in several steps of the purification process.

Sample	[Protein] (mg/mL)	Endotoxin		gDNA	
		(EU/mL)	(EU/μg of pDNA)	(ng/mL)	(ng/μg of pDNA)
Primary isolation					
Alkaline lysis	3.64	7409	202.21	66530	1815.78
$(NH_4)_2SO_4$ precipitation	0.40	691.25	18.87	12750	347.84
CDI monolith chromatography					
Peak 1 (oc)	Undetectable	6.56	0.18	23.80	0.65
Peak 2 (sc)	Undetectable	3.84	0.10	20.70	0.57
Peak 3 (RNA)	Undetectable	0.31	–[a]	17	–[a]

[a] No pDNA is present in these fractions.

E. coli clarified lysate with the CDI monolith, in comparison with the efficiency of a native plasmid control sample purified with a commercial kit based on anion-exchange chromatography, using the cell lines COS-7, Hela and A549 (Fig. 5(A), (B), and (C), respectively). To complete the information, Fig. 6 shows the percentage values of transfection efficiency of the three different plasmid samples applied in the same conditions at three cell lines. The supercoiled pDNA sample was able to transfect a higher number of COS-7 cells (59%), whereas the native plasmid sample (oc + sc) transfected less quantity (26%), and the oc plasmid sample transfected the lowest quantity (16%) (Fig. 5(A)). Likewise, for the other cell lines, the transfection efficiency obtained with the sc plasmid fraction was almost four times higher than the obtained with the oc isoform fraction, and two times higher when compared with the efficiency achieved with the pDNA control sample, as schematized in Fig. 6. Although the levels of transfection efficiency are lower for the Hela and A549 cells, which is dependent on the type of cell line used [47], the magnitude order of transfection efficiency between the plasmid samples is kept the same. This outstanding difference is in conformity with other studies that have shown the effect of pDNA topology on transfection efficiency [10], and also have obtained higher transfection efficiency with the sc plasmid isoform [17,18].

3.4. Applicability of the CDI monolithic column for different pDNA sizes

To verify the applicability of the CDI monolith to recognize and isolate plasmids with different sizes, some chromatographic runs were performed. The objective was to observe whether the retention behavior of each plasmid used depends on its size. These experiments were performed by applying the same linear decreasing gradient of ammonium sulfate ranging from 2.5 to 0 M (Fig. 7).

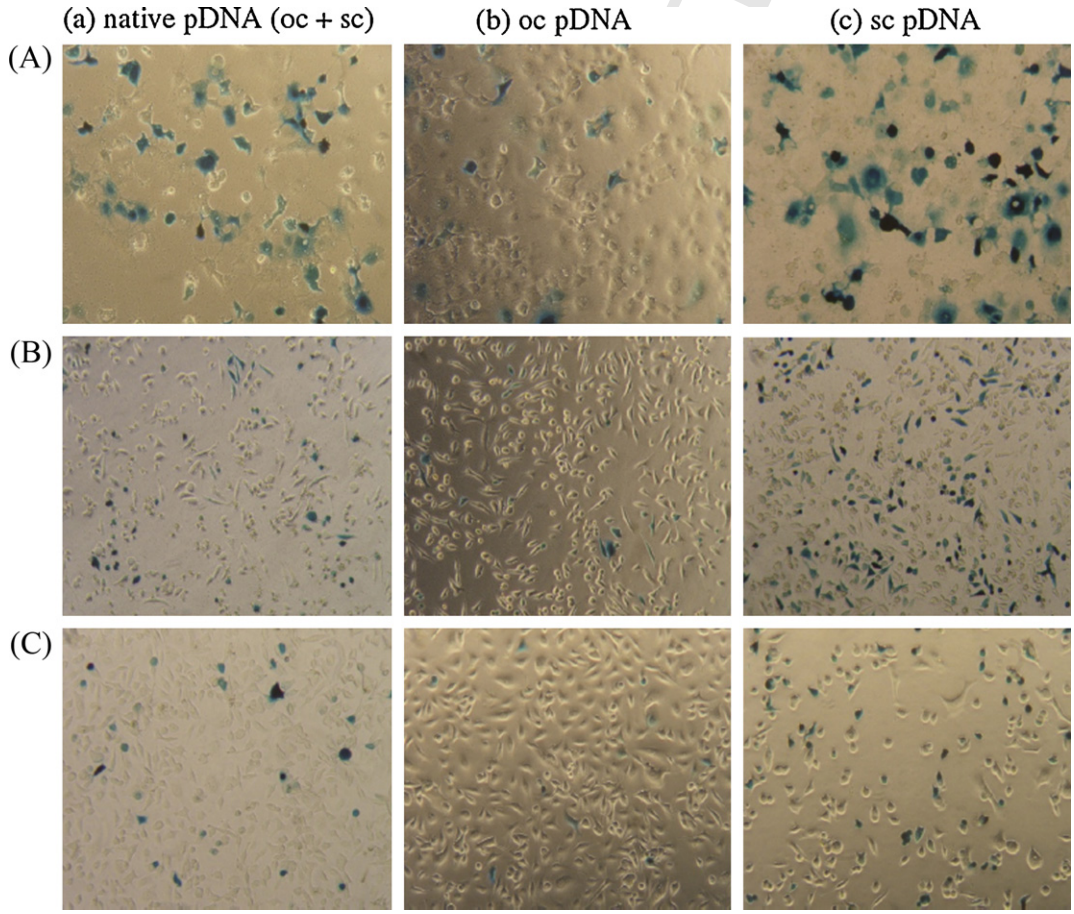

Fig. 5. Typical results of *in vitro* transfection experiments with COS-7 (A), Hela (B) and A549 (C) cells, using different plasmid samples with the same concentration (30 μg/mL). (a) Native pDNA (oc + sc) control sample obtained with a Qiagen kit; (b) oc plasmid and (c) sc plasmid obtained by chromatography with CDI-monolithic support.

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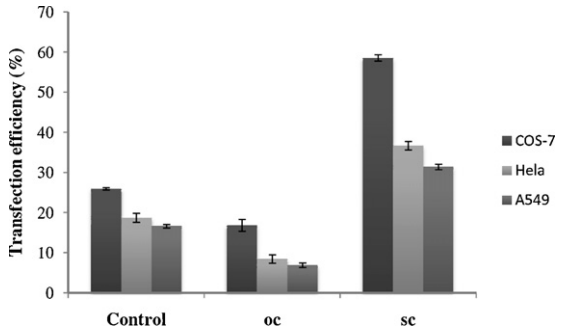

Fig. 6. Representation of transfection efficiency achieved by application of pDNA control sample purified with a Qiagen Kit, and two other samples of oc and sc pDNA purified with CDI-monolith to different cell lines ($n = 3$).

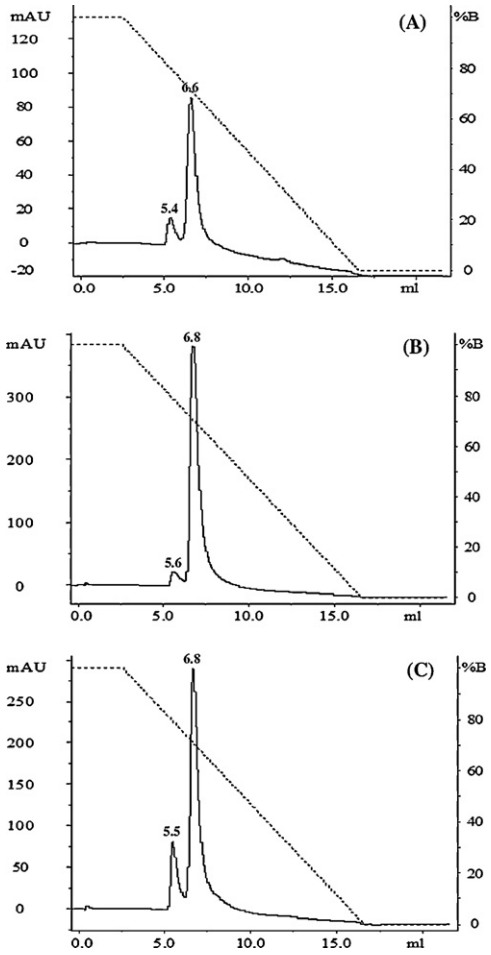

Fig. 7. Chromatographic profile of plasmids pUC19 (A), pVAX1-*LacZ* (B) and pVAX1-*LacZ*gag (C) obtained in CDI monolith. Mobile phase—buffer A: 50 mM Phosphate buffer pH 8.0; buffer B: 2.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 8.0. Elution was performed at 1 mL/min by decrease of ammonium sulfate concentration with a linear gradient in the eluent from 2.5 to 0 M, as represented by the dashed line.

The selected plasmids, pUC19 (2.7-kbp), pVAX1-*LacZ* (6.05-kbp) and pVAX1-*LacZ*gag (7.4-kbp), were obtained by a pre-purification with a Qiagen kit. Initially, each naked plasmid (oc + sc) was injected onto the CDI column previously equilibrated with 2.5 M $(NH_4)_2SO_4$ to ensure that the plasmid is completely retained. After 15 min of linear gradient, all the plasmids were eluted in two peaks due to the presence of two isoforms, but the retention time was identical for the three plasmids (Fig. 7(A)). Therefore, these results indicate that the CDI monolith is able to purify other pDNA molecules with different sizes. The recovery was not affected by the pDNA increasing sizes (data not shown). Thus, the insertion of one or more genes of interest in the same plasmid to be therapeutically applied seems possible, with no significant effects on the purification performance established with the CDI monolithic support.

4. Conclusions

The variety of incurable diseases such as tuberculosis, malaria and HIV that often are resistant to drugs, causing several million deaths per year worldwide, leads to a great need for stable new vaccines. At present, scales of production and efficiency are modest; however, if DNA vaccines prove to be effective, the ultimate scale will be large. In light of these considerations, the need to explore adequate chromatographic matrices arises as a fundamental step in the achievement of sc pDNA with the purity degree required for pharmaceutical applications. The implementation of CDI monolithic chromatography for this purpose was based on the development of specific interactions with nucleic acids from a *E. coli* host, allowing for selectively removing the oc plasmid variant and RNA molecules, as well as for reducing the gDNA and endotoxins to 0.57 ng gDNA/μg pDNA and 0.1 EU/μg of pDNA, respectively. *In vitro* transfection studies using the sc pDNA purified with this methodology have shown a four times higher efficiency than the transfection rate achieved with the oc isoform and the double when comparing with the pDNA purified with a commercial kit, regardless of the cell line used. Additionally, for the sc pDNA purification this monolithic support represents an advantageous alternative to conventional supports due to fast separation and consequent short contact time, favoring the structural stability of the target molecule. In reality, this monolith has shown good capacity and selectivity to separate several nucleic acids and efficiently purify the sc plasmid isoform. In accordance, a pDNA yield of the 89% with an extremely reduced level of impurities (endotoxins and gDNA) was achieved, which was reflected in a good transfection level of the sc plasmid sample.

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Chapter 4

Concluding remarks and future trends

DNA-based therapies using non-viral vectors such as pDNA, which can encode more than one trangene of interest and are less pathogenic than viral vectors, have been considered the most useful and promising technologies for the treatment of several diseases. Knowing that pDNA can be obtained in large scale by a simple and economic manufacturing process using the *E. coli* host, it is required the development of an adequate and efficient purification method to isolate the sc pDNA, the most biologically active plasmid isoform, and to eliminate the host impurities, which can origin adverse effects and inflammatory responses to the patients if present in quantities higher than those recommended. The recent application of amino acid-affinity chromatography has given good results on pDNA purification. Aiming the development and implementation of new affinity chromatographic strategies that allow the enhancement of the process to obtain the sc pDNA conformation, the study and understanding of involved interactions as well as the elution conditions that allow the sc isoform biorecognition by histidine and arginine-agarose matrices becomes essential.

As it was described, the fundamental studies of synthetic oligonucleotides with different sizes showed that the retention mechanism is largely affected by the elution conditions imposed, favoring or disfavoring different kinds of interactions. In general, high salt concentrations play a major role in the retention, favoring ring-stacking and hydrophobic interactions between particular oligonucleotide bases (mainly the guanine base) and histidine ligands. On the other hand, the increase of the molecular size of adenine and guanine oligonucleotides favored the appearance of secondary structures that changed the retention behavior, suggesting the involvement of van der Waals or electrostatic interactions with phosphate groups of nucleotides, which decrease the retention for high salt concentrations. Also the retention pattern of oligonucleotides with increasing temperature didn't follow a hydrophobic behavior, affecting only the oligonucleotides secondary structure. The pH influence was significant for oligonucleotides retention under low salt concentrations, favoring the ionic interactions.

The fundamental studies using arginine-agarose matrix were performed under mild salt conditions, showing that this matrix may be a good alternative to purify nucleic acids comparing with histidine-agarose. Although electrostatic interaction could be the principal phenomenon explaining the retention of single-stranded oligonucleotides, it was also proved that the interaction and retention of double-stranded oligonucleotides on arginine support significantly decreased, as a result of the diminished bases exposure. These results become evident that arginine also participates in multiple hydrogen bonds, mainly with the guanine base, and in hydrophobic interaction because the oligonucleotides retention increased when the temperature was increased, regardless of the secondary structures. Also the effectiveness

of oligonucleotides elution using a competition strategy with free arginine positively charged in the elution buffer reinforced the presence of interactions other than electrostatic effects.

The results obtained provided valuable information for the future enhancement and implementation of histidine and arginine or other amino acids as ligands in chromatography to perform a more targeted and efficient purification of several nucleic acids. On the other hand, knowing the ideal conditions to promote the specific interactions under mild salt concentrations with arginine-agarose, it could be possible to adapt and adjust the purification strategy using other affinity ligands that present similar composition to arginine, as for example lysine amino acid.

Lysine chromatography developed a biorecognition by the sc pDNA conformation, allowing the efficient separation from the oc isoform with a slight increase of sodium chloride concentration. Experiments of competitive elution using different amino acids revealed that the predominant interaction between pDNA and lysine support is ionic and not hydrophobic. However, the specific recognition of sc isoform showed the involvement of other interactions including hydrogen bidentate interactions, van der Waals and hydrophobic interactions, mainly with the nucleotide bases more exposed than in the oc isoform due to the supercoiling phenomenon. This theory was strengthened when only the sc isoform was more retained by testing a temperature increase. Thereafter, the applicability of lysine affinity chromatography to properly purify sc pDNA, eliminating *E. coli* impurities as well as other ineffective plasmid isoforms present in a complex clarified lysate was further confirmed. Furthermore, a strict quality control of the purity degree of the recovered plasmid was necessary to confirm the efficiency of lysine-chromatography. Thus, the final plasmid product presented an homogeneity higher than 97% of sc isoform with no detection of RNA and proteins and a sufficient reduction of the gDNA and endotoxins to recommended levels. The sc pDNA recovery at the end of the process was yielded in 46% and the transfection experiments on COS-7 eukaryotic cells was almost three times higher than the transfection rate achieved with oc isoform and the double comparing with the naked pDNA purified with a commercial kit.

In addition, a comparative study of the binding behavior of different nucleic acids individually injected on the three amino acid matrices, already applied for plasmid purification (histidine- arginine- and lysine-agarose), was accomplished under the influence of different environmental conditions, such as the composition and ionic strength of elution buffer and temperature. Although sc pDNA was efficiently isolated with all the matrices used, it was evident that in some cases preferential interactions with other nucleic acids were found. Histidine and lysine-agarose strongly retained RNA showing to be ideal technologies mainly to purify this nucleic acid. Given that lysine chromatography requires mild salt conditions becomes a purification method more economic than histidine-agarose, mainly for industrial scale. On the other hand, arginine-agarose preferentially retained sc isoform comparatively

to the other plasmid isoform and nucleic acids retention, becoming this support more adequate to sc pDNA purification. In general, temperature variation, competitive elution and fundamental studies allowed the identification of the dominant interactions inherent to biorecognition of pDNA molecule by the amino acid-affinity matrices. Indeed, these ligands allowed to an enhancement in the specificity and efficiency to purify plasmid DNA in a single unit operation, but the limitations associated with conventional supports remain unresolved.

The application of the non-grafted glycidyl methacrylate-based monolith with functional groups of Carbonyldiimidazole (CDI) for pDNA purification revealed the occurance of a specific recognition of the sc isoform that was totally isolated from oc isoform. The similar elution conditions to that used with histidine-agarose and the presence of canbonyldiimidazole groups suggested that the imidazole ring is the major responsible in the specific recognition of the bases of sc isoform, which are more exposed than the oligonucleotide bases of oc isoform, due to the supercoiling phenomenon. This new chromatographic technology based on monoliths showed to be an interesting alternative to the conventional supports due to its excellent mass transfer properties that allowed a fast and efficient plasmid isoforms separation without flow rate dependence. In fact, the characterization of this robust monolith proved the possibility to achieve higher binding capacity for pDNA (12 times higher than the capacity obtained for histidine-agarose support) which was favored for the sc plasmid conformation, increased plasmid concentrations and decreased linear velocities. Moreover, the dissociation constant value obtained was near to 10^{-8} M, revealing a good affinity interaction between the ligand and pDNA, which indicates that CDI monolith presents satisfactory affinity properties as chromatographic support.

Finally, the inclusion of the CDI monolith in the global pDNA manufacturing process to purify sc isoform from host impurities was demonstrated. This integrative approach resulted on extremely efficient purification step with a global yield of pDNA recovery of 89%, among which 74% is correspond to the sc isoform purification step. The removal of host impurities was also efficient, meeting all the regulatory requirements and resulting on high transfection efficiency of eukaryotic cells with sc pDNA sample (59% in COS-7 cells). This work also showed that the CDI monolith is a selective support that presents the additional advantage of a fast separation, resulting in a short contact time with pDNA, which favors the structural stability of target molecule. On the other hand, this monolithic support can also be applied for purification of other plasmids with different sizes, under identical elution conditions. Thus, the insertion of one or more tansgenes of interest in the same plasmid to improve the further therapeutic effect seems to be possible, with no significant effects on purification strategy already established with the monolithic support.

Overall, this doctoral research work revealed that amino acid-based affinity chromatography is a powerful and versatile methodology for nucleic acids purification, mainly for the sc pDNA topology, achieving a suitable purity degree for DNA-based therapies. The knowledge of the

involved interactions in the specific biorecognition of sc pDNA isoform and the understanding of the chromatographic conditions responsible by favor or disfavor the underling interactions are essential to control and manipulate the selective retention and/or elution of the different plasmid topologies and host impurities.

Besides selectivity and specificity obtained with amino acids affinity ligands, the application of the innovative monolithic technology in the pDNA purification field brought a great improvement of the speed, resolution and capacity of the chromatographic operation, being a promising association for plasmid purification technology. Hence, this work provided valuable and helpful information concerning amino acid-affinity chromatography and chromatographic supports that can be useful in the future pDNA bioseparation either for preparative and analytical processes.

By this way, as a future approach, it could be interesting to prepare and characterize new affinity chromatographic supports based on the immobilization of amino acids or their derivatives on different monoliths in order to find the support that can recognize and purify the sc pDNA isoform from the remaining lysate constituents with high selectivity and productivity. Furthermore, the molecular understanding of the biorecognition phenomenon is also important and could be improved through the identification of the interactions occurring between the sc pDNA and the amino acids, the determination of affinity constants, and characterization of the nucleotide regions involved in these interactions by SPR-biosensor and NMR spectroscopy.

The importance of this work could greatly increase if a clinical plasmid is used, becoming interesting to further evaluate the final product quality and gene expression in *in vitro* and *in vivo* studies.