UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Purificação de uma vacina de DNA plasmídico para prevenção ou tratamento do cancro do colo do útero

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Dissertação para obtenção do Grau de Mestre em
Ciências Biomédicas
(2º ciclo de estudos)

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Covilhã, junho de 2016

"The ultimate measure of a man is not
where he stands in moments of
comfort, but where he stands at
times of challenge and controversy."

- Martin Luther King, Jr

To the most important people of my life,

My parents…

Thank you for everything.

Acknowledgments

Firstly, I would like to thank to my supervisors Professor Doctor Ângela Sousa and Professor Doctor Fani Sousa for all guidance and knowledge transmitted throughout this year. Your support was the key to my progress and to achieve the goals of this work.

To University of Beira Interior and Health Sciences Research Centre, for providing all the conditions to develop this research project.

To all Biotechnology and Biomolecular Sciences group members, for the promptitude to join me into the group and help me whenever I needed.

To my lab colleagues, specially to Ana Margarida Almeida and Patrícia Pereira for sharing with me your knowledge, for always being ready to listen and help me with your experience, allowing me to overcome the constant barriers of scientific research.

To all my friends, namely João Brás, Jorge Ferreira, Marta Pereira, Rui Vieira, Luís Espínola, Sandra Antunes, Cláudia Antunes, Paulo Veloso, Marta Maciel, Diana Dias and Tânia Mendes. You are the best, you are my second family. Thank you.

To all my family, you are the most precious people in the world, specially my parents. I owe you everything I am. Thank you for believe in me and to do everything to see me happy. I love you.

To Marisa, for all love and support over the years. Even far away, you are always present. Thank you for being always by my side.

Resumo alargado

A infeção pelo Vírus do Papiloma Humano (HPV) é sexualmente transmissível e está associada ao desenvolvimento de vários tipos de cancro, entre eles o cancro do colo do útero, a segunda maior causa de morte em mulheres a nível mundial. A patogenicidade do vírus advém da capacidade de alterar o ciclo celular através das oncoproteínas E6 e E7, responsáveis pela proliferação descontrolada das células infetadas e consequente desenvolvimento de massas tumorais. Apesar da existência de três vacinas disponíveis no mercado (Cervarix, Gardasil e Gardasil 9), a sua função é apenas preventiva, através do desenvolvimento de respostas imunes humorais, não tendo a capacidade de tratar pacientes já infetados aquando da sua administração. Além desta desvantagem, as vacinas preventivas não abrangem imunidade contra todos os tipos de vírus do HPV. Como tal, o desenvolvimento de uma vacina terapêutica é considerada uma estratégia promissora que tem vindo a ser cada vez mais estudada.

O crescente conhecimento acerca dos mecanismos das doenças juntamente com a facilidade em manipular o DNA, tem aumentado o desenvolvimento na área do DNA recombinante ao longo das últimas décadas, nomeadamente nas abordagens de terapia génica e de vacinas de DNA. O baixo custo destas terapias torna-as acessíveis e cada vez mais exploradas pela ciência. Assim, a utilização de vetores virais e não virais para a entrega de genes, tem vindo a ganhar importância como meio de prevenção e tratamento, através da expressão de proteínas alvo. Entre os vetores não virais mais explorados, destaca-se o DNA plasmídico (pDNA) pela sua segurança, fácil manipulação e possível produção em grande escala. As vacinas de DNA fazem uso deste vetor e das suas vantagens, no sentido de desencadear uma resposta preventiva e terapêutica eficaz contra doenças infeciosas. A expressão de proteínas antigénicas leva ao desenvolvimento das respostas imunitárias humoral e celular, evitando a progressão da doença e permitindo a sua regressão. Para se tornarem eficazes, estas vacinas de DNA requerem elevadas quantidades de pDNA superenrolado (sc), considerada a forma biologicamente ativa do pDNA, com elevado grau de pureza de forma a aumentar a eficiência de transfeção das células alvo e da expressão das proteínas antigénicas.

O desenvolvimento de um processo biotecnológico para a produção da vacina de pDNA HPV E6/E7^{MUT} foi estabelecido, sendo fundamental explorar estratégias de purificação eficientes, aplicando suportes cromatográficos vantajosos e inovadores de forma a obter o pDNA sc com o grau de pureza recomendado pelas agências reguladoras. A cromatografia de afinidade tem vindo a ser explorada para a purificação de pDNA, nomeadamente através da utilização de aminoácidos como ligandos. Este tipo de ligandos permitem explorar interações específicas e seletivas com o pDNA sc, à semelhança do que acontece em sistemas biológicos no bioreconhecimento entre proteínas e ácidos nucleicos. Este bioreconhecimento resulta na obtenção de um produto biofarmacêutico final com elevado grau de pureza. Devido às

limitações das matrizes convencionais, os monolitos surgem como suportes cromatográficos cada vez mais explorados. Através da sua estrutura polimerizada, formam canais tridimensionais, interconectados entre si e de largo diâmetro, possuem uma elevada capacidade de ligação para biomoléculas com grandes dimensões (como o pDNA) e permitem realizar processos de purificação rápidos e eficientes, mesmo em suportes de pequenas dimensões.

Desta forma, o presente trabalho teve como objetivo explorar as características e a versatilidade dos suportes monolíticos, modificados com dois ligandos semelhantes, a lisina e a cadaverina, no sentido de tentar obter a purificação da isoforma sc do pDNA, removendo contaminantes provenientes do processo de produção e extração do pDNA a partir das células bacterianas, como as isoformas menos funcionais do pDNA (linear e circular aberta) e os constituintes do hospedeiro (DNA genómico, proteínas e endotoxinas).

Após vários estudos do comportamento de retenção e eluição do pDNA sc presente na amostra de lisado por variação da concentração de NaCl com diferentes tampões no monolito de cadaverina, verificou-se que tanto o tampão Tris-EDTA como o Fosfato-EDTA mostraram alguma seletividade na separação entre o pDNA e o RNA. No entanto, foram necessários mais testes combinando com estes tampões a manipulação de pHs, para obter a purificação do pDNA sc. Foi otimizada uma estratégia de eluição através de um gradiente por passos com concentrações crescentes de 1.05 para 1.5 M de NaCl em tampão Tris-EDTA, pH 6.0, que permitiu isolar o pDNA sc com um grau de pureza de 97.89% e uma recuperação de 98.70%. Com o sucesso obtido no suporte monolítico de cadaverina, as melhores condições foram também testadas no monolito de lisina. A estratégia otimizada para o monolito de lisina consistiu num gradiente por passos com concentrações crescentes de 0.45 M para 1 M de NaCl em tampão Tris-EDTA, a pH 6.0, sendo que os resultados obtidos neste suporte em termos de grau de pureza e rendimento de recuperação do pDNA sc foram semelhantes aos resultados alcançados no monolito de cadaverina (97.53% e 96.10% respetivamente). Para verificar se as amostras obtidas nos monolitos de cadaverina e lisina com melhor valor de pureza e rendimento para o pDNA sc cumprem os requisitos exigidos pelas agências reguladoras, foi realizada uma análise detalhada por PCR em tempo real, micro BCA e ensaios de LAL com o objetivo de quantificar impurezas como o DNA genómico, proteínas e endotoxinas, respetivamente. As amostras selecionadas apresentaram níveis de impurezas abaixo dos limites recomendados pela Food and Drug Administration. Embora o monolito de lisina permita a purificação do pDNA sc aplicando menores quantidades de sal, o que se torna vantajoso devido ao menor impacto ambiental, o suporte de cadaverina permitiu obter melhores níveis de pureza e recuperação, tal como melhor eficiência na remoção de impurezas. Assim, pode concluir-se que as estratégias de purificação otimizadas para ambos os suportes permitem obter devidamente o pDNA sc para aplicação como um produto biofarmacêutico.

Palavras-chave

Cromatografia de afinidade, HPV, monolito de cadaverina, monolito de lisina, vacinas de DNA plasmídico.

Abstract

Human papillomavirus (HPV) is a common sexually transmitted virus responsible for malignant progression of cervical cancer. High-risk HPVs have been reported in 99% of cervical cancer cases worldwide, the second most common cancer in women. The cervical cancer development is associated with HPV E6 and E7 protein production, which can interfere with apoptosis and deregulate cell cycle proliferation. Nowadays, prophylactic vaccines are available and commercialized towards HPV eradication. However, these vaccines only stimulate the immune system against future infections, being ineffective when the woman is already infected. Furthermore, not all types of HPV virus are covered by this type of vaccines. DNA vaccines are advantageous compared to conventional vaccines by inducing both cellular and humoral immune responses, leading to prevention and treatment of a target infection. Thus, the development of a DNA vaccine able to produce E6 and E7 antigens arises as a promising therapeutic pathway to control HPV infection. Plasmid DNA (pDNA) has been explored as a versatile non-viral vector to deliver therapeutic genes due to its biosafety, low cost and easy manufacture, which make it a desirable vector to deliver therapeutic genes. In order to guarantee the purity of the pDNA vaccine, it is necessary to explore several chromatographic methods. Our research group has been focused on the purification of mutated pDNA HPV-16 E6/E7, to prevent oncogenecity of E6 and E7 viral proteins, in order to achieve the required purity levels imposed by regulatory agencies for therapeutic applications. In addition, interest around innovative monolithic supports has recently increased due to their advantageous features, like high binding capacities of large molecules and excellent mass transfer properties. Thus, the aim of the present work was to explore two monolithic columns modified with lysine and cadaverine ligands, in order to develop suitable purification strategies to isolate supercoiled (sc) pDNA isoform, which is the conformation with most biological interest for therapeutic applications. Through the manipulation of NaCl concentrations, buffer nature and pHs, the chromatographic conditions were optimized to isolate sc pDNA in cadaverine and lysine monolithic columns. The results obtained with cadaverine and lysine monoliths from purity degree (97.89% and 97.53%, respectively) and recovery yield (98.70% and 96.10%, respectively) of sc pDNA, revealed that the best elution strategy consists on an increasing stepwise gradient of NaCl in 10 mM tris-EDTA (pH 6.0). It was also observed that lower pH values led to stronger interaction between ligands and biomolecules, favoring the sc pDNA purification. The results of genomic DNA, endotoxins and proteins quantification, revealed that all impurities had levels below to those imposed by regulatory agencies. These data suggest the applicability of two supports to purify sc pDNA in order to be applied on a DNA vaccine against HPV.

Keywords

Affinity chromatography, cadaverine monolithic column, HPV, lysine monolithic column, plasmid DNA vaccines.

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List of Acronyms

µg	Microgram
µL	Microliter
µm	Micrometer
APCs	Antigen-presenting cells
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CR	Conserved regions
CTL	Cytolytic T lymphocite
DC	Dendritic cells
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E6AP	E6 Association Protein
EDTA	Ethylene-diamine tetraacetic acid
EMEA	European Medicines Evaluation Agency
EU	Endotoxin units
FDA	Food and Drug Administration
g	Gram
gDNA	Genomic DNA
h	Hours
H-bond	Hydrogen-bond
HCl	Hydrochloric acid
HPV	Human Papilloma Virus
IMAC	Immobilized metals affinity chromatography
K_2HPO_4	Dipotassium phosphate
kbp	Kilo base pairs
kDa	Kilo Daltons
kg	Kilogram
KH_2PO_4	Monopotassium phosphate
L	Liter
LAL	Limulus amebocyte lysate
LCR	Long Control Region
M	Molar
mAU	Miliabsorbance units
mc-DNA	Mini circular DNA
MHC	Major histocompatibility complex
Min	Minute

mL	Milliliter
mM	Milimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nanometer
ºC	Celsius
oc	Open circular
OD_{600}	Optical density at 600 nm
ORFs	Open reading frames
pb	Base pairs
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
PEI	Polyethylenimine
Phosphate-EDTA	10 mM sodium phosphate and 10 mM EDTA
pKa	Acid dissociation constant
PLL	Poly-L-Lysine
pRb	Retinoblastoma tumor suppressor protein
RNA	Ribonucleic acid
rpm	Rotations per minute
sc	Supercoiled
SDS	Sodium dodecylsulfate
TAE	Tris-acetate-EDTA
Tris	Tris-(hydroxymethyl)aminomethane
Tris-EDTA	10 mM Tris and 10 mM EDTA
UV	Ultraviolet
V	Volts
VLP	Virus-like particles
w/w	Mass/mass

Chapter 1 – Introduction

1.1 Human papillomavirus

Infection by human papillomavirus (HPV) is the leading cause of cervical cancer, the second most common cause of cancer mortality in women worldwide [1] and one of the most common sexually transmitted infections [2]. With a 99.7% of prevalence in cervical carcinomas, this virus has been also related with other anogenital cancers (vaginal, anal, vulvar and penile), such as head and neck cancers [3, 4].

The cervical carcinogenesis caused by HPV is the main studied malignancy related with this virus, once high-risk sexual behavior as the age of first sexual intercourse or number of partners, nutritional and immune status are related with appearance of uterine infections [2]. Presently there are over than 200 HPV genotypes identified [5], and 90% of cervical cancers are triggered by HPV high-risk types 16, 18, 31, 33, 45, 52 and 58, being the types 16 and 18 responsible for 70% of the cases worldwide [3]. The most strongly associated to cervical cancer is the type 16, with a presence of around 60% in all cervical cancer samples [6].

Most of HPV genotypes belongs to three main genera: alpha papillomaviruses predominantly isolated from genital lesions and also beta and gamma papillomaviruses that are mainly isolated from cutaneous lesions [7]. Table 1 shows the main genera, several identified types and their association with human diseases. Within HPV types that can infect genital tract there are two main groups: high risk types that are associated with anogenital cancers and non-oncogenic low risk types associated with genital warts [8].

Table 1 - Main identified HPV types that belong to different genera and their association with human diseases (adapted from [9]).

Genera	HPV type	Group	Disease
Alpha	HPV16	Mucosal high-risk	Cervical squamous cell carcinoma Cervical adenocarcinoma Oropharyngeal cancer
	HPV18		Cervical squamous cell carcinoma Cervical adenocarcinoma
	HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59		Cervical squamous cell carcinoma
	HPV6, 11	Mucosal low-risk	Benign genital lesions Respiratory papillomatosis
	HPV13, 32		Oral focal epithelial hyperplasia
	HPV2, 3, 27, 57	Cutaneous benign	Skin warts
Mu	HPV1		Skin warts
Beta	HPV 5 and 8	Cutaneous	First beta HPV types isolated from squamous cell carcinoma of epidermodysplasia verruciformis individuals
	HPV9, 12, 14, 15, 17, 19-25, 36-38, 47, 49, 75, 76, 80, 92, 93, 96, 98-100, 104, 105, 107, 110, 111, 113, 115, 118, 120, 122, 124, 143, 145, 150- 152, 159		Likely associated with squamous cell carcinoma in epidermodysplasia verruciformis patients as well as immuno-compromised and immuno-competent individuals
Gama	HPV4, 48, 50, 60, 65, 88, 95, 101, 103, 108, 109, 112, 115, 116, 119, 121, 123, 126-142, 144, 146-149, 153 -158, 161 -170		Unknown

Although there is no effective treatment for cervix cancer, the prevention can be done through two complementary approaches, vaccination to prevent HPV infection and screening to detect and treat cervical precancerous lesions [10]. Cytology screening, despite its limitations in terms of sensitivity and reproducibility, has been gaining importance as one of the most important activities of public health prevention worldwide, due to significant reductions of cervical cancer incidence and mortality [10].

1.1.1 Virus structure and genome organization

HPVs belong to Papillomaviridae family and different HPV types share some characteristics such as the presence of a double-stranded circular DNA, associated with histone-like proteins, within an icosahedral capsid formed by two late proteins (L1 and L2) and composed of 72 capsomeres, each one with five monomeric units to form a pentamer, corresponding to the major protein capsid, L1. Each virion measures 50-60nm in diameter and replicates their genomes within the nuclei of infected host cells [7, 11, 12]. The structure of HPV is represented on figure 1.

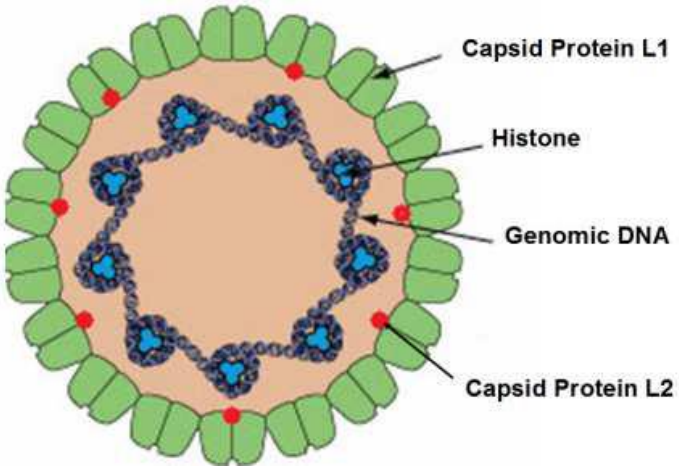

Figure 1 – General representation of the structure of HPV (adapted from [12]).

Although the viral genome can vary slightly in size between different HPV types, the genomes of all HPVs have approximately 8000 base pairs in size, while for the particular case of HPV-16, the size is 7904 base pairs (GenBank accession number NC_001526) and its genome can be divided into three major functional regions: early, late and long control (noncoding) regions. [11, 13, 14]. There are eight open reading frames (ORFs): the early genes constitute over 50% of the viral genome and encode six early proteins (E1, E2, E4, E5, E6 and E7) that are involved in the proliferation of infected cells, their lateral expansion and oncogenesis; and the late genes (L1 and L2 structural proteins). The name of proteins is based on their temporal expression pattern in the viral life cycle [13, 14]. The genome organization of HPV is schematized on figure 2.

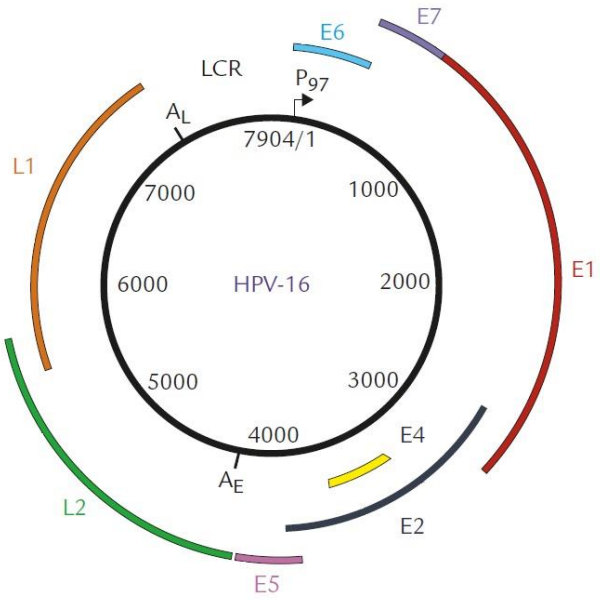

Figure 2 - HPV genome organization. The HPV-16 genome structure contains a long control region (LCR) and eight genes that are necessary for different stages of the virus life cycle. The LCR comprises binding sites for cellular transcription factors, as well as for the viral E1 and E2 proteins that control viral replication and gene expression (adapted from [15]).

Additionally, another segment of about 850 base pairs (10% of the HPV genome), named long control region (LCR), has no protein-coding functions but is responsible for the origin of replication and have enhancer and silencer sequences that are important in the regulation of RNA polymerase II initiated transcription from viral early and late promoters and thus regulate DNA replication [14].

1.1.2 HPV life cycle

The HPV life cycle begins with infection of stem cells in the basal layer of the epithelium. To establish a persistent infection, HPV needs to overcome some barriers. Firstly, HPV re-programs the host cell's DNA replication machinery to replicate their own genome and achieve a high copy number amplification of viral DNA [1, 16]. Second, it is necessary to eliminate host defense mechanisms that limit viral survival, such as clearance of infected cells by apoptosis, immune responses of the host, and eventually replicative senescence of infected and abnormally proliferating host cells [16]. LCR, which harbors non-coding sequences, control HPV oncoproteins expression in a temporal way [1]. After the entry in the cells, the virus requires the expression of E1 and E2 genes to maintain a low copy number of the genome. These proteins bind to the viral origin of replication and recruit cellular DNA polymerases and other proteins necessary for DNA replication [17]. In the suprabasal layer, the expression of genes E1, E2, E5, E6 and E7 contributes to the maintenance of the viral genome and induces cell proliferation, increasing the number of HPV-infected cells in the epithelium, resulting in a higher number of cells that will eventually produce infectious virions [17, 18].

In the more differentiated cells of this same layer of the epithelium occurs the activation of differentiation-dependent promoter and maintenance of gene expression E1, E2, E6 and E7. Furthermore, there will be activation of the expression of E4 gene, whose product will induce amplification of the viral genome replication, greatly increasing the number of virus copies per cell, at the same time that occurs the expression of genes L1 and L2 [18, 19]. In the granular layer, the products of late genes, the major and minor proteins of the viral capsid, L1 and L2 respectively, gather to assembly of the viral capsids and formations of virions, which reach cornified layer of the epithelium and are released [18]. Figure 3 shows HPV life cycle.

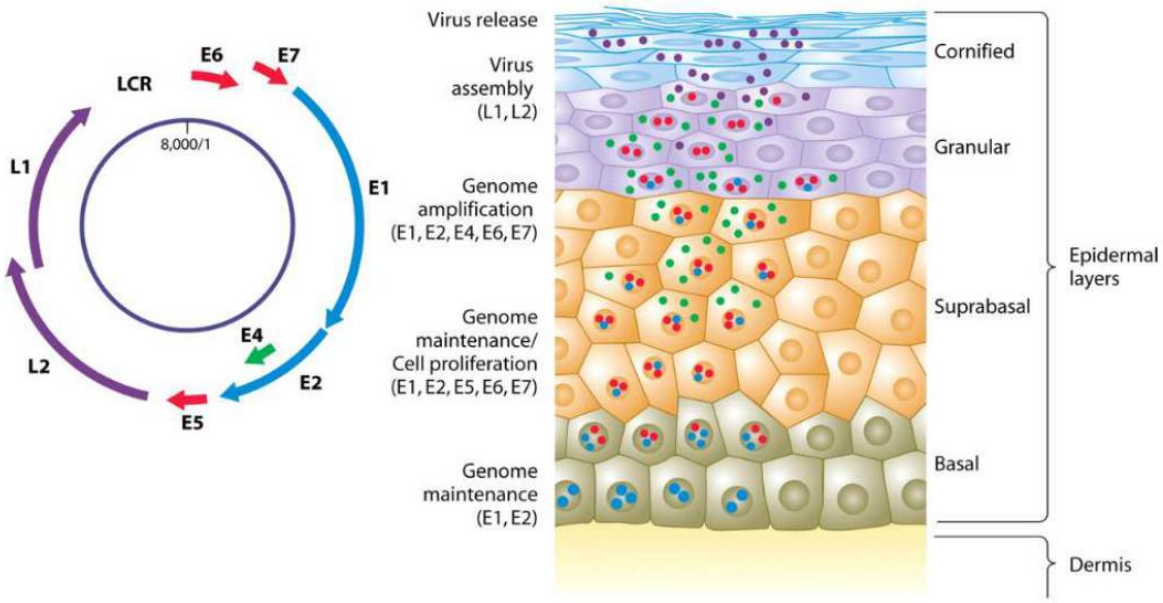

Figure 3 – The HPV life cycle on keratinocyte (adapted from [18]).

The assembly of virions occurs in the upper differentiated layers of the infected squamous epithelium in cells that, normally, would have permanently stopped to proliferate [1, 16]. To solve this problem and to promote viral replication, the HPV E6 and E7 oncoproteins are able to re-introduce an S-phase like milieu under differentiating conditions of the host cell [1]. The interruption of cellular mechanisms that regulate apoptosis and the cell cycle results in dysregulated cell cycle proliferation, delayed cellular differentiation, increased frequency of spontaneous and mutagen-induced mutations, and increased chromosomal instability. Thus, the overexpression of the viral oncoproteins E6 and E7 drives and maintains the neoplastic process [13]. The E6 prevents the activity of tumor suppressor p53 whereas the E7 inhibits the retinoblastoma tumor suppressor protein (pRb), which controls cell division by blocking the activity of transcription factors [20]. The expression of E6 and E7 along the time is responsible for the continued cancer phenotype [21]. Table 2 presents the HPV oncoproteins and their functions.

Table 2 - HPV oncoproteins and their functions (adapted from [12]).

Protein	Function
L1	Major capsid protein: contains the major determinant required for connection to cell surface receptors. It is highly immunogenic and has conformational epitopes that induce the production of neutralizing type-specific antibodies against the virus.
L2	Minor capsid protein: L2 contributes to the binding of virion in the cell receptor, supporting its uptake, transport to the nucleus, and delivery of viral DNA to replication centers. Moreover, E2 helps the packaging of viral DNA into capsids.
E1	Viral DNA replication
E2	Control of viral transcription, DNA replication, and segregation of viral genomes.
E4	Favor and help the HPV genome amplification, besides regulating the expression of late genes, controlling the virus maturation, and facilitating the release of virions.
E5	Enhance the transforming activity of E6 and E7; Promotes fusion between cells, generating aneuploidy and chromosomal instability; Contribute to immune response evasion
E6	Bind and degrade the tumor-suppressor protein p53, inhibiting apoptosis; Interact with proteins of the innate immune response, contributing to immune evasion and persistence of virus; Activate the expression of telomerase.
E7	Bind and degrade the tumor-suppressor protein pRb; Increase CDK activity; Affects the expression of S phase genes by directly interacting with E2F factors and with histone deacetylases; Induce a peripheral tolerance in cytotoxic T lymphocytes (CTL) and downregulate the expression of TLR9, contributing to immune response evasion.

1.1.2.1 E6 oncoprotein

The HPV E6 protein has 151 amino acids, 18 KDa in size and contains two C-terminal zinc fingers with four Cys-X-X-Cys motifs, that in case of high-risk mucosal HPV types contains a PDZ-binding motif [22]. Oncogenic HPV E6 inactivates PDZ-containing proteins, localized on membrane-cytoskeleton interfaces, inducing loss of cell polarity and promoting cellular transformation through the disruption of cell junctions [1]. The E6 protein does not show sequence homology to other viral or cellular protein and it inactivates p53 through the interaction between E6, p53 and other cellular E6-associated protein (E6AP), also known as UBE3A, leading to complete degradation of p53 [23, 24]. Since HPV depends on the cellular DNA synthesis machinery and must stimulate S-phase progression for the replication of its genome, overexpression of p53 inhibits viral replication [25]. To degrade p53, the E6 proteins from high-risk HPV types first bind to a cellular E3 ubiquitin-protein ligase, the E6AP, which, in a complex with E6, is then able to bind the p53 protein. E6AP, a component of ubiquitin-protein pathway, targets proteins for degradation through a complex of enzymes, which in this case transfers ubiquitin to lysine residues of the target protein. With the aid of ubiquitin-protein ligase, the proteasome initiates p53 proteolysis. HPV-16 E6 also binds the transcriptional co-activators CBP/p300 and decreases the ability to activate p53- responsive promoter elements, even without E6AP involvement [1]. E6 thereby enables infected cells to bypass the normal growth arrest signals at the G1/S and

G2/M checkpoints and progress through the cell cycle unhindered, leading to the accumulation of genomic defects that contribute to malignant transformation [26]. Moreover, E6 also interferes with other pro-apoptotic proteins such as Bak, FADD and procaspase 8 to prevent apoptosis [20]. The high-risk HPV-16 E6 oncoprotein also promotes cellular immortalization through the transcriptional up-regulation of the catalytic subunit of human telomerase, hTERT, by several mechanisms including association with the transcriptional activator c-Myc or the E6AP-dependent degradation of a putative transcriptional repressor of the hTERT promoter, NFX1-91, interfering with maintenance of telomere [1]. In summary, E6 is responsible for a wide range of pathological effects as inhibition of apoptosis, disruption of cell adhesion, telomerase activation, decreased genomic stability, p53 inactivation and reversal of epithelial differentiation [23].

1.1.2.2 E7 oncoprotein

The HPV E7 oncoprotein is composed of 98 amino acids and has three functional domains known as conserved regions (CR1, CR2 and CR3). CR1 is related with cellular transformation and pRb degradation but it is not responsible for pRb binding, while CR2 has a Leu-X-Cys-X-Glu motif and a casein kinase II phosphorylation site that functions as a pRb-binding site. CR3 domain is involved in pRb and other host cellular proteins association, in metal binding and may function as a dimerization domain [23, 26]. The pRb tumor suppressor is responsible by cell cycle regulation promoting the transition from G1 into S phase. In normal cells, pRb is hypophosphorylated in early G1 and becomes hiperphosphorylated towards S phase. In its hypophosphorylated form, pRb binds E2F transcription factor and represses transcription from promoters containing E2F sites. E2F-pRb complexes can be disrupted by E7 protein, promoting cell cycle progression through inactivation of pRb [25]. This leads to arrest the regulation of cell cycle driven by this factor and targets the related pocket protein family members, p107 and p130 which are responsible for regulating E2F-mediated transcription of S-phase genes, for ubiquitin-mediated proteolysis [1, 18]. Furthermore, E7 profoundly disrupts the pRB-signaling axis to favor replication of viral genome, by the ability to stimulate the S-phase genes cyclin E and cyclin A, overcoming cellular growth arrest signals mediated by cyclin-dependent kinase inhibitors, such as $p21^{CIP1/WAF-1}$ and $p27^{KIP-1}$ [26]. Interestingly, E7-expressing cells contain increased steady-state levels of p53. This occurs in response to DNA damage and p53 acts as repressor or activator of targets responsible for the inhibition of pRb phosphorylation. The presence of high-risk E6 protein acts to reduce the elevated levels of p53 induced by E7 [25, 27].

1.1.3 Preventive and therapeutic vaccination

Infection by HPV continues to cause significant morbidity and mortality worldwide, making prophylactic cervical cancer vaccines an important focus for cervical cancer prevention [28]. Preventive HPV vaccines aim to avoid HPV infection by inducing a neutralizing antibody

response, through humoral immune response [29]. However, despite of the great accessibility of these vaccines worldwide and their potential to decrease the incidence of disease in the future, prophylactic vaccines are not able to treat already infected patients neither patients with precancerous lesions or cervical cancer [28, 30]. The L1 encoded protein has been used to identify HPV capsid proteins. Therefore, a vaccine produced by recombinant viral L1 capsid protein, self-assemble to form virus-like particles (VLPs), will be able to induce high levels of neutralizing antibodies [20]. Although referred to as "virus-like", VLPs do not contain the viral genome or specific viral genes and are absolutely non-infectious and non-oncogenic [31]. Actually, HPV prophylactic vaccines are made with recombinant technology taking advantage from the production of abundant L1 proteins in a host with L1 gene encapsulated [32]. There are three available vaccines on the market to prevent infection with HPV. Cervarix, a bivalent vaccine, containing antigens for the high-risk HPVs 16 and 18, the quadravalent vaccine Gardasil, which contains antigens against four HPV types, including HPVs 16, 18, 6 and 11, and also Gardasil 9, against HPVs 16, 18, 6, 11, 31, 33, 45, 52 and 58, which can protect against around 85% of HPV related infections [10, 31]. Table 3 summarizes the main features of these available vaccines.

Table 3 - Features of currently available vaccines (Adapted from [10]).

	Bivalent	Quadrivalent	Nonavalent
Brand name	CERVARIX	GARDASIL	GARDASIL 9
Manufacturer	GlaxoSmithKline Biologicals, Rixensart, Belgium	MERCK & CO., INC., Whitehouse station, NJ, USA	MERCK & CO., INC., Whitehouse station, NJ, USA
Approval year by FDA	2009	2006	2014
HPV types	16, 18	16, 18, 6, 11	16, 18, 6, 11, 31, 33, 45, 52, 58
Target population	Females aged 9-25 years	Females aged 9-26 years Males aged 9-26 years	Females aged 9-26 years Males aged 9-15 years
Dose and schedule	Three doses, 0.5 ml/dose at 0, 1, and 6 months	Three doses, 0.5 ml/dose at 0, 2, and 6 months	Three doses, 0.5 ml/dose at 0, 2, and 6 months

As described above, the current available vaccines are not able to treat an already infected patient with HPV, so they should be given before sexual debut and exposure to HPV to get all protection benefits from the vaccine [32]. L1 and L2 are not good targets for therapeutic vaccines once they are not expressed in pre-cancerous cells, so effective therapeutic HPV vaccines are needed. The E6 and E7 proteins are strongly expressed in tumor cells and they are essential to maintain the induction and cellular transformation, arising as a promising target to therapeutic vaccines in order to prevent and treat this disease by cell-mediated immunity. This approach could have a significant impact by reducing mortality and morbidity caused by HPV [28, 29]. Among ongoing investigations for therapeutic approaches against HPV, DNA vaccines

are considered a potentially valuable form of antigen-specific immunotherapy and a promising approach due to their easy production, safety profile and stability [33]. Therefore, research in this area should be made, considering the advantages of DNA vaccines (analyzed below) and using E6 and E7 oncoproteins as antigenic targets. Given that, our research group is developing processes to produce and purify a DNA vaccine for the treatment of cervical cancer.

1.2 DNA-based therapy

On the last three decades, there was a great evolution of therapeutics obtained through recombinant DNA technology, owing to the growing interest by DNA therapy as a strategy to treat several human disorders, such as slowing the progression of tumors, fight viral infections and stop neurodegenerative diseases [34, 35]. The investigation about genetic information has greatly increased in 1944, when Avery and co-workers proved that DNA contains human genetic information [36]. Later, Watson and Crick discovers the double helix structure of DNA in 1953 [37], thus bringing together two important discoveries that led to improvements on understanding the mechanisms of some diseases and consequently develop gene therapy and DNA vaccines. The rapid expansion of knowledge in disease mechanisms combined with the facility to manipulate DNA has allowed a great development on DNA-based therapy. The low cost of DNA therapy may play an important role in the reduction of morbidity and mortality worldwide given their accessibility even for developing countries.

1.2.1 Gene therapy

Gene therapy consists on the delivery of exogenous DNA to target cells, in order to achieve therapeutic effect, by replacing a dysfunctional gene for a functional one that would allow the expression of the required proteins, controlling the disease progression [38]. By this way, gene therapy can solve some limitations of protein drugs, including high cost of manufacturing, low bioavailability, and repeated parenteral administration [39]. This approach can be classified according to the nature of targeted cells as germ line gene therapy or somatic gene therapy. However, the first approach raises some concerns related with genetic alterations of future generations, whereby only in some exceptional cases is allowed the use of gene therapy. On the other way, treated somatic cells do not affect nobody else, so the treatments on this type of cells are commonly tested. The success of gene therapy depends on the ability to overcome some obstacles to efficiently express target genes on the cell nucleus without being degraded. The key to this problem can be the use of vectors as gene delivery systems, that will be explained below [35, 40]. The main therapeutic indications for gene therapy among clinical trials are cancer, monogenic, infectious and cardiovascular diseases, wherein cancer diseases represents 64% of all 2240 gene therapy clinical trials documented in 2015, as shown in figure 4 [41].

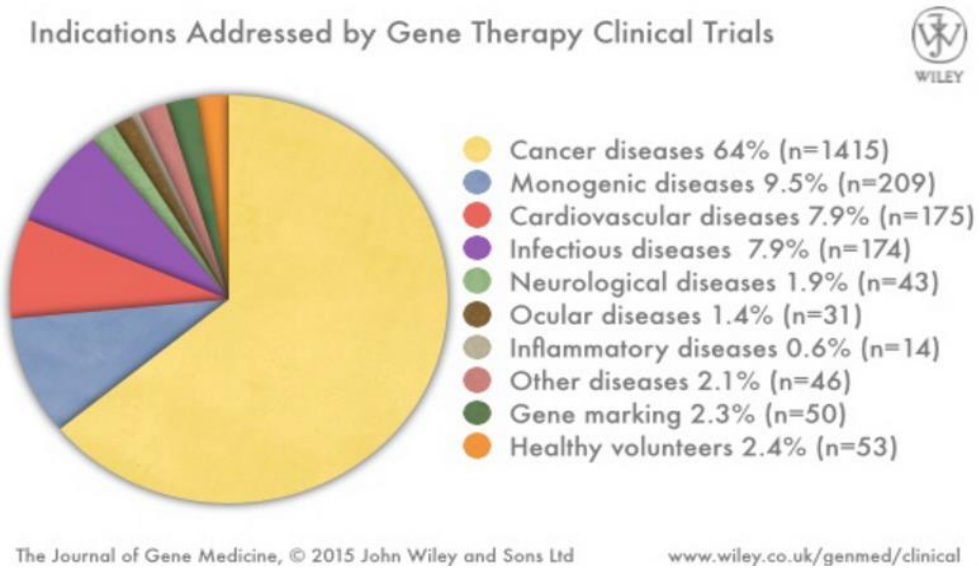

Figure 4 – Gene therapy clinical trials until 2015 (adapted from [41]).

1.2.2 DNA vaccines

Traditional or prophylactic vaccines have the purpose of protecting people against infections, but when they were firstly created, the knowledge about immune system and immunological mechanisms was poor. Thus, the vaccines available were not able to prevent severe infections [42]. With the increase of knowledge about immunological mechanisms, more efficient vaccines were obtained to prevent serious infections such as HPV, as it is the case of the three available vaccines mentioned above. However, from a moment at which a patient is infected, cellular and long-lived memory immune responses are needed.

Due to its usefulness, DNA-based therapy can be applied to develop a versatile vaccine capable of triggering humoral and cellular immune responses, performing simultaneously preventive and therapeutic actions and thus eliminating the major problem from prophylactic vaccines. To reach these goals and effects, therapeutic DNA vaccines have been developed. DNA vaccine is defined by Food and Drug Administration (FDA) as purified DNA vector preparations containing one or more DNA sequences capable of inducing or promoting an immune response against a specific pathogen [43]. DNA vaccines comprise genetically engineered DNA, containing information that will be expressed by the cellular machinery of the patient, for the production of an antigenic protein that will induce a strong and long-lasting immune response and allowing the treatment of the disease [44, 45]. After injection on muscle, DNA is taken up by host cells, such as muscle cells, monocytes or dendritic cells, to translate genetic information and produce the antigen for presentation to CD8 T cells through their major histocompatibility complex (MHC) class I pathway. On the other way, antigen-presenting cells (APCs) can capture proteins secreted by transfected cells to express antigenic peptides to CD4 T cells through MHC class II pathway. Furthermore, APCs loaded with antigens migrate to lymph nodes to activate humoral

immunity through B cells as well as CD4 and CD8 T cells [46, 47]. This complete mechanism leads to a strong and solid immune response and it is presented in figure 5.

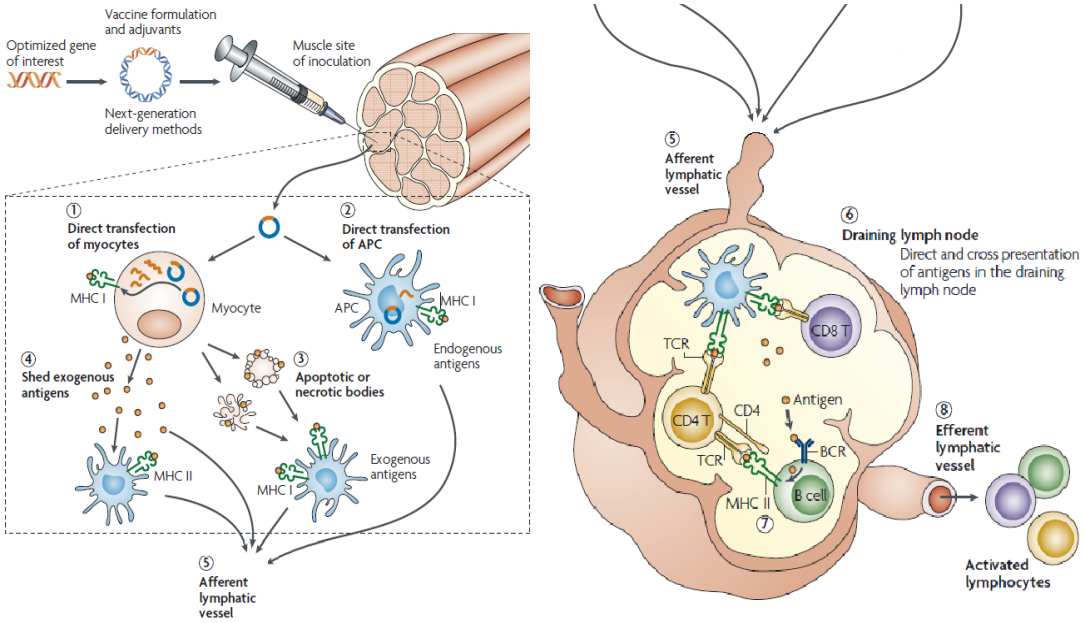

Figure 5 - Immunological mechanisms of DNA vaccines. Plasmid enters the nucleus of transfected cells and initiate gene transcription, which is followed by formation of antigens as proteins or as peptide strings. The host-synthesized antigens can become subject of immune surveillance in the context of MHC I or MHC II of the vaccinated individual. (adapted from [48]).

Besides this capacity to efficiently stimulate the immune system that would be able to initiate a therapeutic response when someone is infected, DNA vaccines have many other features that make it a major research target. The main advantages of DNA vaccines, beyond those already described, are summarized in table 4.

Table 4 - The main advantages of DNA vaccines (adapted from [45]).

Advantages of DNA vaccines
Inexpensive
Long-term persistence of immunogenicity
Subunit vaccination with no risk for infection
Ease to develop and produce
Immune response focused only on antigen of interest
Stability of vaccine for storage and shipping
Safety and facility to handle
Heat stability

1.2.3 Gene delivery systems

Recombinant DNA, as described above, is a promising technology to be exploited in the development of therapeutic approaches for certain diseases. Nevertheless, the transfer efficacy of exogenous DNA to cells, organs or tissues, without biodegradation, is crucial to achieve high gene expression levels. Currently, the most used and more efficient systems to transfer recombinant DNA to cells are viral vectors, but several limitations associated with this method promoted the search for safer procedures, namely by using the non-viral vectors [39]. Other biological approach based on bacterial gene transfer vectors is also being studied [49]. In geral, an ideal gene delivery vector should be easy to produce at industrial scale, with low cost associated, be able to express its genetic information sustainably, being immunologically inert. In addition, it should have the capacity to encode large size sequences, only deliver the genetic material onto target cells, either be dividing or non-dividing cells. Lastly, it should remain in episomal position or integrate into a specific region of the genome [50]. The viral, non-viral and bacterial vectors will be better described below.

1.2.3.1 Viral vectors

Viruses are the main gene delivery system used in DNA therapy trials due to its ability to infect the host cells, being very efficient for *in vivo* transfection and consequently leading to increased gene expression [41, 51]. Viruses are evolved organisms, able to infect cells and explore the cellular machinery to achieve their replication. When viruses are used as vectors, the genome is modified to avoid expression of viral genes and eliminate their pathogenicity, but preserving their high efficiency in gene transfer, making it more safe [52, 53]. The main type of viruses used for gene therapy are adenovirus, adeno-associated virus, herpes simplex virus, lentivirus and retrovirus. The main features, the advantages and disadvantages of each one are described in table 5.

Table 5 - The main advantages and disadvantages of viruses used for DNA therapy (adapted from [54]).

Vector	Advantage	Disadvantage
Adenovirus	Very high titers (10^{12} pfu/mL) High transduction efficiency *ex vivo* and *in vivo* Transduces many cell types Transduces proliferating and non-proliferating cells Easy production at high titers	Remains episomal Transient expression Requires packaging cell line Immune-related toxicity with repeated administration Potential replication competence No targeting Limited insert size: 4–5 kb
Retrovirus	Integration into cellular genome Broad cell tropism Prolonged stable expression Requires cell division for transduction Relatively high titers (106–107 pfu/mL) Larger insert size: 9–12 kb	Inefficient transduction Insertional mutagenesis Requires cell division for transfection Requires packaging cell line No targeting Potential replication competence
Adeno-associated virus	Integration on human chromosome 19 (wild-type only) to establish latent infection No targeting Prolonged expression Transduction does not require cell division Small genome, no viral genes	Not well characterized Requires packaging cell line Potential insertional mutagenesis High titers (10^{10} pfu/mL) but production difficult Limited insert size: 5 kb
Herpes simplex virus	Large insert size: 40–50 kb Neuronal tropism Latency expression Efficient transduction *in vivo* Replicative vectors available	Cytotoxic No targeting Requires packaging cell line Transient expression, does not integrate into genome Moderate titers (104–108 pfu/mL)
Lentivirus	Transduces proliferating and nonproliferating cells Transduces hematopoietic stem cells Prolonged expression Relatively high titers (106–107 pfu/mL)	Safety concerns: from human immunodeficiency virus origin Difficult to manufacture and store Limited insert size: 8 kb Clinical experience limited

Besides viral vectors are very efficient, there are several disadvantages such as acute immune response that can be fatal, mutagenesis, carcinogenesis, toxicity, and anti-vector immunity, which limits later and repeated administrations. On the other way, viral vectors are very difficult to produce in large quantities, being too expensive, and some safety questions still persist for human use [40, 51, 53]. Thus, the use of non-viral vectors is emerging as a promising approach among the scientific community.

1.2.3.2 Bacterial vectors

The bacterial delivery systems use bacteria to transfer DNA into host cells, a process called bactofection [55]. This technique was first used in 1980, when Schaffner cloned genetic information from bacteria to mammalian cells [56]. Bactofection consists on the whole entry of bacteria into target cells, carrying the DNA. This entry process occurs through phagocytosis

(by macrophages or dendritic cells), or through pinocytosis or zipper-like mechanism (by non-phagocytic cells). The process continues with endosomal escape and further DNA expression. At the same time, different mechanisms start, through pathogen-associated molecular patterns present in the bacteria that lead to maturation and migration of dendritic cells to secondary lymph nodes, amplifying the immune response against the antigen. The bacteria transfected should not be pathogenic and this is a promising approach to express DNA-encoded proteins into different cell types as phagocytic and non-phagocytic cells [49, 55, 57]. Nevertheless, there are some problematic issues related with this approach as potential toxicity, rapid clearance by immune system response and even autoimmune reactions, so this vector needs further improvements and more studies to be safely applicable [55].

1.2.3.3 Non-viral vectors

As noted above, viral vectors have several limitations to their use, although their high transfection rate. On the other hand, non-viral vectors have low transfection efficiency [58] but this disadvantage is overcome for all the advantages that this system presents. With biocompatible chemical agents, non-viral vectors are better in terms of safety, availability, stability, relative ease of large-scale production and especially lack of intrinsic immunogenicity and no limitation in DNA size, making it a very studied approach and more effective than other gene delivery systems [51, 59]. Over the years, plasmid DNA (pDNA) has gained interest as non-biological vector to the direct injection of naked DNA into the muscular tissues. However, naked DNA has poor transfection rate and gene expression, once it is quickly degraded *in vivo* by phagocytic cells or extracellular nucleases. Moreover, the hydrophilic poly anionic nature of the DNA macromolecule and its large size avoid it to penetrate passively through the cell membrane [60, 61]. Thereby, naked DNA injection is a simple and safe method but to overcome the low transfection capacity, DNA is combined with physical or chemical methods to carry the therapeutic gene into the targeted cells, protecting it from degradation by nucleases and making sure that it is transcribed inside the cell [60].

1.2.3.3.1 Physical methods

The naked DNA administration by needle injection is the simplest and safest physical approach to gene delivery. The first case of successful expression *in vivo* of naked DNA in myofibers was reported in 1990 [62], and even with low transfection efficiency, this event led to the development of therapeutic DNA vaccines. The physical damage caused by needle injection is responsible for DNA uptake that occurs substantially in this area [63]. Despite these features and due to poor efficiency on DNA spontaneous uptake, as described above, other approaches have been investigated as alternative to needle injection.

Electroporation is a physical method capable of temporarily disrupt the integrity of cell membranes through an electric field, allowing therapeutic drugs and macromolecules to enter in the cell by pores opened in the membrane [64]. This technique is safe and efficient compared

to other non-viral methods [63], however, as all of them, they have their drawbacks. Actually, this strategy has limited effective range of space between the electrodes which makes difficult the transfection in a large tissue area, inability to achieve internal and deep organs and there is the possibility of tissue damage due to high voltage applied [65].

Another physical system is the gene gun or "biolistic" gene transfer that uses high-voltage electronic discharge or inert gas such as helium to bombarding target cells with DNA-coated gold particles [65]. The advantages of this approach are long-lasting gene expression, the absence of toxicity, capacity to encapsulate DNA molecules of various sizes, possibility to repeat the process, facility to produce and ability to reach organs like heart, brain, liver and muscle. However, more improvements are needed to face front to low penetration of metal particles, which causes poor efficiency when transfected, being necessary surgery for deep tissues [40, 66].

1.2.3.3.2 Chemical vectors

Chemical vectors are a frequently studied method for non-viral gene delivery, mainly focusing two approaches involving cationic lipids and cationic polymers, where the DNA is taken into particles to be protected and more easily target the host cells [63].

Cationic lipids have low toxicity, can incorporate hydrophilic and hydrophobic drugs, do not activate immune system and are able to deliver the encapsulated compounds in the target site. Moreover, the production are inexpensive [63, 65]. Cationic lipids comprise a hydrophobic tail and hydrophilic head positively charged connected with a linker structure. The interaction between the positively charged head group and negatively charged phosphate groups of nucleic acids results in a uniquely compacted structure named lipoplex. The production of lipoplexes occurs by mixing liposomes and pDNA in an adequate buffer. The external positive charged lipids protect pDNA against nucleases and favor the interaction with the target cell membrane. To achieve an efficient transfection there are some characteristics that affect this process: structure, size, charge ratio between DNA and liposome, presence of the helper liquid, chemical structure of the cationic lipid and cell type [63, 65]. With all these requirements, the transfection efficiency is still poor and need more improvements, mainly due to the rapid plasma clearance, toxicity and short duration of gene expression, which are important drawbacks of this approach [63].

Cationic polymers are also used in DNA therapy due to its capacity to compact large genes in nanostructures, that together with masking the negative DNA charges, form polyplexes and can improve transfection efficiency, being typically more stable than lipoplexes [67]. There are several cationic polymers under investigation with different advantages and disadvantages, but the two better described and studied are poly-L-lysine (PLL) and polyethylenimine (PEI). PLL is recommended for *in vivo* applications because it is biodegradable. However, this polymer

requires modifications to overcome poor transfection ability and rapid plasma clearance. A successful transfection rate requires the application of a tropic agent to avoid the lysosomal degradation of polyplexes. The linking of polyethylene glycol and palmitoyl groups reduces the toxicity of this delivery system [38, 63]. PEI has a proton sponge effect given by non-protonated amine groups, important for gene delivery efficiency. Different transfection efficiencies are mainly related with different molecular weights of PEI, being that low molecular weight polyplexes present high transfection efficiency and low toxicity. Besides that, the efficiency and toxicity can also be influenced by the degree of branching, ionic strength of the solution, zeta potential and particle size. The main drawback of this polymer is its non-biodegradable nature, which leads to increased toxicity in cellular environment [38, 63, 65]. Although these are the two most commonly used polymers, others like chitosan have shown significant levels of gene transfer activity [68].

To conclude, all systems have their disadvantages, so the main challenges to improve currently available delivery systems still are the enhancement of intracellular long-time expression, the improvement of extracellular targeting and the reduction of toxicity and side effects on human body [59].

1.3 Plasmid DNA

Over the years, pDNA has been gaining interest among biopharmaceutical products namely because it is one of the most studied vectors for DNA vaccination. As described above, several advantages make this product the preferred vector for DNA-based therapy, as DNA vaccines, whereas this vector does not trigger immune responses against itself, it is easy to manipulate and safe to produce on a large-scale [69]. The importance of pDNA among non-viral vectors is shown by almost 25% of clinical trials based on DNA, where naked/plasmid DNA is responsible for 17.5% and lipofection, which also uses pDNA, for 5%, in a total of 502 clinical trials [41].

Plasmids are normally produced on *Escherichia coli* (*E. coli*) by extrachromosomal self-replication. These double-stranded DNA molecules are covalently closed, negatively charged when pH > 4 and have a size ranging from 1 to 100 kilo base pairs [70, 71]. The capacity to encode high amounts of DNA is a great advantage compared to viral vectors. Plasmids can present various conformations, being the supercoiled (sc) isoform the pDNA conformation produced by *E. coli* and considered the more effective isoform in transferring encoded genes due to its compact and stable structure. But other isoforms can appear in result of temperature or pH variations and enzymatic or chemical cleavage, such as open circular (oc) (one strand cleavage), linear (double strand cleavage), denaturated and oligomeric pDNA [70, 72, 73]. To be useful in DNA vaccination, it is necessary large amounts of pDNA with high purity degree to achieve the desired effects. So, to reach this aim, optimized upstream and downstream processes have to be considered.

1.3.1 Upstream process

To have a viable final therapeutic product, it is necessary a process that allows the production of large amounts of pDNA. The vector design is essential for the successful production and further target gene expression. Thus, in order to construct a small pDNA, all non-essential sequences should be removed and translational pause sites should be added, contributing also to better gene expression. The plasmid molecule typically contains a replication origin (responsible for efficient plasmid replication on bacterial host), a selection marker (for example an antibiotic resistance gene to guarantee stable inheritance of plasmids during bacterial growth), a promoter (to drive high-level expression of the gene of interest in eukaryotic cells), a therapeutic gene (coding for target product) and a transcription terminator poly A signal (to stop target gene transcription and to protect translated RNA from degradation) [74, 75]. The map of a DNA vaccine plasmid construction is represented on figure 6.

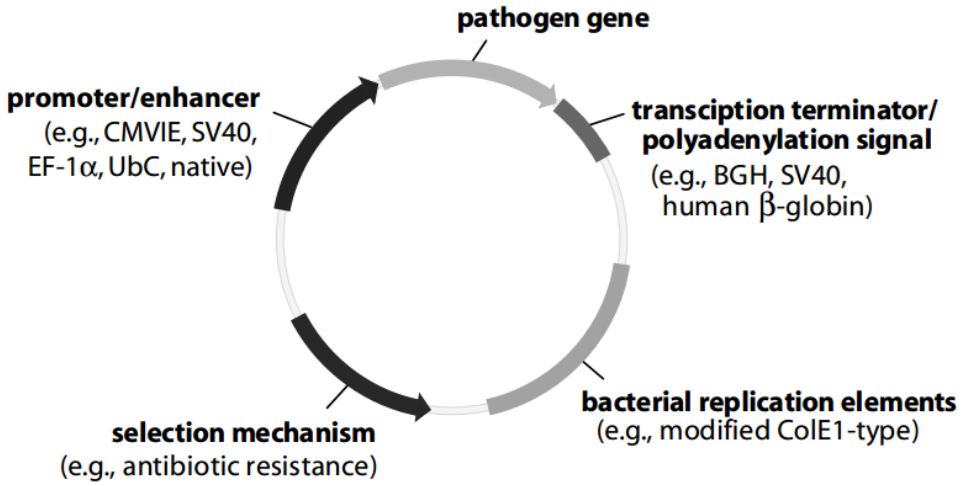

Figure 6 - Map of a DNA vaccine plasmid construction (adapted from [75]).

The most used bacterium to produce pDNA is *E. coli*, once this host has rapid growth, it has been widely studied and successfully modified to replicate high copy numbers of plasmid, increasing the yield from the production process and being able to be applied at industrial scale, simplifying the downstream process and improving the final product [75]. After optimization of the growth conditions for the selected host, fermentation process should achieve the desired optical density in order to have high plasmid quantities, typically 1g of pDNA per kg of wet weight biomass after centrifugation [69]. Thus, the conditions are met to advance to downstream process and purify pDNA to achieve a biopharmaceutical product.

1.3.2 Downstream process

Downstream process starts with a crucial step and the one at which most problems occur. It is the process of cell lysis to obtain a clarified lysate in order to be further purified. After recovered from the medium, the cells must be disrupted to release the pDNA. Among several mechanical and chemical methods for cell lysis, such as homogenization, freeze/thaw or detergent-based extraction, the most used method is alkaline lysis through sodium hydroxide (NaOH) and sodium dodecyl sulfate (SDS), which together lead to the disruption of cell membrane and consequent release of intracellular components such as pDNA, genomic DNA (gDNA), RNA, proteins and endotoxins [75]. High pH levels cause the denaturation of gDNA, cell wall material and most of the cellular proteins. To avoid the hydrogen bond disruption from pDNA, pH should be lower than 12.5, preventing the separation of the complementary strands in order to allow the complete renaturation of pDNA in the following neutralization step, with potassium acetate [70]. In the neutralization step occurs a decrease of pH that leads to the precipitation of cell debris, proteins, gDNA and some RNA [69]. Although, during the process of alkaline lysis, most of proteins and gDNA are precipitated, the final clarified lysate still has several contaminants. Thus, to prepare pDNA sample for further purification process, the clarification method has still another step based on the use of a caotropic salt to precipitate

high molecular weight RNA and proteins [76]. Finally, the clarified sample is ready for the purification process through chromatography. The scheme of entire pDNA process development is presented in figure 7, including upstream and downstream processes.

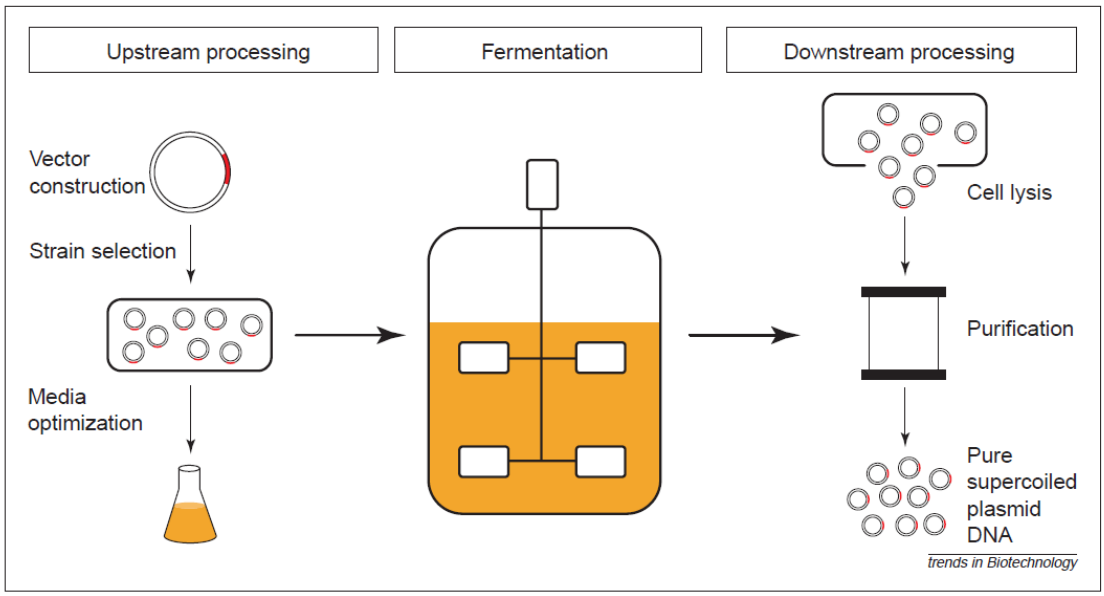

Figure 7 - The main stages of pDNA process development (adapted from [76]).

1.3.2.1 Plasmid DNA purification

After the recovery of the clarified lysate, the sample still has several impurities that should be removed, in order to achieve the required purity of the biopharmaceutical product based on the quality criteria imposed by regulatory agencies, such as Food and Drug Administration (FDA) or European Medicines Evaluation Agency (EMEA) [69]. Thus, the required specifications of pDNA to pharmaceutical application are colorless and clear solution in terms of appearance, more than 97% of sc plasmid homogeneity, non-detectable proteins by BCA-assay (< 3 µg/mg pDNA), non-detectable RNA on 0.8% agarose gel electrophoresis, gDNA < 2 µg/mg plasmid (via real time PCR) and endotoxins < 10 EU/mg plasmid (via LAL assay) [77]. Given that, a process able to eliminate contaminants derived from *E. coli* is mandatory. Liquid chromatography is the current essential technology for separation of pDNA from other molecules, in order to improve the purification process. There are various modalities of chromatography based on different size, charge, hydrophobicity and affinity of molecules present on a mixture. This technique is also important as analytical tool to control pDNA quality during all downstream process and in the final formulation. The contaminants present in the *E. coli* extract have some similar characteristics to the pDNA, such as the negative charge (RNA, gDNA and endotoxins), size (gDNA and endotoxins) and hydrophobicity (endotoxins). The removal of endotoxins as contaminants have additional importance, once it can produce symptoms of toxic shock syndrome *in vivo* and it can reduce transfection efficiency in *in vitro* studies [70, 77]. The main chromatographic modalities applied in pDNA purification are size exclusion, anion exchange, hydrophobic interaction, and affinity.

Size exclusion chromatography exploits the difference of biomolecules size for their separation. The large biomolecules, as gDNA and pDNA, are the first to pass through the support, eluting close to the exclusion limit of the matrix. On the other way, smaller biomolecules such as RNA, endotoxins and proteins have greater ability to enter in pores of matrix beads, being retarded. This approach is able to separate gDNA and pDNA from smaller molecules, but has some drawbacks as limited capacity and selectivity for pDNA isoforms [78, 79].

One of the most popular chromatographic techniques is ion exchange chromatography, due to the rapid separation of biomolecules that occurs due to their opposite charge compared to the stationary phase. In the case of pDNA, the negatively charged phosphate groups allow the interaction with positively charged functional groups of the matrix. The support is usually modified with tertiary or quaternary amines and the elution of retained biomolecules is performed by competition through the increase of salt concentration. However, some biomolecules share similar binding affinities with pDNA, because of similar chemical composition and structure, such as gDNA and RNA, which causes a drawback for this technique due to the co-elution of these molecules with pDNA [70, 78].

On the other way, hydrophobic interaction chromatography explores the hydrophobicity of single-strand nucleic acid impurities, pDNA and endotoxins, promoting the separation on basis of hydrophobic interactions between non-polar regions on biomolecules and immobilized hydrophobic ligands. The binding to the column is achieved with high salt concentrations on mobile phase and the elution by decreasing the salt concentration, which weakens the hydrophobic interactions. Nevertheless, this technique is limited by the use of high salt concentrations that demand high costs and has environmental impact [70, 78].

Affinity chromatography has been explored over the past years as a promising technique able to overtake the drawbacks of other chromatographic principles such as the referred above. This technique is based on specific interactions that occur in natural biological processes to purify biomolecules, taking advantage of their biological function and chemical structure [79, 80]. Once the specific interactions between the ligand and biomolecules can be provided by electrostatic or hydrophobic interactions, van der Walls forces and hydrogen bonding, the elution step can be performed through different strategies such as changing the pH, ionic strength or polarity, and using a competition agent. By this way, the selection of the ligand immobilized in the column plays an important role for the success of pDNA purification [78]. Nevertheless, the biological origin of the ligands tends to be considered a disadvantage of the method, as well as the associated low binding capacity, prompting to the development of synthetic ligands in order to achieve the selectivity of natural ligands combined with the high capacity, durability and cost effectiveness of synthetic systems [79]. Affinity chromatography comprises several approaches that can be used in the pDNA purification, all of them with some advantages and disadvantages. The main features of immobilized metal-ion affinity chromatography, triple-helix affinity chromatography, polymyxin B affinity chromatography,

protein-DNA affinity chromatography and amino acid-DNA affinity chromatography are described on table 6.

Table 6 - Affinity chromatography methods for pDNA purification (adapted from [80]).

Affinity type	Principle	Specific binding	Advantages	Limitations
Immobilized metal-ion	Chelating ligands charged with divalent metal ions specifically interact with aromatic nitrogen atoms through π-π orbital overlap	Single-stranded nucleic acids (particularly purine bases)	Efficient resolution of RNA from gDNA and pDNA; High endotoxin removal; Separation of denatured pDNA	pDNA in the flowthrough; Incomplete RNA capture in complex mixtures; Co-elution of all DNA isoforms
Triple-helix	Specific sequences present on pDNA are recognized by an immobilized oligonucleotide, forming a triple-helix	Double-stranded DNA	Discrimination of different plasmids based on their sequence; sc pDNA isolation in one chromatographic step; Reduction of RNA, gDNA and endotoxin contamination levels; Possibility for scale-up	Loss of pDNA during wash step; Low yields; Slow kinetics of triple-helix formation; Long chromatographic run times
Polymyxin B	Immobilized polymyxin B specifically recognizes the lipid structure of endotoxins	Endotoxins	Elimination of endotoxin contamination from pDNA preparations;	Non-specific interaction of ligands with pDNA; Poor yields; Toxicity of polymyxin B
Protein-DNA	A protein or protein complex immobilized on the matrix specifically recognizes a DNA motif	pDNA	Discrimination of different plasmids based on their sequence; pDNA isolation from clarified lysates; Elimination of proteins and RNA from preparation	Relatively low yields; Contamination with gDNA
Amino acid-DNA	Multiple interactions occur between immobilized amino acids and nucleic acids	sc pDNA	sc pDNA purification in a single chromatographic step; Efficient elimination of RNA, gDNA, proteins and endotoxins	Elution with high salt concentration (in some cases)

Amino-acid affinity chromatography has been gaining interest and it is increasingly used for pDNA purification, mainly due to the natural occurrence of protein-DNA complexes in biological systems and consequent safety use for biopharmaceutical applications [79].

Our research group has shown the versatility of amino-acid ligands such as arginine, histidine, lysine, methionine and tyrosine for the isolation of sc pDNA isoform under the required purity degree. Sousa and co-workers describe the use of arginine-agarose matrix by affinity

chromatography as a strategy for pVAX1-*LacZ* purification with satisfactory results and using mild elution conditions, resulting on the recovery of 79% of sc pDNA and a purity degree higher than 99% [81]. On the other hand, the use of histidine-agarose matrix for pVAX1-*LacZ* purification showed efficiency on the removal of *E. coli* contaminants, with a sc pDNA homogeneity of 100%. However, the recovery was lower compared to the arginine support, with an overall yield of only 40% [82]. Lysine-agarose matrix also showed great efficiency on removing contaminants from *E. coli* lysate, with 100% purity of sc isoform of pVAX-*LacZ*, but similar to histidine support, the recovery yield was 46% [83]. Despite differences on recovery yields, all of these amino-acid ligands showed specific interactions with different biomolecules, resulting in a final sc pDNA sample with high purification degree. However, all these agarose supports show some limitations such as low binding capacity for large biomolecules, working only at low flow rates and the possibility of sc pDNA degradation owing to the extensive retention time [84]. Therefore, it is necessity to explore alternative chromatographic supports for pDNA purification at industrial level.

1.3.3 Monolithic technology

To take advantage of natural and safe composition of amino acids as specific ligands immobilized in the stationary phase, and its selective interaction with target biomolecules present in the mobile phase, amino acid affinity chromatography has been intensely explored. Previous studies with lysine [83], histidine [82] and arginine [81] amino-acids as affinity ligands in agarose matrices showed success at sc pDNA purification. However, owing to limitations already described and related with conventional supports, a new approach based on monolithic supports have been gaining influence on chromatography [85, 86]. Subject of study over the last few years, monoliths are considered the fourth generation of chromatographic supports, offering characteristics such as solid, macroporous, chemical and physically stable stationary phases. Besides that, the low nonspecific adsorption, good flow properties, high binding and mass transfer capacity offer fast separation of large molecules. The main advantage of monoliths is the arrangement between convective flow and high interconnectivity, allowing fast separations with extremely short beds [79, 85, 87]. Monoliths are polymerized as a single homogeneous piece that can be set in several dimensions, characterized by a highly interconnected network of channels with large diameters, resembles several stacked sheets. Problems related with scale-up and scale down variations in packing quality and the need to repack a column due to the inadvertent introduction of air bubbles are eliminated with the monolithic structure. This continuous bed can be prepared as a disk and cylindrical or conical tubes. For the ideal purification of larger biomolecules such as pDNA, monoliths should be short and support higher flow rates, in order to reduce back pressure, unspecific binding and product degradation, without sacrificing resolution [84, 87]. All the described features, together with the advantages in using amino acid or derivatives as ligands, makes monoliths the material of choice for amino-acid affinity chromatography in order to achieve efficient pDNA purification

and overcoming the limitations of conventional matrices, as already established in several studies with the histamine-, arginine- and histidine-based monoliths [88-90]

Chapter 2 – Global aims

In this work, the main objective is to produce and purify a DNA vaccine for prevention and treatment of cervical cancer associated to the HPV infection. To accomplish this, the final sample of sc pDNA should present the purity degree recommended by regulatory agencies. Thus, after the suitable production of pDNA in the recombinant *E. coli* host, a chromatographic process will be optimized in order to separate host components and non-functional pDNA isoforms from sc pDNA. Lysine affinity chromatography was already explored by Sousa and co-workers [83], to purify sc pDNA with high specificity by using conventional chromatographic supports. However, the overall yield of the process was low, probably due to the limitations of conventional matrices. Therefore, in the present work it will be evaluated the amino-acid affinity chromatography through lysine amino acid and cadaverine ligand (derived from lysine amino acid), immobilized in monolithic columns.

Firstly, several assays will be performed at cadaverine monolithic column in order to understand the retention behavior of pDNA isoforms in this support. After this study, the purification conditions will be optimized and adjusted in order to purify the sc pDNA from the *E. coli* lysate, by manipulating salt concentrations, buffer nature and pH values. Finally, the best elution conditions will be tested on lysine monolithic column to compare the performance of both supports and understand the main differences between them.

Overall, the main goal is to improve the recovery of sc pDNA comparing to the results previously achieved with conventional chromatographic supports, maintaining the high purity of sc pDNA.

Chapter 3 – Materials and methods

3.1 Materials

All chromatographic experiments were carried out in two analytical chromatographic columns, with a bed volume of 0.1 mL, and modified with lysine and cadaverine ligands, respectively, kindly prepared and provided by BIA Separations (Ajdovščina, Slovenia). The Qiagen Plasmid Purification Maxi Kit was from Qiagen (Hilden, Germany). Sodium chloride, ethylene-diamine tetraacetic acid (EDTA) and sodium phosphate dibasic were purchased from Panreac (Barcelona, Spain), sodium dihydrogen phosphate monohydrate was purchased from Sigma Aldrich (St.Lois, MO, USA), HEPES was purchased from Fisher (Porto Salvo, Portugal), and tris-(hydroxymethyl)aminomethane (Tris base) was purchased from Merck (Darmstadt, Germany). In chromatographic experiments, all solutions were freshly prepared using deionized water ultra-pure grade, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and analytical grade reagents. The filtration of elution buffers was performed with 0.20 µm pore size membrane (Schleicher Schuell, Dassel, Germany), followed by ultrasonic degassing. The iQ SYBR Green Supermix from BioRad (Hercules, CA, USA) was used for gDNA quantification.

3.1.1 Plasmid DNA

Addgene plasmid 8641, the 8.702 kbp HPV-16 E6/E7 DNA vector [91], was obtained on Addgene (Cambrige, MA, USA). This vector has the human beta-actin mammalian expression promoter and also an ampicillin resistance gene (figure 8).

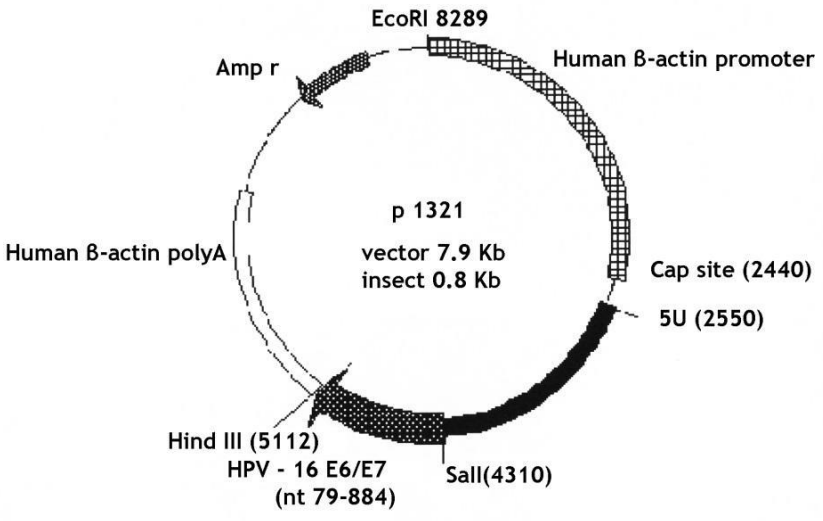

Figure 8 - Schematic representation of HPV-16 E6/E7 pDNA (plasmid 8641, adapted from [92]).

E6 and E7 genes were mutated in several points to prevent the oncogenic potential from these proteins, avoiding the recognition by the tumor suppressor proteins, p53 and pRb. The mutated genes were synthesized by NZYtech (Lisbon, Portugal).

3.2 Methods

3.2.1 Bacterial growth conditions

HPV-16 E6/E7^{MUT} pDNA amplification was obtained by *E. coli* DH5*α* fermentation. Bacterial growth was performed at 37 ºC and 250 rpm shaking. The medium used was Terrific Broth (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4) supplemented with 100 µg ampicillin/mL. Growth was suspended at the late log phase (OD_{600}~7), cells were recovered by centrifugation at 3900 g for 10 min at 4 ºC, and pellets were stored at -20 ºC.

3.2.2 Alkaline lysis with Qiagen Kit

Plasmid DNA was obtained by alkaline lysis using the Qiagen plasmid maxi kit according to the manufacturer's instructions. After cell lysis, the purification starts with cellular debris elimination followed by pDNA binding to Qiagen anion-exchange columns under appropriate low-salt and pH conditions. RNA, proteins and low-molecular-weight impurities were removed by a medium-salt wash while the pDNA was eluted in a high-salt buffer and then concentrated by isopropanol precipitation. The final pDNA (oc + sc) sample was loaded in the new monolithic supports to study the binding and elution profiles.

3.2.3 Modified alkaline lysis

After the bacterial growth, in order to obtain a lysate sample, it is necessary to perform cell lysis using the modified alkaline method [93], as described by Diogo *et al.*, 2000 [94]. Bacterial pellets, resulting from the centrifugation of 250 mL of growth medium, were ressuspended in 20 mL of solution I (50 mM glucose, 25 mM tris-hydroxymethil aminomethane (Tris) and 10 mM ethylene-diamine tetraacetic acid (EDTA) at pH 8.0). Alkaline lysis was performed by adding 20 mL of solution II (200 mM NaOH and 1% (w/v) SDS) and 5 minutes of incubation at room temperature. The solution II was neutralized by adding 20 mL of solution III (3 M potassium acetate at pH 5.0) and followed by 20 minutes of incubation in ice. The elimination of cellular debris, gDNA and proteins was performed through double centrifugation at 20 000 *g* (30 minutes, 4 ºC) with an Allegra™ 25R Centrifuge (Beckman Coulter, Miami, FL, USA). The concentration and reduction of the impurity content was performed according to a previously published method [94], where nucleic acids present in supernatant were precipitated by adding 0.7 volumes of isopropanol. After 30 minutes incubation in ice, the precipitated was recovered by centrifugation at 16 000 *g* (30 minutes, 4 ºC). Further, the pellet was dissolved in 4 mL of 10 mM Tris-HCl and 10 mM EDTA (Tris-EDTA) buffer, pH 8.0. Proteins and high-molecular-weight

RNA were precipitated by adding ammonium sulfate at pDNA solution up to a final concentration of 2.5 M. After 15 minutes of incubation in ice, impurities were removed by centrifugation at 16 000 *g* (20 minutes, 4 ºC). Lastly, the supernatant was desalted by passing through PD-10 desalting columns according to the manufacturer's instructions, using 10 mM Tris-EDTA solution as the elution buffer (pH 8.0).

3.2.4 Affinity chromatography

The equipment used to perform the chromatographic experiments was an AKTA *püre* (GE Healthcare, Biosciences Uppsala, Sweden), consisting of a compact separation unit and a personal computer with UNICORN 6.3 software. Initially, cadaverine monolith was tested in order to understand the pDNA retention/elution behavior in the column and to optimize the elution strategy to purify the sc pDNA isoform from the lysate sample. The evaluated conditions were the manipulation of NaCl concentration, the buffer composition (Tris-EDTA, Sodium phosphate-EDTA and HEPES) and pH (6.0 – 8.0). The best conditions were obtained when the column was equilibrated with 1.2 M NaCl in 10 mM Tris-EDTA buffer, pH 6.0, at a flow rate of 1 mL/min. Lysate sample resulting from modified alkaline lysis was injected onto the column using a 100 µL loop in the same flow conditions. Unbound species were eluted in the flowthrough and the ionic strength was increased to 1.5 M NaCl in 10 mM Tris-EDTA buffer, pH 6.0 by a stepwise gradient, being eluted the bound species. Thereafter, lysine monolith was tested by using the best elution conditions previously studied to compare the behavior and the performance of both columns. All experiments were carried out at room temperature and the absorbance was continuously monitored at 260 nm. Recovered fractions were concentrated and desalted with Vivaspin® 6 Centrifugal Concentrators (Vivaproducts, Littleton, MA, USA) at 1300 *g* in the SIGMA 3-18K centrifuge (SIGMA Laborzentrifugen GmbH, Germany). After chromatographic experiments, the columns and AKTA *püre* system were cleaned with Milli-Q water and 0.5 M of NaOH.

3.2.5 Agarose gel electrophoresis

Pooled fractions from each chromatographic experiment were analyzed by horizontal electrophoresis using 15-cm-long 0.8% agarose gel (Hoefer, Holliston, MA, USA) stained with 0.012 µL/mL greensafe premium (NZYTech, Lda. – Genes and Enzymes, Lisbon, Portugal). Electrophoresis was carried out 120 V, for 40 minutes in TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). The gel was visualized under UV light in a FireReader (Uvitec Cambridge, Cambridge, UK).

3.2.7 Supercoiled plasmid DNA quantification

The evaluation of RNA, oc pDNA and sc pDNA molecules present in the clarified lysate and in sc pDNA purified fractions was accomplished through the application of a modified quantification method by using the CIMac™ pDNA analytical column [88]. This process was performed using an

AKTA purifier system (GE Healthcare Biosciences, Upssala, Sweden). As it is presented in figure 9, a calibration curve was performed with pDNA standards of 1 to 50 µg/mL. The pDNA standards were prepared through dilution of the highest concentration of pure pDNA with 200 mM Tris-HCl (pH 8.0) and the concentration was confirmed by Ultrospec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech, Cambridge, England). CIMac^{TM} pDNA analytical column was equilibrated with 600 mM NaCl in 200 mM Tris-HCl (pH 8.0). After injection of 20 µL of sample at 1 mL/min, a linear gradient from 600 mM to 700 mM NaCl in 200 mM Tris-HCl (pH 8.0) was applied for 10 minutes, leading to the elution of all pDNA species. The analysis of peaks obtained during analytical assays allowed the calculation of purity and recovery. Purity degree was defined as the percentage of the sc pDNA peak area related with total area of all peaks from analytical chromatogram. The recovery yield was calculated by the ratio between the obtained sc pDNA concentration and the sc pDNA concentration present in the lysate sample.

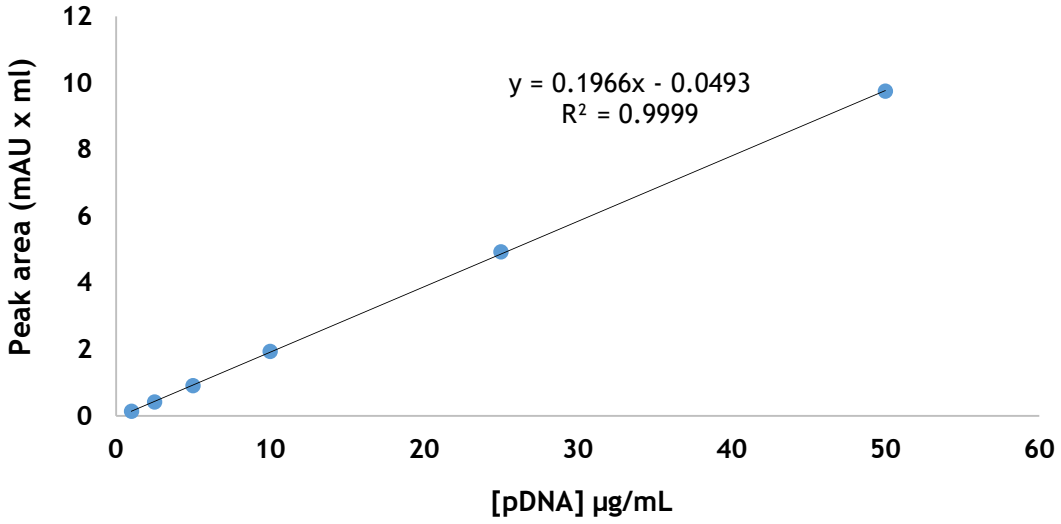

Figure 9 - Calibration curve with pDNA standards (1 - 50 µg/mL).

3.2.8 Protein quantification

Protein content was assessed by the micro-BCA (bicinchoninic acid) protein assay kit from Pierce (Rockford, USA). Briefly, 50 µL of each sample was added to 200 µL of BCA reagent in a microplate. After 30 minutes of incubation at 60 ºC, absorbance was recorded at 595 nm in microplate reader (Biochrom, Cambridge, United Kingdom). The calibration curve (figure 10) was constructed with Bovine Serum Albumin (BSA) (St. Louis, MO, United States of America) as a standard protein (20-2000 µg/mL).

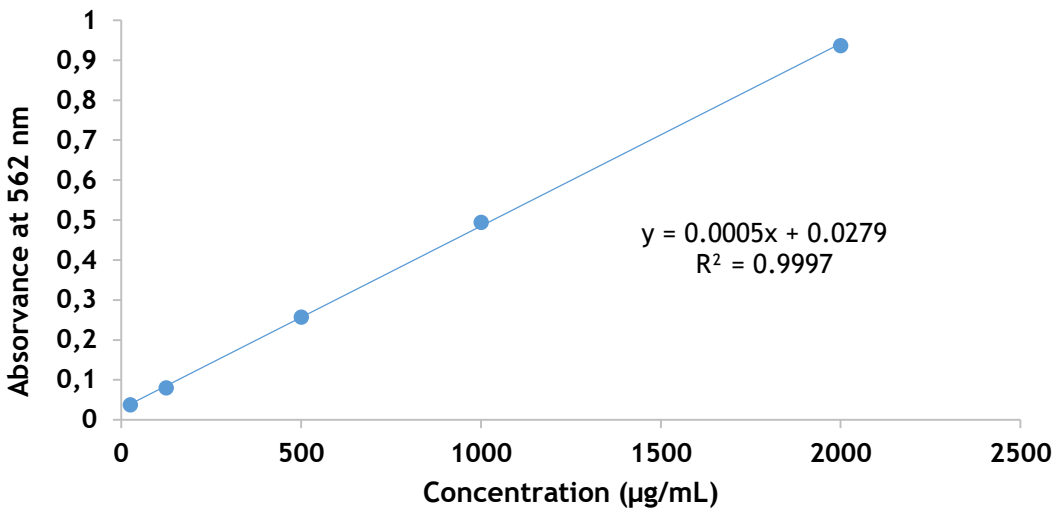

Figure 10 – Reference curve Bovine Serum Albumin standards (20 – 2000 µg/mL).

3.2.9 Genomic DNA quantification

Genomic DNA quantification was performed through real-time polymerase chain reaction (PCR) in a iQS Multicolor Real-Time PCR Detection System (BioRad), such as described by Martins *et al.* [95]. Sense (5'-ACACGGTCCAGAACTCCTACG-3') and antisense (5'-CCGGTGCTTCTTCTGCGGGTAACGTCA-3') primers were used to amplify a 181-bp fragment of the 16S rRNA gene. PCR amplicons were quantified by following changes in fluorescence of the DNA binding dye Syber Green I. The calibration curve to achieve the gDNA concentration was constructed by a serial dilutions of the *E. coli* DH5α gDNA sample, purified with the Wizard gDNA purification kit (Promega) in the range of 0.005 to 50 ng/mL, as it is presented in figure 11.

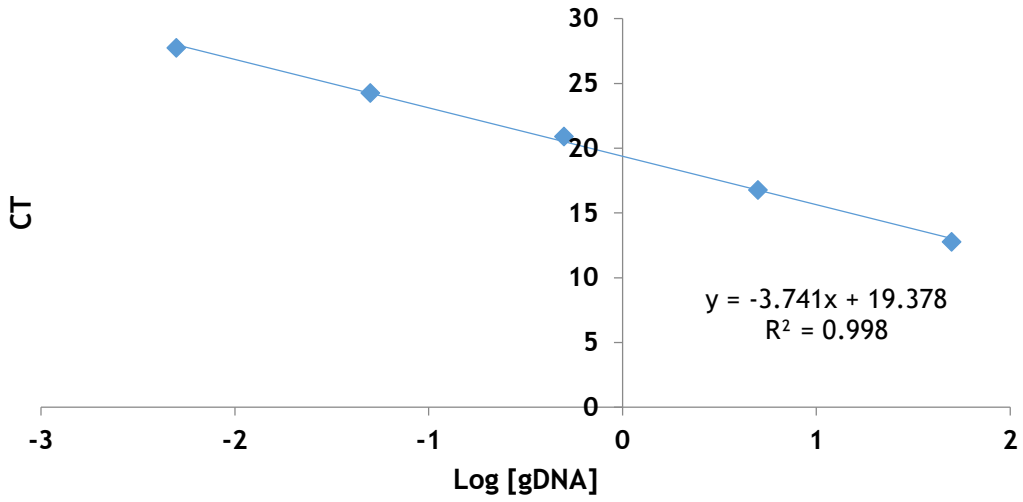

Figure 11 – Reference curve *E. coli* DH5α genomic DNA standards (0.005 – 50 ng/mL)

3.2.10 Endotoxin quantification

Endotoxin contamination was assessed by using the ToxinSensor™ Chromogenic Limulus Amoebocyte Lysate (LAL) Endotoxin Assay Kit from GenScrip (USA, Inc.) according to manufacturer's instructions. A calibration curve was constructed with 10 EU/mL stock solution provided with the kit (0.01-0.1 EU/mL), and it is represented on figure 12. Samples to analyze and samples from the kit were diluted, or dissolved respectively, with non-pyrogenic water, which was also used as the blank, in order to avoid external endotoxin interference. All tubs and tips or diluents used to perform this quantification must be endotoxin-free.

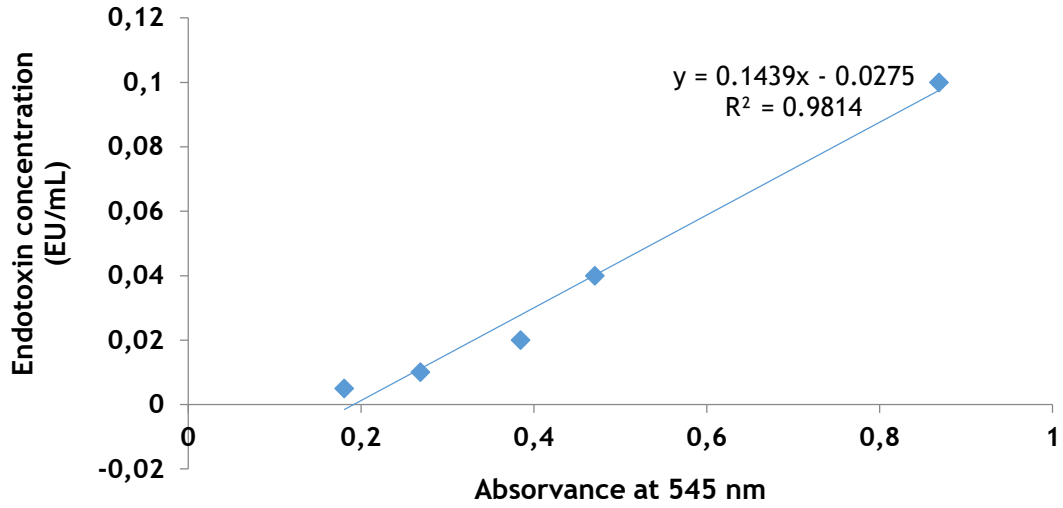

Figure 12 – Reference curve endotoxins standards (0.01 – 0.1 EU/mL).

Chapter 4 – Results and discussion

The approaches through DNA-based therapy have been gaining importance among scientific investigation. Due to advantages related with recombinant DNA, both gene therapy and DNA vaccines have been subject of intensive study. HPV is responsible for genital tract lesions and malignant progression of cervical cancer, namely through the production of E6 and E7 oncoproteins capable to interfere with normal cell cycle. A vaccine able to produce E6 and E7 viral antigens arises as a promising therapeutic pathway to control HPV infection, once these vaccines can stimulate not only humoral but also cellular immune responses. Plasmid DNA presents technical features as biosafety, low cost and easy manufacturing process, which makes it a desirable non-viral vector to deliver therapeutic genes. In order to guarantee the purity of a pDNA vaccine, it is necessary to explore an efficient and highly specific chromatographic technique. Amino acid-affinity chromatography takes advantages of specificity and selectivity of amino acid ligands to purify nucleic acids on basis of their biological function or individual chemical structure. In addition, interest around monolithic supports has recently increased because they present several advantageous features, like high binding capacities of large molecules and excellent mass transfer properties. With this in mind, the aim of this work was to characterize and explore two similar monolithic columns modified with lysine and cadaverine ligands, in order to develop suitable purification strategies to isolate sc pDNA isoform from the lysate sample, which it is the conformation with most biological interest for therapeutic applications, respecting the specifications of regulatory agencies.

4.1 Obtained samples from alkaline lysis with Qiagen Kit and modified alkaline lysis

The alkaline lysis is an important process where most problems can occur. The pDNA recovery from recombinant *E. coli* cells, associated with the elimination of most contaminants, leads to a more efficient purification process through chromatography. However, in this work not only the oc and sc isoforms of pDNA were obtained. Besides these pDNA conformations, in the alkaline lysis process with the Qiagen kit, also the linear pDNA isoform was detected in the sample by agarose gel electrophoresis. The same problem occurred in the modified alkaline lysis, and the final lysate sample presented three pDNA isoforms and RNA (figure 13).

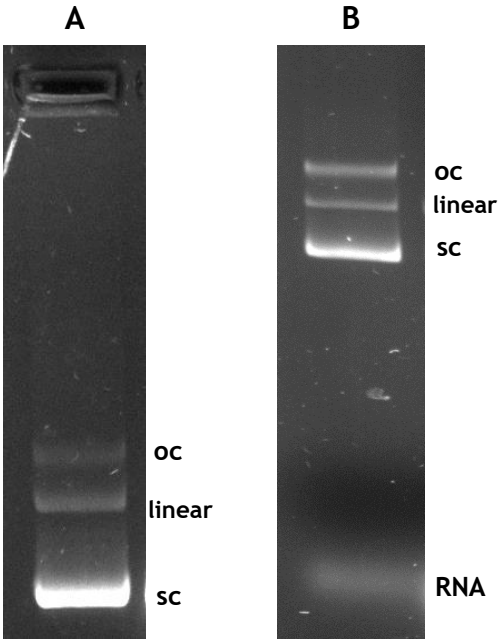

Figure 13 - Agarose gel electrophoresis from the sample obtained with alkaline lysis with Qiagen kit (A), and with the modified alkaline lysis (B).

During the alkaline lysis, a pH above 12.5 on NaOH solution is responsible for disruption of cell wall membranes and can irreversibly denature plasmids by disruption of hydrogen bonds between base pairs, avoiding pDNA renaturation with the addition of potassium acetate to neutralize the mixture [70]. However, solution's pH was minutely controlled before to be added to *E. coli* pellets in order to perform alkaline lysis procedure, and never exceed 12.0 pH value. Thus, the problem could be the resuspension buffer, composed by 50 mM Tris-HCl and 10 mM EDTA ph 8.0 (at Qiagen kit, this solution had also 100 µg/mL of RNase A), due to the incapacity of maintaining the pH under 12.5 as recommended, when NaOH is added to this solution of resuspended cells [96, 97].

4.2 Cadaverine monolithic column

4.2.1 HPV E6/E7^{MUT} plasmid DNA isoforms separation

The first chromatographic application of the lysine amino acid, immobilized in a conventional support, to purify the sc pDNA isoform from *E. coli* lysate sample was performed by Sousa and co-workers [83]. However, as described in chapter 1, conventional supports have some limitations that must be overtaken. Thus, the immobilization of this ligand in a support with promising features, such as monoliths, may be the solution to achieve higher yields in the chromatographic process. On the other hand, cadaverine is a ligand derived from the decarboxylation of lysine amino acid [98], so it could be also a promising approach to achieve the purification of the biomolecule of interest, taking advantage of interactions that this ligand can promote with the pDNA. Thus, two monolithic supports were prepared with lysine and cadaverine immobilized ligands, tested and explored in the present work.

Given that the pDNA purification strategy described with the lysine-agarose matrix was based on increasing gradients of NaCl in Tris-EDTA buffer at pH 8.0, this work started with screening tests by using the cadaverine monolithic column under the same elution conditions. Once this ligand was never tested before, initially it was performed a linear gradient, to understand not only the behavior and the involved interactions between this ligand and the pDNA in NaCl retention/elution conditions, but also the functionality of the column to retain all the injected sample after the immobilization procedure. Firstly, the cadaverine monolith was equilibrated with low ionic strength (10 mM Tris-EDTA buffer, pH 8.0 at room temperature). After the injection of 100 µL of the pre-purified sample (resultant from alkaline lysis with Qiagen kit (oc + linear + sc isoforms)) onto the column, a linear gradient from 0 M to 1.3 M of NaCl in 10 mM Tris-EDTA buffer, pH 8.0, was performed during 10 minutes. The resultant chromatogram is shown in figure 14.

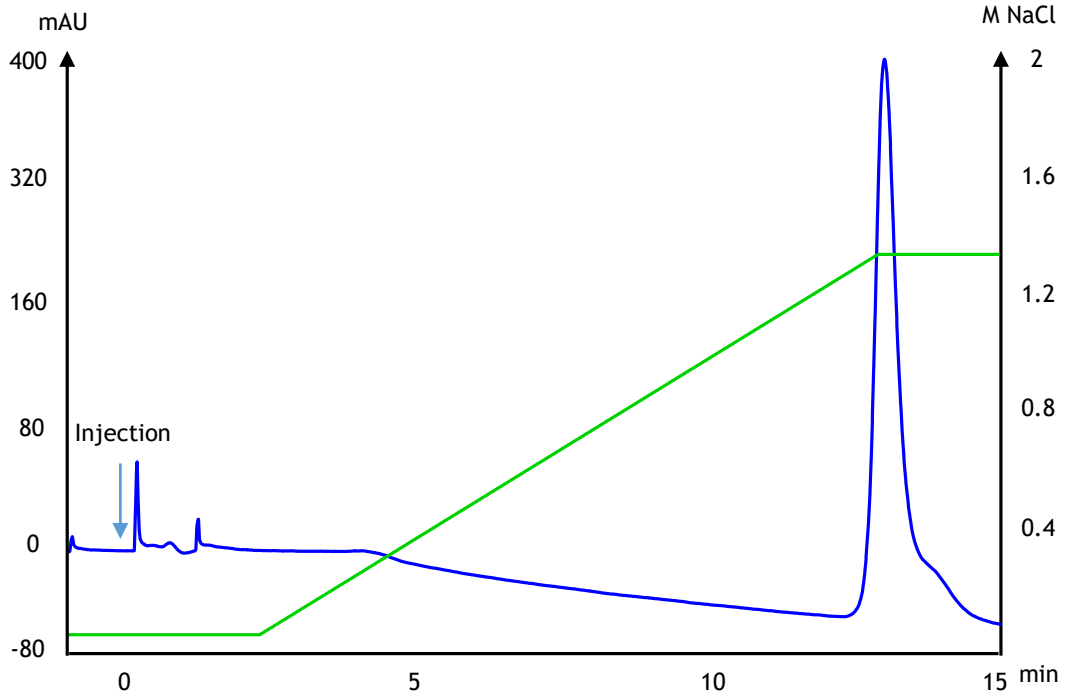

Figure 14 – Chromatographic profile of retention of pDNA isoforms in the cadaverine monolithic column. A pre-purified pDNA sample (oc + linear + sc) was injected onto the column and a linear gradient was performed at 1 mL/min by increasing the NaCl concentration from 0 M to 1.3 M in 10 mM Tris-EDTA buffer, pH 8.0, during 10 minutes (as depicted by green line).

As it is visible in Figure 11, the injected sample was totally retained to the monolith under low ionic strength conditions and the elution of pDNA occurred approximately at 1.2 M of NaCl in 10 mM Tris-EDTA buffer, pH 8.0, during the increased linear gradient with NaCl. Thus, it is observed that cadaverine ligands have affinity for oc, linear and sc pDNA isoforms, resulting in a strong interaction that was only weakened by competition with high ionic strength. Favorable electrostatic interactions are involved in this biorecognition due to the attraction between positively charged cadaverine derivative amino acid, at pH 8.0 (this ligand has a pKa of 9.13) [99], and the negatively charged phosphate groups of pDNA isoforms, as it commonly occurs in

protein-DNA complexes in biological systems to guarantee their stability [100]. The presence of high NaCl concentrations leads to the reduction of the pDNA amount bound per unit area of matrix surface, due to the weakening of electrostatic attractions. The neutralization of negatively charged phosphate groups in the pDNA backbone results in the decrease of repulsive forces that leads to the pDNA compaction. The reducing of hydrodynamic diameter of pDNA occurs in result of this compaction and the slightly retained molecules can simply flowthrough the monolith pores [101, 102].

The preliminary result of pDNA retention profile to cadaverine monolith was satisfactory. However, given that the sc pDNA is the target molecule because it is the biologically active conformation, it is important to verify if this column allows the isolation of sc pDNA isoform. Thus, more binding and elution studies were required to understand if cadaverine monolithic column had the desired selectivity. Two different elution strategies were tested by an increased stepwise gradient of NaCl, with the same buffer and pH previously used. The first one started with the column equilibration at 10 mM Tris-EDTA buffer (pH 8.0) to promote the total sample binding at the monolithic column, followed by two steps of increased ionic strength to achieve pDNA isoforms separation. The second one consisted on column equilibration with some ionic strength to promote the immediate elution of contaminants on flowthrough after sample injection, and also the retention of the target molecule that would be eluted at the second step with the increase of ionic strength. Due to the efficiency of both referred strategies, the methodology with only two chromatographic steps was optimized, once it offers advantages of low time-consuming, avoiding pDNA conformational changes. After several experiments, the best elution conditions for the sc pDNA isolation are presented in the chromatogram of figure 15.

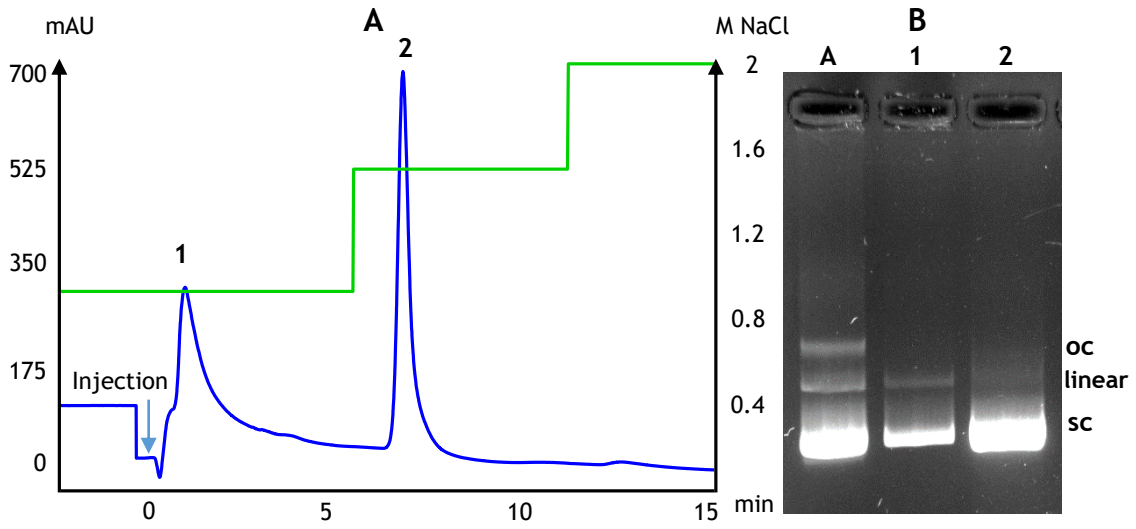

Figure 15 – (A) Chromatographic profile of pDNA isoforms in cadaverine monolithic column. A stepwise gradient was performed at 1 mL/min from 0.92 M to 1.5 M NaCl in 10 mM Tris-EDTA, pH 8.0 (as depicted by green line). Injection sample: 100 µL of pre-purified pDNA (oc + linear + sc). (B) Agarose gel electrophoresis of obtained peaks. Lane A: pDNA sample injected onto the column (oc + linear + sc); lane 1: linear and sc pDNA; lane 2: sc pDNA.

Cadaverine monolithic column was equilibrated at a flow rate of 1 mL/min with 0.92 M of NaCl in 10 mM Tris-EDTA buffer, pH 8.0 (at room temperature). After the injection of 100 µL of pre-purified plasmid sample, a first peak of unbound species was eluted at the equilibrium conditions, followed by a second peak of the retained molecules with 1.5 M of NaCl in 10 mM Tris-EDTA buffer, pH 8.0. The obtained peaks in the chromatogram (figure 15 – (A)) were concentrated, desalted and analyzed by agarose gel electrophoresis (figure 15 – (B)). As visible at lane 1, not only linear pDNA was eluted in the first peak, but also some quantity of sc pDNA, while the second lane corresponds to peak 2, which only had sc pDNA. In order to obtain the purification of target molecule at peak 2, the recovery of sc pDNA was sacrificed, with a partial elution of this biomolecule at peak 1 together with linear pDNA isoform. On the other hand, the decrease of salt concentration at equilibration step, in order to promote higher sc pDNA recovery, would result in a contamination of peak 2 with linear pDNA isoform. During the chromatographic run, the oc pDNA was not eluted, suggesting their retention into the monolith. The behavior of oc pDNA into the column may be due to its large spacial conformation, with higher hydrodynamic radii, leading to the blocking of small column channels and consequentially to a steady pressure increase [103]. This event was observed for the most performed experiments, and in some assays, also the linear pDNA was retained.

The difficulty to obtain the purification of sc pDNA led to explore other elution strategies that would be applicable in this chromatographic process, in order to improve the selectivity of cadaverine ligand. Several studies showed the pH influence in arginine and histamine ligands to efficiently isolate the sc pDNA [88, 89], so this condition was also tested in the cadaverine monolithic support. Firstly, several assays were performed at pH 9.0, manipulating the NaCl concentrations in order to achieve the pDNA isoforms separation. Results are shown in figure 16.

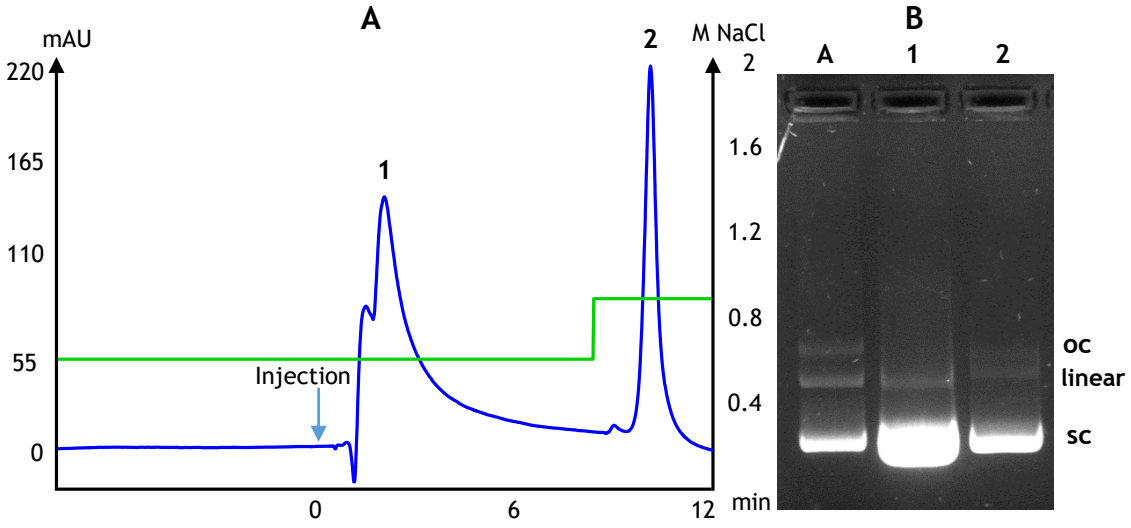

Figure 16 – (A) Chromatographic profile of pDNA isoforms in cadaverine monolithic column. A stepwise gradient was performed at 1 mL/min from 0.7 M to 0.9 M NaCl in 10 mM Tris- EDTA, pH 9.0 (as depicted by green line). Injection sample: 100 µL of pre-purified pDNA (oc + linear + sc). (B) Agarose gel electrophoresis of obtained peaks. Lane A: pDNA sample injected onto the column (oc + linear + sc); lane 1: fraction corresponding to peak 1; lane 2: fraction corresponding to peak 2.

Cadaverine monolithic column was equilibrated with 0.7 M of NaCl in 10mM Tris-EDTA buffer, pH 9.0, (room temperature) and a flow rate of 1 mL/min. After the injection of 100 µL of pre-purified plasmid sample, a first peak was obtained (Figure 16 – (A)). Then, NaCl concentration was increased to 0.9 M of NaCl in 10 mM Tris-EDTA buffer, pH 9.0, being eluted the retained species (peak 2). As it is visible in figure 16 – (B), the obtained peaks do not present the isoforms separation, as it happened at pH 8.0. Thereby, peak 2 has oc and linear pDNA isoforms as contaminants, revealing that the use of pH 9.0 was not a good approach. Furthermore, the interaction between ligands and pDNA molecules was weaker, as it was shown by the lower NaCl concentration used for equilibration and elution steps. Regarding the oc pDNA, its quantity, observed through agarose gel electrophoresis, was increased throughout the assays performed in the monolith. The accumulation of retained oc pDNA molecules led to a steady pressure increase on monolithic support after each sample injection. When the column was saturated, oc pDNA started to be eluted in sc pDNA elution peak and the pressure remained stable. When necessary, to restore the initial conditions of monolithic column, regeneration with 0.5 M of NaOH is required.

Moreover, the chromatographic behavior of cadaverine monolithic column was still evaluated at pH 6.0 elution conditions. After several experiments in order to obtain ideal NaCl binding and elution conditions, it was observed stronger interactions with the pDNA sample but still lack of selectivity. Figure 17 shows an example of the obtained results.

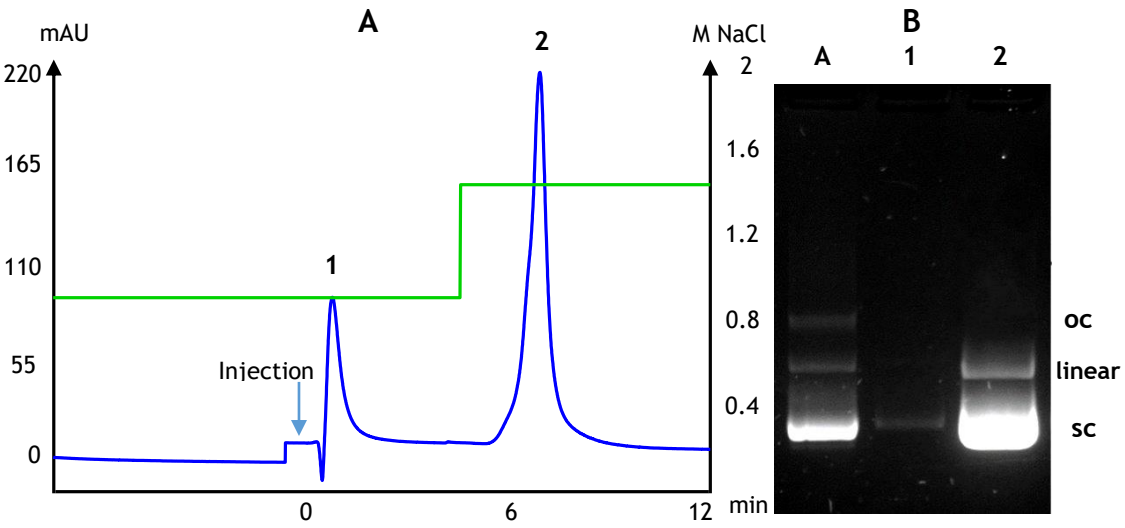

Figure 17 – (A) Separation of pDNA isoforms in cadaverine monolithic column. A stepwise gradient was performed at 1 mL/min from 1 M to 1.5 M NaCl in 10 mM Tris- EDTA, pH 6.0 (as depicted by green line). Injection sample: 100 µL of pre-purified pDNA (oc + linear + sc). (B) Agarose gel electrophoresis of obtained peaks. Lane A: pDNA sample injected onto the column (oc + linear + sc); lane 1: fraction corresponding to peak 1; lane 2: fraction corresponding to peak 2.

The cadaverine column was equilibrated with 1 M of NaCl in 10 mM Tris-EDTA buffer, pH 6.0 (at room temperature), and after injection the pre-purified pDNA sample (100 µL) a first peak

was obtained (figure 17 – (A)). The increase of NaCl concentration to 1.5 M by a stepwise gradient resulted in the elution of retained molecules (peak 2). The separation of pDNA isoforms was not achieved because peak 2 had a contamination of linear pDNA together with sc pDNA and in the peak 1 there was still a small elution of sc pDNA (figure 17 – B). As it happened at pH 8.0, to guarantee the desired purity level of sc pDNA at peak 2, a partial loss of this isoform must occur together with linear pDNA at peak 1. However, it was observed a stronger interaction between ligands and pDNA molecules, once the total elution of retained species occurred at 1.5 M of NaCl (0.5 M higher compared to the results obtained at pH 9.0). Considering that the pKa of cadaverine is 9.13, lower working pHs lead to the increase of effectiveness positive charges of cadaverine and consequently to a stronger interaction with pDNA. On the other hand, higher pH values (near the pKa of cadaverine) can neutralize the cadaverine charge, resulting in weaker ionic interactions [89]. Nevertheless, the desired separation was not yet reached.

4.2.2 Purification of supercoiled plasmid DNA from the clarified *E. coli* sample

Once the sc pDNA isolation was not achieved, even with the pH variation, the possibility to test different buffers was considered due to some published studies that already proved the influence of mobile phase buffer in the pDNA interaction with amino acid ligands [104]. This strategy was tested with clarified *E. coli* sample obtained through modified alkaline lysis, once the presence of other biomolecules in addition to pDNA could led to different types of interactions.

Several experiments were performed by manipulation of NaCl concentrations, with the use of three different buffers, namely 10 mM Tris-EDTA pH 8.0, 10 mM sodium-phosphate in 10 mM EDTA (Phosphate-EDTA) pH 8.0 and also 100 mM HEPES pH 7.4. The chosen pHs for this preliminary studies about buffer efficiency are the most described on literature. The main objective of this buffer screening was the isolation of sc pDNA from other biomolecules, although in some cases the pDNA recovery has to be sacrificed in order to maintain the purity degree. After the concentration and desalting of resultant peaks from chromatographic assays, samples were analyzed through a 0.8% agarose gel electrophoresis. The results of optimized conditions for each buffer are presented in figure 18.

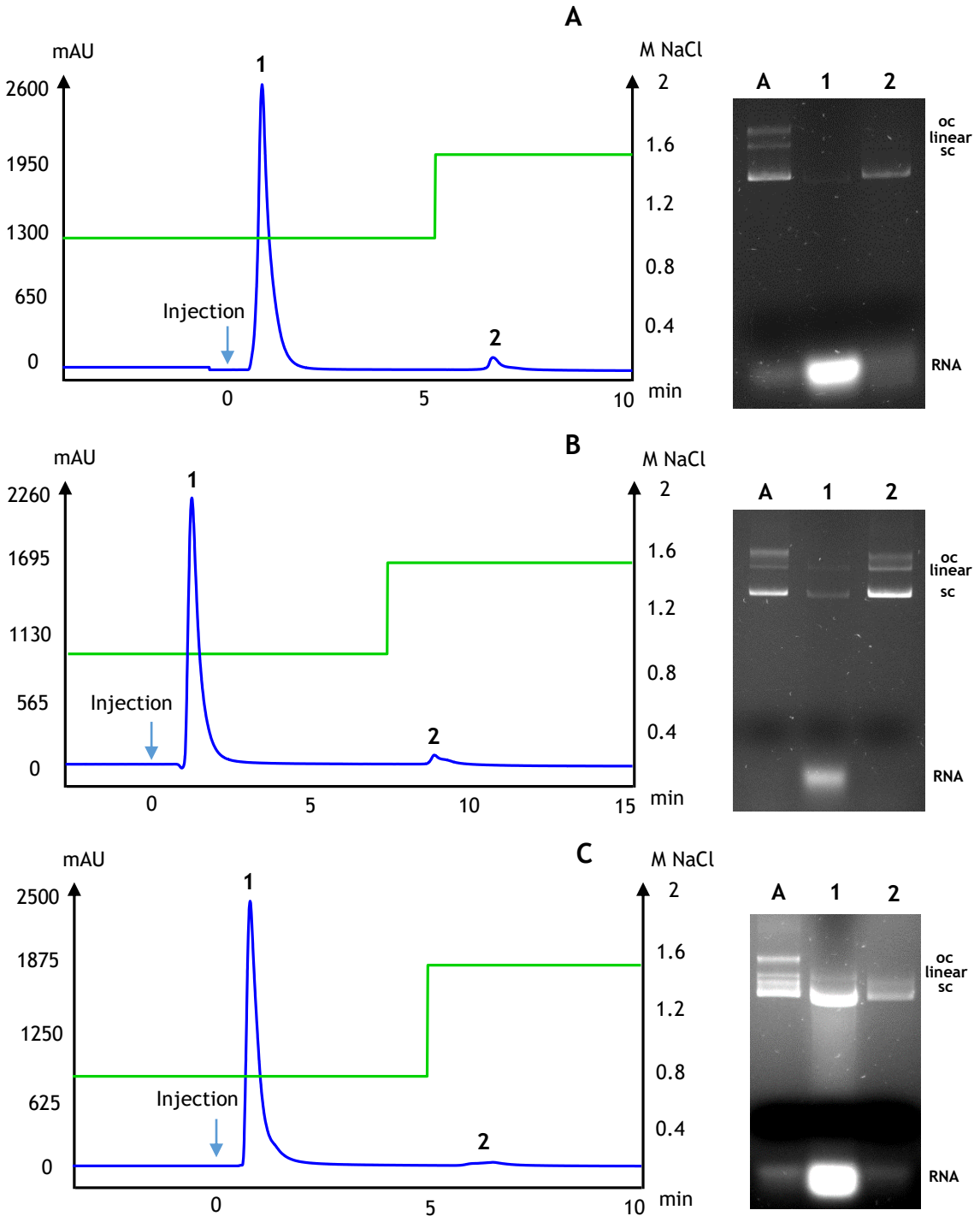

Figure 18 – Chromatographic profiles of the injection (100 µL) of *E. coli* lysate sample in the cadaverine monolithic column, by using Tris-EDTA (A), Phosphate-EDTA (B) and HEPES buffers (C). Agarose gel electrophoresis of obtained peaks is presented in the respective chromatogram; Lane A: lysate sample injected onto the column; lane 1: fraction corresponding to peak 1; lane 2: fraction corresponding to peak 2.

A selection of optimized conditions to isolate sc pDNA with Tris-EDTA, Phosphate-EDTA and HEPES buffers (at room temperature) was made in other to compare their results. As illustrated in figure 18 (A), Tris-EDTA showed promising results not only because the loss of sc pDNA on the first peak was not significant in the case of this screening study (figure 18 (A) – lane 1), but also because RNA was present in low quantities at peak 2 (figure 18 (A) – lane 2). The results

for phosphate-EDTA buffer were similar to those obtained with Tris-EDTA, as represented in figure 18 (B). Contrariwise, HEPES buffer was not a good approach once the sc pDNA isoform and RNA were eluted together mostly in the first peak and also in the second peak (figure 18 (C)), conditioning the achievement of the desired selectivity. As described by Gaspar *et al.*, despite of high binding events between mini circular DNA (mcDNA) and arginine dipeptides by using HEPES buffer, the strength of mcDNA-ligand interaction was the weakest for this buffer among the three tested buffers (Tris, Phosphate and HEPES) [104]. This phenomenon is due to HEPES be a zwitterion at pH 7.4, leading to disruption of electrostatic potentials and consequentially repelling of mcDNA nucleotides, phenomenon that could also be involved in the present work between the cadaverine monolithic column and pDNA nucleotides.

In order to improve the purification of sc pDNA from lysate sample, a combination of Tris-EDTA and Phosphate-EDTA buffers with pH manipulation was required. Given that, several chromatographic experiments were performed by manipulating the NaCl concentrations in 10 mM Tris-EDTA buffer, and in 10 mM Phosphate-EDTA buffer, for the pH values of 8.0, 7.0 and 6.0. For each buffer and pH, many assays were tested in order to find the best condition and concentration of NaCl to elute contaminating species at peak 1 and sc pDNA at peak 2. Since the interaction strength between biomolecules and the cadaverine ligand differ according to the buffer and pH used, the NaCl concentration needed in the stepwise gradient was different for each pH value as well as for each buffer. After chromatographic experiments, obtained peaks were concentrated, desalted and analyzed through agarose gel electrophoresis to choose de best elution conditions. Experiments for each optimal condition were repeated and peaks resultants from the injection of 200 µL of clarified *E. coli* sample were recovered, concentrated and desalted for further analysis on CIMac pDNA analytical column.

4.2.3 Recovery and purity quantification of recovered peaks

The recovered peaks obtained from optimal conditions for each buffer and pH were analyzed in terms of recovery and purity of sc pDNA by using the CIMac pDNA analytical column [88]. This analytical method starts with the equilibration of analytical column with 600 mM of NaCl in 200 mM Tris-HCl buffer (pH 8.0). Then, 20 µL of sample recovered from one peak was injected onto the column and a linear gradient from 600 mM to 700 mM of NaCl was performed during 10 minutes, leading to the elution of pDNA retained species. Analytical chromatograms were treated in order to register obtained peak areas of each analyzed sample. The pDNA concentration was assessed by using a calibration curve. The recovery yield of sc pDNA was evaluated by the ratio between the sc pDNA concentration obtained in the purified sample and the sc pDNA concentration present in the lysate sample. The analysis of peak areas resultant from the elution of sc pDNA and impurities allow to calculate the purity degree of recovered peaks through the ratio of sc pDNA peak area and the total area of all peaks present in the analytical chromatogram. Table 7 presents the values of purity and yield for all buffers and pHs tested, as well as the elution condition for each peak.

Table 7 - Analysis of purity and recovery yields of the sc pDNA isolated from *E. coli* lysate through of cadaverine-affinity chromatography.

Buffer	Peak	pH	Elution (M NaCl)	Recovery (%)	Purity (%)
Injected sample	-----	-----	-----	-----	4.24
Phosphate - EDTA	1	6.0	1.20	84.50	-----
Phosphate - EDTA	2	6.0	1.50	43.50	54.61
Phosphate - EDTA	1	7.0	1.00	52.90	-----
Phosphate - EDTA	2	7.0	1.50	48.20	46.85
Phosphate - EDTA	1	8.0	0.85	42.10	-----
Phosphate - EDTA	2	8.0	1.50	41.90	41.43
Injected sample	-----	-----	-----	-----	3.83
Tris-EDTA	1	6.0	1.05	1.30	-----
Tris-EDTA	2	6.0	1.50	98.70	97.89
Tris-EDTA	1	7.0	0.95	28.20	-----
Tris-EDTA	2	7.0	1.50	84.40	78.60
Tris-EDTA	1	8.0	0.75	13.50	-----
Tris-EDTA	2	8.0	1.50	73.00	68.63

The presented results confirm the influence of pH values in the sample retention. The lower pH leads to the use of higher NaCl concentration in order to assure the isolation of sc pDNA, for both tested buffers. Moreover, the analysis of table 7 showed high purity and recovery yields of sc pDNA with the use of Tris-EDTA buffer, compared to those obtained with Phosphate-EDTA buffer. Tris-HCl can promote higher ligand-DNA interaction due to its lower equilibrium dissociation constant, when compared to Phosphate [104], probably because the phosphate ions present in the buffer can compete with phosphate groups of nucleic acids by the ligand. With a purity degree for sc pDNA of 97.82% and a recovery yield of 98.7% for 10 mM Tris-EDTA buffer, pH 6.0, this was the best condition obtained for this study and respective chromatogram and agarose gel electrophoresis are represented in figure 19.

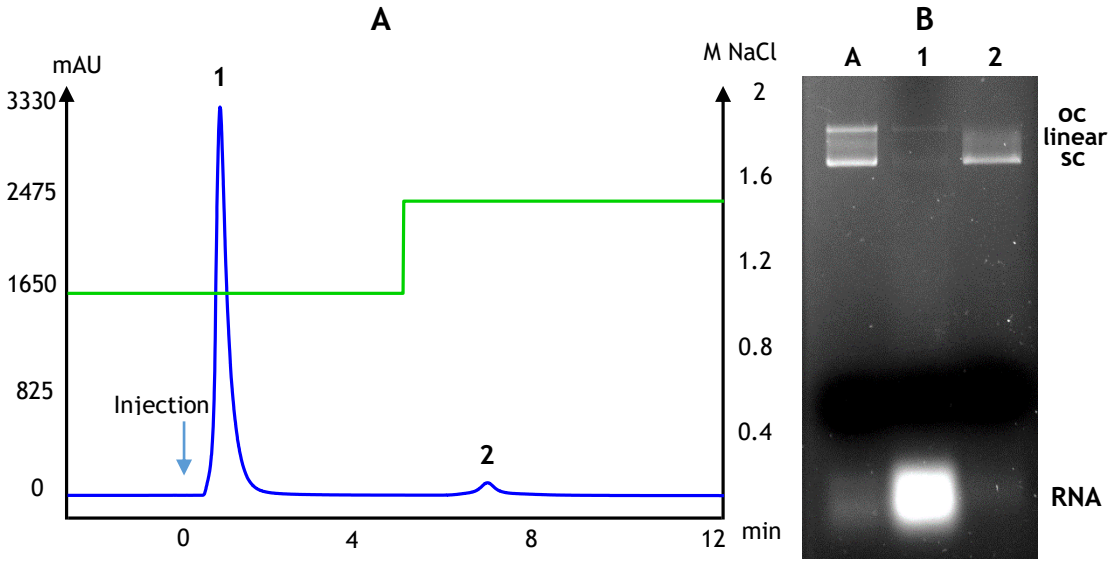

Figure 19 – (A) Chromatographic profile of the *E. coli* lysate sample injected in the cadaverine monolithic column. A stepwise gradient was performed at 1 mL/min from 1.05 M to 1.5 M NaCl in 10 mM Tris-EDTA buffer, pH 6.0 (as depicted by green line). Injection volume: 100 µL. (B) Agarose gel electrophoresis of obtained peaks. Lane A: lysate sample injected onto the column; lane 1: fraction corresponding to peak 1; lane 2: fraction corresponding to peak 2.

Cadaverine monolithic column was equilibrated with 1.05 M of NaCl in 10 mM Tris-EDTA buffer, pH 6.0 (room temperature). Immediately after the injection of lysate sample, contaminants (RNA and oc pDNA) were eluted in the peak 1 (figure 18 – (A)). The NaCl concentration was further increase to 1.5 M and the elution of sc pDNA occurred in the peak 2, due to the higher ionic strength applied. The resulting peaks were analyzed by agarose gel electrophoresis (figure 18 – B). RNA is low molecular weight biomolecule, negatively charged and single stranded, with high base exposition [105]. Likewise, the bases of sc pDNA isoform are more exposed due to the deformation induced by torsional strain (supercoiling phenomenon), while oc isoform bases are less exposed, being eluted with low NaCl concentrations. The RNA and sc pDNA base exposition could lead to a similar interaction of RNA and pDNA with cadaverine ligand, as occurred in a study performed by Sousa and coworkers with lysine amino acid [83]. However, the plasmid used in this work is much size-larger than RNA, being the RNA biomolecule eluted early in the chromatographic experiments (peak 1 – figure 19 (A) and lane 1 – figure 19 (B)), once its interactions are easily broken with low NaCl concentrations. The sc pDNA purification in peak 2 (figure 19 (B) – lane 2), resulted from the higher bases exposition that leads to a stronger interaction with cadaverine ligand [86]. The increase of ionic strength through NaCl concentration led to the differential elution of RNA and pDNA species due to the reduction of electrostatic interactions [106]. The presence of electrostatic interactions is related with the amine groups of cadaverine, which make it a multiple donor, despite the use of a single chain atom for binding. Thus, hydrogen complex interactions (bidentate) are promoted with pDNA bases, namely guanine. Furthermore, positively charged amine groups of this ligand promote cation-π interactions with aromatic rings of pDNA [107]. As cadaverine does not have carboxyl group, the interaction with pDNA is strong, once the lack of its negative group avoid some

repulsion of pDNA phosphate groups [88]. The carbon lateral chain of cadaverine could also favor van der Waals and hydrophobic interactions [106].

4.3 Lysine monolithic column

4.3.1 Preliminary tests with *E. coli* lysate

As mentioned on subchapter 4.2, several studies were performed with the lysine-agarose matrix and it was observed the success of this amino acid ligand for sc pDNA purification [106]. However, it is known that conventional matrices have some limitations that needs to be overcome, in this case by the promising approaches related with the use of monolithic supports. After a detailed study about the behavior of cadaverine ligand and its capacity to interact with biomolecules in the presence of different types of buffers and pHs, the best conditions were carried out for lysine monolithic support in order to do a comparison of both ligands. The main difference between these ligands is the presence of a terminal carboxyl group in the lysine ligand, which can lead to some repulsion effect with negatively charged species present in clarified *E. coli* lysate. So, it is expected that NaCl concentrations needed to elute bound species are lower than at previous assays.

To start the study with the monolithic support, a preliminary test (figure 20) was performed to confirm the success of column immobilization with lysine amino acid. Thus, a linear gradient was applied with the buffer chosen in the cadaverine monolith, Tris-EDTA at pH 6.0.

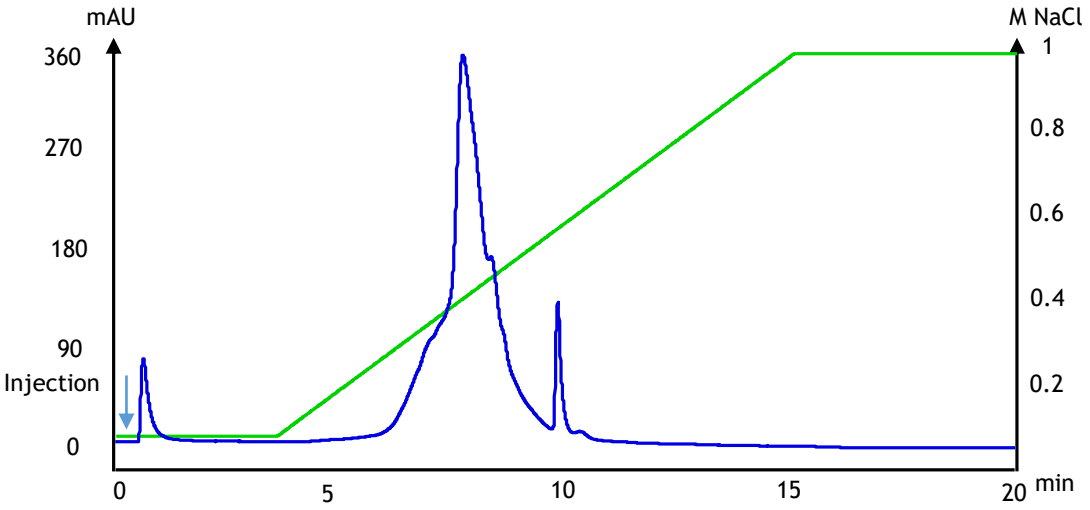

Figure 20 - Chromatographic behavior of lysine monolithic after the injection of the clarified lysate sample and applying a linear gradient at 1 mL/min by increasing the NaCl concentration from 0 M to 1 M in 10 mM Tris-EDTA buffer pH 6.0, during 20 minutes (as depicted by green line).

The column was equilibrated with 10 mM Tris-EDTA, ph 6.0 (room temperature). After the injection of 100 µL of clarified lysate sample onto the column, obtained from modified alkaline lysis, a linear gradient from 0 M to 1 M of NaCl was performed during 20 minutes. The sample

was totally retained by lysine ligands (agarose gel electrophoresis was not shown) being the initial peak resultant from the effect of sample injection. Comparing to the chromatogram obtained on cadaverine support in figure 14, it is possible to affirm that interactions promoted between the lysine ligand and nucleic acids was weaker as expected, since the total elution of sample species occurred at approximately 0.55 M of NaCl.

To compare the influence of pH on the interaction strength between biomolecules and lysine monolithic support, similar experiment was performed at pH 7.0 and 8.0 (data not shown). The experiments of the linear gradient from 0 M to 1 M of NaCl in 10 mM Tris-EDTA buffer with both pHs showed that increasing pH leads to weak interactions between the species present in the *E. coli* clarified lysate and lysine ligands, decreasing the retention time. Moreover, there was no binding from the sample to lysine amino acid ligands at pH 8.0, occurring the elution of the injected sample on the flowtrough. Thus, these results showed the presence of weaker ionic interactions than those present on cadaverine ligand, probably due to the presence of carboxyl group at lysine ligand, which could lead to some repulsion of negatively charged biomolecules.

4.3.2 Lysine amino acid affinity for supercoiled plasmid DNA

Given the last results, they were gathered the conditions to continue the study. The design of an elution strategy must be prepared considering the influences on retention behavior of biomolecules [108]. After the success obtained with the linear gradient at pH 7.0 and 6.0 on sample retention, two strategies were optimized by using stepwise increasing gradients of NaCl, in order to achieve the recovery and purification of sc pDNA. Preliminary experiments of stepwise gradients performed with the initial equilibration of the column with some NaCl concentration (first elution step) led to a compromised recovery and purification of sc pDNA. So, with lysine amino acid ligand, it was need an additional equilibration step with 10 mM Tris-EDTA buffer, for both pHs tested, in order to bind all species present in the *E. coli* lysate sample onto the column after the injection. The optimization of NaCl conditions through a stepwise gradient for the purification of sc pDNA at pH 7.0 is shown in figure 21.

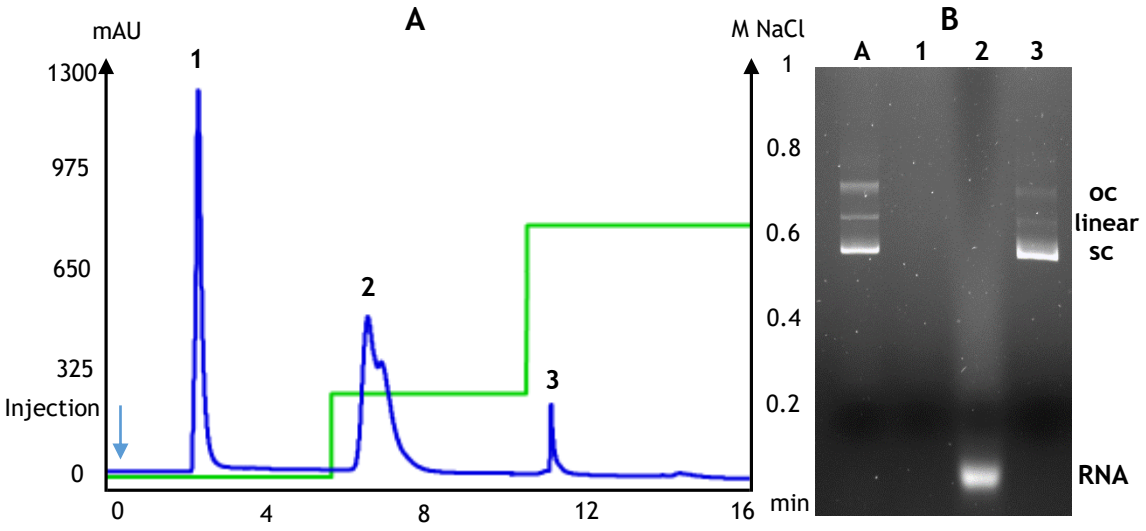

Figure 21 – (A) Chromatographic profile of the *E. coli* lysate sample injected in lysine monolithic column. A stepwise gradient was performed at 1 mL/min from 0 M to 0.2 M and to 0.6 M NaCl in 10 mM Tris-EDTA buffer, pH 7.0 (as depicted by green line). Injection volume: 100 µL. (B) Agarose gel electrophoresis of obtained peaks. Lane A: lysate sample injected onto the column; lane 1, 2 and 3: fractions corresponding to peak 1, 2 and 3, respectively.

Figure 21 (A) shows the chromatogram obtained with the optimizing stepwise gradient for pH 7.0 (room temperature), with three steps performed at 0 M, 0.2 M and 0.6 M of NaCl in 10 mM Tris-EDTA buffer. Monolithic column was equilibrated at 1 mL/min with 10 mM Tris-EDTA buffer at the first step (binding step). Then, a second step (figure 21 (A) – peak 2) was performed with the increase of NaCl concentration to 0.2 M, in order to wash RNA and other impurities. Finally, the third step (figure 21 (A) – peak 3) was the elution step of sc pDNA at 0.6 M of NaCl. Agarose gel electrophoresis from each peak is represented in figure 21 (B), and it is possible to see that although some quantity of oc and linear pDNA was retained on the column, as happened for cadaverine monolithic column, it occurred some oc and linear pDNA elution for this pH conditions on the peak of interest (figure 21 (B) – lane 3), compromising the purification of sc pDNA.

After some experiments of the elution strategy at pH 6.0, the optimized conditions for the purification of target molecule are presented in figure 22.

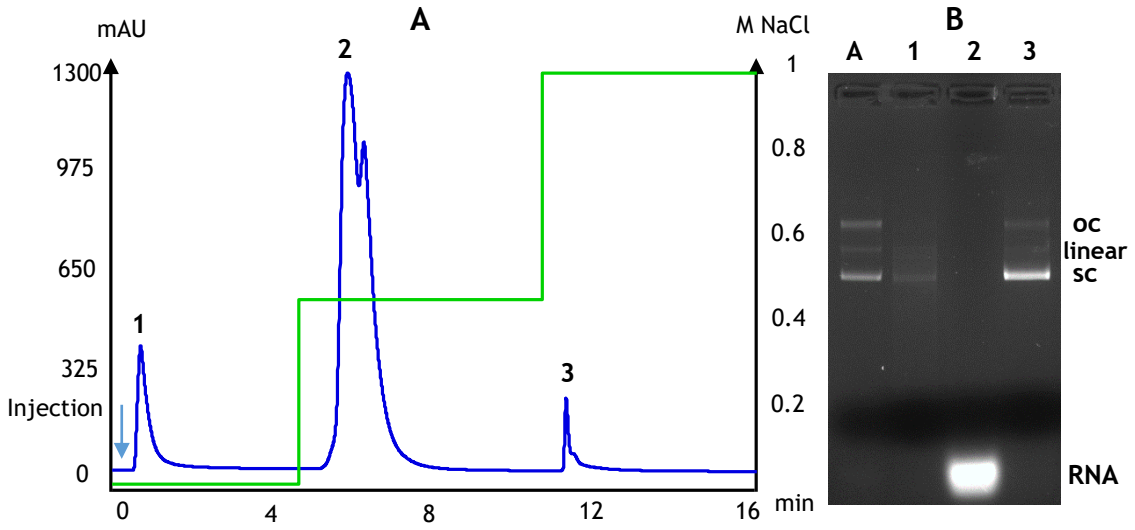

Figure 22 – (A) Chromatographic profile of the *E. coli* lysate sample injected in lysine monolithic column. A stepwise gradient was performed at 1 mL/min from 0 M to 0.45 M and to 1 M NaCl in 10 mM Tris-EDTA buffer, pH 6.0 (as depicted by green line). Injection volume: 100 µL. (B) Agarose gel electrophoresis of obtained peaks. Lane A: lysate sample injected onto the column; lane 1, 2 and 3: fractions corresponding to peak 1, 2 and 3, respectively.

As it happened before, also for this pH condition was applied three different steps of 0 M, 0.45 M and 1 M of NaCl in 10 mM Tris-EDTA buffer (at room temperature). The injection of 100 µL from clarified *E. coli* lysate was performed after column equilibration with 10 mM Tris-EDTA at pH 6.0. Then, the elution of RNA and other contaminants was performed at 0.45 M of NaCl. The final step for the elution of sc pDNA isoform was achieved with 1 M of NaCl (Figure 22 (A)). At lane 1 of the agarose gel electrophoresis (figure 22 (B)) is possible to observe a tenuous band of sc pDNA which is insignificant given the total amount of this isoform recovered on peak 3. RNA was totally eluted in the second step, and observing the lane 3 is possible to affirm that sc pDNA is the main present isoform. Comparing to the optimal chromatographic experiment done at pH 7.0, sc pDNA is less contaminated by other isoforms on the present conditions.

The preferential retention of sc pDNA are related with its negative charge due to the presence of phosphate groups, suggesting the involvement of electrostatic interactions with lysine ligands that are positively charged at pHs below the pKa (10.53) [109]. Also cation-π interactions can be established between the positively charged amine of lysine and the aromatic rings of nucleic acids. The evidences that the lysine ligand promotes hydrogen complex interactions (bidentate) preferentially with guanine base [110], reinforce the presence of hydrogen bounds between the lysine amino groups and the more exposed bases of sc pDNA conformation due to the supercoiling phenomenon. In addition, van der Walls forces and water-mediated bonds can be promoted, although hydrogen-mediated bonds are the predominant forces [106, 110]. This affinity of lysine amino acid by the nucleic acid bases could explain the differential retention of biomolecules present in the lysate sample.

4.3.3 Recovery and purity analysis through CIMac plasmid DNA analytical column

The sc pDNA recovery and purity of samples eluted from lysine monolithic column at pH 7.0 and 6.0 were assessed, in order to compare with the results obtained from cadaverine support. So, these samples were injected into CIMac pDNA analytical column through a process already described, in order to analyze peak areas from resulting chromatograms. The obtained results are presented in table 8.

Table 8 - Analysis of purity and recovery yields of the sc pDNA isolated from *E. coli* lysate through the lysine-affinity chromatography.

Peak	pH	Elution (M NaCl)	Recovery (%)	Purity (%)
Initial sample	6	---	---	3.32
1	6	0.45	8.70	---
2 (sc pDNA)	6	1.00	96.10	97.53
Initial sample	7	---	---	5.29
1	7	0.20	7.50	---
2 (sc pDNA)	7	0.60	94.20	75.04

The results obtained from recovered samples provided by purification of sc pDNA through lysine monolithic column showed, one more time, stronger interactions between nucleic acids and ligands for lower pH values, which can help to achieve the selectivity and purification of some biomolecules by the increase of retention time. The best recovery and purity results were obtained for pH 6.0 (96.10% and 97.53%, respectively), as happened at cadaverine monolithic column. Besides the good recovery yield obtained at pH 7.0 (94.20%), the purity value is low (75.04%). A detailed comparison between two supports tested in this work will be done on the next chapter.

4.4 Comparison of results obtained through lysine and cadaverine monolithic columns

With sc pDNA recovery and purity values analyzed for the best elution conditions established in cadaverine and lysine monoliths, it is suitable to compare them in order to understand which were the ligand with best performance in this study. Table 9 resumes the best results for each monolith.

Table 9 – Comparison of purity and recovery yields of the sc pDNA isolated from *E. coli* lysate through of lysine and cadaverine affinity chromatography, with the use of NaCl in 10 mM Tris-HCl and 10 mM EDTA buffer.

Peak	pH	Elution (M NaCl)		Recovery (%)		Purity (%)	
-----	-----	Cadaverine	Lysine	Cadaverine	Lysine	Cadaverine	Lysine
1	6	1.05	0.45	1.30	8.70	-----	-----
2	6	1.50	1.00	98.70	96.10	97.89	97.53
1	7	0.95	0.20	28.20	7.50	-----	-----
2	7	1.50	0.60	84.40	94.20	68.63	75.04

The achieved results were too similar in terms of recovery yield and purity, for the same used conditions of pH (6.0) and type of buffer (10 mM Tris-EDTA). This fact could be related to the similar chemical constitution of two ligands. As mentioned initially, cadaverine results from decarboxylation of lysine amino acid. Thus, it is possible to affirm that the lack of carboxyl group could only increase the interaction strength between ligands and nucleic acids, once the NaCl concentration needed for the elution of sc pDNA from the lysate sample in the lysine monolith was 1.0 M and in the cadaverine was 1.5 M. The recovery yield of both supports was similar for the elution strategies at pH 6.0 (98.70% and 96.10% for cadaverine and lysine ligands, respectively). Previous studies of affinity chromatography with amino acids showed recovery yields of 55% for lysine conventional matrix [83], 83.5% for arginine monolith [89] and 97% for histamine monolith [88]. Thus, it can be affirmed that the current study obtained good results for recovery yield with the use of both cadaverine and lysine ligands immobilized into a monolithic column, for experiments performed with Tris-EDTA buffer at pH 6.0. The results obtained at pH 7.0 were not so satisfactory and the sc pDNA recovery achieved with cadaverine monolith was low (84.40%) compared to the lysine monolith (94.20%). The influence of pH in sc pDNA purification could be related with ligand total protonation. In the case of lysine and cadaverine ligands, with pKa of 9.13 and 10.53 respectively, the low pH value allows the increase of positive charges available for the interaction with negatively charged nucleic acids, leading to stronger interactions and consequently best selectivity, improving the sc pDNA purification. Regarding the purity degree, the most satisfactory results were also attained on the experiments performed at pH 6.0. Once regulatory agencies recommend more than 97% of sc pDNA purity, this goal was achieved with success for the elution conditions optimized in both supports. For cadaverine ligand, the sc pDNA purity was 97.89%, while for lysine amino acid was 97.53%. Both results accomplish the requirements of sc pDNA purity for a suitable biopharmaceutical, able to be used in therapeutic trials as DNA vaccines. The results obtained

with the elution strategy with pH 7.0 do not meet the imposed requirements, once the purity degree was very low. For instance, the purity of sc pDNA in the cadaverine monolith was 68.63%, while in the lysine support was 75.04%. Although lysine monolith required low salt concentrations, which was advantageous in terms of environmental impact, cadaverine support allowed better purity degree and recovery yield.

4.4.3 Assessment of supercoiled plasmid DNA quality

Despite the satisfactory purity and recovery yield of sc pDNA achieved with optimized elution strategies of increasing NaCl stepwise gradient in Tris-EDTA buffer at pH 6.0, other features are determinant for the product approval by regulatory agencies. The quality criteria imposed by regulatory agencies is summarized on table 10.

Table 10 – Criteria imposed by FDA for pDNA quality (adapted from [111]).

Characteristics	Specifications
Appearance	Clear / colourless solution
Plasmid Homogeneity	>97% sc
Proteins	Not detectable, by BCA assay
gDNA	<2 µg/mg plasmid, assessed by PCR
Endotoxins	<10 EU/mg plasmid, assessed by LAC assay
RNA	Not detectable, on 0.8% agarose gel

Contaminants such as proteins, endotoxins and gDNA need to be evaluated. Thus, a detailed analysis and quantification of these impurities were performed in the samples with best sc pDNA purity and recovery values (table 11), resultants from the best purification strategies.

Table 11 – Proteins, gDNA and endotoxin quantification in injected samples and peaks of interest.

Sample	Protein	gDNA		Endotoxin	
	(µg/mL)	(µg/mL)	(µg/mg of pDNA)	(EU/mL)	(EU/mg of pDNA)
E. coli lysate	79.20	113.73	2.97	2.31	60.28
sc peak (cadaverine column)	Undetectable	8.56	0.51	Undetectable	Undetectable
E. coli lysate	66.20	98.72	1.85	2.50	46.85
sc peak (lysine column)	Undetectable	6.49	0.39	0.23	13.87

The presence of impurities in the pDNA sample for therapeutic studies could led to irreversible secondary effects. The immune reactions related with protein contamination of a biopharmaceutical are involved in neurological and allergic responses or autoimmune diseases. The production of hormones, cytokines and antybodies trigger physiological processes that leads to negative biological effects in humans [112]. Thus, regulatory agencies imposed a non-detectable level of proteins in sc pDNA samples. The measurement of protein levels was done through LAL micro-BCA protein assay kit, as described on chapter 3. Lysate sample from *E. coli* presented moderate levels of proteins, as 79.2 µg/mL and 66.2 µg/mL (table 11), which were removed during the purification processes through affinity chromatography. The purified sc pDNA is in accordance to the requirements of FDA (table 10), once the proteins are undetectable for both samples purified through cadaverine and lysine monoliths (table 11).

Another problem that comes from the use of *E. coli* as host for pDNA production, is the presence of gDNA. The fragments of host genome could encode an oncogene, with possible activation or inactivation on eukaryotic cell genome after an efficient transfection [113]. The possibility of tumor development by the person receiving the treatment, makes this impurity a controlled target by regulatory agencies, and should be present in quantities below of 2 µg/mg of plasmid (table 10). The detection of gDNA in lysate and purified samples was performed through real time PCR. Despite the presence of gDNA with 2.97 µg/mg of pDNA on the lysate sample injected for chromatographic experiments with lysine monolith, this value was reduced for 0.51 µg/mg of pDNA after the purification process (table 11). In relation to the sc pDNA sample purified through cadaverine monolith, the injection lysate had 1.85 µg of gDNA/mg of pDNA, a value reduced to 0.39 µg/mg of pDNA through affinity chromatography. Both purified samples had gDNA in quantities below to the limits applied by FDA.

On the other way, the presence of endotoxins at pDNA vaccines could result in events of fever, systemic inflammation, endotoxin shock, tissue injury and death, due to the extreme activation of immune system [114]. Endotoxins are provided from the cell wall of gram negative bacteria and composed of high negatively charged lipopolysaccharides that confers immunospecificity to the molecule [113, 114]. The measurement of endotoxins was performed through LAL endotoxin assay kit, the most sensitive and widely used method. At cadaverine monolith, the endotoxins level was reduced from 60.28 EU/mg of plasmid at lysate sample to undetectable levels at sc pDNA sample (table 11). Although the levels obtained at purified sample were according to requirements imposed by FDA (table 10) on this monolithic support, the sample purified through lysine monolithic column presents 13.87 EU/mg of plasmid, while the *E.coli* injected lysate had 46.85 EU/mg of plasmid (table 11). The removal of endotoxins contamination on lysine monolith was not so efficient compared to cadaverine monolith, however, the values are closed to those recommended by FDA.

Chapter 5 - Conclusions and future perspectives

Nowadays, millions of people are diagnosed with cancer and most of them die due to viral infections. The inefficiency of some current available treatments together with people resistance to some drugs, increases the necessity to develop new approaches able to reverse the alarming numbers of deaths per year. Actually, pDNA vaccines emerge as a suitable tool for application with high security and efficiency when compared to conventional vaccines. However, the successful use of DNA vaccines on human body requires a purified product in order to reduce adverse effects. Several purification techniques were studied, namely though chromatographic experiments, being that affinity chromatography takes advantage among all by the use of amino acids as ligands. These molecules allow a specific biorecognition by the sc pDNA, the isoform with most biological activity, and takes advantage of biological interactions present between nucleic acids and proteins. Thus, the purpose of this work was the optimization of a chromatographic purification process by exploiting two similar ligands, namely lysine and cadaverine immobilized in monolithic supports, in order to remove bacterial contaminants (such as gDNA, endotoxins, proteins, and other pDNA isoforms) and obtain high purity degree and recovery yield of sc pDNA for biopharmaceutical application.

Preliminary studies were performed for the first time in cadaverine monolithic support, through an increasing linear gradient of NaCl in 10 mM Tris-EDTA buffer, in order to understand the chromatographic behavior of a pDNA pre-purified sample under ionic elution conditions. Despite strong interaction of biomolecules with the support, the low selectivity between pDNA isoforms led to explore different types of buffers in the purification of sc pDNA from the lysate sample. Promising results for pDNA isoforms and RNA separation were obtained through increasing stepwise gradient of NaCl in 10 mM Tris-EDTA or 10 mM Phosphate-EDTA buffers, ph 8.0, while HEPES buffer at pH 7.4 was excluded for further tests because showed similar elution conditions for RNA and pDNA isoforms. Even with the good results for the referred buffers, sc pDNA purification were not totally achieved with the established conditions and more experiments were performed, by exploiting the effect of pH manipulation. Lower pHs increase the positive charges of ligands, leading to a stronger interaction with biomolecules, both for Tris-EDTA and Phosphate-EDTA buffers, which led to easily separation of contaminants from the sc pDNA. After a complete analysis of both buffers at pH 6.0, 7.0 and 8.0, the best result was obtained with an increasing stepwise gradient from 1.05M to 1.5 M of NaCl in 10 mM Tris-EDTA buffer at pH 6.0, with a recovery yield of 98.70% and a purity degree of 97.89% for sc pDNA. These results allowed to carried out these conditions for lysine monolith support. Different conditions were

tested, being optimized an increasing stepwise gradient from 0.45 M to 1 M of NaCl in 10 mM Tris-EDTA buffer to isolate sc pDNA isoform. The results were similar to those obtained for cadaverine monolith, with purification degree of 97,53% and a recovery yield of 96.10%. Tris-EDTA buffer at pH 6.0 showed to be the best condition to use in these supports. Thereafter, recovered samples from both supports at the described conditions were analyzed to confirm the quality of sc pDNA, by assessing impurities such as gDNA, endotoxins and proteins. In general, these parameters presented levels below from those imposed by regulatory agencies to approve a biopharmaceutical product. Summing up, both cadaverine and lysine monolithic supports showed applicability for the efficient purification of sc pDNA, using low salt quantities, which is important for issues related with environmental impact. Although lysine monolith has required low salt concentrations, cadaverine support allowed better purity degree and recovery yield, as well as better efficiency on impurities removal.

However, more studies could be done with these monolithic supports, to understand the possibility of the sc pDNA purification in a preparative scale, namely through dynamic binding capacity tests.

Despite the great progress in the development of DNA vaccines, the achievement of purified sc pDNA in large quantities is not enough. The evaluation of E6 and E7 gene expression through *in vitro* tests is very important to estimate the efficiency of sc pDNA transfection in eukaryotic cells. The success of this study would allow to advance for *in vivo* trials.

Concluding, the monolithic supports immobilized with lysine and cadaverine ligands prove their capacity to overcome the problems related with conventional matrices, due to their advantageous features of mass transfer properties and high binding capacities. The rapid process of purification favors the stability of target molecule and prevent costs related with time consuming experiments.

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