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Monoliths for purification of a DNA vaccine against Influenza

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Specially dedicated to…

Fernanda, Daniel and Luís,

who I owe all that I am

The difficulty lies not so much in developing new ideas as in escaping from old ones.

John Maynard Keynes

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List of publications

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III. **Purification of influenza deoxyribonucleic acid-based vaccine using agmatine monolith**
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Journal of Chromatography A (2013) 1307, 91-98.

II. **Screening of gellan gum as an ionic and hydrophobic chromatographic matrix for biomolecules purification**
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List of scientific communications

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I. **CDI (CarbonylDiImidazole) monolithic disk: Effect of pDNA size in isoforms purification and Dynamic Binding Capacity**
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II. **Purificação cromatográfica de uma vacina de DNA contra Influenza utilizando derivados de aminoácidos imobilizados em monolitos**
D. Bicho, A. Sousa, F. Sousa, J.A. Queiroz, C.T. Tomaz
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III. **Purification of a plasmid DNA-based vaccine against Influenza using a CDI-Agmatine chromatographic support**
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IV. **Nova abordagem para purificação de uma Vacina de DNA contra o Influenza**
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II. **Two Different approaches for separation of pDNA isoforms using a CarbonylDiImidazole monolith matrix**
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III. **Purification of a new chromatographic approach using agmatine as ligand for the separation of pDNA isoforms**
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IV. **Agmatine as a ligand for chromatographic purification of supercoiled plasmid DNA-based vaccine against Influenza**
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Resumo alargado

O vírus influenza é responsável por uma infeção muito contagiosa que afeta o sistema respiratório, convencionalmente designada por Gripe. Este vírus é constituído por uma cadeia simples de RNA "negative sense" pertencente à família Orthomyxoviridae. O vírus influenza apresenta vários antigénios de superfície como a hemaglutinina (HA) e a neuraminidase (NA). A HA é o glicopéptido responsável pela entrada do vírus nas células do hospedeiro e tem correlação direta com a proteção contra o vírus, uma vez que induz a produção de anticorpos neutralizantes. Vários estudos têm demonstrado que o insert HA contido nas vacinas de DNA usadas esperimentalmente permitem conferir protecção cruzada contra várias estirpes do vírus, o que justifica a sua escolha para este projecto. Por outro lado, a NA é responsável pela saída do vírus da célula infetada permitimdo a sua propagação. É de referir ainda a proteína M2, um canal proteico transmembranar responsável pela replicação do vírus.

Segundo dados da organização mundial de saúde em dezembro de 2015, as infeções respiratórias são mais frequentemente causadas por influenza do tipo A e B, sendo que a elevada propagação do vírus pelos vários continentes contribui para a ocorrência de mutações e aparecimento de novas estirpes. Assim, todos os anos vários milhares de pessoas são hospitalizadas devido a complicações da gripe, havendo uma taxa de mortalidade considerável, em especial em indivíduos com idade superior a 65 anos e com patologias preexistentes. Deste modo, a prevenção passa pelo reforço anual do sistema imunitário o que é conseguido de forma mais rápida e efetiva mediante a aplicação de vacinas. O objetivo da vacinação é controlar ou diminuir os sinais de infeção e é especialmente importante para indivíduos de elevado risco.

As vacinas convencionais contra influenza são preparadas sazonalmente e são específicas para uma determinada estirpe, ou seja, a imunidade gerada não é particularmente ampla e a sua eficácia depende do grau de correspondência entre a vacina e a estirpe circulante. Como alternativa, a utilização do DNA plasmídico (pDNA) como uma vacina não viral tem-se tornado numa potencial estratégia terapêutica para prevenir ou tratar determinadas doenças de forma menos invasiva e segura comparando com os vetores virais.

A vacinação de DNA consiste na injecção de DNA bacteriano geneticamente modificado com o gene de interesse que expressa proteínas antigénicas responsáveis por desencadear uma resposta imunológica, evitando a progressão da doença. Estas vacinas oferecem uma série de vantagens quando comparadas às vacinas clássicas, em termos económicos e técnicos. O controlo de qualidade é mais fácil e a comercialização não necessita de uma rede de refrigeração, pois estas vacinas são estáveis à temperatura ambiente e podem ser liofilizadas. Além disso, as vacinas de DNA induzem uma resposta imunitária celular através da produção de linfócitos T auxiliares (CD4+) e linfócitos T citotóxicos (CD8+) e também uma resposta humoral por produção de linfócitos B.

O pDNA usado na produção de vacinas deve conter vários elementos funcionais para garantir a sua propagação (origem de replicação procariótica) e seleção em microrganismo bacterianos

(marcador de seleção, como por exemplo a resistência a um antibiótico). Adicionalmente, deve apresentar elementos responsáveis por uma elevada expressão nos hospedeiros eucarióticos (promotor de expressão eurariótica, sinal de poliadenilação, sítio de múltipla clonagem) e ativação do sistema imunitário (gene de interesse). O pDNA para fins terapêuticos deve estar em conformidade com as especificações das agências reguladoras, seguindo rigorosos critérios de qualidade em termos de uso de reagentes tóxicos ou de origem animal, bem como de pureza relativamente aos restantes constituintes dos lisados celulares. Contudo, as semelhanças existentes entre o pDNA e os seus contaminantes comuns, como proteínas, endotoxinas, DNA genómico e RNA, podem complicar a sua separação, pelo que o processo de purificação deverá ser extremamente eficiente. Portanto a preparação destas vacinas de DNA requer o desenvolvimento de processos de produção e purificação que permitam obter grandes quantidades de plasmídeo na sua forma mais compacta e biologicamente ativa (isoforma superenrolada (sc)).

Hoje em dia qualquer processo para obtenção de vacinas de DNA requer a utilização de métodos cromatográficos para obtenção do plasmídeo sc na sua forma mais pura. A cromatografia é uma técnica quantitativa que tem por finalidade geral a identificação e a separação dos componentes de uma mistura. Os compostos presentes são distribuídos entre uma fase estacionária e uma fase móvel e a separação ocorre porque os compostos têm diferentes afinidades com a fase estacionária e com a fase móvel, portanto deslocam-se com diferentes velocidades. Os métodos cromatográficos exploram propriedades do pDNA como tamanho, hidrofobicidade, carga e a afinidade das suas bases para os ligandos, de modo a promover a separação das impurezas com a maior eficiência possível. A flexibilidade deste tipo de processo advém da grande variedade de suportes e ligandos que podem ser usados, tendo em conta as características da molécula a separar.

Em relação aos suportes cromatográficas existentes, alguns problemas ainda precisam de ser ultrapassados, como a baixa capacidade de ligação dos suportes, o tempo do processo de purificação das biomoléculas e os rendimentos de recuperação. Apesar dos bons resultados obtidos com as matrizes convencionais há necessidade de utilizar novas matrizes com melhor performance. Os monolitos poderão ser uma alternativas às matrizes convencionais uma vez que mostram várias vantagens em relação a estas, nomeadamente: elevada capacidade de ligação devido às excelentes propriedades de transferência de massa; separação mais rápida o que leva a uma baixa degradação; resolução independente do caudal; fácil manuseamento e elevada reprodutibilidade. Desta forma, o trabalho apresentado nesta tese consistiu na utilização de colunas monolíticas com a finalidade de purificar a isoforma sc da vacina de DNA NTC 7482-41H-VA2 HA com 6.471kbp que contém o codão de iniciação e sequência necessária para expressar a HA do vírus influenza.

Numa fase inicial, foram realizados vários ensaios com plasmídeos modelo de diferentes tamanhos (até 14 kbp) utilizando o monolito CarbonylDiImidazole (CDI) não derivatizado. Este ligando foi escolhido pelas suas semelhanças com a coluna histidina agarose previamente utilizada com sucesso na purificação de um plasmideo modelo. Plasmídeos maiores

necessitaram de menor concentração de sal (sulfato de amónio) para ligar ao monolito devido ao maior número de interações entre estas biomoléculas e o monolito. A caracterização do suporte foi possível através da determinação da capacidade dinámica de ligação (DBC) por variação de parâmetros como o tamanho do plasmídeo, força iónica, caudal e o pH da fase móvel. Verificou-se que quanto maior concentração de sal usada, menor a repulsão electrostática dos grupos fosfato no pDNA, o que torna estas moléculas mais compactas aumentando a DBC. Por outro lado, observou-se que para o mesmo caudal, plasmídeos maiores possuem menor DBC, uma vez que ocupam maior área de superfície dentro dos canais do monolito. Por fim, constatou-se que a diminuição do pH também tem uma influência positiva na DBC.

No seguimento do trabalho, foi também testado um supporte monolítico imobilizado com agmatina para a purificação da vacina de pDNA contra o vírus influenza. A agmatina é naturalmente sintetizada a partir do aminoácido arginina na forma descarboxilada, e é conhecida pela sua intervenção em inúmeros processos biológicos. As semelhanças estruturais da agmatina com o seu precursor arginina, previamente utilizado como ligando numa coluna de agarose para purificar sc pDNA, foram a razão de escolha para o propósito aqui referido. A agmatina foi pela primeira vez usada como ligando em processos cromatográficos, verificando-se que possui a particularidade de funcionar sob dois modos de interação, o que permitiu a aplicação de duas estratégias de purificação: gradiente por diminuição da concentração de sulfato de amónio ou por aumento da concentração de cloreto de sódio. Os melhores resultados foram obtidos com a estratégia que usa sulfato de amónio, atingindo-se um grau de pureza de 98 % e um rendimento de 51,8 %. Adicionalmente, o método apresentou uma elevada DBC (5.656 mg/mL) e uma redução significativa das impurezas do hospedeiro quando comparadas com o lisado injectado no monolito. A eficiência de transfeção nos fibroblastos transfectados com o pDNA sc purificado com esta estratégia foi de 73 %.

Por último, a implementação de uma abordagem baseada em cromatografia de troca iónica utilizando o monolito com o ligando etilenodiamina (EDA) para a purificação do pDNA sc com expressão do gene HA do vírus influenza foi também bem sucedida. Este último ligando funciona por troca aniónica e possui semelhanças estruturais com o ligando DEAE, previamente utilizado como método analítico para quantificação de impurezas e da isoforma sc em amostras de pDNA. O processo permitiu uma redução significativa das impurezas provenientes do hospedeiro *E. coli* obtendo-se um grau de pureza de 97,1% e um redimento de 47%. Após a verificação dos padrões de qualidade do pDNA sc purificado, foram realizados ensaios *in vitro* com 2 tipos de células (A549 e CHO). A expressão da HA determinada por imunofluorescência permitiu verificar uma maior eficiência de transfecção nas células CHO com cerca de 70,6 %, enquanto as células A549 apenas registaram uma eficiência de transfecção de 61,4 %. De entre todos os métodos cromatográficos utilizados, o EDA permitiu obter um processo mais rápido e com menor impacto económico.

No global, este projeto de doutoramento demonstrou que os monolitos possuem um grande potencial de aplicação em processos cromatográficos para a purificação de vacinas de DNA

obtidas a partir de lisados complexos de *E. coli* seguindo as exigências das agências reguladoras. Apesar de os monolitos utilizados com os ligandos acima referidos apresentarem valores de rendimento e purificação relativamente semelhantes às matrizes particuladas convencionais, a elevada capacidade dos monolitos permite uma maior rapidez e eficiência do processo de purificação.

Palavras-chave

Agmatina, EDA, CDI, capacidade dinâmica de ligação, cromatografia, influenza, DNA plasmídico superenrolado, monolito, transfecção, vacina de DNA.

Abstract

Influenza viruses (of the *Orthomyx-oviridae* family) are enveloped, negative-stranded, RNA viruses with segmented genomes responsible for a significant human respiratory disease named flu. Researchers have made efforts to fight this contagious disease that still shows high levels of morbidity and mortality. The best option for reducing the impact of this viral infection is through vaccination. Even though traditional influenza vaccines are safe and usually effective, they only provide protection against the dominant strains of a given year, and thus need to be annually updated. This limitation, together with the use of embryonated chicken eggs as the substrate for vaccine production, is time-consuming and could involve potential biohazards in the growth of new strains of the virus. In the last years, the expansion of efficient plasmid DNA purification processes has fostered new therapeutic applications, concretely gene therapy and DNA vaccination. The latter is a promising alternative to conventional vaccination since it induces all three arms of adaptative immunity (antibodies, helper T cells, cytolytic T-lymphocytes), providing cross-strain protection. Moreover, DNA vaccines need to be obtained in high quantities, can be easily stored and the production process is generic, in contrast to the complicated process needed for conventional vaccines. However, there are still bottlenecks in the large scale manufacturing of this and other DNA pharmaceuticals, mainly at downstream processing level. It is known that a large quantity of the highly pure biologically active supercoiled (sc) plasmid DNA (pDNA) with pharmaceutical grade is necessary to implement this technology. For that purpose, the application of chromatographic operations has demonstrated good results since there are simple, robust, versatile and high reproducible. However, there are still some bottlenecks associated with conventional matrices namely their low binding capacity and diffusivity for pDNA samples. Owing to these limitations, monolithic supports have emerged as interesting alternatives due to the versatility of their structural characteristics, high binding capacity and the excellent transfer mass properties. Thus, in the present project it is proposed the production and purification of pDNA NTC 7482-41H-VA2 HA with 6.471kbp expressing the influenza virus protein hemagglutinin (HA) with new and more efficient processes based on monolithic supports.

With these in mind, the non-grafted CarbonylDiImidazole (CDI) monolithic column was explored in order to study the interaction behavior of different plasmids with different sizes. The biorecognition of the intact and undamaged plasmid form, the supercoiled (sc) isoform, was studied on each plasmid with different sizes (at least up to 14 kbp). The characterization of the monolithic support was also evaluated by dynamic binding capacity (DBC) manipulating different salt concentration, flow rates and pH values. The strategy applied in this work showed that the isoforms of plasmids with different sizes can be separated using the CDI monolithic disk. These results showed that it was possible to have a selective separation of the sc isoforms of different plasmids. Higher size plasmids needed a lower ammonium sulphate concentration to bind to the monolith. On the other hand, capacity studies proved that at the same flow rate,

the largest plasmid seems to have a lower capacity value whereas the smallest plasmid showed the best capacity value. In addition, high salt concentrations increase the DBC. Finally, the effect of diminishing the pH also had a positive consequence in the breakthrough experiments. These results were useful for the implementation of a new chromatographic strategy based on monolithic supports.

An agmatine monolithic disk was also tested in the purification of the sc isoform of a pDNA-based vaccine against influenza. This was the first application of agmatine (decarboxylated arginine) as ligand in a chromatographic process. Due to the role of this molecule in biological processes different interactions between the ligand and other biomolecules of interest can be exploited. Accordingly, two different purification strategies were used by applying either a descending ammonium sulphate or an ascending sodium chloride elution gradient. The best strategy was obtained using ammonium sulphate with a purification degree over 98 % and a recovery yield of 51.8 %. Moreover, this method presented a high binding capacity (5.656 mg/mL) and all the host impurities were significantly reduced or undetectable when compared with the injected lysate. Furthermore, the transfection efficiency of fibroblast cells using the sc pDNA purified with this strategy was also high, reaching an efficiency of 73%. All these results proved that this agmatine-functionalized monolith is a versatile column for pDNA purification.

Additionally, an ion exchange interaction chromatography approach using an ethylenediamine (EDA) monolith for the purification of a sc pDNA expressing the influenza virus HA protein was successful. The applied process exhibited a significant reduction of the *E. coli* host impurities while achieving a sc pDNA purity degree of 97.1 % and a step yield of 47 %. Finally *in vitro* experiments using A549 and CHO cells were performed. A strong intracellular fluorescence was observed in the transfected CHO cells with the purified sc isoform, presenting 70.6% of transfection while A549 cells showed a weaker signal for a transfection efficiency of 61.4%. The HA expression was recognized by a mouse monoclonal antibody directed to the HA protein with high viability and high transfection efficiency.

Overall, this doctoral research work revealed that monoliths have the potential to be further applied in chromatographic processes for purification of a pDNA influenza vaccine from complex lysates under the requirements of the regulatory agencies.

Keywords

Agmatine, EDA, CDI, chromatography, DNA vaccine, dynamic binding capacity, influenza infection, supercoiled plasmid DNA, monolith, transfection.

Thesis overview

This doctoral thesis is structured in four main chapters. The first chapter is divided into two sections and consists of a literature revision related to the purposed objectives. The first section highlights the importance of supercoiled DNA molecule as a novel vaccination approach, the relevance of purifying these molecules, as well as the main challenges and concerns regarding DNA purification. In the second section different strategies are discussed to choose the best DNA vaccine to be applied in the treatment and prevention of influenza. Specifically, it is given an update of influenza DNA vaccines developments, all the involved techniques, their main characteristics, applicability and technical features in order to obtain the best option against influenza infections. This section is presented as a review article (Paper I – "Influenza plasmid DNA vaccines: progress and prospects").
The second chapter includes the global aims of this doctoral work and its development purpose.
The third chapter includes the work developed during the PhD study in the form oforiginal research articles, organized as follows:

Paper II - Effect of chromatographic conditions and plasmid DNA size on the dynamic binding capacity of a monolithic support

Paper III - Purification of influenza deoxyribonucleic acid-based vaccine using agmatine monolith

Paper IV - Application of ethylenediamine monolith to purify a hemagglutinin influenza deoxyribonucleic acid-based vaccine

Finally, the fourth chapter summarizes the concluding remarks obtained during this research work, regarding the application and characterization of monolithic chromatographic supports for influenza plasmid DNA purification and the understanding of the biorecognition phenomena of the plasmids with the used ligands. Furthermore, some future work is suggested to complement the important findings achieved in this study.

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Abbreviations

AC	Affinity chromatography
AEC	Anion-exchange chromatography
bp	Base pair
CsCl	Cesium chloride
DNA	Deoxyribonucleic acid
EMEA	European Agency for the Evaluation of Medical Products
FDA	Food and Drug Administration
gDNA	Genomic deoxyribonucleic acid
HA	Hemagglutinin
HIC	Hydrophobic interaction chromatography
kbp	Kilo-base pair
ln	Linear
MCS	Multiple Cloning Site
mRNA	Messenger ribonucleic acid
oc	Open circular
ORI	Origin of Replication
pDNA	Plasmid deoxyribonucleic acid
polyA	Polyadenylation signal
RNA	Ribonucleic acid
RPLC	Reverse-phase liquid chromatography
sc	Supercoiled
SDS	Sodium dodecyl sulfate
SEC	Size-exclusion chromatography

Chapter 1

Introduction

1. Gene therapy

Genes are the functional unit of heredity and represent specific base sequences that encode instructions to make proteins. Although they get a lot of attention, proteins perform the majority of life functions. When genes are altered, the encoded proteins are unable to carry out their normal functions, and this unbalance may result in genetic disorders. In this sense, the advances in biotechnology have brought gene therapy to the forefront of medical research (Orkin, 1986; Gardlik *et al*., 2005). The most common form of gene therapy basically delivers genomic material (transgene) into specific cells with the purpose of generating a therapeutic effect by correcting an existing abnormality or providing the cells with a new function. Despite the potential of this approach other mechanisms of action are used, including:

- ✓ Swapping an abnormal gene for a normal gene through homologous recombination;
- ✓ Repairing an abnormal gene through selective reverse mutation;
- ✓ Regulation of a particular gene could be altered (degree to which a gene is turned on or off) (Rubanyi, 2001).

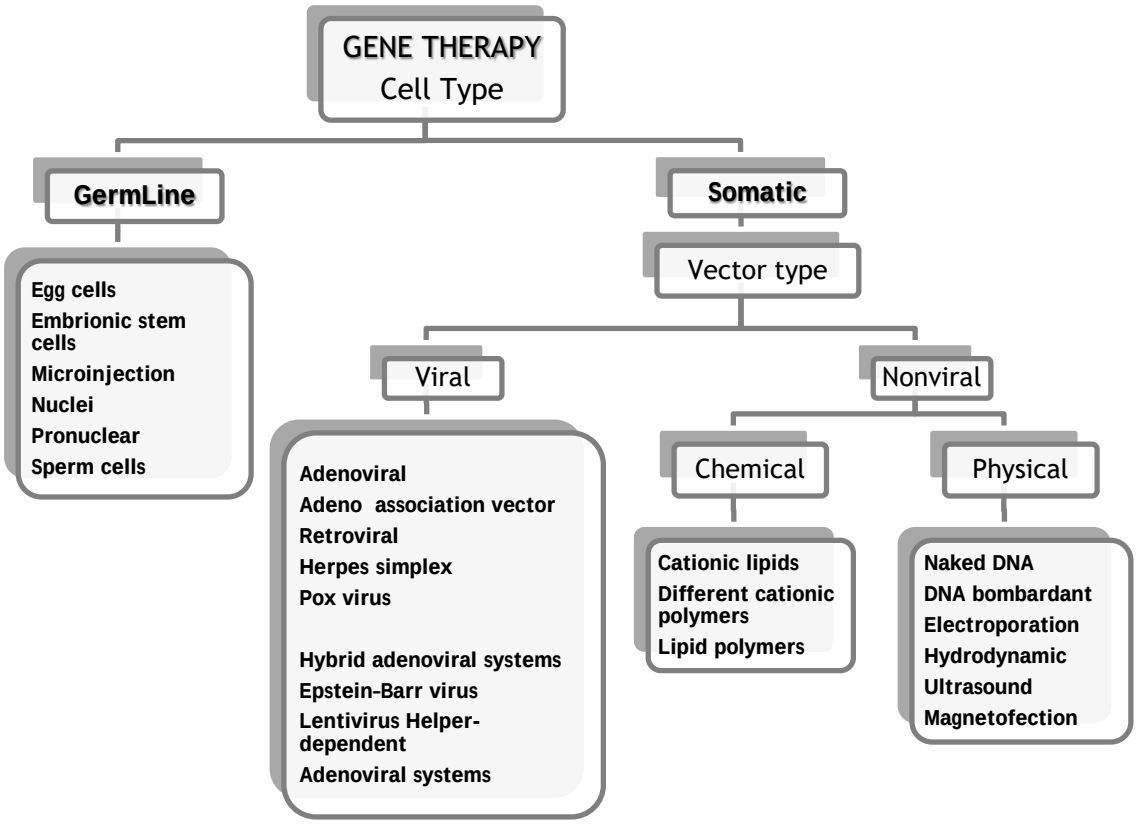

Figure 1: Diagram of the different gene delivery systems (adapted from Nayerossadat *et al*., 2012).

Gene therapy is different from traditional drug-based approaches which may treat symptoms but not the underlying genetic problems. Thus, in theory, any disease based in a genetic defect could be treated with this therapy, but in fact only the genetic disorders caused by mutations in single genes tend to be good candidates for gene therapy whist diseases involving many genes and environmental factors tend to be poor candidates (Brown *et al.*, 2001). However, nowadays diverse pathologies with different patterns of inheritance and acquired diseases are also targets to gene therapy. To date, thousands of disorders have been treated by several hundreds of protocols applying this methodology (Sousa *et al.*, 2014; Stone, 2010). As seen in figure 1 there are two major gene therapy categories: Germline gene therapy and somatic gene therapy. The first one refers to the improvement of genetic abnormalities by direct manipulation of germline cells. Although germline gene therapy may have a great potential, it cannot be used because it is ethically forbidden (MacDonough, 1997; Resnik, 2001). Currently, human gene therapy has been limited to somatic cell alterations and there is a remarkable development in the field. This methodology involves the insertion of genes into diploid cells of an individual where the genetic material is not passed on to future generations.

Anyway, the process of gene therapy remains complex and many techniques need further developments. The challenge of developing successful approaches for any specific condition is considerable and several parameters must be taken into account (Gardlik *et al.*, 2005). Two of these parameters are the identification of the faulty gene and there must be available a working copy of the involved gene. Additionally, specific cells that need treatment must be identified and accessible. Finally, means of efficiently delivering the gene to the cells must be also available.

1.1 Gene Delivery

During the 90s, both several vectors expressing genes in mammalian cells and new methods of direct gene transfer were developed (Azevedo *et al.*, 1999). However, no delivery system has been designed that can be applied to all kinds of cells *in vitro* and *in vivo* with no limitation and side effects. Of all challenges the one that is most difficult to address is the problem of how to get the new, or replacement, gene into the target cells. Thus, a carrier molecule called vector must be used for the above purpose (Gardlik *et al.*, 2005). The ideal gene delivery vector should be very specific, capable of efficiently delivering one or more genes of the size needed for clinical application, unrecognized by the immune system and be purified in large quantities at high concentration. Once the vector is inserted into the patient, it should not induce an allergic reaction or inflammation. It should be safe not only for the patient but also for the environment. Finally a vector should be able to express the gene for as long as is required, generally the life of the patient (Glenting and Wessless, 2005; Kennedy, 2010).

Two kinds of vectors have been employed as vehicles for gene transfer. Viral vectors for gene transduction and non-viral vectors for gene transfection, such as plasmids and liposomes. However, each vector has its own advantages and disadvantages; none of these types of vectors

has been found to be ideal for both safe and efficient gene transfer and stable and sufficient gene expression.

1.1.1 Viral vectors

One of the most successful gene therapy systems available today are viral vectors. These include retrovirus, adenovirus (types 2 and 5), adeno-associated virus, herpes virus, pox virus, human foamy virus, and lentivirus (Huang *et al.*, 2011). Viruses have evolved a way of encapsulating and delivering their genes to human cells in a pathogenic manner. Scientists have tried to take advantage of this capability and to manipulate the viral genome in order to replace it with the working human gene so that their replication becomes deranged and it makes them safer (Gardlik *et al.*, 2005). Nevertheless, this system has some problems, such as marked immunogenicity with induction of inflammatory system leading to degeneration of transducted tissue toxin production, insertional mutagenesis and their limitation in transgenic capacity size (Karate and Aeri, 2010). During the past few years some viral vectors with specific receptors have been designed to transfer the transgenes to some other specific cells, which are not their natural target cells (Wickham, 2003). Thereby, nonviral vectors have emerged as an alternative to address this shortcoming. This technology although less efficient in gene transduction presents a beneficial cost-effectiveness, it is easily produced, and more importantly, presents less induction of the immune system (Audouny *et al.*, 2001).

1.1.2 Nonviral vectors

Nonviral systems comprise all the physical and chemical systems. They are less efficient than viral systems in gene transduction, but their cost effectiveness, availability, and less induction of immune system have made them more effective for gene delivery.

Physical methods applied for *in vitro* and *in vivo* gene delivery are based in making transient penetration in the cell membrane by mechanical, electrical, ultrasonic, hydrodynamic, or laser-based energy so that DNA entrance into the targeted cells is facilitated. Some physical methods comprehend electroporation (creation of electric field-induced pores in plasma membrane), sonoporation (ultrasonic frequencies to disrupt cell membrane), magnetofection (use of magnetic particle complexed with DNA), gene guns (shoots DNA-coated gold particles into cells by using high pressure) and receptor-mediated gene transfer (Stone, 2010). On the other hand, chemical systems are more common than physical methods and generally comprise nanomeric complexes, which include compaction of negatively charged nucleic acid by polycationic nanomeric particles, belonging to cationic liposome/micelle or cationic polymers. The success of the non-viral DNA-based therapy, namely by using plasmids, has been shown to be very much dependent on various extra- and intracellular barriers that dramatically affect the efficacy of the gene delivery systems (Pathak *et al.*, 2009).

After the administration, the plasmid intake and tissue distribution is inefficient due to the extracellular barriers (such as the endothelial barriers), the degradation or inactivation of pDNA by serum proteins or tissue and blood nucleases and lipid bilayer membranes. Also, the size of

the DNA molecule and existing repulsion between the negative charges of the cell membrane and pDNA represents an issue (Anderson *et al.*, 2009). However, even after reaching the target cell, the pDNA meets additional intracellular barriers which need to be crossed before it arrives at its intracellular target region, the nucleus. Some of which are: cell uptake, endosomal escape, intracellular trafficking and nuclear delivery (Anderson *et al.*, 2009). Despite these novel strategies bring significant advances for the systemic delivery of DNA, the *in vivo* delivery is a highly challenging task, especially if administered systemically via intravenous injection. Nevertheless, as referred, various efforts have continually been made to improve the nuclear uptake of naked DNA.

1.1.2.1 DNA vaccines

The role vaccines play in protecting large, healthy populations from diseases is fundamentally different from that of conventional drugs, which are predominantly used to cure a relatively small number of people with specific diseases. Evidently, the evolution of vaccines and vaccine production methods are intimately tied to each other. Presently a wide array of vaccination technologies is being developed. Their purpose is to create safer and more efficacious techniques such as DNA vaccines produced in *Escherichia coli* (*E. coli*).

The first DNA vaccine was described in 1990 by John Wolf who used a plasmid with the reporter gene encoding the α-galactosidase protein which was expressed after direct inoculation into a mouse muscle (Wolff *et al.*, 1990). This study was focused on the evaluation of the factors that determine the gene transfer efficiency and the corresponding immunogenicity conferred by the inoculation of the plasmid. Later, on further studies, the inoculation of DNA encoding an immunogenic protein of influenza virus conferred protective immunity in mice (Ulmer *et al.*, 1993). From these results, the understanding of the immune mechanism induced by this type of vaccines sparked the interest from the scientific community.

DNA vaccines are based on a recombinant DNA technology that involves transferring a gene encoding a protein into a bacterial plasmid but having an expression in eukaryotic cells (Osborn, 2011). As an emerging technology, plasmid vectors are critical to the development of a wide range of DNA vaccines, including therapeutic vaccines for infectious diseases like influenza (Resnik and Langer, 2001). Plasmids are advantageous because they have greater biological safety, an easy and low cost production, present relative stability and a genomic capacity from 2 to 19 kilobases that can be transferred to the muscle cells. However, some studies with larger plasmids have been performed because the size tends to increase with future requirements for multigene vectors (Kong *et al.*, 2006).

The advance of DNA vaccines used to treat the avian flu is responsible for underscoring the importance of these types of vaccines and the need to optimize the processes of production. DNA vaccines can be produced more rapidly (in as little as two or three weeks) than conventional vaccines currently used to protect against the flu and could possibly save thousands of lives if a global outbreak occurs (Eisenstein, 2011).

The administration of a single dose of plasmid can provide a broad spectrum of immune responses, including the activation of T lymphocytes CD8+ and CD4+ T, which secrete cytokines and have a regulatory function in the production of antibodies. However, the successful DNA immunization depends mainly on the nature of the antigens, the frequency and route of administration, the administered DNA concentration, the cellular location of the antigen encoded by the plasmid (secreted, membrane-bound or cytoplasmic), the age and health of the species of the host to be vaccinated (Kennedy, 2010).

2. Plasmid technology

Plasmids are double stranded chains of deoxyribonucleotides linked together by a shared phosphate between two deoxyribose sugars on adjacent nucleotides. However, the anti-parallel chains are covalently attached and linked together with hydrogen bonds between purine and pyrimidine nitrogenous bases. These circular molecules should have the following essential elements: i) An expression promoter for mammalian cells; ii) polyadenylation signal (polyA) of transcript (mRNA), iii) selection marker; iv) a prokaryotic origin of replication v) multiple cloning site which is inserted the gene of interest. Other sequences are also important, namely the intron that increases the activity of the promoter, signal peptide and nucleotide sequences with immunostimulatory function as shown in figure 2 (William, 2009). It is also important to state that several modifications can be performed into the plasmid to assure an improvement in the immunological response. These improvements include the incorporation of immunostimulatory sequences, interleukin codification sequences and viral genes, thus improving the cell propagation (Zheng *et al.*, 2005).

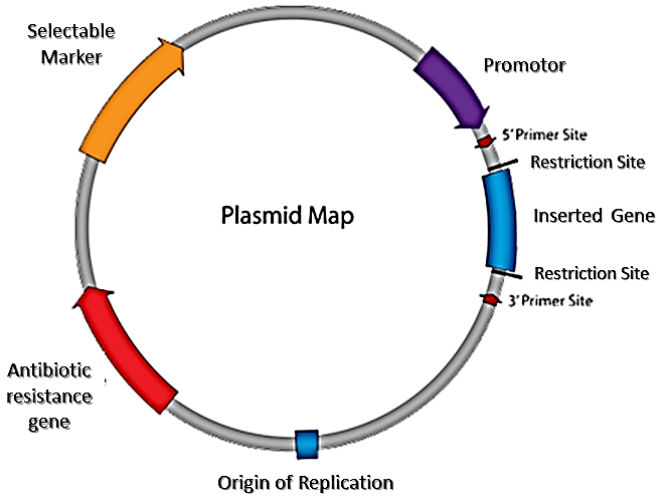

Figure 2: Illustration of a plasmid vector construction for DNA vaccination (adapted from Prazeres *et al.*, 2001).

As referred, the clinical use of pDNA, its functionality and manufacturing is severely related to the organization of its genetic elements. Thus, pDNA molecules contain one unit responsible

for its propagation in the microbial host and another unit that drives the expression of the therapeutic gene in the eukaryotic cells, as explained in table 1 (van Gaal *et al*., 2006).

Table 1: Genetic elements for plasmid DNA vaccines (adapted from Prather*, 2003;* van Gaal *et al., 2006;* Vázquez *et al., 2005*).

Vector Element	Description
Origin of Replication (ORI)	DNA sequence which allows initiation of replication within a plasmid.
Antibiotic Resistance Gene	Allows the selection of bacteria with the plasmid of interest.
Multiple Cloning Site (MCS)	Short segment of DNA which contains several restriction sites for the easy insertion of DNA. In expression plasmids, the MCS is often downstream from a promoter.
Insert	Gene, promoter or other DNA fragment with a specific function.
Promoter Region	Drives transcription of the target gene.
Selectable Marker	Ensure stable inheritance of plasmids during bacterial growth since their presence only enables the propagation of the microbes hosting the pDNA.
Primer Binding Site	A short single-stranded DNA sequence used as an initiation point for PCR amplification or sequencing. Specific sequence used for the verification of plasmids.

In the early 60s, several research groups devoted themselves to the study of the circular DNA topology. It was in 1965 that Vinograd and his co-workers observed, by electron microscopy, that the DNA circular polyoma virus could take various conformations and the most common had a more compact nature being called supercoiled (sc) pDNA. This isoform is the most appropriate isoform for therapeutics application due to its coiled and highly order structure. However, there are also other isoforms resultant from nicks in the sc chains. The breakage of one sc DNA strand leads to loss of the molecular coiling, resulting in the open circular (oc). By the contrary the damage of both strains favors the appearance of the linear pDNA isoform (ln) as shown by figure 3. Notice that 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).

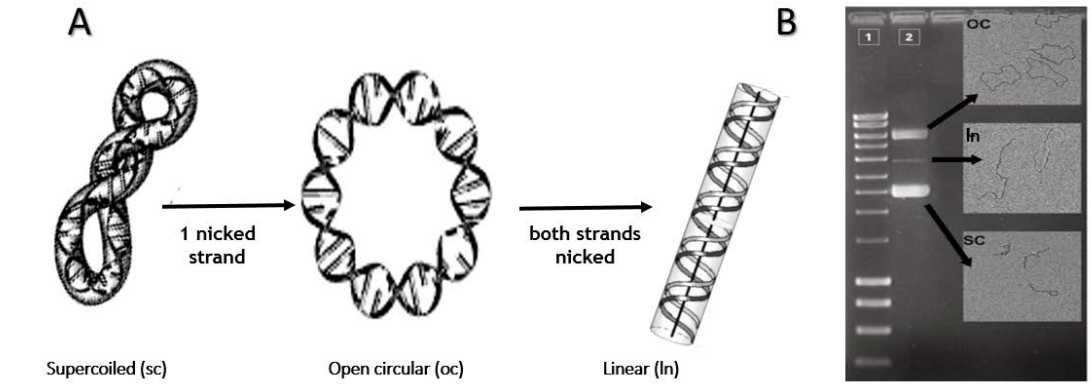

Figure 3: Plasmid topologies: A - Schematic representation; B- Agarose gel electrophoresis with electron microscopy images (adapted from Molloy *et al.*, 2004).

2.1 Fermentation

The process of pDNA manufacturing generally starts on with the construction and selection of the appropriate expression vectors and their production in bacterial microorganisms. It is followed by the selection and optimization of the fermentation conditions (upstream processing), cell growth, clarification and finally by the isolation and purification steps (downstream processing) as presented in figure 4.

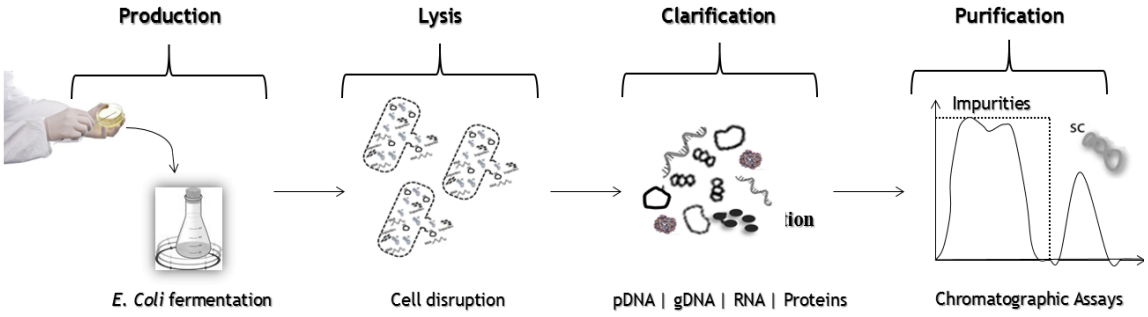

Figure 4: Schematic representation of the main unit operations to be considered during the manufacturing process of sc pDNA.

Even though the choice of the microbial host strain can affect the quality of the final pDNA product, there are no specific guidelines about the most convenient genotypic or phenotypic characteristics of bacterial strains should present to produce pDNA. Therefore, the preference for *E. coli* is based on its ability to sustain the stability of this biomolecule, its compatibility with subsequent purification steps, a low risk of promoting genetic modifications and its high cellular density (Vázquez *et al.*, 2005). Notwithstanding, the vast majority of plasmid DNA vectors currently used in clinical trials were propagated by growing *E. coli* in a bioreactor, other alternative host systems have been proposed (Ow *et* al., 2006). The judicious selection

of the plasmid vector and its host strain, combined with the growth-conditions optimization (media and conditions), can result in plasmid yields as high as 100 mg and a reduction in the RNA content during cell lysis (Carnes, 2005).

However, the major challenge of working with bacteria remains in achieving a scalable and economical means of producing large quantities of sc pDNA, thus both fulfilling the safety and potency demands of gene-therapy vectors and the inherent 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). This challenge is due to the low concentration, of plasmid DNA which represents less than 3 % of the total contents of an *E. coli* extract. Additionally, *E. coli* as a gram-negative bacteria contains highly immunogenetic endotoxins in its outer membrane and these chemical species can eventually be a problem in pDNA purification (Glenting and Wessels, 2005). Other host impurities such as genomic DNA (gDNA), RNA or proteins can possibly originate adverse effects and inflammatory responses to the patients, if present in quantities higher than those recommended (table 2).

Table 2: Specifications of *E. coli* lysate cell content and accepted levels of host impurities in the final purified sc pDNA for DNA vaccination (adapted from Stadler *et* al., 2004; Klinman *et al*., 2010).

***E. coli* Lysate**		**Purified pDNA**	
Components	**Content (%)**	**Range of acceptance**	**Method of quantification**
Proteins	55%	< 3 µg/mg pDNA	BCA test
RNA	21%	<0.2 µg/mg pDNA	Analytical HPLC
gDNA	3%	< 2 µg/mg pDNA	TaqMan-PCR
Endotoxins	3%	< 0.010 EU/µg	LAL test
pDNA	< 3%	> 97% sc pDNA	Analytical HPLC
Others	15%	-	-

Although the plasmid conformation is maintained predominantly in the sc form, during production by fermentation, the subsequent steps of extraction, purification and storage can lead the molecule to suffer cuts. So, FDA has some recommendations for DNA vaccines production, namely a high content of the sc pDNA conformation (which should be more than 97%). This recommendation is based on the awareness that the supercoiled isoform has the highest biological activity of all the conformations which is advantageous because it promotes the necessary unwinding and strand separation during replication, transcription and recombination (Schleef, 2001).

2.2 Cell lysis and clarification

A sequence of unit operations must be set up after fermentation, in order to eliminate impurities and to obtain a final pDNA which fulfills the required specifications. The first critical

step in the downstream processing of plasmid DNA is cell lysis. In this step, all the intracellular components, including plasmid DNA, RNA, gDNA, endotoxins and proteins, are released. The release and recovery of large amounts of intact sc pDNA is crucial if the purpose is to obtain high overall process yields.

Many different methods and technologies are available for the isolation of genomic DNA. In general, all methods involve disruption and lysis of the starting material followed by the removal of proteins and other contaminants, being the final one the recovery of the DNA. The disruption of *E. coli* cells can occur by three different methods: mechanical, chemical and enzymatic lysis. However, the chemical lysis is the most widely used, because the mechanical method employs ultrasound, which increases the temperature, being aggressive for the sc pDNA structure, while the enzymatic strategy is not accepted by the regulatory agencies due to the adverse effects of the administration of pharmaceuticals with residues of animal-derived materials, such as RNases (Stadler *et al.*, 2004; Ferreira *et al.*, 2000). The chemical method usually chosen is based on alkaline lysis, relying on the disruption of the 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). Even though during the lysis process, several impurities are removed (cell debris, most of gDNA, endotoxins, part of the RNA and some proteins) a large amount is still mixed with the pDNA isoforms.

After the cellular disruption and lysis, clarification and concentration steps are used to remove cell debris and structurally unrelated impurities, such as proteins and low molecular weight nucleic acids, while simultaneously concentrating and conditioning the plasmid-DNA preparations for the purification step (Prazeres *et al.*, 2000).

2.3 Purification

The final step of the main unit operation for the production of sc pDNA for therapeutic use is the purification process. Mostly chromatographic approaches are used to separate supercoiled plasmid DNA from structurally related impurities (relaxed and denatured pDNA, gDNA, RNA and endotoxins). Nevertheless, the main challenge of this process lies with developing effective and economical cellular purification protocols since sc pDNA is quite similar in size and structure to the contaminants released upon cellular lysis and also labile, therefore, requiring mild processing conditions (Stuve *et al.*, 2007). To satisfy the regulatory guidelines for DNA vaccines, the pDNA must be highly purified which leads to expensive operational costs and also a high number of complex steps in extraction, isolation, purification and formulation of pDNA, inducing a structural stress which can result in damage to the supercoiled plasmid molecules (Sousa *et al.*, 2010). With this in mind, chromatography appears as a downstream method highly used for this purpose. This processes can advantageously explore some properties of pDNA such as size, charge, hydrophobicity, accessibility of the nucleotide bases and the topological limitations imposed by supercoiling and/or affinity (Sousa *et al.*, 2008).

3. Chromatographic purification of pDNA-based vaccine

The use of laboratory-scale production of pDNA-based vaccines is currently time consuming and cost ineffective. However, together with the impressive progress achieved in pDNA vaccination and the increased demand of the market; a large scale, intensified plasmid purification process needs to be introduced. Liquid chromatography is one of the most widely used methods in biotechnology, both at analytical, preparative and industrial level. Due to its simplicity, robustness, versatility, and high reproducibility, it can be applied in the purification of a variety of biomolecules, including pDNA. This method is usually used after clarification and concentration operations where the impurity load and the process volume have been already reduced. In any typical pDNA vaccine production process, chromatographic purification is mandatory for the removal of impurities from the pDNA solution and consequently to assess if the achieved purity has clinical grade for quality and safety purposes (Sagar *et al.*, 2003; Ferreira, 2005). As referred above, such purification should include the separation of the necessary sc pDNA from the other topologies and important impurities.

Different chromatographic techniques have been developed for pDNA purification in accordance with their chemistry including: size exclusion (SEC), anion exchange (AEC), hydrophobic interaction (HIC), reversed phase (RPLC), or affinity chromatography (AC) as evidenced in table 5.

Amongst the chromatographic strategies employed for pDNA purification, some of them present advantages which lead to more efective downstream processes. The good specificity and selectivity of conventional matrices allows the purification of the sc pDNA isoform, and the use of mild salt conditions without organic compounds, avoiding the loss of pDNA integrity. However, in general, considerable co-elution is the major problem associated with the similarities between pDNA and impurities in the majority of the chromatographic methods. Therefore, some problems of these matrices remain to be solved, namely the low capacity of available supports and the recovery yield. On the other hand, the average pore size of common chromatographic supports is smaller than or approximately the same size as the radius of gyration of a pDNA molecule. Consequently, pDNA cannot access pores in standard chromatographic media. Instead, it can only bind to the outer surface of highly porous supports which constitute the stationary phase. Hence, only approximately 0.2–2 g pDNA bind per litre of resin in contrast to proteins where the loading can range from 10 to 100 g per litre of resin. This means that a substantial amount of chromatographic media is needed in a large-scale plasmid DNA separation for vaccine applications (Sagar *et al.*, 2003). So, there is a need to use chromatographic supports with a better performance.

In the next section 4 it will be discussed the structural evolution of several chromatographic matrices 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 conventional ones.

Table 3: Chromatography methods used for pDNA purification (adapted from Sousa *et al.*, 2008).

Method	Description	Advantage	Limitation	Ref.
SEC	Distinguishes clarified lysate components on the basis of their size.	Ideal for the polishing step. It can be used alone or with other chromatographic methods.	Long chromatographic run; Limited capacity; High dilution; Co-elution of pDNA isoforms and gDNA.	Ferreira, 2005; Li *et al.*, 2007
AEC	Based on the interaction between the negatively charged phosphate groups on the DNA backbone and the positively charged groups of the stationary phase.	Application in preparative and analytical scales; Most used technique for pDNA separation, purification and quantification. Many pDNA purification kits apply this technique.	Lack of selectivity of the adsorbents leading to co-elution of sc pDNA and other isoforms; Low resolution of pDNA with impurities: endotoxins and RNA.	Prazeres *et al.*, 2001; Ferreira *et al.*, 2000 Stadler, 2004
HIC	Takes advantage of the higher hydrophobicity of single stranded nucleic acids and endotoxins over other biomolecules, interacting strongly with HIC supports than double stranded nucleic acids.	Efficient pDNA purification from impurities; Application at preparative, analytical and industrial scales.	Elution at high salt concentration; Non-binding of pDNA and difficulty in the separation of isoforms.	Diogo *et al.*, 2005
RPLC	Interaction based on their polarity. Hydrophobic regions of the molecules bind to non-polar immobilized ligands.	Separation of pDNA from crude lysate with the possibility of isolating the sc isoform.	Elution using a gradient of organic solvent (non-polar).	Wysoczynski *et* al., 2013
AC	Consists on a highly specific molecular recognition that relies on a strong but reversible interaction similar to many found in biological systems	Possibility of choosing specific ligand directed to nucleic acids; Selective purification of sc pDNA; Efficient elimination of impurities; High yields.	Expensive ligands; Limited lifetime; In some cases, high salt concentrations are needed.	Sousa *et al.*, 2012; Pfaunmiller *et al.*, 2013

3.1 Chromatographic supports for pDNA-based vaccine purification

Different chromatographic beads were developed in the 1980's, mostly based on dextran and agarose, but also using cellulose, polyacrylamide, and methacrylate (Janson and Hedman, 1982). By this stage it was apparent that much advancement was made in the design of chromatographic supports since their early primitive beginnings, however chromatography supports still suffered from many inadequacies by the end of the 1980s. Therefore, chromatographic materials were polydisperse, had insufficiently wide pores, large particle size, and suffered from low mechanical strength. These problems remained unresolved throughout the 1990s with few new developments made in further enhancing the design of these supports. Ligand chemistry did progress somewhat with the creation of bimodal ligands (Frechet *et al.*, 1994).

Accordingly, when choosing the characteristics of the material to be used in the chromatographic process the nature of the mobile phase should not only be adapted to the features of the stationary phase but also to the characteristics and stability of the target molecule, which in our case is pDNA. Latest developments show that the best suitable stationary phase should ideally be solid, macroporous, chemical and physically stable, exhibit low nonspecific adsorption with high binding capacity and mass transfer, and maintain good flow properties (Billen and Desmet, 2007).

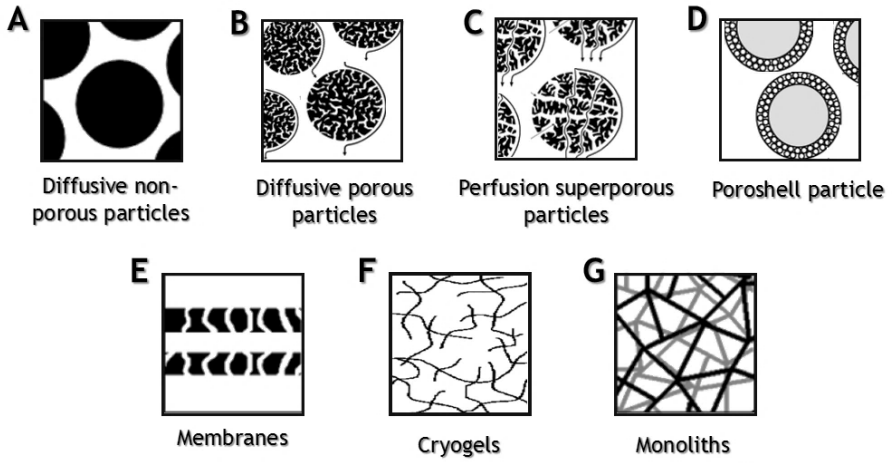

Figure 5: Structural representation of the different chromatographic supports. (A) Diffusive nonporous particles; (B) diffusive porous particles; (C) perfusion superporous particles; (D) poroshell particle; (E) membrane sheets; (F) cryogels; (G) monoliths (adapted from Sousa *et al.*, 2012 and Billen and Desmet, 2007).

The evolution of this research area allows the classification of the most common supports into packed particles or continuous beds (Table 4). Among the packed particles it is found nonporous, porous, superporous packed particles and pore shell matrices while the continuous beds are represented by membranes, cryogels and monoliths (figure 5). The recent developments on chromatographic supports will allow a rapid purification of the desired pDNA conformation with low sample loss and high purity.

Table 4: Types of chromatographic supports and their main characteristics (adapted from Billen and Desmet, 2007).

Chromatographic support	Characteristics	Ref.
Packed Particles		
Diffusive non-porous (A)	Especially useful for quality control, on-line monitoring and purity evaluation of biomolecules. The absence of pores (Figure 5A) eliminates the mass transfer resistance and intraparticulate diffusion effects Rapid separation and analysis with high efficiency. Generates high pressure drops.	Lee, 1997 Gustavsson *et al.*, 2004
Diffusive porous (B)	Created to overcome the limitations of non-porous particles on the basis of porous matrices that increase the contact surface area of the biomolecules. Small pores stagnate the mobile phase and molecules only penetrate by molecular diffusion increasing mass transfer resistance. Shear forces or surface incompatibility may cause irreversible loss of biological activity of pDNA.	Gustavsson *et al.*, 2004 Freitas *et al.*, 2007
Perfusion superporous (C)	Contain a network of large through-pores that allow intraparticle mass transport by molecular convection Good mass transfer resistance properties Comparatively with agarose nonporous matrices, superporous revealed a twofold increase of the purification factor and concentration of the desired product, at flow velocities five times higher. The pore number is not sufficient to overcome the diffusion limitation and the void volume between the particles.	Faman *et al.*, 2002 Gustavsson *et al.*, 1999
Poroshell (D)	Small highly porous particles. Low backpressures. Especially used for protein separation or gas identification in the analytical field.	Billen and Desmet, 2007
Continuous beds		
Membranes (E)	Flat disks bearing a large number of through-pores with similar and optimized diameter. Used for filtration or concentration and as an alternative to perform polishing purification. Membrane adsorbers enhance chromatographic resolution, efficiency, as well as productivity. High cost per volume of adsorbent and lower throughput due to lower unit surface areas.	Przybycien *et al.*, 2004 Haber *et al., 2004*
Cryogels (F)	Produced by polymerization of specific materials, combining the advantageous mass transfer properties and permeability of rigid supports. Ability to process crude solutions that contain non-clarified cell homogenates as well as entire cell suspensions suitable to extract the target molecule.	Noppe *et al.*, 2007
Monoliths (G)	Polymerized rigid beds highly porous with high binding capacity Excellent mass transfer properties. Short contact time with biomolecules which reduces biomolecule degradation. Emerged as an excellent solution for pDNA purification because of the high number of binding points.	Danquah and Forde, 2008

3.1.1 Monoliths

Monoliths are considered the fourth generation of chromatographic stationary phases. They are also called continuous beds, consisting in a single piece of highly porous organic or inorganic solid material into a column in form of disk, rods or tubes. The structure of monoliths avoids generation of shear forces, thereby contributing to high functional recoveries, even for labile biomolecules such as live virus vaccines, DNA plasmids, and large proteins (Strancar *et al.*, 2002). This structural feature eliminates some problems related to the scale-up and scale-down variations of conventional matrices, the packing quality and the need to repack a column due to the inadvertent introduction of air bubbles. Monoliths also differ from conventional supports with respect to their hydrodynamic properties, in particular by having low mass transfer (Zöchling *et al.*, 2004 and Sousa *et al.*, 2012).

Monoliths have been widely used for both analytical and preparative purposes. The most essential feature of this chromatographic support is that all the mobile phase is forced to flow through its large pores. Therefore, pore size plays an important role in providing spaces for both ligand attachment and plasmid mobility.This important feature allows their usage in pDNA purification which has been highly investigated. Some research groups have been using different functionalities of the monolith such as triethylamine (Ongkudon and Danquah, 2010), carbonyldiimidazole (Sousa *et al.*, 2011a) or metal affinity to purify pDNA (Shin *et al.*, 2011).

Another important feature of these matrices is that the flow passes through them by convection, allowing rapid separations in short beds (Sousa *et al.*, 2012). Thus, the association between the excellent mass transfer of monoliths and the easy accommodation of pDNA in their large pores justifies their high binding capacity for this biomolecule (Sousa *et al.*, 2011a). A pratical example was observed when a non-grafted CDI monolithic disk was used for the separation of a model plasmid from bacterial cells (Sousa *et al.*, 2011b). According to this work, the isolation of the sc pDNA sample allowed the achievement of the required purity degree for pharmaceutical applications. The results were similar when compared to pDNA isolated with the commercially established methods, but the separation time could be substantially decreased due to the convective flow and high interconnectivity of the monolith. Moreover, a successful separation of pDNA from RNA was obtained without any addition of RNAse. Studies have been developed to obtain highly pure DNA vaccines with a therapeutic purpose using single monolithic chromatographic processes. The work of Soares and co-workers (Soares *et al.*, 2013) used a modified monolithic disk immobilized with arginine to purify HPV 16 E6/E7 pDNA vaccine. The results had a good sc pDNA isoform purification profile, confirmed by agarose gel electrophoresis, with an impurity level within the acceptable limits. In another study, Smrekar and co-workers (Smrekar *et al.*, 2013) tested the monolith gBuMA + DEAE to purify pDNA with a recovery above 80%. It was also accomplished the removal of more than 99% of RNA, endotoxins and gDNA. Another group of authors presented the applicability of monoliths in weak anion-exchange chromatography, namely in the purification of bacterial and eukaryotic gDNA (Forcic *et al.*, 2005). Again, a simple and fast chromatographic procedure was developed. The method was very robust as the columns could be efficiently regenerated and used several times.

The sample volume that could be applied on the short chromatographic media was not restricted, and various volumes were loaded onto the column and did not affect the chromatographic profile. Also, there was no need to add toxic organic solutions or enzymes. On the other hand, CIM monolithic quaternary amine (QA) was used for the separation and purification of siRNAs and dsRNAs intended for therapeutic applications (Romanovskaya *et al*., 2013). These anion exchange columns showed to be a good alternative to standard purification methods (e.g. LiCl precipitation) and obtained highly pure RNAs because they efficiently remove contaminants from enzymatically produced siRNA. They can also, be applied to resolve a mixture of dsRNA molecules up to 1 kb under non-denaturing conditions.

Additionaly, Sousa and co-workers (Sousa *et al.,* 2014*)* studied the physicochemical properties of the ligand histamine in a sc pDNA purification method with emphasis on the elution strategy that allowed the highest selectivity and efficient removal of other impurities combining the ionic strength with pH. The strategy with ascending sodium chloride gradient revealed that 97% of sc pDNA was recovered with a purity degree of 99 %. However, the ammonium sulfate step had a lower recovery yield (79%) with a purity degree of 92 %.

Concluding, chromatographic processes using monoliths can be performed in flowthrough mode, in large scale and with a reduction in the purification time, which makes these supports very attractive for plasmid purification on industrial scale. Additionally, monolithic supports have improved the resolution and capacity of conventional matrices due to the high quantity of accessible binding sites, reinforcing their utility and the step forward in chromatographic processes that they represent.

Even though there are some works using monolithic supports to purify pDNA vaccines (Almeida *et al.*, 2015; Smrekar *et al*., 2013 and Soares *et al*., 2013), the biologically active sc isoform must be obtained in high quantities in order to be applied as a therapy. Therefore, the main goal of this project will focus on the purification of pDNA against influenza using monolithic matrices.

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Paper I

Influenza plasmid DNA vaccines: progress and prospects

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Influenza Plasmid DNA Vaccines: Progress and Prospects

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Abstract: Current influenza vaccines have long been used to fight flu infectious; however, recent advances highlight the importance of produce new alternatives. Even though traditional influenza vaccines are safe and usually effective, they need to be uploaded every year to anticipate circulating flu viruses. This limitation together with the use of embryonated chicken eggs as the substrate for vaccine production, is time-consuming and could involve potential biohazards in growth of new virus strains. Plasmid DNA produced by prokaryote microorganisms and encoding foreign proteins had emerged as a promising therapeutic tool. This technology allows the expression of a gene of interest by eukaryotic cells in order to induce protective immune responses against the pathogen of interest. In this review, we discuss the strategies to choose the best DNA vaccine to be applied in the treatment and prevention of influenza. Specifically, we give an update of influenza DNA vaccines developments, all involved techniques, their main characteristics, applicability and technical features to obtain the best option against influenza infections.

Keywords: Gene expression, Influenza, Flu infection, Plasmid DNA, Purification, Vaccines.

INTRODUCTION

In recent years, researchers have made efforts to fight the highly contagious respiratory disease caused by influenza. This virus is responsible both for high mortality and morbidity in the infected population and various strategies have been used for its prevention. The preferred approach for this type of pathology is vaccination, which dramatically reduces the impact of the infection caused by influenza. Due to the antigenic evolution of influenza strains, annually a committee decides which strain of this virus is chosen to be incorporated into the vaccines. In this selection diverse variables are considered. These include the prediction of which strains will most probably cause the next epidemic, their similarity to the predicted circulating strain, their ability to cause the correct humoral immunity, and finally their appropriateness to large scale production [1]. Afterwards, the vaccines are produced and the inactivated viruses are inserted and grown in embryonated eggs with high effectiveness but its manufacturing takes between 6-9 months [2, 3]. This approach is time consuming, which in a pandemic case becomes unacceptable. Owing this limitation together with its complex manufacturing processes, zoonotic risk and limited immunological protection, it is clear that different approaches to influenza vaccination should be developed [4]. These new methodologies should provide a faster and more effective immunization, in case of a new influenza outbreak.

Consequently, DNA vaccination presents many attributes that make it a suitable technique to be used as a novel approach to treat influenza. This promising technology can be an useful and powerful alternative to conventional therapies due to its low cost production, stability, simplicity of formulation and safety [5, 6]. It consists of a circular double DNA chain (plasmid DNA, pDNA) containing one or more DNA sequences of specific influenza proteins capable of inducing and/or elicit an immune response against flu infections. The manufacturing process is independent of egg supplies and the vaccines can be easily stored by lyophilizing them in order to increase the DNA stability at room temperature contrary to conventional vaccines [7]. Moreover, recombinant vaccines produced in bacteria, free of other viral and cellular components, are expected to have less immunological complications than virus vaccines, such as pyogenic reactions and Guillain-Barre syndrome.

The development of this technology began when researchers were confronted with the poor immunogenicity of conventional vaccination. As evidence of the promising nature of this therapy, in recent years several researchers have developed pDNA vaccines that progressed through various stages of animal [8-10] and human trials [11-16]. Finally, the current limitations faced in the production of conventional influenza vaccines together with auspicious results obtained using pDNA vaccines enable this technique to be applied as a viable alternative to typical influenza vaccination.

CROSS-NEUTRALIZING PROTECTION OF INFLUENZA DNA VACCINES

Understanding the mechanisms of pDNA vaccine protection against influenza infection is crucial to further develop this technique towards clinical application. As known, DNA based influenza vaccines do not need the replication of the whole influenza virus. Because this technology only encloses

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important antigens of influenza, when designing a new pDNA vaccine the gene inserted into the vector must be carefully chosen to elicit the right immune response against to flu virus. Several influenza surface antigens are responsible for its mechanism of action: hemagglutinin (HA), neuraminidase (NA), M2 matrix protein, nucleoprotein (NP) or nonstructural protein (NS1). HA and NA are the most abundant surface proteins of this virus and are required for it to enter and exit of the host cells, while M2 is responsible for its replication [17]. NP supports the progress of the polymerase during the elongation phase [18], while NS1 selectively enhances the translation of viral mRNAs and may also regulate the synthesis of viral RNAs [19]. Several studies have been performed to evaluate these antigens as potential pDNA vaccine candidates. Confirming this premise, several studies showed that HA antigen is involved in the protection against influenza. In fact, pDNA vaccines encoding this gene are able to induce long-term memory with a high titer of neutralizing antibodies (anti-HA) [9, 10, 20-23]. Actually, antibodies directed to HA molecules neutralize the infectivity of the virus. As an example, Wei and co-workers [24] showed that cross protective titers can be achieved to viruses that circulated over 90 years ago. Furthermore, other study have shown that alternative DNA priming with inactivated influenza vaccines induce cross-reactive antibodies in 18-60 years old healthy adults to different strains marginally improving the immune responses of conventional vaccines [14, 24]. The concept of cross-neutralization of different influenza strains present in pDNA vaccines might be of great significance in future approaches to prevent influenza virus. Hence, it works not only by enhancing the protection against this virus in case of pandemics but also by reducing the disease severity [25]. Studies in Balb/c mice [17] revealed the complete protection against a H5N2 avian influenza strain when applying a DNA vaccine expressing a fusion protein consisting of H1N1 HA and M2 with enhanced HA-specific CD8+ and M2 specific T cell responses. This therapeutic approach including HA and M2 resulted in the inhibition of the virus replication. On the other hand, Jamali *et al.* [26] showed in a diabetic mouse model that the immunization using a NP pDNA vaccine protocol was not able to induce optimal cell-mediated immunity in diabetic mice. Despite antibodies against NA do not prevent the attachment and entry of influenza into the cells, they limit the spreading of the virus and increase the immunity against it. Another study with mice has demonstrated that NA of the seasonal H1N1 virus induces cross-reactive antibodies reducing its lethality [27]. Studies in other species reinforce the presence of cross-reactive neutralization after vaccination with pDNA encoding H1N1 influenza HA and boosting with seasonal vaccine in ferrets [24]. Also, adjuvanted influenza DNA vaccines in rabbits [28] presented well-tolerated safety profile.

HUMORAL AND CELLULAR IMMUNE RESPONSES STIMULATION

The limitations of traditional vaccines against influenza virus are well known and described [29]. This approach only has the capacity to stimulate humoral immunity, thus being ineffective to stimulate cell-mediated immunity. Therefore, it only activates one arm of the immune system, contrarily to DNA vaccination which is capable of activating both types of immune response (Fig. **1**). Briefly, pDNA based vaccines involve the inoculation of a plasmid vector into eukaryotic cells in order to transcribe its genetic material into mRNA, translate it and finally express the encoded protein (indirect route). After expression of the antigen of interest, these antigens molecules are presented to the immune system via major complex of histocompatibility class I (MHC 1) [24, 30-33]. Then the secreted antigens can directly stimulate B cells which in turn produce antibodies or can be captured and processed by antigen presenting cells (APCs), which mediate the display the MHC 2. This process has emerged as a strong and efficient method of eliciting both humoral and cellular immune responses and is similar to the natural processing that occurs on viral infection. To confirm this experimental data, DNA vaccination assays against influenza were performed in several animal models [13, 22-24, 33-36] showing that it stimulates cell-mediated immunity more efficiently than the native infection improving the fight against heterologous strains of influenza.

DNA-BASED VACCINES PRODUCTION

In order to withstand as a commercial alternative to traditional vaccination, DNA-based vaccines must be straightforwardly massively produced, allow the maximum expression of the encoded antigens and not show any hazards to the human hosts. One of the major concerns when working with pDNA is not only the risk of insertion into the human genome but also the induction of immunologic tolerance by the antigens expressed inside the host body [37]. Although the plasmid design is relatively easy, a variety of regulatory elements must be taken into account in order to potentially induce protective immune responses. Usually, pDNA expression vectors include a bacterial backbone and the mammalian expression components. Thus, these elements must be carefully selected if the purpose is to obtain sophisticated products that confer higher levels of immunogenicity. Regarding the eukaryotic elements, the plasmid should have an origin of replication (ORI) that allows the initiation of the replication process with high copy number of plasmids in *Escherichia coli (E. coli)*. A bacterial marker of selection must also be selected to assure the exclusive replication of the pDNA of interest. However, to further minimize the resistance to endogenous microbial flora of the patient, it is not allowed by the Food and Drug Administration (FDA) the insertion of penicillin or other Ā-lactam markers into the pDNA vector. RNA-OUT selectable marker has been studied as a substitute of Ā-lactam markers showing to be equivalent or superior to kanamycin resistant plasmid for expression and immunogenicity [38]. On the other hand, eukaryotic elements are chosen for the plasmid backbone with the main purpose of express the antigen in mammalian hosts. Thus, promotors/enhancers, introns and other sequences have been studied. Firstly, a strong promoter is needed in order to activate mRNA transcription in the mammalian cells. Recently, non-carcinogenic promoters like human cytomegalovirus (CMV) have been used due to their expression capacity in a wide range of mammalian cells [39]. A polyadenylation site (PolyA) insertion is also important to improve the stability and translation of mRNA. Also, the insertion of a kozak sequence next to the AUG initiator codon (ACCAUGG) within mRNA may facilitate its recognition by eukaryotic ribo-

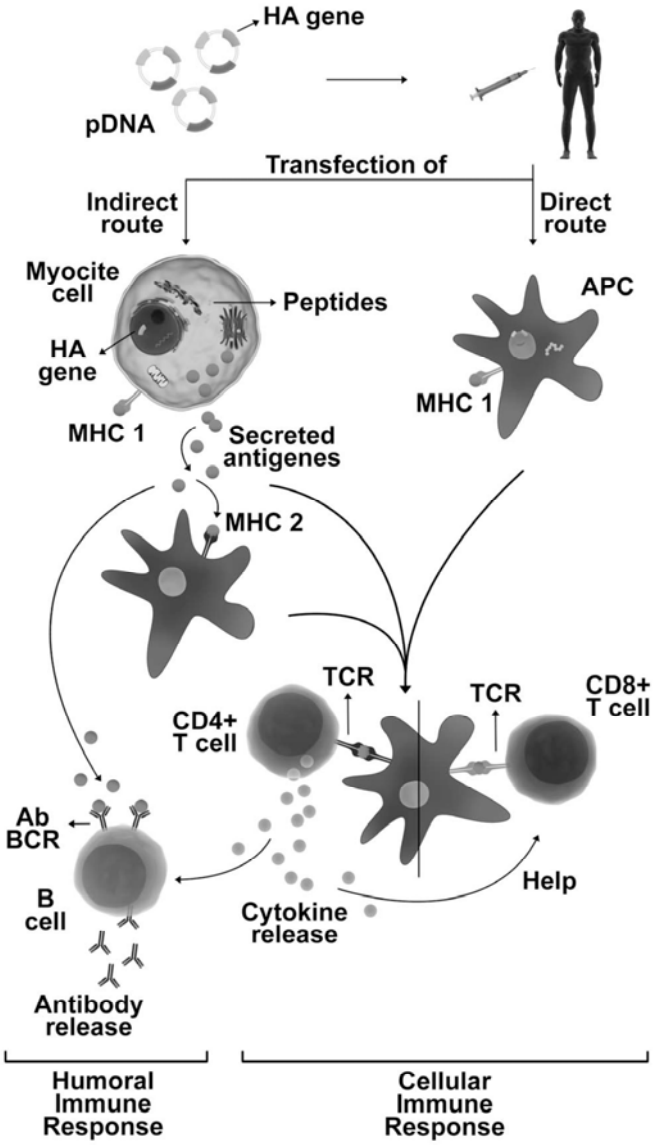

Fig. (1). Schematic representation of humoral and cellular immunity induction by pDNA vaccines. After injecting the DNA vaccine, myocytes (indirect route) and APCs (direct route) are transfected. Following the expression of the antigens of interest, these molecules are presented to the immune system via MHC 1. The antigens may also be secreted by myocytes or by the formation of apoptotic bodies that will lead to antigen presentation by APCs via MHC 2. APCs migrate to the lymph node and activate CD4+ and CD8+. Free antigens still lead to B-cell activation. APC: antigen presenting cell; MHC: complex of histocompatibility; BCR: B-cell receptor; TCR: T-cell receptor; Ab: antibody.

somes, enhancing the initiation of the translation. Finally, a cloning site to insert the heterologous gene of interest is needed. This gene represents a highly conserved region of the superficial antibody-generating structures of a given pathogen, providing a means to broadly activate the immune system [40]. Another major concern related with pDNA based vaccines is the short duration of the expression of the gene of interest which means that several doses of these vaccines must be applied. But due to the clinical safety of this therapy it is possible to administer multiple doses of DNA vaccines without triggering an immune reaction to the plasmid vector. Similarly, improved transgene expression has been observed in vectors with eRNA41H RIG-I agonist inserted into their backbones. These vectors have demonstrated to improve HA-specific serum antibody titers and HA-specific antibody binding avidity after naked DNA immunization [41]. Therefore, through the years several improvements were developed to increment the quality, yield and antigen expression of DNA vaccination approaches with the objective of ensuring the efficacy, safety and cost-effectiveness of future pDNA vaccine products.

REMOVAL OF HOST IMPURITIES FROM THE pDNA VACCINES

Prokaryotic production of DNA vaccines is an extremely convenient manufacturing process and its biggest challenge lies on the development of a scalable and economically viable means of production that meets clinical needs. Several bacterial impurities must be removed from the high quality

pDNA in downstream processes. Given that when using *E. coli* as a host, only 3% of the total fermentation products represent pure pDNA, numerous microbial growths are required to attain the necessary quantity of this biomolecule to provide a proper immune response. This fact also shows that the presence of impurities is massive when comparing to the pDNA quantity and so they must be selectively removed without causing substantial supercoiled (sc) pDNA losses. The sc pDNA isoform represents the biologically active form and the one with the best transfection efficiency, being the other forms of pDNA the result from damages inflicted to the sc form. So, the purification phase is of extreme importance because its failure will originate adverse reactions to the organism receiving the vaccine. These include undesired immunological and biological responses. Commonly, specific conditions are required to purify pDNA vaccines due to the structural and chemical similarities between pDNA and fermentation impurities (genomic DNA, RNA, endotoxins and proteins). This feature represents one of the major bottlenecks in the purification process. The removal of endotoxins needs extra care since they can produce toxic shock syndrome and dramatically reduce transfection efficiencies [42, 43]. Nevertheless, despite the difficulties faced in optimizing the purification strategy, this step must be kept simple in order to avoid several steps that could cause damage the sc pDNA molecules. Thus, the development of more robust and productive purification processes based on chromatographic techniques becomes essential. Several chromatographic methods based on properties as size, charge, hydrophobicity or affinity have been developed to purify sc pDNA. Accordingly, liquid chromatography became an important purification technique of therapeutic pDNA, presenting a variety of interactions with this biomolecule to explore. Nevertheless, the separation and purification of pDNA is faced with a number of limitations that are related with the available stationary phases such as low capacity and recovery yields but also low reproducibility and high contact time between the matrices and molecules which increase the biomolecule degradation. Therefore, better downstream purification processes are essential to produce licensable pDNA on an industrial scale. To date, no industrial approaches were described for the purification of high doses of pDNA influenza vaccines.

Current pDNA purification techniques are based in commercial kits that are not scalable for industrial purposes. However, many studies have been developed to obtain highly pure DNA vaccines using single chromatographic runs. These methods are able to achieve the pDNA purity degree demanded by the regulatory agencies (sc pDNA content higher than 97%). In 2009, Carnes *et al.* [44] compared the purification process of an influenza H5 hemagglutinin DNA vaccine plasmid either by alkaline lysis or acidic autolytic extraction with a final polishing step made by a Mustang Q membrane. The results showed identical isoform profiles, confirmed by agarose gel electrophoresis, and identical expression after transient transfection. Another work used a L-methionine-agarose matrix [45] to purify a p53-based DNA vaccine with an impurity level within the acceptable limits. Also, HPV 16 E6/E7 pDNA was purified using a modified monolithic disk immobilized with arginine [46]. In this approach it was obtained the sc DNA isoform with the purity degree required for pharmaceutical applications.

The implementation of unit operations with the purpose of using pDNA as a therapeutic vaccine needs to be well characterized, obtained by a standard bioprocessing method and verified *in vitro* using transfection to a cell line to measure the level of the products expressed by the gene of interest. This is a promising alternative to current therapies and will help to mitigate the costly production processes of conventional vaccines, thus helping to protect people against influenza outbreaks and other threatening diseases.

ROUTES OF PLASMIDS ADMINISTRATION FOR DNA VACCINATION

The location at which the substance is applied in the body is one of the most important aspects for the success of the DNA vaccine strategy. The usual target for influenza vaccination is the deltoid muscle. However, this tissue contains limited number of APCs, i.e. dendritic cells and macrophages, and lacks MHC 2 expressing cells, resulting in a reduction of humoral and cellular immune responses [47]. Additionally, the vaccines using naked DNA administered intramuscularly require multiple doses and large quantities of pDNA. Contrarily, the skin, that acts as a protective barrier against environmental pathogens, is an ideal alternative site for vaccination because it contains a rich network of APCs, which includes macrophages, Langerhans cells (LCs), and dermal dendritic cells (DCs). These cells will take up the antigen and migrate to the proximal lymph nodes where the T and B cells are activated and will initiate the adaptive immune responses [48]. Thus, different methods for dermal delivery of plasmids and other type of vaccines have been investigated. These include intradermal or subcutaneous gene delivery by topical cutaneous injection [49], microneedle patches [50] or tattoo vaccination [51, 52]. An example for the first method of application is the topical delivery of pDNA vaccine expressing keratinocyte growth factor-1 (KGF-1) in mice to increase the strength and thickness of the skin [49]. The results showed the need to do multiple applications of the pDNA vaccine and microdermabrasion to enable a proper transfection. In another study in humans [53], a pDNA vaccine complexed with polyethylenimine was applied topically in murine pulmonary mucosa, being tested as a potential solution against influenza. With this experimental method, it was provided a significant protection against this challenging respiratory infection, eliciting cellular and humoral protective responses in animal models. Dry-coated microneddles are another choice to improve conventional intramuscular injection. Song *et al.* [54] presented a transdermal delivery method in mice using microneddles coated with an influenza HA DNA vaccine. This method induced and enhanced antibody responses particularly by IgG2a, improved the protective efficacy and increased memory of humoral and cellular immune responses. Also, Sullivan *et al.* [50] showed that microneedle patches offer an attractive approach to administer influenza vaccines with improved safety and immunogenicity. The biolistic gene gun is a method that increases the epidermal delivery of DNA where the LCs and keratinocytes are transfected directly by bombarding gold particles coated with pDNA through the skin [55]. However, several studies with other type of vaccines

have preferred special routes of administration, such as the vaginal [56] to treat infections transmitted through intercourse, the intranasal [57] to protect against influenza virus and the gastric mucosa [58] to fight digestive infections. Additionally, electroporation has been a widely studied method for the delivery of pDNA vaccines. The application of high voltage pulses in a tissue with a duration of milliseconds disrupted the cell membrane and created pores that persisted for hours and allowed the entry of macromolecules, such as drugs, peptides and pDNA vaccines to the cytoplasm [59]. Although, the delivery method is important to assure the DNA vaccine efficiency, the gene transfer mechanisms that allow the translocation of the pDNA from plasma membrane to nuclear space must be also considered.

GENE TRANSFER TECHNOLOGIES FOR pDNA VACCINES

There is a great number of delivery routes for naked pDNA that have been investigated in order to enhance the stimulation of immune responses. It is well known that naked pDNA administered intravenously quickly decays from the sc to the open circular isoform, thus increasing the susceptibility to degradation by cytosolic nucleases. To overcome this problem, nanotechnology has emerged to facilitate the internalization of pDNA vaccines. This technology is currently applied for drugs delivery, but is already being studied for the delivery of these vaccines and gene therapy. Nanoparticles are used because they present a similar size to cellular components which enables them to enter in the living cells using the cellular endocytosis mechanism. As an example, Gaspar *et al.* [60] showed its usefulness by developing a chitosan-arginine-nanodevice, which represents a remarkable improvement in the cellular uptake of pDNA because it represents an efficient strategy to overcome cellular barriers and maximize its inherent potential as a non-viral gene delivery system. On the other hand, the presence of adjuvants also increases the potency of the vaccine since they contradict the low immunogenicity of pDNA vaccines [61]. Such adjuvants can be delivered either as separated plasmids or as additional genes encoded by the plasmid. Upon vaccination, co-administration of influenza pDNA with IL-15 and IL-18 increased the antibody production, T cell responses and cytokine production [62]. Cells transfected with the adjuvant-encoding plasmid can also express and secrete the molecular adjuvant into the surrounding region, affecting local APCs and cells in the draining lymph node. Fagone *et al.* [63] tested the ability of pDNA to encode NP or HA of an influenza strain previously mutated for the expression of HMGB1. They revealed that a HMGB1-encoding plasmid enhanced the immunogenicity and protective efficacy of a pDNA vaccine against the virus. It was observed an increase not only in the proliferative response of CD4+ T-cells but also in the CD8+ T-cell response. So, HMGB1 augmented the protective effect of this pDNA vaccine, which is proved by the significant increase in the survival rate in the co-immunized animals compared with the controls. Even though the delivery of pDNA vaccines to the nuclear compartment can be made by several methods, the most effective delivery system must be identified and optimized to this specific purpose in order to encourage the further development of new formulations.

Table 1. Recent and ongoing clinical trials of influenza DNA vaccines using different antigens, strains and delivery methods.

Clinical Trial Registration	Antigen	Strain	Delivery Method	Results	Ref.
---	HA	A/New Caledonia/20/99 (H1) A/Panama/2007/99 (H3) B/Jiangsu/10/2003	Particle mediated epidermal delivery (gene gun)	A complete phase 1 trial that demonstrated reduction in the symptoms of the disease and improved viral shedding in subjects who received a trivalent DNA-based seasonal influenza vaccine. Cell mediated immune responses were not measured.	[10]
NCT00709800 and NCT00694213	HA, NP and M2	A/Vietnam/1203/04 (H5) A/Puerto Rico/8/34 (H1N1) A/Hong Kong/8/68 (H3N2).	Intramuscular injection	The monovalent H5 DNA vaccine together with Vaxfectin® achieved protective antibody responses in more than 47% of subjects, and H5-specific T-cell responses were detected in at least 75% of subjects.	[15]
NCT00776711 and NCT01086657	HA	A/Indonesia/5/05 (H5)	Intramuscular injection	Influenza DNA vaccination priming increased the magnitude of protective antibody responses (HAI) and in some cases induced hemagglutinin-stem-specific neutralizing antibodies.	[13]
NCT00408109 and NCT00489931	HA	A/Indonesia/5/05 (H5)	Intramuscular and intradermal injection	DNA vaccine encoding H5 was safe and immunogenic. It showed no significant difference between intramuscular and intradermal injection.	[12]
NCT01086657	HA	A/Indonesia/5/2005 (H5)	Intramuscular injection	H5 DNA priming enhanced antibody responses after a MIV boost when the prime-boost interval was 12-24 weeks.	[11, 14]

CLINICAL STUDIES USING INFLUENZA DNA VACCINE

DNA vaccines have been able to induce immune responses to the encoded antigens in animal studies [25, 26]. The promising results obtained with pDNA vaccine have encouraged the progress of this innovative vaccination mechanism into clinical trials. As seen in (Table **1**), the first report of phase I trial with influenza DNA vaccines was conducted by Jones *et al.* [11]. This study intended to use a trivalent DNA vaccine (expressing hemagglutinin from different seasonal influenza virus strains) to protect individuals against a controlled influenza infection. The formulation was well tolerated and no serious adverse reaction appeared. Serological responses showed that all the subjects had antibody responses to the vaccine and the disease symptoms were reduced. Another completed phase I clinical trial was conducted by Smith *et al.* [16] and used intramuscular injections of Vaxfectin® (cationic lipid-based formulation) adjuvanted pDNA vaccine to treat influenza. This study demonstrated that all doses were well tolerated without serious vaccine-related adverse events. The monovalent vaccine expressing the HA protein showed that between 47–67% of the subjects expressed H5-specific T-cell responses but trivalent vaccines expressing also NP and M2 proteins showed 75–100% responses. Very similar results were reported by Ledgerwood *et al.* [14] which used a DNA/monovalent inactivated vaccine (MIV) that was safe and enhanced H5-specific antibody titers. In a following study [13], intramuscular and intradermal administration of H5 DNA vaccine were compared and no statistical significant difference was registered related to safety, antibody or T cells responses. Finally, the shortest prime-boost interval was defined [12, 15] in order to improve the antibody response to influenza A. DNA priming with a MIV boost showed better response, with a positive HAI (hemagglutination inhibition) titer, than DNA and MIV prime-boost interval or MIV-MIV prime-boost. Thus, pDNA well tolerated and safe alternative vaccines are expected to be capable of replacing the current vaccine strategies.

Table 2. Advantages and drawbacks of both traditional and pDNA influenza vaccines.

Traditional Influenza Vaccines			
Advantages	**Ref.**	***Disadvantages***	**Ref.**
Induces robust humoral immunity	[29]	Ineffective to stimulate cell-mediated immunity	[29]
Has effective immunity against a particular strain of a pathogen	[4]	Propagation in embryonated chicken eggs	[3]
Provides high effectiveness in healthy individuals	[2]	The protection conferred by it is low and disappears after a certain period of time	[4]
Most used method for the prevention of an influenza outbreak	[2]	Preservation at low temperatures (2ºC – 8ºC).	[6]
Confers protection against the three virus strains (H1N1, H3N2 and influenza B)	[2]	Minimum of six months for the propagation of the virus, purification, inactivation and production of the vaccine	[3]
		Displays limited protection against closely genetically related virus but not against heterologous subtypes	[4]
		Safety issues related with the viral vector or attenuated viruses	[13]
Influenza plasmid DNA vaccine			
Advantages	**Ref.**	***Disadvantages***	**Ref.**
Highly effective, stable, safe and cost-effective	[5]	Can induce immunologic tolerance by the antigens expressed inside the host body	[38]
Activate both types of immune response humoral and cellular immune responses	[17]	May need additional technologies such delivery devices, formulation or heterologous boost.	[47]
Leads to strong and long-lasting immune responses (cross-strain protection)	[14]	Plasmid does not recognize specific targets in the cells, so can suffer degradation before it enters the cells	[60]
Absence of specific immune responses to the plasmid itself	[25]	Potential host autoimmunity	[38]
Easy and rapid production (~two weeks)	[1]	Risk of malignancy	[31]
Storage in the lyophilized form at room temperature	[5]	Need conjugation or encapsulation of the plasmid to avoid degradation and improve immunity	[13, 60]
Vaccines can induce broad protection against divergent influenza viruses.	[24]		
No issue related to prior-exposure pathogen arise	[40]		
A mixture of plasmids could be used to form a broader spectrum vaccine	[37]		

CONCLUSION AND FUTURE DIRECTIONS

As shown above, influenza DNA vaccination can represent a safe alternative to current commercial vaccines. Despite all the referred advantages there are still some drawbacks of this therapy that also need to be overcome (Table **2**). Although the good immunogenicity of pDNA biomolecules, they are especially susceptible to degradation by nucleases, thus affecting the expression of the transgenes. To make up for this limitation the plasmid construction can be altered either by targeting the expressed antigen to professional APCs and thereby ensuring the efficient MHC-I and MHC-II compartment activation, by including adjuvants or by using specific delivery methods. Even though it has not been proved, the presence of prokaryotic protein subunits in pDNA vaccines backbone can trigger an allergic reaction or cause the mutation of the elements in the plasmid. So, minicircle DNA without unnecessary bacterial material has emerged as an alternative to conventional pDNA vaccination, because of its easiness to manipulate, produce and transfect [64, 65]. This strategy only encodes an antigen expression cassette (promoter, antigen and polyA region) and has shown to be immunogenic, inducing both a cellular and humoral responses which means that it can be used as a potential strategy to combat influenza infections. In this fight, as previously referred, current vaccines need to be updated every year in order to be able to protect against newly emerging seasonal or pandemic flu strains. To fulfil this objective, the development of universal vaccines with the most conserved portions of influenza virus such as M2 [66], NA [67] or NP [26] are been designed. These antigens, with low drift, should be able to recognize and neutralize several strains without the usual selection of the strains to be inserted in the annual vaccine. However, antibodies against these proteins are not generally protective without adjuvants or other components that dramatically enhanced immune response [68]. Despite the improvements in vector design and the use of molecular adjuvants, there is still a clear requirement for an efficient method of administration of DNA vaccines [69]. A physical method for drug and gene delivery that temporarily increases skin permeability is electroporation which allows the passage of large molecules of much lower doses with high effectiveness [70]. Also, in order to have well stablished procedures that follow the regulatory agencies requirements, pDNA vaccines and downstream processes platforms that are well suited must be created in order to address the emerging or suspected infectious disease threats even before they become pandemics. Advances in DNA vaccination indicate that in the future superior product profiles will be available that might replace conventional vaccines. So, this strategy can save time and economic resources eliminating many of the hurdles that limit the rapid development and deployment of a vaccine to combat influenza infections.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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Chapter 2

Global aims

The principal aim of this project was to develop new and more efficient purification processes for DNA vaccines encoding the influenza virus protein hemagglutinin by taking advantage of monolithic chromatographic supports. To pursuit this goal the selection of the appropriate ligand is extremely important in order to obtain the most biologically active isoform of this biomolecule, sc pDNA. The interactions between the chosen ligand and pDNA are the key to establish the best chromatographic conditions to efficiently achieve a final product highly pure with clinical application.

The biggest challenge of choosing the right loading and elution conditions resides in the similarities between the impurities of the *E. coli* host and pDNA in terms of chemical composition and structure (gDNA, RNA) or charge (endotoxins). Having in mind this global aims, the specific tasks developed in this work were:

1) Selection of the molecules to be used as ligands for the purification of the hemagglutinin DNA-based vaccine;
2) Study of the influence of different plasmid sizes in the chromatographic downstream processing;
3) Screening of the binding and elution conditions on pDNA isoforms (flow rate, ionic strength and pH of the mobile phase) and its impact on the chromatographic behavior;
4) Evaluation of the support capacity to efficiently separate sc pDNA from complex lysates following the specification of the regulatory agencies.
5) Assessment of the biological effectiveness of the final sc pDNA product by monitoring the expression of hemagglutinin.

Chapter 3

Paper II

Effect of chromatographic conditions and plasmid DNA size on the dynamic binding capacity of a monolithic support

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Research Article

Effect of chromatographic conditions and plasmid DNA size on the dynamic binding capacity of a monolithic support

DNA therapies are becoming recognized alternatives for the treatment and prevention of severe pathologies. Although most current trials have used plasmids <10 kbp, in the future larger plasmids would be required. The purpose of this work was to study the chromatographic behavior of nongrafted carbonyldiimidazole monolithic disks using plasmids with different sizes under hydrophobic conditions. Thereunto, the purification of several plasmids was performed. Higher size plasmids needed lower ammonium sulfate concentration, due to the greater number of interactions between the plasmids and monolith. The dynamic binding capacity experiments for the different plasmids revealed a lower capacity for bigger plasmids. It was also verified that the increase of salt concentration from 2.5 to 3 M of ammonium sulfate increased the capacity. At the highest salt concentration, a slight improvement in the capacity using lower flow rate was observed, possibly due to compaction of plasmid molecules and its better organization on the monolith channels. Finally, a low pH also had a positive effect on the capacity. So, this monolithic support proved to be appropriate to purify the supercoiled isoform of different plasmids with different sizes, providing a valuable instrument as a purification technique.

Keywords: Affinity chromatography / Dynamic binding capacity / Monoliths / Supercoiled plasmid DNA
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1 Introduction

Gene therapy and DNA vaccination have gained some relevance mainly because of the ability of cells to uptake foreign genetic information and to express encoded proteins [1]. These therapies require large quantities of highly pure supercoiled (sc) plasmid DNA (pDNA), which is the most efficient isoform for gene expression and immune responses induction [2]. Thus, according with regulatory agencies, pDNA for pharmaceutical applications should be free from bacterial host impurities and the plasmid homogeneity must be >97% of sc isoform [3]. Extensive efforts have been made to develop efficient and reliable processes in order to accomplish these requirements [3]. The purification strategy should also be applicable to plasmids with different sizes, since this parameter tends to change with future requirements for multigene vectors, including extensive control genes, which involve larger plasmids (>10 kbp) [4]. New downstream methods for pDNA purification have emerged mainly based on chromatographic techniques exploiting different types of interactions [5,6]. Despite the good results achieved using affinity chromatography with amino acid as ligands [1, 7–9], some problems remain to be solved. The low recovery yields and slow diffusion that leads to degradation of target biomolecules [10, 11] and low capacity are still the main limitations.

The dynamic binding capacity (DBC) describes the amount of sample that will bind to a chromatographic support under defined conditions, i.e. the maximum plasmid–ligand adsorption. The breakthrough curve can be used to determine not only the column capacity but also how much plasmid has been wasted during the adsorption phase, processing time, and production rate [12]. Thus, its determination is essential to optimize the purification process and extend the column lifetime.

Progress has been made to overcome the limitations of conventional matrices, among others the development of superporous stationary phases, ligand immobilization into the bead surface, and application of monolithic supports and adsorptive membranes [1, 13]. Monolithic columns have shown a better performance than conventional supports and for that, nowadays, they hold an impressively strong position in biomolecule separation. Convective Interaction Media (CIM™) is a continuous rigid matrix with a highly porous polymer, which allows the mass transport enhancement of large pDNA molecules. Therefore, the monolith allows the efficient purification of large biomolecules due to its highly

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Abbreviations: **$(NH_4)_2SO_4$**, ammonium sulfate; **CDI**, carbonyldiimidazole; **DBC**, dynamic binding capacity; ***E. coli***, *Escherichia coli*; **FDA**, Food and Drug Administration; **oc**, open circular; **pDNA**, plasmid DNA; **sc**, supercoiled

accessible binding sites [14]. These supports have low backpressure even under high flow rates, short contact time reducing the biomolecule degradation, simple handling, and flow-independent resolution [15, 16].

Our previous work [17] showed that nongrafted carbonyldiimidazole (CDI) glycidyl methacrylate monolith has specific recognition for sc isoform at different flow rates, exhibiting a satisfactory capacity for the model plasmid pVAX1-*LacZ*. The present work showed the CDI applicability in the purification of plasmids with different sizes. Additionally, the effects of several parameters in the DBC of the CDI monolith were also explored, including the pDNA size, flow rate, $(NH_4)_2SO_4$ concentration, and pH of the mobile phase.

2 Materials and methods

2.1. Materials

Experiments were performed using the nongrafted CDI poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith [18], packed into a CIM^{TM} disk housing of 0.34 mL (3 mm thick and 12 mm diameter), provided by BIA Separations (Ajdovščina, Slovenia). All salts, ammonium sulfate [$(NH_4)_2SO_4$] from Panreac (Barcelona, Spain), sodium phosphate monobasic anhydrous and sodium phosphate dibasic from Sigma-Aldrich (Madrid, Spain), were of analytical grade. The solutions were freshly prepared using deionized water of ultra-pure grade from a Milli-Q system from Millipore (Billerica, MA, USA), filtered through 0.20 μm pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. Four different size plasmids were used in the experiments: pcDNA3-myc-FLNa S2152A (14 kbp), Addgene plasmid 8983, (pcDNA3-based plasmid) [19] with both ampicillin and neomycin resistance (Addgene, Cambridge, USA); pEGIP (10.292 kbp) [20], and Addgene plasmid 26777, with high copy number (Addgene), EF1α promoter and puromycin and ampicillin resistance genes; pVAX1-*LacZ* (6.05 kpb) designed for the development of DNA vaccines (Invitrogen, Carlsbad, CA, USA), and used as a model plasmid with Human cytomegalovirus (CMV) early promoter/enhancer and kanamycin resistance gene; finally, pUC19 (2.686 kbp; Invitrogen), a standard DNA cloning vector for *Escherichia coli* (*E. coli*) with ampicillin resistance and a *Lac* promoter. *Escherichia coli* DH5α host strains were used for harboring the pcDNA3-based plasmid, pUC19 and pVAX1-*LacZ* plasmids. pEGIP vector was obtained with Stbl3 *E. coli* strain.

2.2 Bacterial growth conditions and plasmid production

Growth was carried out in a 250 mL shake flask at 37°C with Terrific broth medium supplemented with 100 μg/mL ampicillin plus 50 μg/mL neomycin for pcDNA3-based plasmid, 100 μg/mL ampicillin plus 25 μg/mL puromycin for pEGIP, 30 μg/mL kanamycin for pVAX1-*LacZ* and 100 μg/mL ampicillin for pUC19. Bacterial cell growth was monitored by measuring the OD_{600} using a Pharmacia Biotech Ultraspec 3000 (Cambridge, UK) and suspended at late log phase ($OD_{600} \approx 10$). The cells were recovered by centrifugation and stored at −20°C.

2.3 Alkaline cell lysis and pDNA isolation

The alkaline lysis [21] used to isolate pDNA from *E. coli* cells was accomplished using a Plasmid Purification Maxi Kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. The final pDNA sample used in the experiments contained around 90% of the sc isoform, while the remaining 10% was open circular (oc) isoform, as revealed by agarose electrophoresis.

2.4 Chromatography assays

All chromatographic experiments were carried out in an ÄKTA purifier system (GE Healthcare Biosciences, Uppsala, Sweden) using UNICORN software, Version 5.11. The monolithic column was equilibrated with three column volumes of the mobile phase. The system was run at three flow rates 0.5, 1, and 1.5 mL/min, and monitored at 260 nm. Partially purified pDNA (300 μg/mL) with the Qiagen kit, was resuspended in 50 mM phosphate buffer (pH 8.0) with the ionic strength adjusted with $(NH_4)_2SO_4$. After the injection of the sample (50 μL), the elution of unbound species was performed with $(NH_4)_2SO_4$ in 50 mM phosphate buffer (pH 8.0) and the bound species were eluted with three column volumes without $(NH_4)_2SO_4$. The fractions (1 mL) were collected, concentrated, and desalted with Vivaspin® concentrators (Sartorius Stedim Biotech, Goettingen, Germany) for further electrophoretic analyses. The experiments were performed at room temperature.

2.5 DBC experiments

The CDI column was also used for DBC experiments using plasmid feedstock prepared with the Qiagen kit. The final pDNA product was resuspended with 50 mM phosphate buffer and $(NH_4)_2SO_4$ was adjusted according to the equilibration buffer. The CDI disk was equilibrated with 2.5 or 3 M $(NH_4)_2SO_4$ in 50 mM of phosphate buffer pH 8.0 with three flow rates, 0.5, 1, and 1.5 mL/min. The DBC experiments studied the effect of salt concentration, plasmid size, flow rate, and pH. The DBC was determined by open-loop frontal analysis [22] using the breakthrough area integration method described by Kaltenbruner and Jungbauer [23]. At the end of each experiment, the amount of pDNA bound per milliliter of support at 10 and 50% breakthrough was calculated. The void volume determined under nonbinding

conditions was discounted to each breakthrough curve. Elution of the bound plasmid was achieved by decreasing the salt concentration in the mobile phase to 0 M in a stepwise manner by reversed flow once the disk is not damaged with this procedure. DBC was calculated with the following equation (Eq. (1)):

$$q = \frac{(V_{\text{breakthrough}} - V_{\text{d}})C_0}{V_{\text{c}}} \quad (1)$$

where $V_{\text{breakthrough}}$ is the volume corresponding to the feedstock loaded at% breakthrough (mL), V_{d} characterizes the void volume from bypass run in milliliter (dead volume), C_0 describes pDNA concentration in the feedstock (mg/mL) and V_{c} represents the monolith bed volume (mL).

2.6 Pressure drop measurements

The pressure drop on CDI disks was calculated using the Konzeny–Carmen equation (Eq. (2)). commonly applied for noncompressible porous beds and previously modified by Podgornik [24]

$$\Delta P = 2v\mu L k_v^2 \frac{1}{d_v^2}\frac{1}{\varepsilon} \quad (2)$$

where v is the mobile phase linear velocity, μ denotes the mobile phase viscosity, L is the bed length (m), k_{v} is a proportional constant reflecting the shape of the pore, d_{v} represents the pore linear dimension, and ε characterizes the monolith porosity.

2.7 Agarose gel electrophoresis and sample purity and quantity analysis

Agarose electrophoresis was run by an electric field in a horizontal gel electrophoresis using a 10 cm long gel stained with 0.1 μL/mL of gel GreenSafe (NZYTech, Lisbon, Portugal). The solutions were prepared by dissolving agarose (Hoefer, San Francisco, CA, USA) in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0), with the final agarose concentration adjusted from 0.5 to 1.0% w/v depending on the plasmid size. The electrophoresis was carried out at 120 V for 35 min and was visualized in a UVITEC system (Cambridge, UK) using Firereader 1D Gel analysis software version 15.15 of UVITEC. DNA concentration and purity was estimated by using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Amersham, Netherlands) where one unit of od 260 nm corresponds to 50 μg/mL pDNA concentration. Purity was expressed as the absorbance ratio at 260/280 nm considering a pure plasmid sample with ratio between 1.8 and 2.0.

3. Results and discussion

3.1 pDNA purification

It was recently described [17] that glycidyl methacrylate based monolithic columns with CDI ligands present significant advantages over the current supports for the separation and purification of nucleic acids. This monolith proved to be effective in the isolation of pDNA isoforms of a model plasmid (pVAX1-*LacZ*) with high-purity degree as required by the Food and Drug Administration (FDA) [25] and high yield. The study revealed affinity interactions between CDI-monolith and pDNA, with a dissociation constant value (K_{d}) of $4.81 \pm 0.21 \times 10^{-8}$ M, showing good affinity as chromatographic support. Therefore, it was necessary to prove the robustness of this method using this strategy with plasmids with different sizes, which were pcDNA3-based plasmid (14 kbp), pEGIP (10.292 kbp) and pUC19 (2.686 kbp). pDNA samples were injected in an agarose gel electrophoresis (Fig. 1A) with their respective digestion in order to verify the different migration profiles. Figure 1B shows the chromatographic profile obtained when a native pDNA sample (oc + sc) was loaded onto the monolithic support. The separation of isoforms was achieved using a stepwise salt gradient. As showed by the agarose gel electrophoresis (Fig. 1B), the oc pDNA isoform (lane 1) was eluted in the first step at high ionic strength (applying 2.50, 2.20, or 2.06 M $(NH_4)_2SO_4$ for the plasmids pUC19, pEGIP, or pcDNA3 based plasmid, respectively). The elution of sc pDNA (lane 2) in the second step was performed at low salt concentration, with only 50 mM phosphate buffer pH 8.0 at 1 mL/min for pEGIP and pUC19 or 0.5 mL/min for the pcDNA3-based plasmid. These results reveal a stronger interaction between the sc isoform and CDI monolith. The higher overall charge density of sc pDNA can be responsible for the interactions with specific groups of the monolith, which allowed the separation of pDNA isoforms. The deformation induced by torsional strain in the sc isoform promotes higher base exposure and the involvement of different interactions thus improving the selectivity for this isoform. As mentioned by Diogo et al. [5] and Giovannini et al. [26], plasmid conformation plays an important role in pDNA purification due to the different interactive forces. Likewise, Sousa et al. [27] studied the preferential and specific interaction between histidine–agarose matrix and pDNA isoforms, proving a tendency of sc pDNA to be eluted under low salt concentration, indicating the ring stacking/hydrophobic interactions and histidine–DNA/RNA direct hydrogen bonding, which are favored at high salt concentrations. Similarly to the histidine agarose matrix, the specific recognition in the CDI monolith could also be explained on basis of the interaction between imidazole ring and pDNA. Besides that, other elementary interactions could be involved [17].

Figure 1B also shows a differential interaction between plasmids with different sizes and CDI groups in the monolith. The salt concentration required to bind pDNA decreased with the increase of the plasmid size. This fact could be

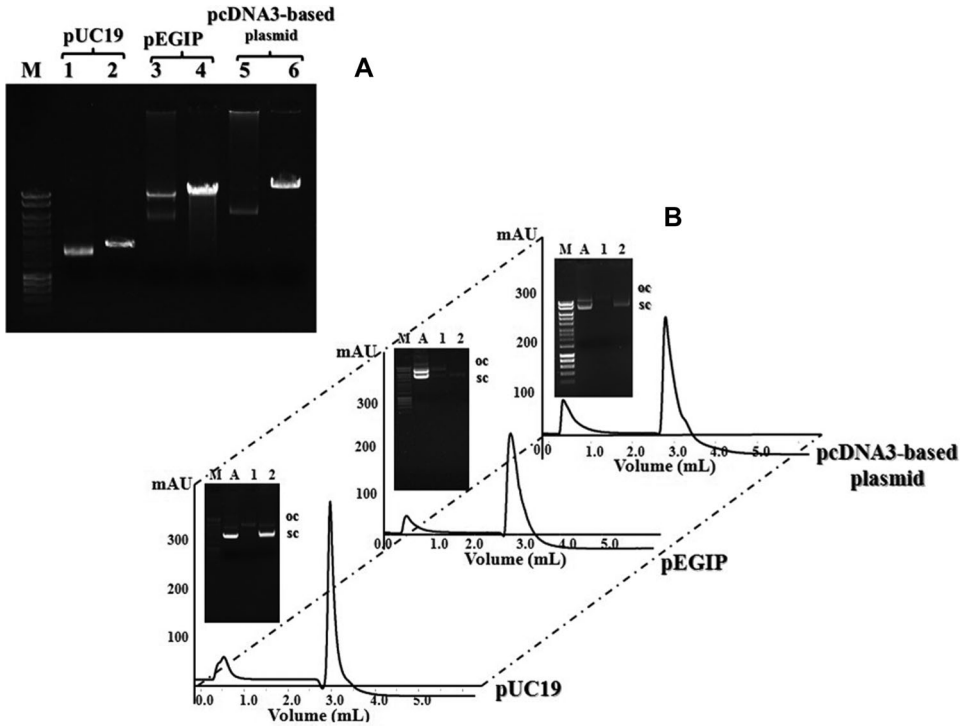

Figure 1. (A) Agarose gel electrophoresis of each plasmid used and respective digestion. M: molecular weight marker (HiperLadder I: 10 kbp); lane 1: pUC19 (oc + sc); lane 2: pUC19 digested with Sma I (ln); lane 3: pEGIP (oc + sc); lane 4: pEGIP digested with Nhe I (ln); lane 5: pcDNA3-based plasmid (oc + sc); lane 6: pcDNA3-based plasmid digested with Hind III (ln). (B) Chromatographic profiles of plasmids with different sizes and their effect on resolution of pDNA isoforms. Different salt concentrations were used for each plasmid namely pUC19, pEGIP, pcDNA3-based plasmid (2.50, 2.20, and 2.06 M $(NH_4)_2SO_4$). Agarose electrophoresis of pDNA isoforms separation is associated with each chromatogram. M: molecular weight marker (HiperLadder I: 10 kbp); A: sample (oc + sc); lane 1: oc; lane 2: sc.

explained by the existence of a greater number of interactions established in larger plasmids, as a consequence of the higher number of exposed bases and contact points to strengthen the binding between plasmids and the binding sites in the monolith. In this case, the ionic strength necessary to bind the plasmid biomolecules is lower.

3.2 Dynamic binding capacity

3.2.1 Effect of pDNA size

As previously described [17], the oc isoform of pVAX1-*LacZ* presented a lower DBC than the sc isoform. This occurs because of the lower degree of compaction of the oc isoform, which increases its superficial contact area, reducing the binding of other biomolecules, and decreasing the DBC. Thus, it became clear that the reduction in size by changing plasmid topology resulted in a capacity improvement [28]. This question led us to test different plasmid sizes in the capacity experiments. As shown in Fig. 2A, the CDI monolith had a lower capacity for the larger plasmids pEGIP (0.203 mg/mL at 10% breakthrough) and pcDNA3-based plasmid (0.141 mg/mL at 10% breakthrough) than for the model plasmid pVAX1-*LacZ* used in a previous study (2.206 mg/ mL at 10% breakthrough) [17]. This means that the lowest DBC was obtained for the largest plasmid (pcDNA3-based plasmid) at 2.5 M $(NH_4)_2SO_4$ with a flow rate of 1 mL/min (Fig. 2A). Additionally, the results presented in Table 1 also show an increase in DBC when smaller plasmids were used, i.e. the monolith presented a higher DBC for pUC19 than for pcDNA3-based plasmid at both flow rates and $(NH_4)_2SO_4$ concentration. Since smaller molecules of sc pUC19 can easily penetrate into the channels of the monolith due to their more compact form, they will cover a smaller area when bound to the support, improving the possibility of binding other biomolecules and, consequently, improving the DBC. Krajnc et al. [29] found a DBC decrease with the increase in plasmid size from 39.4 to 62.1 kbp under ionic conditions using NaCl. They showed a maximum DBC of 12.4 mg/mL for 39.4 kbp plasmid using 0.4 M NaCl, while that for 62.1 kbp was 8.4 mg/mL using 0.5 M NaCl. However, in another study Krajnc et al. [11] referred that although there are some scattering, the slight decrease on the DBC with the increase of molecules size is not a significant trend. On the other hand, Yamamoto and Kita [30] who worked with CIM QA monoliths and several proteins found that DBC increases with the increase of protein size, using ionic conditions. In fact, these different studies proved that DBC of monoliths is affected by experimental conditions and biomolecules used. In our work, the main difference exists in the salt used leading to multiple interactions, mainly hydrophobic, which implies a different behavior of pDNA within the monolith. The $(NH_4)_2SO_4$ added to the sample can compact the molecules; however even under salt conditions, large molecules occupied more binding sites reducing the DBC.

Most of the breakthrough curves of DBC present a typical S shape and a gradual stealthy movement until the column is saturated. The characteristic shape of this curve depends on plasmid concentration, ligand density, conductivity, pH inlet, flow rate, diffusion mechanisms, column diameter, and

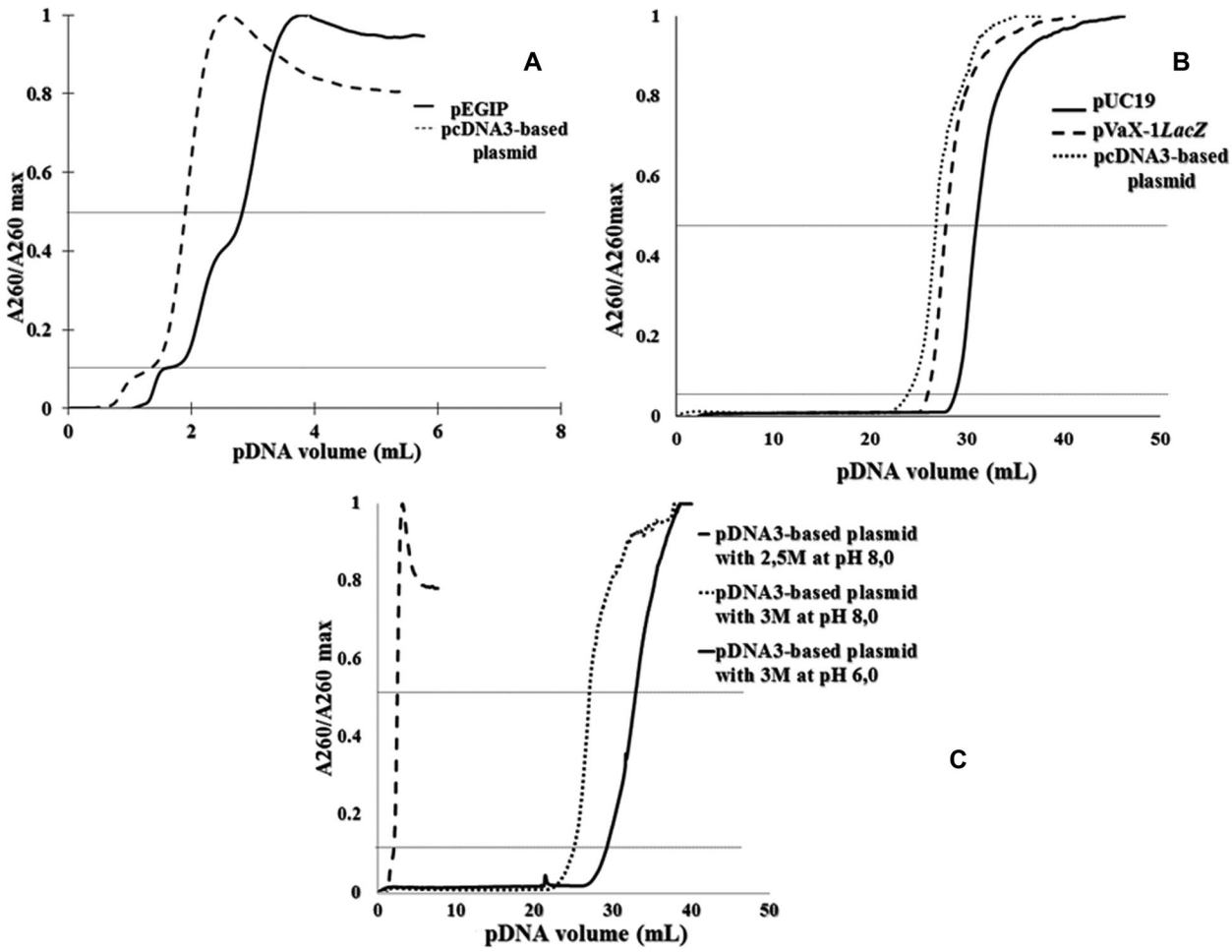

Figure 2. Breakthrough curves of sc pDNA (0.05 mg/mL) in CDI disk at room temperature using 1 mL/min flow rate. (A) Influence of large plasmids in the DBC, pEGIP (10.292 kbp), and pcDNA3-based plasmid (14 kbp), with 2.5M $(NH_4)_2SO_4$. (B) Plasmid size effect on the DBC, pUC19 (2.686 kbp) pVAX-1LacZ (6.05 kbp), and pcDNA3-based plasmid (14 kbp) using 3M of $(NH_4)_2SO_4$ concentration. (C) Effect of salt concentration (2.5 and 3 M of $(NH_4)_2SO_4$) and pH (8.0 and 6.0) in the DBC of pcDNA3-based plasmid (14 kbp).

Table 1. Effect of plasmid sizes on 10 and 50% DBC of nongrafted CDI monolith. The sc isoforms of pUC19 (2.686 kbp) pVAX1-*LacZ* (6.05 kbp), and pcDNA3-based plasmid (14 kbp) were loaded with 3 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer at flow rate of 0.5 mL/min (A) and 1 mL/min (B)

Breakthrough (%)	Flow rate	DBC (mg/mL)		
		pUC19	pVAX1-*LacZ*	pcDNA3-based plasmid
10	0.5 mL/min	5.317	5.086	4.902
50	(A)	5.891	5.478	5.319
10	1 mL/min	4.246	3.295	3.598
50	(B)	4.502	4.217	3.891

bed height [31, 32]. Nevertheless, as Fig. 2A demonstrates, the plasmid size also seems to influence the shape of breakthrough curves and consequently the DBC. As depicted in Fig. 2A, large plasmids (pEGIP and pcDNA3-based plasmid) present a noticeable difference in the breakthrough curve shape when compared to (pVAX1-*LacZ* with 6.05 kbp) [17]. Theoretically, the channel size of an adequate support should be at least five times larger than the average size of the biomolecule for an increase in the DBC [33]. However, the average pore radius for a CDI monolith is between 600–700 nm and the usual plasmid size varies between 150 and 250 nm [34], which means that these results could be affected by the channel size of the monolith and also by the plasmid size, as described by Watson [35]. This could affect the passage of larger plasmids through the monolith and reduce the DBC. Still, hydrophobic interactions could be involved in the curve behavior of bigger plasmids under 2.5 M $(NH_4)_2SO_4$ as shown in Fig. 2A, once this salt concentration could not allow the complete penetration through the narrow channels of the support, which seemed to cause a temporary obstruction and consequently deformation of the curve. High salt concentration has several effects on the binding kinetic of pDNA molecules and their access to the internal surface area of the monolith. The geometry of pDNA molecules depends

Table 2. Impact of size and salt concentration on the registered CDI monolith pressure and viscosity values at flow rate of 1 mL/min

Pressures (MPa)	2.5M $(NH_4)_2SO_4$			3M $(NH_4)_2SO_4$		
	pVAX1-LacZ	pEGIP	pcDNA3-based plasmid	pUC19	pVAX1-LacZ	pcDNA3-based plasmid
Lowest[a)]	0.4	0.6	0.25	0.5	0.5	0.4
Highest[a)]	0.8	1.55	0.45	1.0	1.15	3.5
Pressure drop (MPa)[b)]	0.860	0.856	0.872	0.997	0.928	0.944
Viscosity, μ (mPa·s)[c)]	3.59	3.57	3.64	4.16	3.87	3.94

a) Achieved by Äkta pürifier system.

b) Calculated from Carman–Konzey equation.

c) The data used for the calculation of the viscosity values in the table where proportional constant $k_v = 8.3$, the pore linear dimension $d_v = 650$ nm, and the porosity $\varepsilon = 60\%$.

dramatically on the quantity of salt, which involves the reorientation of the molecules [33].

These limitations are evidenced in Table 2 where the pressure values for each plasmid are shown for two $(NH_4)_2SO_4$ concentrations at 1 mL/min. Even when the pressure drop is considerably lower for monoliths than for classic particulate stationary phases [36], in the present work it was visible that a pressure increases with the plasmid size from pUC19 to pcDNA3-based plasmid using 3 M $(NH_4)_2SO_4$, although the pressure drop has been slightly higher for pUC19. This is in agreement with Krajnc et al. [29], demonstrating that the passage of large molecules through the monolith channels depends on the molecular size. Table 2 also shows an increase in pressure drop for experiments with 3 M $(NH_4)_2SO_4$ when compared with 2.5 M for pVAX1-*LacZ*. This can be explained by a major entrapment of the plasmid into the monolith channels due to its compressed form in presence of high salt concentrations.

Figure 2B revealed that a higher DBC was achieved for pUC19 (4.246 and 4.502 mg/mL for 10 and 50% breakthrough curve, respectively). A higher DBC for smaller plasmids similar to that reported earlier for membranes is evident [37] where the capacity was reduced with the increasing vector size. As previously described, smaller plasmids have a greater compaction degree leading to a lower occupation of the binding sites and more available sites for other biomolecules to bind thus increasing the DBC.

3.2.2 Effect of ionic strength

As described above, salt concentration in the equilibrium buffer influences sc pDNA purification. In fact, depending on the plasmid size, a different salt concentration must be used to isolate the main plasmid isoforms. Thereby, taking into account the use of 3 M $(NH_4)_2SO_4$ in pUC19 plasmid (Fig. 2B) it was decided to evaluate the effect of salt concentration on pVAX1-*LacZ* and pcDNA3-based plasmid. Keeping the same initial conditions (0.5, 1, and 1.5 mL/min flow rate, 50 μg/mL plasmid concentration), it was used 3 M $(NH_4)_2SO_4$ for DBC determination (Table 3). The results showed that the DBC obtained with this salt concentration are more industrially useful than the ones using 2.5 M $(NH_4)_2SO_4$ (Fig. 2C) making the CDI monolith an appropriate matrix for the recovery of pDNA, with the purity degree required by FDA for therapeutic applications. Apparently, the increase of salt concentration improved the DBC by more than 20-fold for the pcDNA3-based plasmid (from 0.234 to 4.902 mg/mL at 10% breakthrough and 0.5 mL/min). pVAX1-*LacZ* also had a DBC increase from 2.671 to 3.295 mg/mL at 10% breakthrough and 1 mL/min (data not shown). Figure 2C shows the DBC increase for the pcDNA3-based plasmid with the increase in salt concentration at 1 mL/min. These results also demonstrate a high improvement in the breakthrough curve shape with salt increment, which promotes the plasmid compaction due to the shielding of electrostatic repulsion [38] stabilizing the pDNA structure. Similarly, Hammermann et al. [39] and Lyubchenko and Shlyakhtenko [40] verified that ionic conditions of the solution can dramatically change and affect the geometry of plasmids molecules forcing them to adopt a highly compact and bent interwound shape, with only an increase from 10 to 100 mM of NaCl concentration. A pDNA capacity increase was also recently reported using compacting agents [41]. In addition, Bencina et al. [18] described that higher NaCl concentrations led to close packing of DNA on the surface column, which masks the destabilizing intermolecular charge repulsion and produces a strong negative electrostatic charge in the plasmids leading to a higher DBC. As a result of low electrostatic repulsion between phosphate groups in the DNA backbone, the molecular apparent size reduces, increasing DNA stability and increasing the outer surface area available for adsorption facilitating the access to the monolithic surface [41, 42]. Another study using ultrafiltration membranes showed that the plasmid transmission through the membrane pores was significantly higher in the presence of salt [37]. Although many studies reported salt effects on plasmid size taking advantage of NaCl in low concentrations, Ueberbacher et al. [43] showed that a higher retention of one protein with the increase of $(NH_4)_2SO_4$ from 0.7 to 1.2 M was also possible. Furthermore, Freitas et al. [44] obtained a higher purification degree of pDNA from a lysate sample using 2.5 M of $(NH_4)_2SO_4$ instead of 1.5 or 1 M. These results indicate the advantage of performing

Table 3. Comparison of DBC for pcDNA3-based plasmid using three different flow rates and two binding salt concentrations

DBC (mg/mL)						
Breakthrough (%)	2.5M $(NH_4)_2SO_4$			3M $(NH_4)_2SO_4$		
	0.5 mL/min	1 mL/min	1.5 mL/min	0.5 mL/min	1 mL/min	1.5 mL/min
10	0.234	0.141	0.129	4.902	3.598	3.591
50	0.289	0.209	0.205	5.319	3.891	3.644

experiments with a higher salt concentration, in order to increase the DBC for large plasmids, due to the reduction of intermolecular repulsion and a more closely DNA packing on the monolith surface.

3.2.3 Effect of flow rate

Due to their physical and chemical constitution, agarose-based matrices present some flow rate limitations when compared with alternative supports. Otherwise, the monolithic matrices present convective mass transport that is only imposed by the flow rate, and is independent of diffusion or molecular size, due to their excellent mass transfer properties and high porosity [9]. Thus, these innovative supports allow fast separations and high DBC of large molecules independent of the flow rate [45, 46]. As it was previously studied, no changes in selectivity were observed during the pDNA isoforms separation at different flow rates [17]. Thereby, the effect of different flow rates (0.5, 1, and 1.5 mL/min) on the DBC with two $(NH_4)_2SO_4$ concentrations was investigated in the present study. According to the obtained results (Table 3), the DBC using 2.5 M $(NH_4)_2SO_4$ was almost unaffected by different flow rates, which is in agreement with most of the works described for the DBC of several monoliths at mild ionic conditions (NaCl concentrations up to 1 M) [15, 18, 47]. However, as it can be seen for the results using 3 M of $(NH_4)_2SO_4$, the DBC increased slightly when lower flow rates were used. This result could be related to the extreme concentration of $(NH_4)_2SO_4$ used in these particular assays, which can favor plasmid compaction. Thus, low flow rates can contribute to the salt effect in decreasing the radius of gyration of plasmids, which will consequently increase the DBC. In previous circular dichroism studies of pDNA structural stability as a function of the elution conditions, a change in the ellipticity of the pDNA spectrum was shown at high $(NH_4)_2SO_4$ concentrations [48, 49]. These studies confirmed that the pDNA structure can change as a function of high salt concentrations. So, the slight increase of DBC at low flow rates in this work can be due to the conformation and arrangement of pDNA with 3 M of $(NH_4)_2SO_4$. Under this condition, the DNA chains became more coiled because the repulsions between phosphate groups were effectively screened. This fact, together with the low flow rate, leads to a better organization of the molecule and a small surface area is occupied increasing the number of binding sites available to bind more molecules [39]. So, the specific shape of the pDNA molecules and the conditions to which the molecule are exposed can influence the DBC.

3.2.4 Effect of pH

Given that the pK_a of the imidazole ring is approximately 6.5, its behavior is highly affected by pH range between 5.0 and 8.0. In this way two different pH values, 8.0 and 6.0, were tested to evaluate the influence of pH in the DBC of the CDI disk. The experiments showed a DBC increase from 3.598 mg/mL at pH 8.0 to 4.199 mg/mL at pH 6.0 at 10% breakthrough. The lower pH makes these CDI groups positively charged, polar and hydrophilic, favoring the interaction with plasmid phosphate groups [50]. Figure 2C shows the pronounced shifting of the breakthrough curve indicating an increase in DBC with lower pH. This behavior is in accordance with the literature since the negatively charged plasmid could bind more effectively to the positive CDI groups at lower pH. Many authors took advantage of the pH dependence of the CDI group of histidine amino acid to substitute its conserved residues in order to identify histidine residues [50] or to purify proteins using immobilized metal ion affinity chromatography (IMAC) [51].

3.3 Pressure drop evaluation

Several parameters highly influence the pressure: linear flow velocity [52] and viscosity of the mobile phase together with porosity [15], size of the particles [53] and bed thickness of the monolith. As Table 2 shows, pressure drop in a CDI monolithic disk was affected by the $(NH_4)_2SO_4$ concentration of the injected sample. In fact, for pVAX1-*LacZ* and pcDNA3-based plasmids an increase in pressure drop with the increase of 0.5 M in the salt concentration was observed. This happened due to the increase in the viscosity of the samples with high salt concentration (data not shown). These data are in agreement with literature [54], and the pressure drop obtained is <1 MPa, which is required for preparative chromatography with high reproducibility.

4 Concluding remarks

In this paper, we report the involvement of multiple interactions, mainly hydrophobic, between the nongrafted CDI

monolith and different plasmids for isoforms purification. The applied strategy showed that isoforms of plasmids with different sizes (at least up to 14 kbp) can be separated using the same chromatographic process. Additionally, at high salt concentration higher DBC values were obtained for lower flow rates. The minimum DBC for the pcDNA3-based plasmid was 0.141 mg/mL using 2.5 M $(NH_4)_2SO_4$ and 1 mL/min at 10% breakthrough. On the other hand, the maximum DBC was 5.891 mg/mL for the smaller plasmid pUC19 at 0.5 mL/min using 3 M $(NH_4)_2SO_4$ pH 8.0 at 50% breakthrough. All plasmids showed the same behavior since DBC increased with higher salt concentrations and lower flow rates. These conditions favor the monolith capacity, which suggests that the DBC may also be influenced by plasmid characteristics, size, and compaction. Furthermore, lower pH in DBC assays should be preferred to obtain higher capacities since the pH-sensitive CDI groups would be in the protonated form providing a binding enhancement of negatively charged pDNA molecules. Given that CDI monolith structure preserves pDNA conformation with high purity degree using only one single chromatographic step, this makes the non-grafted CDI disks an attractive option for pDNA purification on a milligram scale.

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Paper III

Purification of influenza deoxyribonucleic acid-based vaccine using agmatine monolith

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ABSTRACT

Lately, researchers have made several efforts to improve vaccine production to fight highly contagious respiratory diseases like influenza. One of the most promising options for reducing the impact of this virus is DNA vaccination. However, a large quantity of highly pure plasmid DNA (pDNA) is necessary to attain this goal. The present work describes the production and purification of the plasmid NTC7482-41H-VA2HA expressing influenza virus hemagglutinin using an agmatine monolith. This ligand was chosen to purify supercoiled (sc) pDNA from complex lysates because of its versatile multimodal character. Its natural intervention in several biological systems together with its similarity with the highly studied arginine ligand allowed the development of a simpler and more specific purification process. Agmatine works under two strategies: descending ammonium sulfate gradient and ascending sodium chloride gradient. Furthermore, pH manipulation revealed an important role in pDNA isoforms selectivity. Dynamic binding capacity (DBC) experiments were performed varying different parameters and showed an increase with pDNA concentration, while high flow rate and high pH had the opposite effect. Sc pDNA was purified with high yield and was efficient with respect to cell transfection and cell viability. This monolith showed to be appropriate to purify the plasmid NTC7482-41H-VA2HA, providing a valuable tool for pDNA influenza vaccines preparation.

1. Introduction

DNA vaccination has largely evolved in the last 20 years to become a competitive alternative to conventional vaccines. This technology is genetically engineered to produce immunological responses against several diseases [1]. Vaccination has remained the best approach for prevention and control of influenza virus. However, current influenza vaccines are only effective against the circulating strains, and therefore must be updated and administered every year. In this way, DNA vaccines are a promising alternative since they induce both humoral and adaptive immunity, providing cross-strain protection against one or more virus proteins [2]. Hemagglutinin (HA) is the most abundant glycoprotein on the surface of influenza virus and plays an important role in the life cycles of this organism. HA suffers several mutations through the years which are responsible for the susceptibility to influenza viruses and are the basis for the frequent update of conventional influenza vaccines [3]. HA-specific antibodies formed after a natural infection reduce the severity of the disease by inhibiting the attachment and entry of the virus in the host cells in a future contact [4,5]. Thus, HA is the gene of choice in the production of pDNA vaccines against influenza.

The purity degree of pDNA is essential to improve gene expression in eukaryotic cells and avoid injurious effects. So, only the most biologically active pDNA conformation, the sc isoform, must be used as a biopharmaceutical product, after being purified with an efficient downstream process [6]. Nowadays, the typical pDNA-based vaccine preparation requires a suitable chromatographic purification for the removal of impurities. The major difficulty in sc pDNA purification relies on its analogous physical, chemical and structural properties with other molecules of the lysate, requiring highly selective chromatographic processes. Currently, there are several conventional techniques that have been used in the purification of sc pDNA such as hydrophobic interaction chromatography (HIC), anion exchange (AIE), size exclusion or affinity chromatography [7]. Our research team has reported a successful application of affinity chromatography using amino acids (histidine, arginine and lysine) as ligands in agarose matrices for specific separation of pDNA sc isoform and also for its purification from *Escherichia coli*

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1570-0232/

(*E. coli*) clarified lysates [8–10]. However, conventional matrices have some limitations that remain to be solved, including their low capacity, low diffusivity and low recovery yields. So, there is a need to use matrices with better performance, such as monoliths, since they show advantages such as high binding capacities due to excellent mass transfer properties and a huge quantity of binding sites for large biomolecules as pDNA, flow independent resolution; and fast chromatographic assays [11–13]. Moreover, the ligands to be immobilized on these chromatographic supports can have several sources [14–16]. Recently, histamine, a derivative of the histidine, was used with high selectivity and specificity for sc pDNA purification due its multimodal interaction [17,18]. Following the same line, agmatine, a cationic biogenic amine derived from arginine, was immobilized into carbonildiimidazole (CDI) disks in order to investigate its performance in purification of an influenza pDNA vaccine. The choice of this arginine derivative lies not only in the natural intervention of agmatine in biological systems [19], but also on its similarity with the arginine monolith previously used in the purification of a human papillomavirus pDNA vaccine [20]. Due to its multimodal character that combines both ionic and hydrophobic interactions, agmatine monolith showed to be effective in the separation of pDNA isoforms under two different elution systems, as well as in the purification of the pDNA influenza vaccine from clarified lysates. The effect of pH, feed concentration and flow rate on the dynamic binding activity (DBC) was also evaluated. Furthermore, the supercoiled (sc) pDNA transfection efficiency and cell viability were tested in fibroblast cells.

2. Material and methods

2.1. Materials

All solutions were freshly prepared, filtered through a 0.20 μm membrane (Dassel, Germany) and degassed ultrasonically. The $(NH_4)_2SO_4$ and NaCl were from Panreac (Barcelona, Spain), sodium phosphate monobasic anhydrous (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4) were from Sigma–Aldrich (Madrid, Spain). Hyper Ladder I (Bioline, London, UK) was used as a DNA molecular weight marker. GreenSafe Premium was purchased to NZYTech (Lisbon, Portugal). The iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used for genomic DNA (gDNA) quantification. The grafted agmatine monolith with 0.34 mL bed volume (average pore size of 1500 nm in diameter) and the CIMac™ pDNA analytical column of 0.32 mL bed volume were prepared and provided by BIA Separations (Ajdovščina, Slovenia). The plasmid NTC7482-41H-VA2HA (6471 bp) was kindly provided by Nature Technology Corporation (Lincoln, USA).

2.2. Fourier transform infrared spectroscopy

The monolith was characterized using Fourier transform infrared spectroscopy (FTIR) by using a Nicolet iS10 interferometer (Thermo Scientific Waltham, MA, USA). After vacuum drying, the dry disk was powered and then mounted on a diamond window and compressed to improve spectrum signal to noise ratio and the spectrum acquired. The acquired data were then processed in Omnic Spectra analysis software and the baseline subtraction was performed.

2.3. Bacterial cell culture, alkaline cell lysis and primary isolation

Fermentation of *E. coli* DH5α harboring the 6471 bp plasmid NTC7482-41H-VA2HA (HA plasmid) was carried out as described in our previous work [21]. The pDNA isoforms sample was obtained from *E. coli* cells by alkaline lysis [22] using the Plasmid Purification Maxi Kit from Qiagen (Hilden, Germany), according to the manufacturer's instructions. On the other hand, the *E. coli* lysate sample was prepared using the method described by Černigoj et al. [18].

2.4. Preparative chromatography

Chromatographic studies were performed with ÄKTA purifier system (GE Healthcare Biosciences, Uppsala, Sweden) using UNICORN software, Version 5.11. The immobilization procedure of agmatine in the monolith consisted on pumping the solution of this amino acid derivative (5 g agmatine sulfate dissolved in 15 mL of 2.5 M NaOH) through a CDI monolith (BIA Separations, Ajdovščina, Slovenia) as previously described [17].

For sc pDNA purification, two elution strategies were established for the agmatine monolithic disk. The first strategy was based on a decreasing $(NH_4)_2SO_4$ stepwise gradient (3 to 0 M in 50 mM phosphate buffer (PB), pH 9.6) and the other strategy was based on ascending NaCl stepwise gradient (0 to 2 M in 50 mM PB, pH 8.0) at room temperature using 1 mL/min. The monolith was equilibrated with 3 column volumes (CVs) of 50 mM PB and monitored at 260 nm. Partially purified pDNA sample (300 μg/mL) with the Qiagen kit, was ressuspended in 50 mM PB (pH 8.0) and the ionic strength was adjusted with $(NH_4)_2SO_4$ or NaCl depending on the used strategy. After the sample injection (50 μL), fractions of 1 mL were collected according to the chromatograms by UV detection at 260 nm. The fractions were concentrated and desalted with Vivaspin® concentrators (Sartorius Stedim Biotech, Goettingen, Germany) for further electrophoretic analysis. The clarified lysate samples (50 μL), with a nucleic acid concentration of 800 μg/mL were loaded into the column at 1 mL/min and the elution was performed differently by descending ammonium sulfate gradient or ascending sodium chloride gradient. Fractions (1 mL) were also concentrated and desalted and kept for further analysis. After each assay, the monolith was regenerated with 5 CV of 1 M NaOH plus 1.5 M NaCl and thoroughly washed with deionized water until the conductivity was restored.

2.5. Agarose gel electrophoresis

Pooled fractions were analyzed by an electric field in a horizontal gel electrophoresis using a 10-cm-long gel of 0.8% agarose in TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) stained with 0.1 μL/mL of gel GreenSafe Premium (NZYTech, Lisbon, Portugal). The gel was visualized with UVITEC system (Cambridge, UK) using Firereader 1D Gel analysis software version 15.15 of UVITEC.

2.6. Analytical chromatography

The CIMac™ pDNA analytical column was used to monitor the quality of sc pDNA according to the modified analytical method described by Mota et al. [23]. The quantity of RNA, oc and sc pDNA present in the clarified lysate was determined. Calibration curves of each component were obtained with concentration of 2–30 μg/mL for RNA and 1–100 μg/mL for pDNA.

2.7. Impurities analysis

Quantification of genomic DNA (gDNA) in the purified sc pDNA solutions was made through real-time polymerase chain reaction (PCR) in a iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), according to the method described by Martins et al. [24]. In turn, protein concentration of the purified samples was measured using micro-bicinchoninic acid (BCA) assay from Pierce (Rockford, USA) according to the manufacturer instructions. Finally, the endotoxins quantification in the pooled fractions was achieved

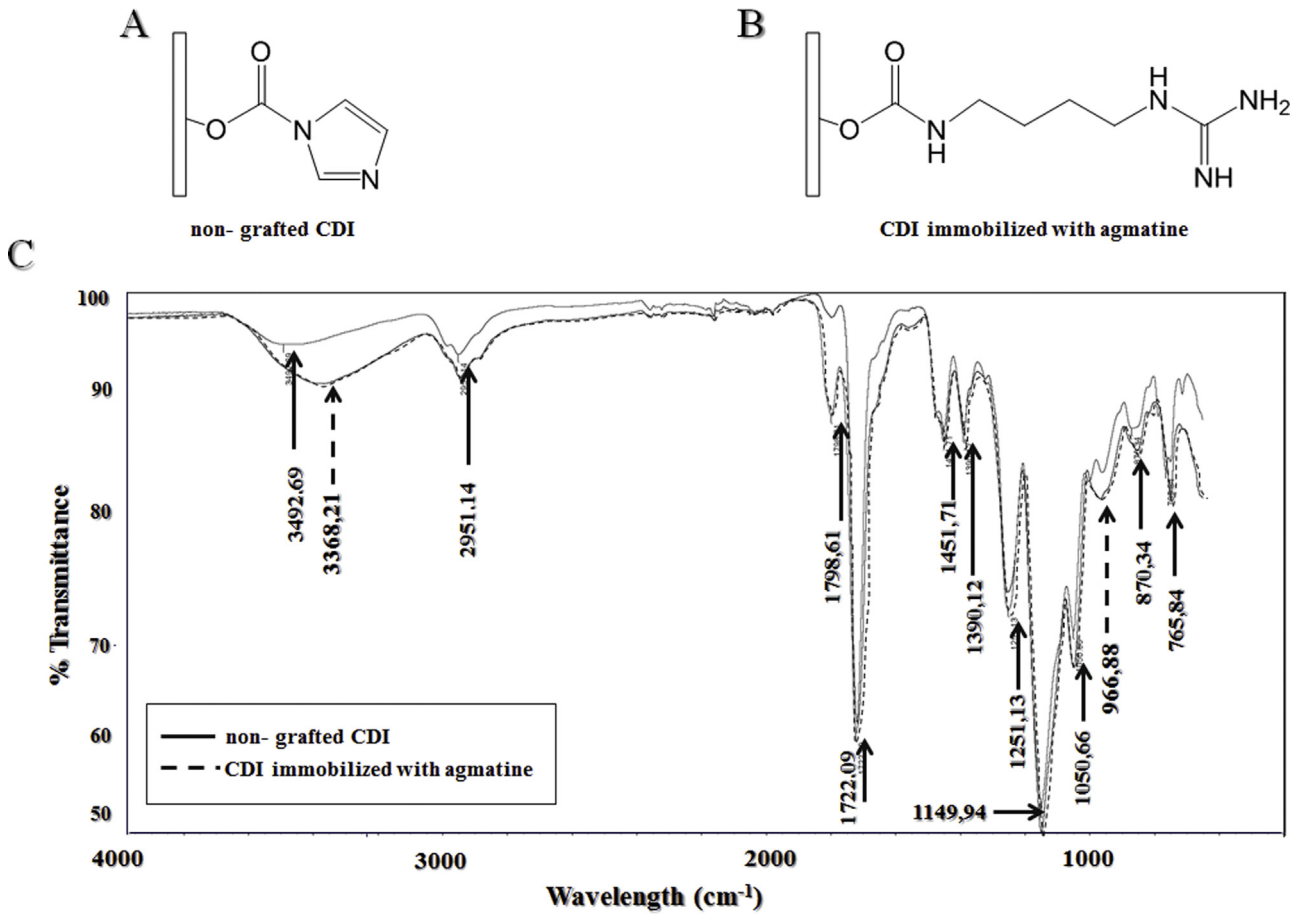

Fig. 1. Schematic representation of (A) Non-grafted CDI monolith and (B) CDI monolith immobilized with agmatine. (C) FTIR spectra of non-grafted CDI monolith and agmatine monolith.

using ToxinSensor™ Chromogenic LAL Endotoxin Assay kit from GenScript (Piscataway, USA).

2.8. Dynamic binding capacity

The DBC characterization of agmatine monolithic disk was conducted varying several parameters such as flow rate (0.5, 1.0 and 1.5 mL/min), feed stock concentration (0.025, 0.05, and 0.1 mg/mL) and pH (6.0, 8.0 and 9.6). The assays were performed at room temperature in ÄKTA purifier system (GE Healthcare Biosciences, Uppsala, Sweden). The pDNA samples were prepared using the Qiagen kit in 50 mM PB at several pH's (6, 8.0 and 9.6). The monolithic column was equilibrated using PB with $(NH_4)_2SO_4$ 3.0 M and thereafter it was overloaded with the plasmid solution under the same equilibrium buffer. The DBC was determined using the breakthrough area integration method described by Jungbauer and Kaltenbrunner [25]. At the end of each experiment, the amount of pDNA bound per milliliter of support at 100% breakthrough was calculated. The void volume determined under non-binding conditions was discounted to each breakthrough curve. Elution of the bound plasmid was achieved by decreasing the salt concentration in the mobile phase to 0 M in a stepwise manner.

2.9. Dynamic light scattering

The plasmid size was determined by dynamic light scattering (DLS). To determine the hydrodynamic diameter by DLS, pDNA molecules were diluted in a dispersant medium (800 μL of 50 mM PB with different concentrations of $(NH_4)_2SO_4$). Size measurements were obtained under 1.0, 2.0 and 3 M of $(NH_4)_2SO_4$ and then performed in a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK), in automatic mode and with a scattered light detection angle of 173°. The reported particle size was obtained as an intensity distribution by cumulative analysis performed in the zetasizer software (version 6.20).

2.10. Transfection experiments

Fibroblast cell line was cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM F12) containing 1% antibiotic and antimycotic, supplemented with 10% fetal bovine serum, at 37 °C in a 5% CO_2 atmosphere incubator. Three days before transfection, 5×10^5 cells per well were seeded on a 6-well plate. On the day of transfection, the serum-containing medium was removed and replaced by antibiotic-free medium, whereas pDNA-Lipofectamine (Invitrogen, Carlsbad, CA) complexes were prepared as described by the manufacturer instructions. The transfection efficiency was determined after 72 h using an immunochemistry analysis with mouse monoclonal anti HA H5N1 IgG primary antibody (abcam, Cambridge, UK). The transfection efficiency of the influenza DNA vaccine purified with agmatine monolith strategies using the two elution strategies was then compared with the same plasmid purified with Qiagen commercial kit. The transfection efficiency was determined as the percentage of transfected cells compared to the total cell number.

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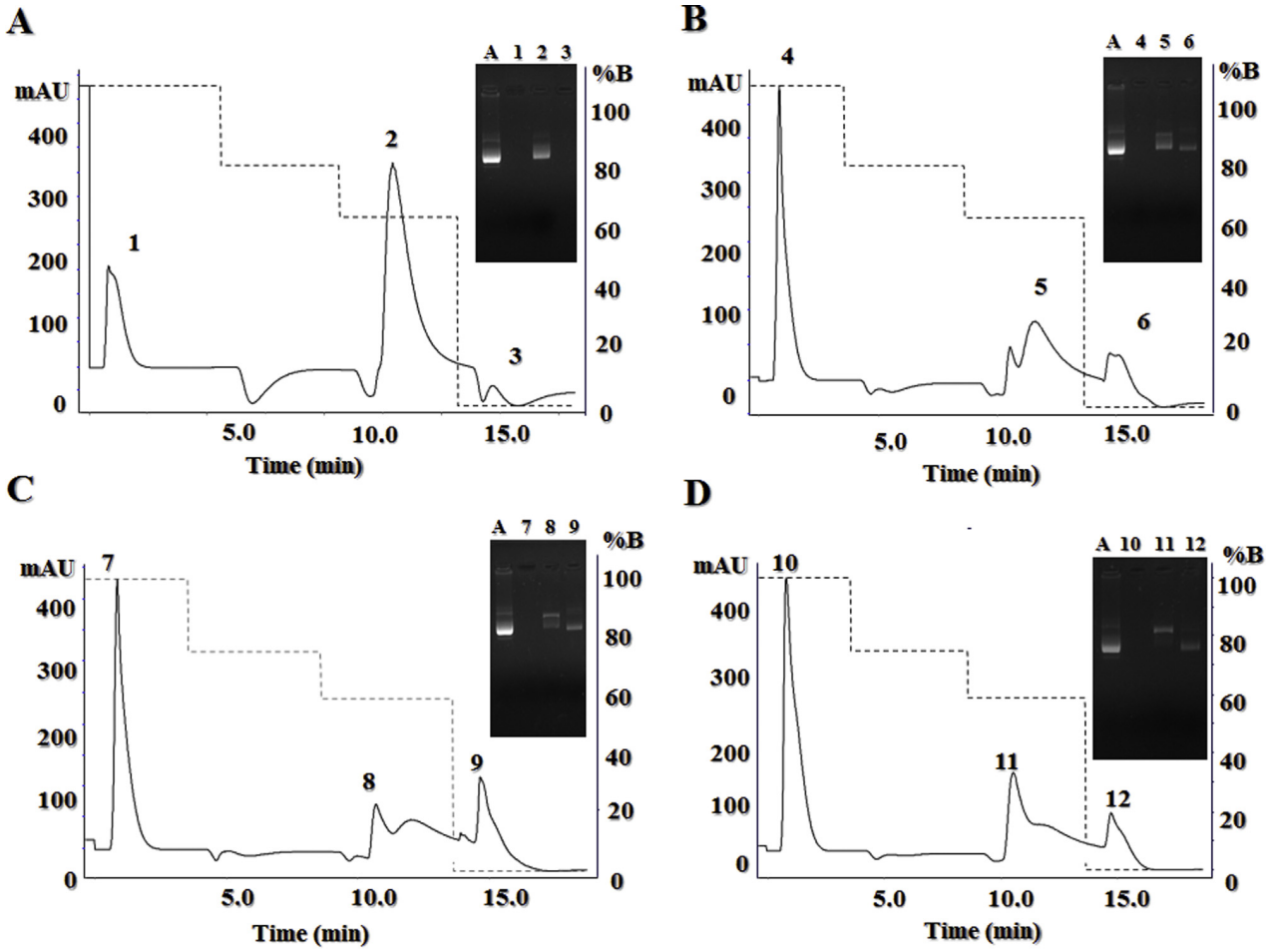

Fig. 2. Chromatographic profile of pDNA isoforms separation using the agmatine monolith at 1 mL/min by decreasing stepwise gradient of $(NH_4)_2SO_4$ in 50 mM phosphate buffer. (A) pH 9.0, (B) pH 9.2, (C) pH 9.4, (D) pH 9.6. Lane A: pDNA sample (oc + sc); lanes 1–12: peaks 1–12; Lanes 2, 5, 8 and 9: sc + oc; Lanes 6 and 12: sc; lane 11: oc.

2.11. Cell viability

The effect of plasmid transfection on cell viability was determined by measuring a fluorescent resorufin product using the resazurin assay. After transfection, 10% (v/v) of fluorescent dye solution was added to each well and incubated for 4 h, in a humidified atmosphere, at 37 °C, 5% CO_2. Then, the fluorescence intensity was measured in a SpectraMax Gemini™ XS spectrofluorometer (Molecular Devices), at λ_{ex} = 545 nm and λ_{em} = 590 nm, respectively. Cells cultured without transfection were used as negative control and ethanol treated cells were used as positive control.

3. Results and discussion

3.1. FTIR

FTIR is a sensitive technique that provides information about molecular structural changes [26,27]. Agmatine monolith (Fig. 1B) displayed intense absorption peaks different from non-grafted CDI monolith (Fig. 1A) at about 765, 966, 1798, 2951 cm^{-1} and a broad band between 3100 and 3500 cm^{-1} as can be seen in the FTIR spectra (Fig. 1C). These absorption peaks are associated to the amino groups in the monolith namely the absorption of NH_2 twisting and wagging deformations, NH asymmetric angular stretching, C—H stretching on the carbon chain of agmatine, axial stretching of NH and the stretching vibrations of the NH_2 and NH groups of primary and secondary amines, respectively.

3.2. Separation of pDNA isoforms

Agmatine is a biogenic amine which is synthesized by the enzymatic decarboxylation of L-arginine by arginine decarboxylase. This amino acid derivative acts as a neurotransmitter/neuromodulator of the central nervous system, mediating growth and cell proliferation [28]. The goal of this work was to investigate the versatility of agmatine as multimodal ligand in a monolithic disk for the purification of an influenza pDNA vaccine. A study by Kallberg et al. [29] stated that a ligand for multimodal chromatography should have

Table 1
Characterization of the dynamic light scattering of sc pDNA under different salt concentrations.

Sample	Feed concentration	Solution	Particle size DLS (nm)
sc pDNA	0.05 mg/mL	50 mM phosphate buffer, pH 8.0	119.4– 125.6
		1 M $(NH_4)_2SO_4$ in Buffer A, pH 8.0	108.4–118.5
		2 M $(NH_4)_2SO_4$ in Buffer A, pH 8.0	77.7–83.6
		3 M $(NH_4)_2SO_4$ in Buffer A, pH 8.0	58.3–65.6

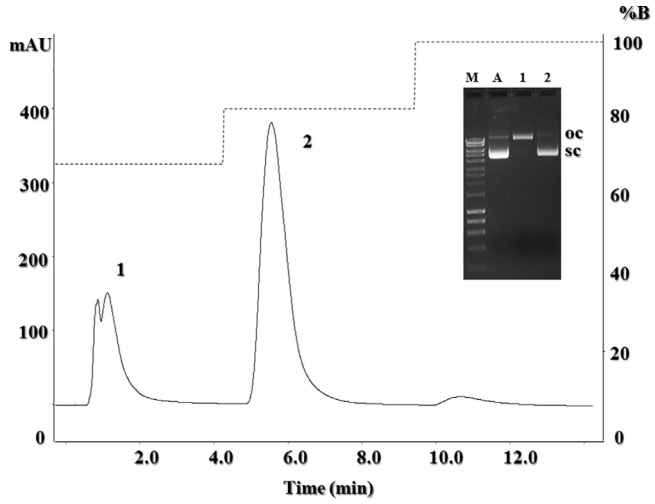

Fig. 3. Chromatographic profile of pDNA isoforms separation using the agmatine monolith at 1 mL/min by ascending stepwise gradient of NaCl in 50 mM phosphate buffer pH 8.0. Lane M: molecular weight marker (HiperLadder I: 10 kbp); lane A: pDNA sample (oc + sc); lane 1: oc; lane 2: sc.

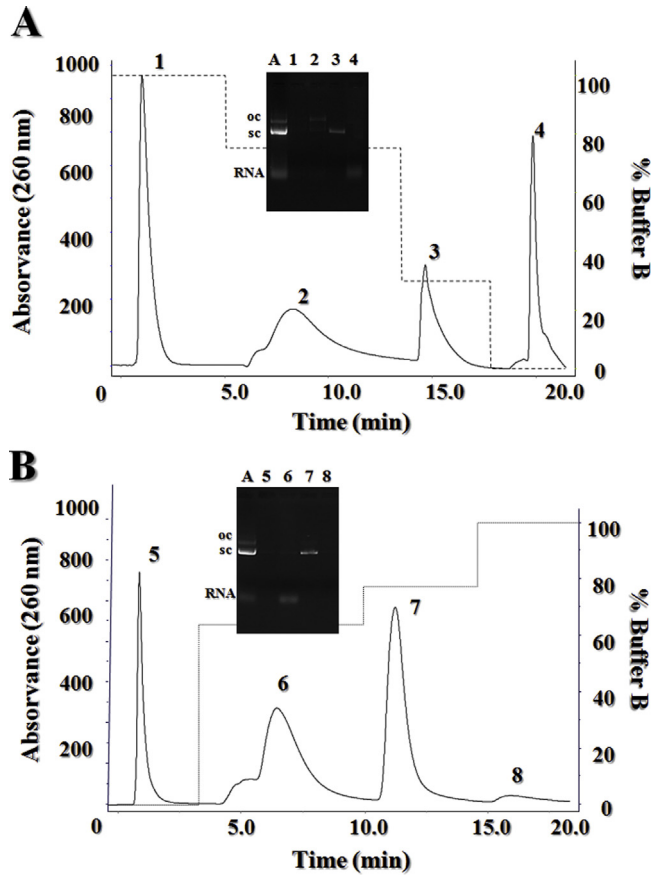

Fig. 4. Chromatographic profile of sc pDNA isoform purification from host impurities using the agmatine monolith at 1 mL/min, by (A) decreasing stepwise gradient of $(NH_4)_2SO_4$ in 50 mM phosphate buffer pH 9.6 and (B) ascending stepwise gradient of NaCl in 50 mM phosphate buffer pH 8.0. Lane A: lysate feed; lanes 1–8: peaks 1–8; lane 2: oc; lane 3: sc; lane 4 and 6: RNA; lane 7: oc + sc.

at least one hydrophobic moiety (such as butyl, phenyl and hexyl groups) and one ionic moiety (as sulfonic, quaternary ammonium, carboxyl and amino groups). In the specific case of agmatine, this molecule presents a combination of a butyl group with ionizable amino groups that allow it to work under both ionic (stepwise gradient of ascending NaCl) and hydrophobic (stepwise gradient of decreasing $(NH_4)_2SO_4$) strategies. Thus, several salt gradients and pHs were tested in order to find suitable conditions for purification purposes on preparative scale. It was observed that the pH variation had an effect on the purification of sc pDNA influenza vaccine. The protonation of the amino groups of agmatine depends notably on pH and affects the retention of pDNA. As shown by Fig. 2A–D, under descending $(NH_4)_2SO_4$ gradient strategy a proper separation of the isoforms was achieved after increasing the pH from 9.0 to 9.6, which decreases the contribution of electrostatic interactions as stated by Sasaki et al. [30].

The chromatographic profile and agarose gel electrophoresis analysis of the eluted fractions of pDNA isoforms (oc and sc) using decreasing $(NH_4)_2SO_4$ stepwise gradient at pH 9.6 is presented in Fig. 2D. After the plasmid injection, a first peak of unbound material was obtained (Fig. 2 D, peak 10, lane 10), and after by decreasing $(NH_4)_2SO_4$ concentration two additional peaks were eluted, respectively at 1.77 M and 0 M. Agarose gel electrophoresis showed that the oc pDNA eluted in the second peak (Fig. 2 D, lane 11) was totally separated from the sc pDNA which appeared in the third peak (Fig. 2 D, lane 12) using 50 mM PB. Since the elution of both pDNA isoforms was only achieved after decreasing the salt concentration, hydrophobic interactions are prevalent between these molecules and the agmatine monolith. However, the relative strength of these interactions is different to each pDNA isoform, being stronger to sc pDNA. In fact, sc bases are more exposed [31] due to their characteristic torsional deformations. As mentioned by Diogo et al. [31] and Giovannini et al. [32], plasmid conformation plays an important role in pDNA purification due to the different interactive forces. Moreover, the composition and ionic environment influences pDNA structure with high salt concentrations favoring the base exposition of sc pDNA and promoting the interaction with agmatine. In contrast, sc isoform at low salt concentrations has its bases less exposed decreasing its retention [16]. DLS experiments (Table 1) showed a decrease in plasmid size from 119.4–125.6 nm to 58.3–65.6 nm, due to a $(NH_4)_2SO_4$ increment from 0 M to 3 M, respectively, which allows a highly condensed form of pDNA. The results are in accordance with several authors [33,34]. that defend that highly ionic conditions can dramatically change the geometry of pDNA molecules, leading to a close packing and reducing the intermolecular charge repulsion with a decrease of the effective diameter of the molecule [35].

When applying the ascending NaCl gradient, the pH variation did not show a noticeable improvement on the separation of pDNA isoforms. This strategy showed not only a weaker retention of pDNA using high pH conditions, but also a lower purification of the sc pDNA isoform. A possible explanation for the low retention under alkaline conditions is the weakening of electrostatic interactions at pH close to the pKa of agmatine (13.0 in aqueous solution [36]). According to Fig. 3, the partial separation of the pDNA isoforms was obtained using three steps in isocratic mode. Firstly, the agmatine monolith was equilibrated with 1.36 M NaCl in 50 mM PB (pH 8.0) and after the sample injection, a peak was obtained in the flowthrough containing the oc isoform (Fig. 3, lane 1). Then, after increasing the salt concentration to 1.65 M NaCl, a second peak appeared containing mostly the sc isoform (Fig. 3, lane 2). Finally, the elution with 2 M NaCl in 50 mM PB (pH 8.0) assures that no plasmid remained attached to the disk. As can be seen, the use of NaCl weakens the electrostatic interactions between agmatine and pDNA molecules which means that this strategy took advantage of the ionic exchange mode of the agmatine ligand to perform pDNA purification. However, the high binding strength was responsible for the lack of separation between oc and sc isoforms, as also mentioned in other works [37].

Table 2
HPLC analysis of concentration, purity and recovery yield of sc NTC 7482-41H-VA2-HA pDNA isolated by agmatine monolith.

Purification strategy	Process step	Volume (mL)	sc pDNA content					oc pDNA content			RNA content		
(μg/mL)	(μg)	% of sc pDNA	Purification factor	Step yield (%)	(μg/mL)	(μg)	% of oc pDNA	(μg/mL)	(μg)	% of RNA			
	Feed sample	0.05	66.7	3.335	32.3	–	–	14.7	0.735	5.4	481.5	24.075	62.3
$(NH_4)_2SO_4$ strategy	oc-peak[a]	0.100	0.13	0.013	0.32	–	–	4.59	0.459	26.6	0.138	0.014	1.6
sc-peak[a]	0.150	11.4	1.710	**98.3**	3.04	**51.8**	0.15	0.015	0.30	0	0	0	
RNA-peak[a]	0.05	0.51	0.025	1.03	–	–	0.17	0.008	0.21	456.58	22.83	98.3	
NaCl strategy	sc + oc -peak[a]	0.100	16.5	1.650	**91.6**	2.84	**50.1**	2.86	0.286	8.6	0	0	0
RNA-peak[a]	0.100	0.095	0.009	0.63	–	–	0.104	0.010	0.23	147.3	0.147	99.0	
sc-peak[b]	0.100	5.52	0.552	**99.6**	3.08	**45.3**	–	–	–	–	–	–	

The correlation coefficients were 0.9981 for sc pDNA, 0.9986 for oc and 0.9875 for RNA.
[a] Corresponding to the first run feed.
[b] pDNA fraction collected after two runs through the CDI-Agmatine disk.

3.3. *Supercoiled pDNA purification from* E. coli *lysates*

The agmatine monolith was also applied to purify sc pDNA of the influenza DNA vaccine from clarified lysates. This ligand possesses the advantage to perform the purification of pDNA under mix mode, combining both ionic and hydrophobic interactions. Both strategies (descending $(NH_4)_2SO_4$ and ascending NaCl gradients) were used for this purpose, while in the arginine-agarose and in the arginine monolith was only possible explore the ascending NaCl gradient [20]. Fig. 4A represents the chromatographic profile and agarose gel electrophoresis of the eluted fractions using the descending $(NH_4)_2SO_4$ strategy, after the injection of a clarified lysate into the monolith. The disk was equilibrated with 3 M $(NH_4)_2SO_4$ in 50 mM PB at pH 9.6, since these were the best conditions obtained in previous experiments. In the first peak after injection, some impurities were detected but they were not visualized by agarose electrophoresis (Fig. 4A, lane 1). The decrease of the salt concentration to 2.2 M and to 1.3 M $(NH_4)_2SO_4$ promotes the elution of oc isoform (Fig. 4A, lane 2) and sc pDNA (Fig. 4A, lane 3), respectively. Finally, after decreasing the ionic strength of the buffer to 0 M $(NH_4)_2SO_4$, a peak corresponding to the most retained RNA species was eluted (Fig. 4A, lane 4). As shown by the results, there was a selective interaction between the different nucleic acids and the agmatine monolith, which enabled the isolation of sc pDNA. This can also be explained by the different degrees of base exposure [31]. RNA molecules are more strongly retained in the support not only because they have more hydrophobic bases exposed to the ligands, since they are a single-stranded chain, but also because they have a smaller size. Thus, these molecules will be better accommodated in the channels of the monolith, which contributes to a higher interaction with the available agmatine groups. On the other hand, oc and sc isoforms are double-stranded molecules that have their phosphate and sugar groups equally packed but, as previously referred, torsional tension of sc pDNA is higher which in turn reflects a bigger exposure of its bases [38]. So, the interaction between agmatine and sc pDNA is higher, being eluted after the oc pDNA isoform.

Comparatively, the ascending NaCl gradient took advantage of the ligand charge to purify the influenza DNA vaccine. After sample injection, no nucleic acids were eluted as can be confirmed in the electrophoresis gel (Fig. 4B, lane 5). However, adding 1.28 M of NaCl to the buffer, a second peak appears corresponding to the eluted RNA (Fig. 4B, lane 6). Later, the buffer ionic strength was increased to 1.55 M NaCl to recover both oc and sc isoform (Fig. 4B, lane 7). Finally, 2 M NaCl was applied to confirm there was no residual nucleic acid bound to the monolith (Fig. 4B, lane 8). The exceptionally strong binding between the nucleic acid and agmatine ligand is responsible for the lack of separation between oc and sc isoforms as also referred by Matos et al. [37]. In this strategy, RNA was firstly eluted as described in previous studies [20,35] due to the lower charge of this single stranded molecules. Since this strategy did not allow the specific separation of oc from the sc isoform, a second run was performed by injecting the pDNA pooled from peak 3 in the agmatine disk under the previous binding and elution conditions.

Despite the gel electrophoresis of the sc pDNA pooled fraction showed to be almost 100% pure, an analytical evaluation was also performed using a CIMac™ pDNA column to assess the amount of sc pDNA, purity and step yield using both purification strategies (Table 2). The analysis of the collected sample in the third step of descending $(NH_4)_2SO_4$ purification strategy (Fig. 4A, lane 3), revealed a single peak containing sc pDNA with only 0.30% of oc isoform and with no RNA. On the other hand, the strategy using NaCl elution conditions was unable to separate oc and sc isoforms in the first chromatographic run, despite RNA was separated in a different peak. Also, the analytical results showed 8.6% of oc content in the sc fraction obtained in the third peak (Fig. 4 B, lane 7). The percentage of purity assessed for sc pDNA was over 97% at the end of both strategies, as required by the regulatory agencies [39]. Moreover, as showed in Table 2, the sc plasmid isolated using the $(NH_4)_2SO_4$ purification strategy, corresponds to a yield of 51.8%, while in the NaCl strategy this yield was 45.3% after the second run, indicating a loss of pDNA in the purification process.

Previous works used amino acids as ligands in the purification of sc pDNA with similar performances. As an example, a histidine agarose matrix [40] showed to be highly specific in the isolation of a sc pDNA using step gradient with $(NH_4)_2SO_4$, with a global yield of 40% and almost 100% purity. Also, lysine ligand [10] allowed the separation and purification of sc pDNA with a step gradient using NaCl and presented a yield of 45% with similar purity. Finally, agarose arginine [35] used with step gradient of NaCl resulted in 79% of recovery yield and a purity near 100%. This means that even it seems to exist an overlap between these supports, the obtained data revealed that each method is unique and provides different results in terms of purity degree and yield, thus showing different selectivity values.

As a comparative multimodal ligand, Cernigoj et al. [18] used a histamine monolith with high selectivity and specificity in the chromatographic purification of plasmid DNA with 94% purity for decreasing of $(NH_4)_2SO_4$ strategy. Nevertheless, only the strategy using a pH gradient with high salt concentration was able to obtain a purity degree higher than 97% as required by the regulatory agencies [39]. On the other hand, both agmatine purification strategies used in this work had slightly better results since the descending $(NH_4)_2SO_4$ strategy showed 98.3% of purity degree while the ascending NaCl strategy demonstrated 99.6%. So, agmatine showed to be effective in the purification of pDNA under mix mode where the $(NH_4)_2SO_4$ elution strategy showed to be more satisfactory when compared to the NaCl or other previous described strategies.

Table 3
Evaluation of host impurities present on sc pDNA sample purified through agmatine monolith.

Sample	Proteins		Endotoxins		Genomic DNA		RNA	
	(μg/mL)	μg/mg	(EU/mL)	(EU/μg of pDNA)	(μg/mL)	(μg/mg of pDNA)	(μg/mL of pDNA)	(μg/mg of pDNA)
Clarified lysate	80.203	0.267	0.09854	0.0013	179.1	588.0	431.7	325.8
sc-peak from $(NH_4)_2SO_4$ strategy	Undetectable		0.01220	0.0001	Undetectable	Undetectable	Undetectable	
sc-peak from NaCl strategy	Undetectable		0.05537	0.0012	Undetectable	Undetectable	Undetectable	
Range of acceptance	<3 μg/mg pDNA		<0.010 EU/μg		<2 μg/mg pDNA		<0.2 μg/mg pDNA	
Method	BCA test		LAL test		TaqMan-PCR		Analytical HPLC	

Table 4
Dynamic binding capacity of agmatine monolithic disk using a NTC 7482-41H-VA2HA plasmid (influenza vaccine) under different elution conditions.

pDNA concentration (mg/mL)[a]	DBC (mg/mL)		
	10%	50%	Total
0.025	1.040	1.232	4.505
0.050	4.172	4.590	5.656
0.100	6.228	6.421	7.208
Flow rate (mL/min)[b]			
0.5	5.603	6.437	7.215
1.0	4.172	4.590	5.656
1.5	4.218	4.265	4.628
pH variation[c]			
6.0	6.816	7.043	8.618
8.0	4.172	4.590	5.656
9.6	2.772	2.901	3.210

[a] Binding buffer: 50 mM phosphate buffer + 3 M $(NH_4)_2SO_4$, pH 8.0 at 1 mL/min.
[b] Binding buffer: 50 mM phosphate buffer + 3 M $(NH_4)_2SO_4$, pH 8.0 with 0.05 mg/mL of pDNA.
[c] Binding buffer: 50 mM phosphate buffer + 3 M $(NH_4)_2SO_4$ with 0.05 mg/mL of pDNA at 1 mL/min.

Since the presence of impurities can lead to undesirable biological effects and may reduce the transfection efficiency [41], the quality of the final sc pDNA product was determined by quantification of proteins (BCA assay), endotoxins (Chromogenic Limulus amoebocyte lysate Endotoxin assay) and gDNA (real-time PCR). The results presented in Table 3 demonstrate a significant reduction in impurities content. The BCA protein assay carried out at the end of both purification strategies indicated that the obtained sc pDNA had undetectable levels of proteins (Table 3). Real-time PCR analysis also showed undetectable levels of gDNA. Low level of this nucleic acid is mandatory due to some concerns related with its presence in plasmid preparations, since there is a risk of necrosis of muscle cells [42]. Endotoxins content in sc pDNA fractions was also extremely low, less than 0.0010 EU/μg (Table 3), which is important to avoid toxicity and undesirable biological effects [43]. In an overall analysis, the agmatine monolith meets the requirements of the regulatory agencies for pDNA influenza vaccine purification, using both elution strategies described in this work.

3.4. Dynamic binding capacity

As previously demonstrated, pDNA concentration affects the DBC of CDI monolithic disks [44]. Thus, DBC for agmatine monoliths with several feedstock concentrations (0.025, 0.05 and 0.100 mg/mL) using 3 M $(NH_4)_2SO_4$ in the eluent buffer at 1 mL/min was assessed. The obtained results (Table 4) showed an improvement in capacity with the increase of pDNA concentrations. For a pDNA concentration of 0.025 mg/mL, the DBC was 4.505 mg/mL, whereas with 0.05 mg/mL the DBC increased to 5.656 mg/mL and finally for 0.100 mg/mL it was 7.208 mg/mL. As referred in our previous work [44], a non-grafted monolithic disk presented a DBC of 3.640 mg/mL using 0.05 mg/mL which means that the DBC improved using the agmatine ligand. The increment of DBC with the feedstock is in agreement with several works [45–47] which suggest that a possible reason for this phenomena is a steric hindrance effect. In fact, pDNA molecules in more concentrated solutions tend to compress, reducing its apparent size [48] and increasing its adsorption to the ligand.

In order to evaluate the DBC under different flow rates (0.5, 1.0 and 1.5 mL/min) several breakthrough curves were achieved at pH 8.0, using 3 M $(NH_4)_2SO_4$ and 0.05 mg/mL of pDNA concentration. Through the analysis of Table 4 it is possible to observe a decrease of the DBC with the increase of flow rate, as described not only in our previous work [49] but also by Zochling et al. [50]. This behavior could be due to the stretching of pDNA molecules under high flow rates, which leads to them occupying a larger space in the monolith, decreasing the DBC [51]. On the other hand, the increased contact time under low flow rates favors the binding between pDNA molecules and the ligand. It is also important to notice that the high salt concentration used in these experiments contributes to a more coiled form of pDNA molecules which also improves the DBC [52,53].

At physiological pH, agmatine is a diamine with two net positive charges, since its pKa value is 13 [54]. Knowing that this ligand is highly affected by pH variations, three different pH values 6.0, 8.0 and 9.6 were tested to evaluate the effect on the DBC. As shown in Table 4, pH 9.6 presented the lowest value of DBC (3.210 mg/mL) whereas the highest DBC value was obtained for pH 6.0 (8.618 mg/mL). At lower pH, the diamino groups of agmatine become more positively charged favoring the interaction with plasmid phosphate groups.

Comparatively, the DBC of histamine for a 5.1 kbp plasmid at pH 5 was 4.0 mg/mL under low salt binding conditions, remaining relatively high (3.0 mg/mL) even in the presence of 1.0 M NaCl due to the multimodal nature of the ligand. However, agmatine monolith presented a higher DBC (8.618 mg/mL) using 3 M $(NH_4)_2SO_4$ at pH 6.

An evaluation of the dissociation constant (K_D) was performed using the adsorption isotherms of agmatine disk under frontal analysis chromatography. The calculations were achieved following the procedure that assumes a Langmuirian behavior [55]. through the equation below:

$$\frac{1}{C(V_R - V_M)} = \frac{K_D}{m_L} \times \frac{1}{C} + \frac{1}{m_L}$$

Where, m_L is the total of binding sites, V_R corresponds to the volume needed to elute an applied concentration of pDNA (C) and V_M is the void volume of the monolithic disk. The three applied pDNA concentrations (0.025, 0.05 and 0.100 mg/mL) of DBC at 50% breakthrough, under pH 8.0 and 1 mL/min, were used to construct a plot and determine K_D and m_L values. The m_L value was 2×10^{-9} mol and the dissociation constant K_D was1.349×10^{-7} M which indicates a good affinity interaction between sc pDNA molecules and the agmatine ligand since the acceptable range for affinity ligands is between $10^{-8} - 10^{-4}$ [55].

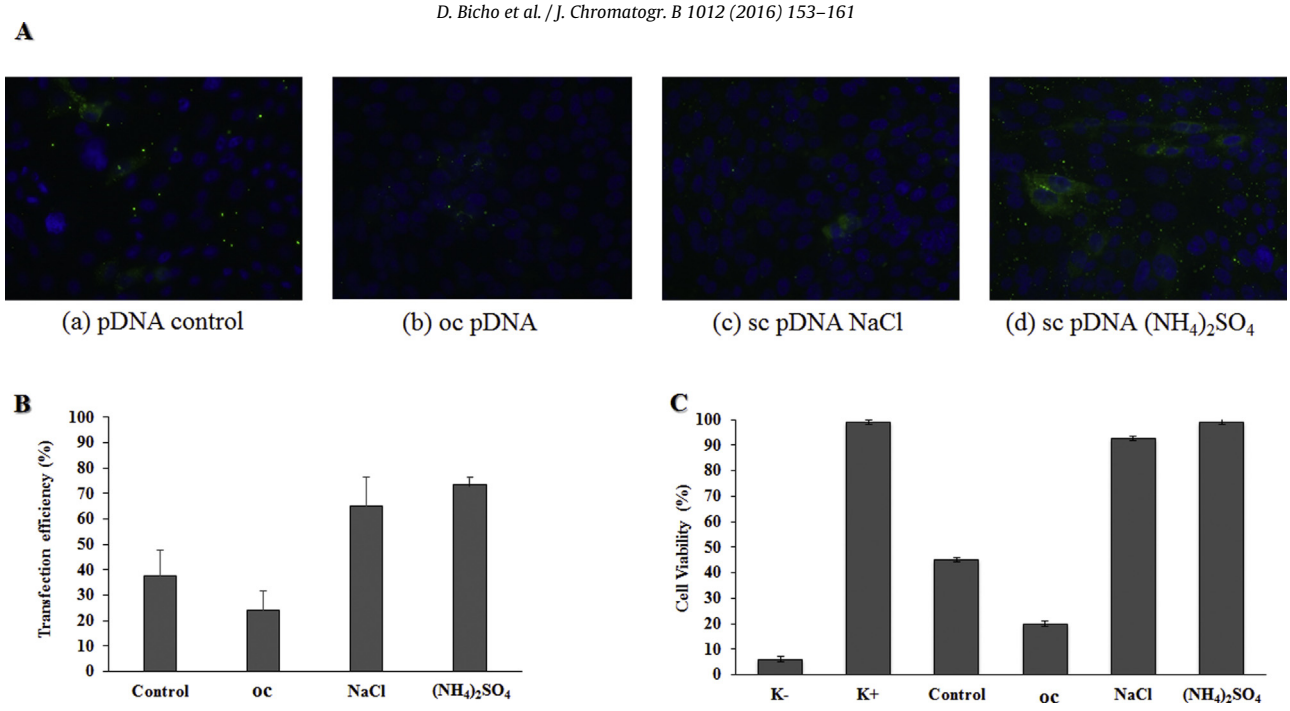

Fig. 5. (A) Fluorescence microscopy analysis of fibroblast transfected cells using different plasmid samples ($n = 3$). HA protein was detected with mouse monoclonal anti HA H5N1 IgG primary antibody (green) and the nucleus with Hoechst stain. (a) Native pDNA (oc + sc) control sample; (b) oc and (c) sc plasmid obtained from NaCl strategy and (d) sc plasmid obtained from $(NH_4)_2SO_4$ strategy with agmatine monolith. (B) Transfection efficiency after 72 h and (C) Cellular viability after application of a pDNA control sample purified with a Qiagen Kit, oc and sc pDNA purified using NaCl and $(NH_4)_2SO_4$ strategies. Positive control (K^+); negative control (K^-). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Transfection

The transfection efficiency of sc pDNA influenza vaccine purified with agmatine monolith from the *E.coli* clarified lysate was compared to the transfection of oc isoform and also the pDNA purified using a commercial kit. As shown in Fig. 5A and B, sc plasmid purified using $(NH_4)_2SO_4$ strategy was able to transfect 73% of fibroblast cells, whereas the sc plasmid purified by NaCl strategy transfected only 65% of the cells. Control pDNA sample purified with a commercial kit had a transfection efficiency of 37% while oc isoform only transfected 23% of cells. This notable difference is in agreement with other studies, that illustrated higher transfection efficiencies for the biologically active sc isoform [56]. Fig. 5C also shows the cells viability after transfection with the different pDNA samples showing that the higher viability was achieved for the sc pDNA influenza vaccine purified with agmatine monolith using the descending $(NH_4)_2SO_4$ strategy.

4. Conclusion

The agmatine monolith showed to be a suitable ligand for the purification of the influenza vaccine. Its multiple non-covalent interactions were able to selectively purify sc pDNA by a decreasing stepwise gradient of $(NH_4)_2SO_4$ and also by an increasing stepwise gradient of NaCl with a purity degree of 98.3 and 99.6%, respectively. A recovery yield of 51.8% was obtained using the $(NH_4)_2SO_4$ purification strategy while the NaCl strategy enabled a recovery of 45.3% after two chromatographic runs. Furthermore, a high DBC was achieved at low flow rates, high pDNA concentrations and low pH. Quality analysis confirmed that the impurity levels (gDNA, RNA, proteins, and endotoxins) followed the specifications of the regulatory agencies. Accordingly, all the host impurities were significantly reduced or undetectable when compared to the injected lysate. Moreover, *in vitro* studies were made to assess the expression of hemagglutinin (protein related with the protection against influenza) and a transfection efficiency higher than 65% was achieved in both strategies. These results proved that this agmatine-functionalized monolith is a multimodal column and can be applied in the purification of a DNA vaccine from complex lysates using two different strategies. The use of decreasing $(NH_4)_2SO_4$ gradient revealed the best results in terms of purity, recovery yield and also transfection efficiency.

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Paper IV

Application of ethylenediamine monolith to purify a hemagglutinin influenza deoxyribonucleic acid-based vaccine

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ABSTRACT

Influenza viruses cause annual epidemics and occasional pandemics and thus represent a significant public health problem together with considerable economic consequences. As current influenza virus vaccines do not provide the best immunological protection, plasmid DNA vaccines have been seen as a potential alternative due to the stimulation of both B- and T-cell responses without the presence of any infectious agent and because they are easily produced, highly stable and safe. From this standpoint, several downstream methods have been proposed to obtain high quantities of pharmaceutical grade supercoiled plasmid DNA. This work describes a rapid process, based on ion exchange chromatography using an ethylenediamine (EDA) monolith for the purification of an experimental DNA influenza vaccine. The purification process allowed a significant reduction of the host contaminants, such as proteins, RNA and genomic DNA and the DNA vaccine recovery with a purity degree of 97.1% and a step yield of 47%. Finally, both transfection efficiency of the influenza vaccine and its cytotoxicity in two types of eukaryotic cell lines were tested in order to compare with the same plasmid DNA purified with a commercial kit.

1. Introduction

Influenza virus is part of the annual vaccination program due to the considerably high mortality rate associated with these infections, especially in individuals with weakened immune system. This infection, commonly known as the flu, is a contagious disease caused by the influenza virus and affects the respiratory system. The best option for reducing the impact of this infection in humans is vaccination [1]. Due to the antigenic drift of influenza, the vaccine needs to be prepared every year with the new strain formed, a process that takes about a year. These traditional vaccines are produced in embryonated chicken eggs and have the potential to be biohazard, present a zoogenic risk, and their preparation is time consuming. Additionally, traditional inactivated or attenuated vaccines generate antibodies that are highly strain-specific and do not provide protection to other strains that are not included in the annual vaccine [2]. To fight these problems, several alternatives have emerged in the last years. Among these, DNA vaccination seems to be a promising alternative, with encouraging results. This therapy uses genetically engineered DNA from bacterial hosts and intents to produce immunological responses against specific antigens. DNA vaccines are potential approaches since they induce all three arms of adaptive immunity (antibodies, helper T cells and cytolytic T-lymphocytes). This technology also provides a cross-strain protection which allows the immunized organism with the pDNA vaccine to be protected against several strains of the influenza virus even if the insert is derived from only one different strain [3].

The bacterial plasmid has an insert with the purpose of producing a protein of interest. Thus, hemagglutinin (HA) was the selected protein in the present study, since it represents a surface antigen responsible for the entrance of the virus in the infected cell [4]. The expression of HA protein induces the production of antibodies against the virus, which confers protection toward the infection. DNA vaccines can be obtained in high quantities, easily stored and the production process is generic, in contrast to the complicated process needed for conventional vaccines [5]. However, there are still bottlenecks in the large scale manufacturing of this and other pharmaceutical DNAs, mainly at the downstream processing level. The purification methods need to consider the organism where the plasmid DNA (pDNA) is produced. Usually, *Escherichia coli* (*E. coli*) is chosen and because pDNA represents less than 3% (w/w) of its content a high quantity of cell impurities needs to be removed. The recovery of the pDNA vaccine has to be in agreement with the criteria demanded by regulatory agencies such as the US Food and Drug Administration (FDA).

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Furthermore, the critical impurities share some characteristics with pDNA such as the negative charge (RNA, genomic DNA (gDNA) and endotoxins), molecular mass (gDNA, endotoxins) and hydrophobicity (endotoxins) [6]. Consequently, several efforts have been done to minimize the amount of these impurities during the steps prior to the purification. Chromatography is a mandatory process in the removal of impurities and purity assessment of pDNA solutions before its subsequent application. The purification step aims the separation of the biologically active supercoiled (sc) pDNA from the other isoforms, together with the removal of all the impurities [7]. Recent years have witnessed a research effort to develop chromatographic methods for pDNA purification, most of them based on the characteristics of these molecules, such as size, charge and hydrophobicity [8]. Anion exchange chromatography (AEC) is one of the most used techniques for pDNA separation, purification and quantification. In fact, many pDNA purification kits are exclusively based on this technology, offering rapid separations with no solvent requirements [8]. AEC is centered in the interaction between the negatively charged phosphate groups of the DNA backbone and the positively charged groups of the stationary phase. Despite the similarity of the overall charge and molecular weight between the different pDNA topologies (as sc, open circular (oc) or linear (ln) isoforms), they possess distinct conformations and consequently, different superficial charge densities. Therefore, the isoforms will have different retention times using an increasing salt gradient strategy [7]. Recent studies with ethylenediamine (EDA), a weak anion exchange ligand, have demonstrated the potentialities of this ligand to isolate ammodytoxins [9], to purify β-glucosidases from contaminants [10] or to preconcentrate environmental Pb(II) [11], but no report for sc pDNA purification has been described. Due to some limitations presented by conventional packed beads, such as low binding capacity and limited flow rates, new developments to surpass them have emerged. Monoliths represent an evolution to those matrices and possess many advantages, namely high binding capacity due to the high quantity of accessible binding sites for large biomolecules such as pDNA, high reproducibility in small and large scale, simple handling and flow independent resolution due to their excellent mass transfer properties [12–14]. The high capacity of these matrices can allow their application in industrial processes for purification of influenza pDNA vaccines. So, the aim of the present work was the application of EDA monolithic disk in order to purify the sc isoform of a therapeutic plasmid against influenza infection from *E. coli* lysate samples. In this work it was also evaluated the purity of the influenza DNA vaccine after this chromatographic process to assure its compliance with all regulatory requirements. The effect of different binding conditions on dynamic binding capacity (DBC) of EDA monolith using a pure plasmid solution was also evaluated at a preparative scale. Finally, the ability of HA pDNA vaccine to induce gene expression in CHO and A549 cells was also tested.

2. Materials and methods

2.1. Materials

All solutions were freshly prepared with analytical grade reagents and with ultra-pure grade deionized water purified from a Millipore Milli-Q system (Billerica, USA). The solutions were filtered through a 0.20 μm membrane (Dassel, Germany) and degassed ultrasonically. The samples injected in the column were filtered through syringe filters, pore size 0.45 μm (Düren, Germany). NaCl, sodium phosphate monobasic anhydrous (NaH_2PO_4) and sodium phosphate dibasic (Na_2HPO_4) were purchased to Sigma Aldrich (Madrid, Spain). Hyper Ladder I (Bioline, London, UK) was used as a DNA molecular weight marker. GreenSafe Premium was purchased to NZYTech (Lisbon, Portugal). The iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used for genomic DNA (gDNA) quantification. The EDA monolith with a 0.34 mL bed volume (3.0 mm thick and 12.0 mm diameter) with average pore size of 1500 nm and the CIMac™ pDNA analytical column of 0.32 mL bed volume (15.0 mm length and 5.2 mm diameter) were gently prepared and provided by BIA Separations (Ajdovščina, Slovenia). The NTC 7482-41H-VA2 HA plasmid (6471 bp) was kindly provided by Nature Technology Corporation (Lincoln, USA).

2.2. Bacterial cell culture, alkaline cell lysis and primary isolation

The plasmid amplification was obtained by the fermentation of *E. coli* DH5α. This process was carried out overnight at 37 °C in a shake flask with 250 mL of Terrific Broth medium supplemented with 30 μg/mL kanamycin. The OD_{600} was used to monitor the bacterial growth and measured in a Pharmacia Biotech Ultraspec 3000 (Cambridge, England). The cells were harvested at 5500 rpm for 15 min and the pellets were stored at −20 °C.

The pDNA isoforms were obtained from *E. coli* cells by alkaline lysis [15] using the Plasmid Purification Maxi Kit from Qiagen (Hilden, Germany) following the manufacturer's instructions. Then, the obtained pDNA sample was ressuspended in 50 mM phosphate buffer, pH 7.5 and used in the chromatographic experiments. On the other hand, *E. coli* lysate samples were prepared in accordance to the method described by Černigoj and co-workers [16], which uses a clarification step with 0.5 M calcium chloride ($CaCl_2$) after the elimination of cellular debris.

2.3. Preparative chromatography

Chromatographic studies were performed in an ÄKTA purifier system (GE Healthcare Biosciences, Uppsala, Sweden) at room temperature with a compact separation unit and a personal computer using UNICORN software, Version 5.11. All experiments were carried out in a 0.34 mL (3 mm thick and 12 mm diameter) EDA cross-linked monolith with poly(glycidyl methacrylate-coethylene dimethacrylate) packed into a CIM disk housing, provided by BIA Separations (Ajdovščina, Slovenia). Prior to the sample application, the column was equilibrated with 50 mM phosphate buffer, pH 7.5 at a flow rate of 1 mL/min. For isoform separation experiments, the pDNA influenza vaccine was pre-purified with Maxi Kit from Qiagen and then injected (50 μL of pDNA with a concentration of 300 μg/mL) onto the column. The elution of bound pDNA was carried out by increasing the ionic strength to 1.85 M NaCl in 50 mM phosphate buffer, pH 7.5. In turn, the lysate samples (25 μL with a concentration of 800 μg/mL) were loaded onto the column in equilibration buffer at a flow rate of 1 mL/min. To promote the selective elution of bound species, the salt concentration was first increased to 1.65 M NaCl in 50 mM phosphate buffer (pH 7.5) and then to 2 M. In both cases the absorbance was monitored at 260 nm. Fractions were pooled according to the obtained chromatograms and were concentrated and desalted using Vivaspin concentrators (Vivascience) before the analysis by electrophoresis.

2.4. Agarose gel electrophoresis

The fractions recovered from each chromatographic experiment, after being efficiently desalinized, were analyzed by horizontal electrophoresis using a 10-cm-long gel of 0.8% agarose in TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) stained with 0.1 μL/mL of gel GreenSafe Premium (NZYTech, Lisbon, Portugal). The gel was visualized with a UVITEC system

(Cambridge, UK) using Firereader 1D Gel analysis software version 15.15 of UVITEC.

2.5. Analytical chromatography

The quality control of the obtained sc pDNA was performed according to a modification of the analytical method described by Mota and co-workers [17,18] using a CIMac™ pDNA analytical column. The quantity of RNA, oc and sc pDNA present in the clarified lysate was determined. Calibration curves for each component were obtained with concentration ranges of 2–30 μg/mL for RNA and 1–100 μg/mL for pDNA. The samples were diluted in 200 mM Tris–HCl (pH 8.0) and the analytical column was equilibrated with 600 mM NaCl in 200 mM Tris–HCl (pH 8.0). After the sample injection (20 μL) a linear gradient was performed until 700 mM NaCl in 200 mM Tris–HCl (pH 8.0) during 10 min at 1 mL/min.

2.6. Impurities analysis

The analysis of the gDNA in the purified sc pDNA was achieved through real-time polymerase chain reaction (PCR) in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), according to the method described by Martins and co-workers [19]. The amplification of the 16S rRNA gene from *E. coli* was accomplished with sense (5′-ACACGGTCCAGAACTCCTACG-3′) and antisense (5′-GCCGGTGCTTCTTCTGCGGGTAACGTCA-3′) primers by following the change in the fluorescence of the DNA binding dye Syber Green (Bio-Rad, Hercules, CA, USA). The gDNA from *E. coli* was obtained with a Wizard gDNA purification kit (Promega, Madison, WI, USA) and several standards with concentration ranging from 0.005 to 50 μg/mL were prepared for the calibration curve. Also, negative controls were run with the standards. On the other hand, protein concentration of the purified samples was measured using the micro-bicinchoninic acid (BCA) assay from Pierce (Rockford, USA), according to the manufacturer's instructions. The calibration curve was prepared using bovine serum albumin standards (0.025–1 mg/mL). Finally, the endotoxins quantification in the pooled fractions of each elution strategy was achieved using ToxinSensor™ Chromogenic LAL Endotoxin Assay kit from GenScript (Piscataway, USA).

2.7. Dynamic binding capacity

The 0.34 mL EDA monolith was used in the determination of the dynamic binding capacity (DBC) of pDNA. The monolith was connected to an ÄKTA Purifier system (GE Healthcare Biosciences, Uppsala, Sweden) and the DBC was determined using the breakthrough area integration method described by Jungbauer and Kaltenbrunner [20]. These experiments were conducted at different pDNA concentrations (0.025; 0.050; and 0.100 mg/mL) and pH values (7.5; 8.5 and 9.0). The column was equilibrated with 50 mM phosphate buffer with the proper pH at 1 mL/min. Non-binding studies were performed using a solution of pDNA (0.050 mg/mL) in 50 mM phosphate buffer with 2 M NaCl at 1 mL/min. Determination of DBC was carried out by recording breakthrough curves and calculating the amount of bound pDNA per mL support at 10%, 50% and 100% breakthrough. The dynamic binding capacity values were calculated by subtracting the value obtained under non-binding conditions. Elution of the bound plasmid was achieved by increasing the NaCl concentration in the mobile phase in a stepwise manner. Afterwards, the support was cleaned and regenerated with 1.5 M NaCl plus 1 M NaOH.

2.8. Transfection experiments

For the transfection experiments, two different cell lines were used and the differences between their expression patterns were registered. CHO (chinese hamster ovary) and A549 (adenocarcinomic human alveolar basal epithelial cells) cells were cultured in DMEM medium in the presence of 10% fetal calf serum (FCS) from Sigma Aldrich (Madrid, Spain), 1% penicillin/streptomycin (Life Technology, Porto, Portugal) and antimycotic (Sigma Aldrich, Madrid, Spain). All the cells were seeded at 1×10^5 cells/well in 1.5 mL of culture medium in a 6-well plates two days before the transfection and were incubated at 37 °C in humidified 5% CO_2 atmosphere. In the transfection day, the cells with approximately 60% confluence were washed with serum free medium. Then, 4 μg/mL of NTC 7482-41H-VA2 HA pDNA was added to the Lipofectamine (Invitrogen, Carlsbad, CA) in OptiMEM medium incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 4 h of incubation the medium was removed and replaced by the complete medium. The transfection efficiency was determined microscopically after 72 h of transfection using an immunocytochemistry analysis with mouse monoclonal anti HA H5N1 IgG primary antibody (abcam, Cambridge, UK). Then, transfection efficiencies of the influenza DNA vaccine (sc and oc isoforms) purified with EDA and the same plasmid purified with a commercial kit were compared. The transfection efficiency was determined as the percentage of transfected cells compared to the total cell number.

2.9. Cell viability

The cytotoxicity of the transfected cells was evaluated by the SensoLyte™ Cell Cytotoxicity Assay kit (AnaSpec Seraing, Belgium) following the manufacturer's instructions. This kit is based on the lactate dehydrogenase (LDH) leakage into the culture medium. The assay estimates the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD by the death cells. The resultant fluorescent signal is proportional to the number of dead cells and can be monitored at 530–560 nm/590 nm. In both cytotoxicity assays, the cultured cells without transfection were used as negative control (k−) and the ethanol treated cells were used as positive control (k+).

3. Results and discussion

3.1. Separation of pDNA isoforms

Plasmid DNA is a large molecule and highly negatively charged. Due to its size and charge, pDNA molecules interact with positively charged EDA. To perform the chromatographic experiments several strategies were applied in order to find the best approach to bind/elute the sc pDNA. The results show that the pH affects the pDNA isoform selectivity. EDA has a pK_a value of 7.56 in the free state [21] but the ligand environment can change its pK_a. Thus, a screening for the best pDNA retention conditions was performed using three distinct pH values (7.0, 7.5 and 8.5). At pH 7.0 (Fig. 1A), a complete retention of DNA (oc + sc) in the monolith occurred with 1.65 M NaCl in 50 mM phosphate buffer and total elution was verified at 1.85 M NaCl with the same buffer. In contrast, when using pH 7.5 the separation of pDNA isoforms was observed (Fig. 1B). However, when using phosphate buffer pH 8.5 there is no binding and both isoforms are collected in the flowthrough (Fig. 1C). The best conditions for sc pDNA purification were the application of a stepwise gradient of NaCl, with concentrations of 1.65 M (82.5%) and 1.85 M (92.5%) in 50 mM phosphate buffer, pH 7.5 (Fig. 1B). After equilibrating the monolith with 1.65 M NaCl, the influenza pDNA was injected and the unbound species eluted due to their

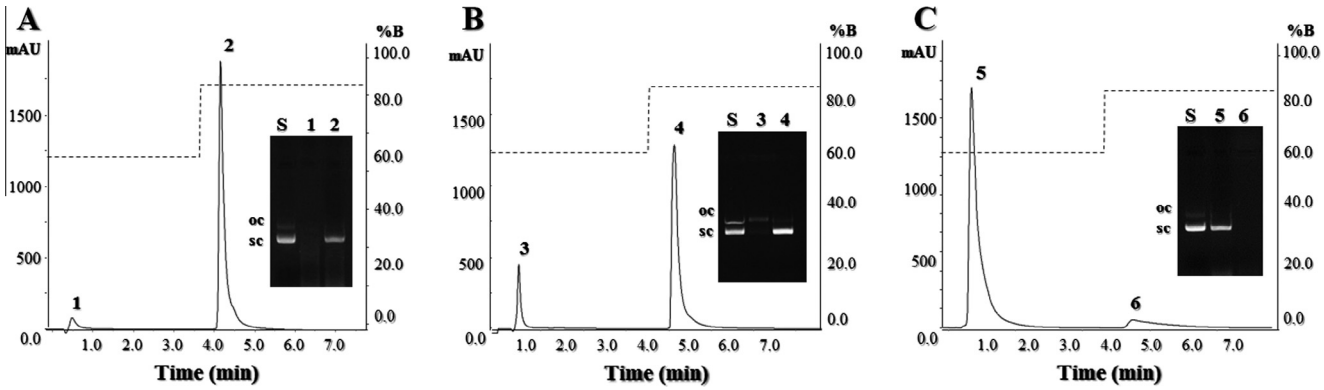

Fig. 1. Chromatographic profile of pDNA isoforms separation using EDA monolith at 1 mL/min by stepwise gradient of 1.65 M and 1.85 M NaCl in 50 mM phosphate buffer. (A) pH 7.0, (B) pH 7.5, (C) pH 8.5. Lane S: pDNA sample (oc + sc); lane 1 to 6: peaks 1 to 6; Lane 3: oc; Lane 4: sc.

lower affinity to the matrix. In this case it was eluted the oc isoform as seen in Fig. 1B, lane 3. The ionic strength of the buffer was then increased to 1.85 M NaCl and the strongly bound sc pDNA isoform was eluted, as shown by agarose gel electrophoresis (Fig. 1B, lane 4). So, the more compact sc isoform, which has higher charge density per surface area, elutes later than the oc isoform. These results suggest that the recognition of sc pDNA by the EDA monolith can be related to its supercoiling phenomenon that induces torsional deformations on this isoform, and consequently increases the exposition of its bases when compared with other isoforms. Therefore, this result is in agreement with the work developed by Mota and co-workers [17] that used a diethylaminoethyl monolith to specific detection and analysis of sc pDNA from *E. coli* lysate samples. Other study published by Nogueira and co-workers [22] showed that the increase in the ionic strength together with the pH manipulation provides better selectivity, particularly because the negatively charged peptides needed to be replaced with high salt concentration, as revealed in the present work using EDA monolith to purify the sc pDNA.

3.2. gDNA and RNA chromatographic profile

After the isoforms separation, the behavior of some impurities was analyzed to further separate sc pDNA from complex lysate samples. In this sense, gDNA and RNA samples were isolated from plasmid-free *E. coli* DH5α and loaded separately in EDA monolith. In Fig. 2A it is represented the elution of gDNA after its injection into the column with 1.65 M NaCl in 50 mM phosphate buffer, pH 7.5. Under these conditions, it was not verified an effective interaction of gDNA with the EDA monolith. This result is surprising because it could be expected an interaction between gDNA and monolith due to the anionic charge of gDNA and the positive nature of the EDA ligand. Thus, it is highly likely that parameters such as the degree of exposure of the nucleotide bases are involved in the establishing of the interaction, as happened with the oc pDNA isoform [23]. In this way, gDNA can be eluted with the oc isoform, and thus not interfering with the sc pDNA recovery. On the other hand, in Fig. 2B, RNA is also eluted in the flowthrough step with 1.65 M NaCl in 50 mM phosphate buffer. As described above, the sc pDNA isoform is expected to be eluted at higher salt concentrations. This behavior could be beneficial since it is intended the recovery of a pure sc pDNA fraction, free of contaminants.

3.3. Supercoiled pDNA selectively purified from complex E. coli lysates

The purification of the sc isoform of NTC 7482-41H-VA2 HA pDNA was performed by testing several retention/elution conditions with stepwise gradients. The EDA monolith was equilibrated with 50 mM phosphate buffer (pH 7.5) at 1 mL/min. After injecting the lysate sample (50 μL), a first peak was observed in the flowthrough step. Then, the ionic strength was increased to 1.65 M NaCl to elute different species still present in the sample and after that a 2.0 M NaCl gradient allowed the recovery of the strongly bound species in the third peak. Fig. 3 represents the chromatographic

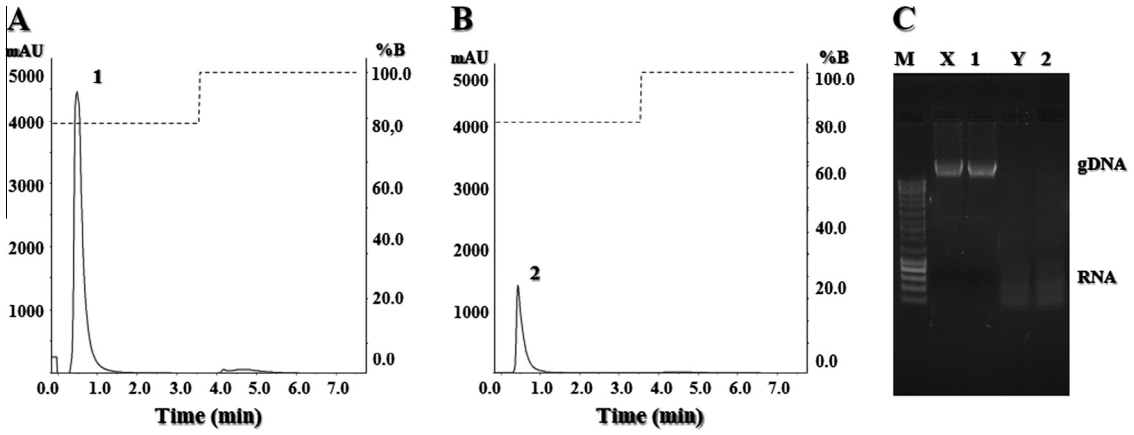

Fig. 2. Chromatographic profile of two *E. coli* components, gDNA (A) and RNA (B), using the EDA-monolithic disk by stepwise gradient of 1.65 M and 1.85 M NaCl in 50 mM phosphate buffer pH 7.5. (C) Agarose gel electrophoresis relative to each chromatogram. M: molecular weight marker (HiperLadder I: 10 kbp); lane X: gDNA sample; lane 1: peak 1 (gDNA); lane Y: RNA sample; Lane 2: peak 2 (RNA).

profile and the corresponding agarose gel electrophoresis. The analysis of the agarose gel electrophoresis showed that the increase of ionic strength allowed the recovery and isolation sc pDNA isoform in the third peak (Fig. 3B, lane 3) with a residual amount of the oc isoform (1.66%, as determined by the CIMac™ pDNA analytical column in the next section) free from other nucleic acids (namely, gDNA and RNA). The oc isoform is not clearly visible in the electrophoresis however, according to the analysis by CIMac™ pDNA analytical column the majority of this isoform is present in peak 2. Also, the first and second peaks (Fig. 3B, lane 1 and 2) correspond to the elution of RNA with different ionic strengths which could be related to the diverse RNA species and different base exposure degree of the RNA molecules [24]. These results are in agreement with the work of Eason and co-workers [25] who used DEAE-Sepharose, a weak anion-exchange matrix, to purify RNA from a plasmid DNA template. The elution profiles on this work show broad peaks over several fractions with the nucleic acids separated after applying a high concentration of NaCl.

3.4. Vaccine quality and purity assessment

The quality of the final pDNA vaccine product was determined using several methods. Firstly, agarose gel electrophoresis was performed to confirm the absence of impurities in the purified samples. Then, CIMac™ pDNA analytical column was applied to verify the absence of RNA in the purified sc pDNA. To quantify the proteins present in the extract it was used the micro-bicinchoninic acid (BCA) assay (Pierce, Rockford, USA). The endotoxins were measured using the ToxinSensor™ Chromogenic LAL Endotoxin assay kit (GenScript, Piscataway, USA) and finally PCR amplified the gDNA in the purified sample. The results from the impurities analysis of sc pDNA purified sample (peak 3, Fig. 3A) are shown in Table 1 while the HPLC analysis using CIMac™ pDNA analytical column is represented in Table 2. The agarose gel electrophoresis (Fig. 3B) showed the absence of RNA in the final plasmid preparation. Despite a remaining amount of 0.659% of RNA was detected in the final product, this result is in accordance to the regulatory agencies, as well as the protein levels (0.073%). The purification process was especially efficient in removing endotoxins, since only 5.164 EU/mg pDNA were detected. These values are satisfactory because the contamination does not exceed the FDA specifications [26].

Table 2 shows the results of DNA purity (97.1%), the purification factor and recovery yield of the chromatographic process. Accordingly, the purity of the plasmid injected in the monolith was about 30%. As shown in Table 2, 2.55 μg of sc pDNA were recovered from 5.43 μg of pDNA present in the feed sample and injected in the EDA monolith. The purification factor associated with this process is 3.31 and the purification step of the sc-peak 3 corresponds to 47.0% yield. This result indicates a loss of pDNA in the purification process with the EDA monolith. To avoid this fact, the ionic strength of the binding buffer can be exploited as a strategy to prevent unnecessary adsorption of low charge density impurities. Smrekar and co-worker [27] described the optimization of an anionic exchange chromatographic step for pDNA purification using CIM DEAE monolith using NaCl between 0.3 and 0.8 M. Previous to the purification the lysate content was adjusted with $CaCl_2$ and the result showed the decrease of RNA content with the increase of the salt concentration. The overall yield of the process was 82% probably due to the adjustment in $CaCl_2$ concentration which allows a better separation of RNA and pDNA.

Table 1
Impurity analysis of NTC 7482-41H-VA2-HA pDNA sample (sc-peak 3) and FDA specifications [27].

Impurity	FDA specification	sc-peak 3
Protein (%)	Preferably <1	0.073
gDNA (%)	Preferably <1	0.287
Endotoxins (EU/mg pDNA)	<40	5.164

The shared characteristics between sc pDNA and other impurities are, as referred before, their negative charge (gDNA, RNA and endotoxins), hydrophobicity (endotoxins) and molecular mass (gDNA and endotoxins) [6]. Therefore, these characteristics considerably constrain the sc purification which in turn reduces the overall step yield of the process. As shown in Fig. 3, the EDA monolith mainly interacts with biomolecules with a high bases exposition like RNA and sc pDNA which means that the main competitor is RNA. This phenomenon happens due to structural and chemical similarities with pDNA, resulting in extensive challenges for the selective purification of pDNA. RNA specifically is responsible for decreasing the capacity or even the global yield of the sc recovery. This means that for different lysate compositions some adjustments in the chromatographic conditions could be needed to maintain the selectivity and to overcome the competition impact of RNA over sc pDNA. Nevertheless, the obtained yield can be considered a reasonable value when compared with similar purification strategies. Previous studies of plasmid purification based on chromatographic strategies, such as triple-helix chromatography [28], or histidine–agarose chromatography [29] or arginine

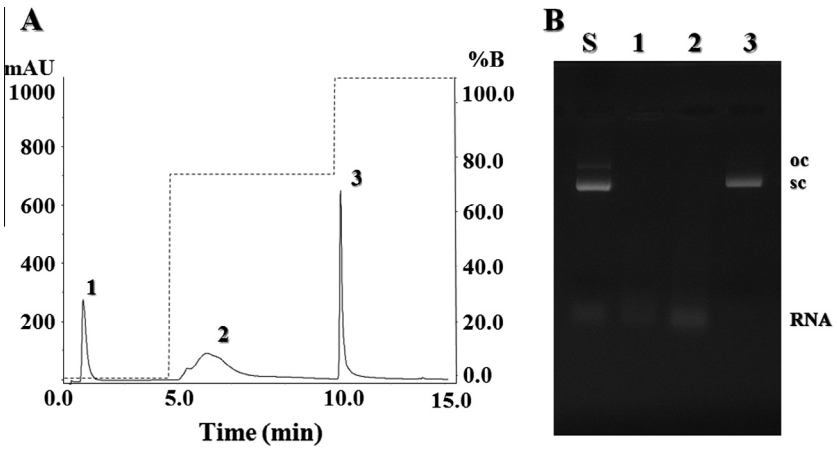

Fig. 3. (A) Chromatogram of the selective purification of sc pDNA isoform from a clarified *E. coli* lysate using EDA-monolithic disk. Sample elution was performed at 1 mL/min by stepwise gradient of NaCl of 0 M; 1.65 M and 2 M NaCl in 50 mM phosphate, pH 7.5. (B) Agarose gel electrophoresis analysis of the samples recovered from each peak of the chromatogram. Lane S: feed sample injected into the column. Fractions corresponding to peaks 1, 2 and 3 are shown in lanes 1, 2 and 3, respectively.

Table 2
Assessment of concentration, recovery yield and purity degree of sc NTC 7482-41H-VA2-HA pDNA isolated by EDA monolith by using the CIMac™ pDNA analytical column.

Process step	Volume (μL)	sc pDNA content				oc pDNA content			RNA content		
		(μg/mL)	(μg)	% of sc pDNA	Purification factor	(μg/mL)	(μg)	% of oc pDNA	(μg/mL)	(μg)	% of RNA
Feed sample	20	271.4	5.43	–	–	50.8	2.54	–	263.0	13.15	–
Peak 1	50	4.7	0.23	0.16	–	11.4	0.57	0.58	58.23	2.91	23.6
Peak 2	50	2.38	0.12	0.16	–	26.97	1.35	42.7	134.12	6.806	55.4
Peak 3 (sc)	50	51	2.55	**97.1**	–	6.13	0.32	1.66	2.27	0.11	0.659
Yield				**47.0**	**3.31**						

The bold data represent the purification degree of sc, yield of sc and purification factor of sc.

monolith chromatography [30] achieved 42%; 45% and 38% step yield, respectively.

The sc pDNA purity degree is crucial to decide if the purified sample is suitable for transfection studies. Briefly, analyzing the results presented in Table 2 and Fig. 3 it is revealed a purity of 97.1% for the sc pDNA purified by the ionic exchange strategy. Based on the criteria recommended by the regulatory agency, the sc pDNA sample presents the homogeneity required for therapeutic applications [31].

3.5. Dynamic binding capacity

The determination of the DBC of a stationary phase is essential for optimization of the purification process. This chromatographic feature represents the amount of target molecules that bind to the support under standard flow conditions and should be determined under specific flow and loading characteristics [32]. It is calculated based on the amount that can be loaded before significant product levels are measured in the breakthrough point. This information about the performance of the support is useful for assessing the loading conditions and column lifetime. In order to determine the DBC of EDA for pDNA, breakthrough curves were performed at different pH (7.5; 8.5 and 9.0) and pDNA concentrations (0.025; 0.050; and 0.100 μg/mL). Plasmid solutions were loaded through the column in binding conditions (without NaCl). The DBC results at 10%, 50% and 100% breakthrough are represented in Table 3, in function of the pH and pDNA concentration. It can be seen that DBC decreases from 2.816 mg/mL at pH 7.5 to 0.968 mg/mL with pH 9.0 at 100% breakthrough. This behavior is easily understandable since pH values are higher than pK_a value (7.56) which make the groups of EDA uncharged, thus disfavoring the interaction with the phosphate groups from the plasmid (negatively charged). Also, Table 3 demonstrates that DBC increases with the increase of pDNA concentration (Fig. 4). For instance, using a pDNA sample with 0.025 mg/mL the DBC at 100% breakthrough was 0.810 mg/mL but increased to 2.816 mg/mL at 0.050 mg/mL and finally to 6.562 mg/mL at 0.1 mg/mL of pDNA.

Table 3
Dynamic binding capacity of EDA monolithic disk using the NTC 7482-41H-VA2 HA plasmid (influenza vaccine) under different conditions.

	DBC (mg/mL)		
	10%	50%	Total
pH variation[a]			
7.5	1.628	1.809	2.816
8.5	0.696	0.994	2.269
9.0	0.100	0.191	0.968
pDNA concentration (mg/mL)[b]			
0.025	0.446	0.563	0.810
0.050	1.628	1.809	2.816
0.100	5.421	6.315	6.562

[a] Binding buffer: 50 mM phosphate buffer, with 0.05 mg/mL of pDNA at 1 mL/min.
[b] Binding buffer: 50 mM phosphate buffer, pH 7.5 at 1 mL/min.

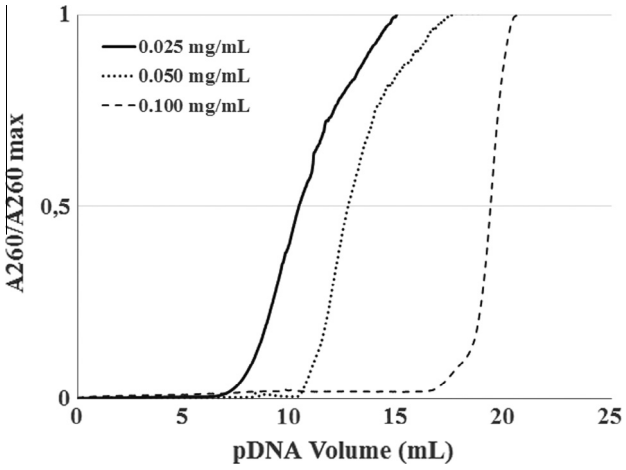

Fig. 4. Breakthrough curves of the effect of sc pDNA concentration (0.025; 0.05 and 0.100 mg/mL) in EDA monolithic disk at room temperature, using pH 7.5 at 1 mL/min flow rate.

The improvement on monolith capacity with the increase of sc pDNA concentration in the feedstock has been widely described. Sousa and co-workers [33] have explained this phenomenon. In concentrated media, pDNA suffers a high compaction degree leading to a significant reduction on the molecular apparent size, consequently increasing the surface area and binding sites on the support for molecules adsorption. These results are in accordance with another plasmid-based work using histidine–agarose chromatography [34].

As it is known, typical results for DBC with ionic matrices are usually higher than the results presented in this work, as showed by Tarmann and co-workers [35]. In fact, CIM DEAE showed a high DBC for pDNA (13.4 mg/mL) without using NaCl on the mobile phase. Interestingly, Krajnc and co-workers [36] obtained the highest capacity value 12.4 mg/mL with a DEAE monolith using 0.4 M NaCl, a value that was almost six fold higher than the capacity measured in the absence of salt. Also, Danquah and co-workers [37] studied a DEAE-Cl functionalized methacrylate resin where it was obtained a DBC of 12.10 mg/mL. On the other hand, Smrekar et al. [38] used a combination of butyl (C4) grafted methacrylate groups and diethylaminoethyl (DEAE) groups and investigated their ionic capacity for pDNA exhibiting a DBC of 4.7 mg/mL.

In the present study frontal analysis was used to quantify not only the column loading but also to determine the dissociation constant (K_D) between the EDA support and the pDNA influenza vaccine. This last parameter was calculated using the breakthrough curves for the different pDNA concentrations referred above. 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.

The calculations were made following the procedure presented in our previous work [39] displaying a m_L value of 1.667×10^{-10} mol and a K_D of 2.696×10^{-8} M. The obtained K_D value is in the acceptable range for affinity ligands between 10^{-8} and 10^{-4} which means a good interaction between sc pDNA molecules and the EDA ligand [40].

3.6. Transfection

In the present study two different cell lines were used to study the transfection efficiency of the several plasmid samples with HA gene derived from a seasonal influenza A virus. Because the non-viral vector present the major limitation of inefficient transfection [41], commercially available lipoplexes to mediate the transfection (lipofectamine) of cultured cells were used. The expression of HA antigen was possible using the immunological method of immunofluorescent staining with anti HA H5N1 IgG primary antibody and Alexa 546 anti-mouse as a secondary antibody. The transfection efficiency was reflected in the expression level in cell lines CHO and A549 with native pDNA control (oc + sc) purified with a commercial kit; oc plasmid isoform and sc plasmid isoform resulting from the purification step of the *E. coli* clarified lysate with the EDA monolithic support.

The quantitative analysis is represented in Fig. 5A. A strong intracellular fluorescence was observed in the transfected CHO cells with the purified sc isoform, presenting 70.6% of transfection while the oc pDNA showed 39.3%. On the other hand, the fluorescent signal for the transfected A549 cells with sc pDNA was substantially weaker, having a transfection efficiency of 61.4% whereas oc only transfected and induced the protein expression in 28.5% of these cells (as it is observed in supplementary information Fig. S1). In both cases, the control test performed with the same plasmid purified with a commercial kit lead to an efficiency of approximately 58.4% in CHO cell and 48% for A549 cells which are appreciably lower than the transfection efficiency achieved by the highly enriched and EDA purified sc plasmid isoform. These results follow the same path of the work of Cherng and co-workers [42] in which the transfection efficiency of the sc isoform is noteworthy higher than for other isoforms. Likewise, other processes developed to achieve a clinical pDNA product revealed that the transfection efficiency is enhanced when using the sc pDNA conformation, namely the work developed by Gaspar et al. [43], which used an arginine–agarose matrix to purify a therapeutic plasmid encoding the tumor suppressor gene p53. This work showed a significant enhancement in the expression using sc pDNA. Thus, the results obtained with EDA monolith confirmed that the process developed presents a good performance in the purification of clinical grade pDNA.

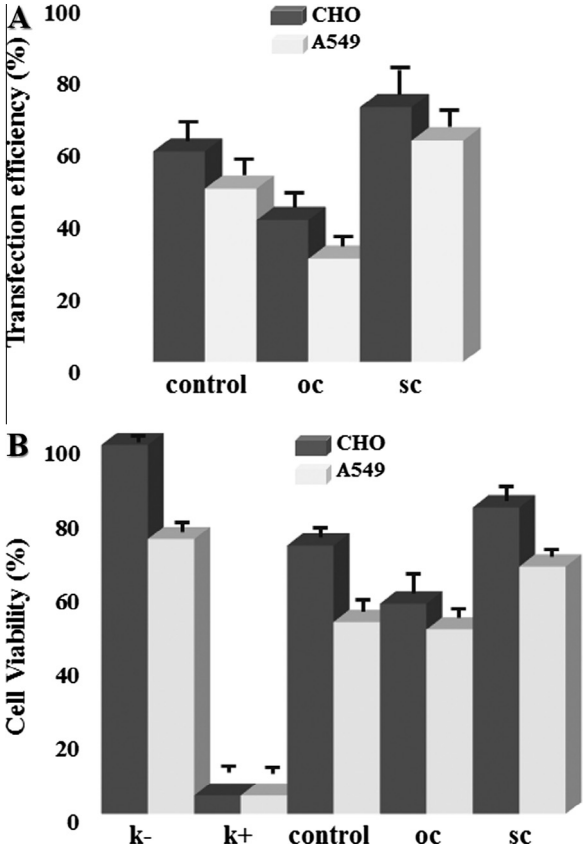

Fig. 5. (A) Transfection efficiency of pDNA control sample purified with a Qiagen Kit, and two other samples of oc and sc pDNA purified with EDA monolith to different cell lines, $n = 3$. (B) Cellular viability of a pDNA control sample purified with a Qiagen Kit, oc and sc pDNA purified with EDA monolith, $n = 3$. Positive control (k+); negative control (k−).

The cell viability profile was assessed after the cellular uptake took place and evaluated the effect of the samples of pDNA in the cells. The results presented in Fig. 5B showed that cells in contact with pDNA-encapsulated liposomes, purified using the EDA monolith, showed higher cell viability than the positive control (k+) (ethanol), but lower than the negative control (k−) (culture medium), during the incubation period (72 h). Therefore, the cell viability was higher for CHO (82.8%) than for A549 (66.8%) cells. Although these results represent a toxicity between 17% and 33%, it is important to keep in mind the impairment of cell viability in transfection procedures using lipofectamine, as related by Nam and co-workers [44].

4. Conclusion

The implementation of DNA vaccines represents a new alternative as therapeutic strategy in the treatment of infectious diseases like influenza. However, due to the singularity of pDNA molecules, its efficient purification is still a challenge. In this work, the application of EDA monolithic support enabled the purification of an influenza pDNA vaccine with differential recognition of the nucleic acids presented in *E. coli* lysates. Additionally, the selective removal of the oc plasmid variant and RNA molecules, as well as the reducing of the gDNA, proteins and endotoxins to 0.287%, 0.073% and 5.164%, respectively, was achieved. Consequently, the purified sc pDNA isoform obtained levels of impurities below that are in accordance to the FDA specifications. The process did not require the use of additives that usually pose a challenge to the validation, thus raising regulatory concerns. Also, *in vitro* transfection studies using the sc pDNA purified with the EDA monolithic methodology have shown the successful expression of the HA gene with higher efficiency than the transfection rate achieved with the oc isoform and with the pDNA purified with a commercial kit, regardless of the cell line used. So, this approach takes advantage of the monolith features to allow the rapid separation of a sc pDNA influenza vaccine with a good capacity, selectivity and high purity degree (97.1%) which was reflected not only in the high transfection efficiency but also in the high percentage of cell viability. In conclusion, our approach provides valuable tool with high specificity and good ability for the efficient isolation of sc NTC 7482-41H-VA2 HA isoform under a pharmaceutical-grade.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2015.09.046.

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Chapter 4

Concluding remarks and future trends

The evolution of vaccines and vaccine production are closely related. New methods to produce vaccines have been created regarding also the great improvement that has been verified. Modern vaccine developments are currently exploiting a wide array of novel technologies intending to create safer and more efficacious vaccines including pDNA produced in *E. coli*. Additionally, the increased interest on this technology is due to its long-lasting immune response and activation of both types of immunity.

In this sense, several vaccines have been produced to fight highly contagious respiratory diseases caused by the influenza virus. Presently, the researchers are focusing in non-viral vectors such as pDNA due to their lower toxicity and prolonged action. Therefore, a large quantity of highly pure sc pDNA is essential. Thus, efficient purification processes should be explored to obtain sc pDNA with the purity degree required for pharmaceutical applications. So, the aim of this study was to develop a suitable strategy for production and purification of a DNA vaccine encoding HA, an influenza protein, taking advantage of the properties of monoliths in the separation of the most biological active sc pDNA isoform.

To finish, a comparison between the three ligands must be made. First, the CDI monolith was the first support to be tested in pDNA purification of different model plasmids with different sizes, by exploiting the interactions that might occur using several pDNA molecules with different sizes. Due to the presence of the aromatic imidazole ring in the structure of CDI, the interactions between pDNA and the support were suggested to be mainly hydrophobic either by ring-staking or hydrogen bonds. A mixture of two pDNA isoforms (sc and oc) of each plasmid was separated by a two stepwise elution using high concentration of ammonium sulphate. As shown by the results, higher size plasmids needed lower ammonium sulfate concentration. This was possibly due to a greater number of interactions occurring between these biomolecules and the monolith, which allowed a stronger binding. Additionally, DBC was influenced by the characteristics of the plasmid: size, and composition. At higher salt concentrations higher DBC values were obtained for lower flow rates. Also, lower pH in DBC assays should be preferred to obtain higher capacities since the pH-sensitive CDI groups would be in the protonated form providing a binding enhancement of negatively charged pDNA molecules.

On the other hand, a single monolith with the ligand agmatine was used to purify the influenza pDNA vaccine, showing great versatility because it presented multimodal interaction. Agmatine showed to be effective as ligand in the purification of pDNA under two strategies: ascending sodium chloride gradient and descending ammonium sulfate gradient. Under descending ammonium sulfate strategy both pDNA isoforms and complex lysate were separated, showing a selective interaction between the different nucleic acids. However, in the ascending sodium chloride strategy, the resolution of the isoforms was lost when complex lysate solutions were loaded. This was possibly due to the exceptionally strong binding between this nucleic acid and agmatine, which may interfere in the ability of the support to differently interact with the two

pDNA isoforms. Moreover, the purity degree obtained in both strategies was higher than 98% with a yield of more than 33% in each approach. Also, the method presented a high binding capacity (5.656 mg/mL). All the host impurities were significantly reduced or undetectable. Moreover, the transfection efficiency of fibroblast cells using the sc pDNA purified with the ammonium sulfate strategy was also high, being reached an efficiency of 73%. All these results proved that this agmatine-functionalized monolith is a versatile column and can be applied in the purification of an influenza vaccine from complex lysates.

Finally, the potential of an ethylenediamine (EDA) monolith for the purification of influenza pDNA vaccine was tested. The purification process allowed a significant reduction of the host contents (proteins, RNA and genomic DNA) and the DNA vaccine was recovered with a high purity degree (97.1 %) and good yield (47 %). As an upside, the process did not require the use of additives that usually pose a challenge to the validation, thus raising regulatory concerns. Also, the data from *in vitro* experiments indicated that the pDNA cloned HA gene could be successfully expressed in two eukaryotic cell lines (A549 and CHO). A strong intracellular fluorescence was observed in the transfected CHO cells with the purified sc isoform, presenting a transfection efficiency of 70.6%. By the contrary, A549 cells presented a weaker signal and a transfection efficiency of 61.4%. The cell viability profile was also assessed with the purpose of evaluating the effect of the pDNA in the cells. These results showed a higher cell viability for CHO (82.8 %) than for A549 (66.8 %) cells. Thus, EDA monolith showed a potential to be further applied in chromatographic processes.

To sum up, three diferent ligands based in different interaction modes were applied. Agmatine monolith even though presented the best recovery yield and DBC under the hydrophobic strategy, it uses high concentrations of ammonium sulfate which represents an environmental impact. EDA monolith for intances, performs under a faster purification method with good purification degree and step yield with less environmental and economical impact which makes it more applicable industrially.

In conclusion, the application of the innovative monolithic technology represents an advantageous alternative in pDNA purification to conventional supports due the rapid separations with short contact time leading to a low degradation rate of the target molecules, good resolution independently of the flow, good capacity and selectivity to the sc plasmid isoform. This work provided new understandings in the purification of pDNA contributing for the future development of new and more robust bioseparation methods.

Despite several efforts to obtain the best results were made during this thesis, further developments need to be performed to better understand the important findings here reported and to achieve more efficient purification methods for pDNA vaccines. In order to improve the recovery yield of this purification process, as a future approach, it could be interesting to prepare and characterize new affinity chromatographic supports, thus refining the binding, and both avoiding losses of the product of interest and the use of harsh conditions in the recovery.

Additionally, new chromatographic matrices such as criogels could be tested with the purpose of finding the best support that can recognize and purify the sc pDNA isoform from the remaining lysate constituents with the highest selectivity and productivity. As an alternative, non-chromatographic techniques such as membranes, aqueous two phase systems or magnetic separations could be applied to reduce downstream expenses that usually account between 70-80 % of the total cost, in a purification process. Evaluation of different production methods of pDNA vaccines, for instance using gram positive bacterias can represent a better altenative to reduce the level of impurities and facilitate the purification process. Also, the *in vivo* evaluation of antigen-specific humoral immune responses in mice after intradermal delivery of the influenza pDNA vaccine must be a valuable methodology. The determination of the expression levels of HA and anti-HA IgG response will give a better understanding not only of the therapeutic function but also of the chromatographic process chosen in the purification of the pDNA vaccine.