Universidade de Aveiro Departamento de Ciências Biomédicas
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Carolina Freitas Pires

Produção e purificação de pDNA como biofármaco com aplicação em oncologia

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ciências Biomédicas, Ramo de Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Mara Guadalupe Freire Martins, Investigadora Coordenadora com agregação do Departamento de Química da Universidade de Aveiro, da Doutora Ana Margarida Nunes da Mata Pires de Azevedo, Professora Auxiliar do Instituto Superior Técnico da Universidade de Lisboa, e do Doutor Augusto Quaresma Henriques Pedro, Investigador Júnior do Departamento de Química da Universidade de Aveiro.

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o júri

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palavras-chave

Biofármaco, DNA plasmídico, cancro da mama, terapia génica, líquidos iónicos, purificação, sistemas aquosos bifásicos.

Resumo

O presente trabalho reporta a utilização de um processo a montante e a jusante para a produção de ácido desoxirribonucleico plasmídico (ADNp) modelo com potencial aplicação em terapia genética, com especial aplicação no tratamento de cancro de mama. Após a produção, estudaram-se sistemas aquosos bifásicos (SAB) como técnica de purificação e recuperação de ADNp. O SAB composto pelo líquido iónico (LI) brometo de tetrabutilfosfónio ($[P_{4444}]Br$) e o sal citrato de potássio ($C_5H_6K_3O_7 \cdot H_2O$) foi identificado como o sistema mais promissor tendo em vista a sua capacidade para promover o isolamento de ADNp com elevada pureza. O comportamento de partição de ADNp e ácido ribonucleico (ARN) mostrou que para concentrações mais baixas de sal e LI, ambas as classes de ácidos nucleicos exibem uma tendência para serem, preferencialmente, particionadas para a fase inferior do SAB, rica em sal. No entanto, e através da otimização da concentração de ambos os componentes formadores de fase, no ponto de mistura de 25% (m/m) de $[P_{4444}]Br$ e 25% (m/m) de $C_5H_6K_3O_7 \cdot H_2O$, o ADNp foi seletivamente recuperado na fase rica em sal com elevada pureza. O ADNp foi recuperado principalmente na sua isoforma superenrolada, e o conteúdo em proteínas totais foi reduzido em 13% quando comparado com a amostra de lisado bacteriano. Além disso, verificou-se que o ADNp purificado apresenta a capacidade de conferir resistência a células de *Escherichia coli* após a transformação por choque térmico, o que vem reforçar que este é purificado com elevada integridade e numa forma funcional.

De uma forma global, foi desenvolvida uma tecnologia promissora baseada em SABs contendo LIs para a purificação de ADNp a partir de lisados bacterianos, na qual a presença do LI é responsável pelo melhoramento da pureza do ADNp em comparação com alguns sistemas convencionais. Estudos futuros deverão compreender a avaliação da atividade biológica do ADNp purificado, bem como a avaliação dos níveis de endotoxinas, visando a obtenção de ADNp com potencial aplicação em terapia genética.

keywords Biopharmaceuticals, plasmid DNA, breast cancer, gene therapy, ionic liquids, purification, aqueous biphasic systems

abstract This work reports the development of an upstream and downstream process to recover a model plasmid DNA (pDNA) with potential application in gene therapy, particularly targeting breast cancer. After the pDNA production, aqueous biphasic systems (ABS) were investigated to as a recovery and purification technique. The ABS composed of the tetrabutylphospnium bromide ($[P_{4444}]Br$) ionic liquid (IL) and potassium citrate salt ($C_5H_6K_3O_7 \cdot H_2O$) was identified as the most promising system promoting the isolation of pDNA with high purity. The partitioning behaviour of pDNA and ribonucleic acid (RNA) showed that for low concentrations of salt and IL, both classes of nucleic acids exhibit a tendency to be preferentially partitioned toward the bottom phase, mainly enriched in potassium citrate salt. However, and by properly engineering the concentration of both phase-forming components, at the mixture point composed of 25 wt% of $[P_{4444}]Br$ and 25 wt% of $K_3C_6H_5O_7$, pDNA was selectively recovered in the salt-rich phase with high purity. It was observed that pDNA is mostly recovered in its supercoiled isoform, and that the content of total proteins was reduced by 13% in comparison with the bacterial lysate sample. Also, purified pDNA exhibited the ability to confer resistance to *Escherichia coli* cells after heat-shock transformation, further supporting that it is purified with high integrity and functionality.

Overall, a promising IL-based ABS technology for the purification of pDNA from bacterial lysates was developed in this work, in which the presence of IL seems to be responsible for upgrading the pDNA purity over some conventional systems. Envisaging the production of pDNA for gene therapy, future work should comprise the evaluation of the biological activity of purified pDNA and the assessment of endotoxin levels.

Index

List of Tables

List of Figures

Figure 15. Phase diagram of $[P_{4444}]Br/K_3C_6H_5O_7$ system at room temperature (± 22 °C).

Figure 16. Agarose gel electrophoresis of nucleic acids in ABS composed of $[P_{4444}]Br$-$K_3C_6H_5O_7$, at different concentrations, with 15 % of pDNA lysate. [A] and [B] represent, respectively, the effect of IL and salt concentrations. 20 µL of each sample were loaded in the corresponding well. Top and bottom phases and the precipitate are represented as "t", "b" and "p", respectively.

Figure 17. Agarose gel electrophoresis of nucleic acids in ABS composed of PEG 400 (24 wt%), $C_6H_5Na_3O_7$ (18.5 wt%), 10 wt% of pDNA lysate and 47.5 wt% of 10 mM Tris-HCl. 20 µL of each phase were loaded in the corresponding well.

Figure 18. Circular dichroism spectra of pDNA samples resulting from: the isolation using a commercial kit (blue); the alkaline lysis method (M4, orange); the bottom phase of the ABS formed by $[P_{4444}]Br$ (25 wt%)/$K_3C_6H_5O_7$ (25 wt%) system (grey).

List of abbreviations

ABS	Aqueous biphasic systems
AC	Affinity chromatography
AEC	Anion-exchange chromatography
ASOs	Antisense oligonucleotide
BC	Breast cancer
BCA	Bicinchonic acid
BIL	Buffered ionic liquid
BSA	Bovine serum albumin
cDNA	circular DNA
cfu	Colony formation units
CD	Circular dichroism
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EMA	European Medicines Agency
ER	Estrogen receptor
EU	European Union
FDA	Food and Drug Administration
gDNA	genomic DNA
HER2	Human epidermal growth factor 2
HIC	Hydrophobic interaction chromatography
HILs	Hydrophobic ionic liquids
HPV	Human papilloma virus
IEX	Ion exchange
IL	Ionic liquids
ISO	International Organization for Standardization
ISPE	International Society of Pharmaceutical Engineers
LB	Luria Bertani
LC	Liquid chromatography
LLE	Liquid-liquid extraction
ln	Linear
mAbs	monoclonal antibodies
MAM-A	Mammaglobin-A
MILs	Magnetic ionic liquids
oc	open circular
OD	Optical density
pDNA	plasmid DNA
PR	Progesterone receptor
sc	supercoiled
SEC	Size exclusion chromatography
SILs	Supported ionic liquids
siRNA	small interfering ribonucleic acids

THAC	Triple-helix affinity chromatography
TLL	Tie-line length
USA	United States of America
UV-Vis	Ultraviolet-Visible
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

1. Introduction

1.1 Breast Cancer landscape

1.1.1 Incidence, mortality, and epidemiology

Cancer is a complex disease involving several signaling pathways, and despite the rising knowledge of cancer biology, its treatment is still a challenge due to the numerous altered metabolic activities in cancer cells.[1] Cell metabolism can lead to cancer in different scenarios: when nutrients are unregulatedly absorbed and used opportunistically, when metabolite-controlled genetic regulation is altered, or even when metabolites interfere with the microenvironment.[2] According to some studies, the growing susceptibility to cancer in households suggests a genetic basis for the occurrence of malignant diseases.[3] Therefore, it is expected that the incidence of cancer in the population will increase due to the heritability of many cancers, which will be reflected in higher mortality rates.[3,4] Cancer incidence and mortality have been growing among the general population, becoming a global burden for society and the most important barrier against life expectancy worldwide in the 21st century. Figure 1 shows how this disease affected the worldwide population in 2020, with 19.3 million new cases of cancer and 9.9 million deaths. Asia, being the continent with more than a half of the global population, accounts for 49.3% of the total cancer cases and 58.3% of the cancer mortality.[4]

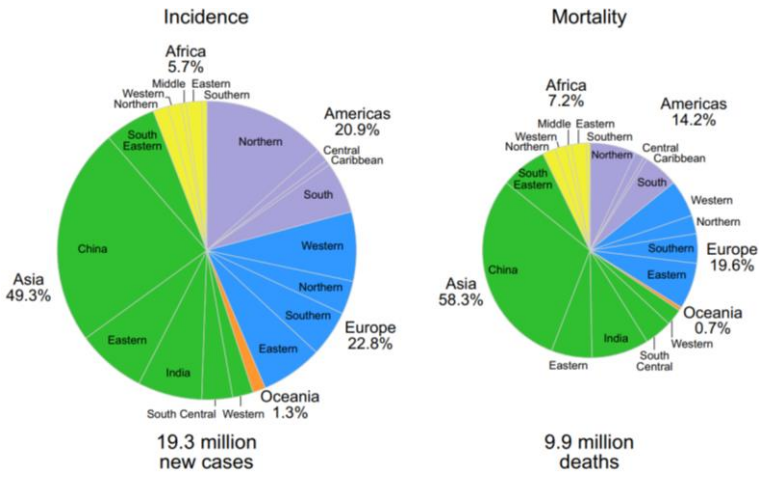


Figure 1. Pie charts representation of the distribution of all-cancer incidence (left) and mortality (right) cases according to world area for both sexes in 2020. Information taken from Bray and co-workers.[4]

Throughout life, the breast tissue undergoes a variety of changes; thus, it is not surprising that the breast cancer (BC) has become the most common malignancy among women.[5] This common female cancer has a high incidence rate around the world, accounting for 2.3 million new cases (11.7%) and 6.9% of deaths of all cancers.[4] According to these data taken from GLOBOCAN[6], there is the need to prioritize the prevention and therapy in order to control cancer with early detection methods and improved alternative treatments. To provide a comprehensive biological aspect of BC, Table 1 lists controllable and uncontrollable risk factors of BC, as well as

preventive measures. Even though some factors are beyond control, like gender and age, there are lifestyle changes that help preventing cancer, namely the adoption of a healthy life based on regular physical activity and healthy eating plans.

Table 1. Factors that influence BC and its prevention. Information taken from the Jordan Breast Cancer Program.[7]

Risk Factors	
Uncontrollable risks factors	**Controllable risks factors**
Female gender	Giving birth (having children decreases incidence)
Age (particularly after 40 years)	No breastfeeding
Genetic factors	Using hormone therapy (for birth control and menopause)
Early menarche (before 12 years)	Eating processed and fatty food
Late menopause (after 55 years)	Lack of regular physical activity
Exposure to radiation	Obesity
Prevention	
Regular physical activity	
Maintain a healthy weight	
Limiting alcohol intake	

1.1.2 Molecular mechanisms involved in cancer onset and progression

BC, the most common malignancy in women worldwide, is classified from a heterozygous population of breast diseases, which develops in tumours and can be of invasive origin if not treated.[5,8] Most BC are sporadic, that is, individuals without a genetic predisposition encounter an aberration in the genome of hereditary cancers. In addition to these defects in inherited genetic material, mutations and epigenetic inactivation from other mechanisms also occur.[5] In the context of non-genetic risk factors, BC can be caused by a woman's personal life, which represents 85% of the cases without inherited mutations from family history.[9] However, it should be emphasized that BC is based on its hereditary susceptibility and sporadic occurrences of its development. All of them have somatic genetic abnormalities, which have been identified in several genes, whose expression can be upregulated.[5,10] Thus, BC is closely related to defects in damaged DNA repair systems or defects in cell cycle checkpoints. This causes the DNA to undergo changes that, when not repaired, increase the susceptibility to this type of cancer due to the genetic defect. Some of the DNA damage response genes, like BRCA1, BRIT-1 and PARP-1, are commonly inactivated through mutation or epigenetic modification.[5] Genetic disorders associated with complex signaling pathways, which allow tumor cells to prevent programmed cell death, can lead to the development of cancer where genetic therapies combined with chemotherapies arise as possible therapeutics.[1]

Gene expression influences the responses of the immune system that play an important role in the remodeling of breast tissue. Immune cells capable of rejecting the tumour, namely T lymphocytes, are not as present in breast cancer as compared to other cancers, and thus the immune system can provide evidence for the progression of BC, being relevant for molecular therapy.[11] A novel biomarker for identifying breast cancer is mammaglobin-A (MAM-A), which is a breast cancer–associated antigen with high tissue specificity and highly expressed in cancer cells.

Considering that this protein is overexpressed in 40% to 80% of primary breast cancers, it is suggested that the development of MAM-A-based vaccine therapies targeting the immune system may be relevant for BC prevention.[12–14] Since it represents a potentially secreted protein, MAM-A can be easily detected in serum, showing a superior clinical utility in relation to other solid tumor markers. Accordingly, it is important to denote that the deregulation of the mammaglobin gene is associated with a specific therapeutic vulnerability of the tumor.[15] Breast cancer is an example of the interaction between the immune system and the tumor in the breast, thus facilitating gene therapy with an introduction of DNA vaccine.[16] In general, MAM-A DNA-based vaccines have demonstrated significant clinical impact in the immune system of patients with BC, highlighting its promising potential outcomes as an effective and safe therapy.[13]

1.1.3 Currently available therapeutic strategies for breast cancer

The area of research for the treatment of cancer has been intensive, in a way that is necessary to investigate what type of gene, proteins or signaling pathways are associated with the development of cancer cells, and thus being relevant to cancer prognosis. The most common treatment for BC is surgery, which is complemented by radiotherapy, chemotherapy and hormone therapy, as presented in Figure 2. However, there are still challenges to overcome in the development of drugs, such as their undesirable side effects, low bioactivity, high dosage and immunogenic response.[17,18]

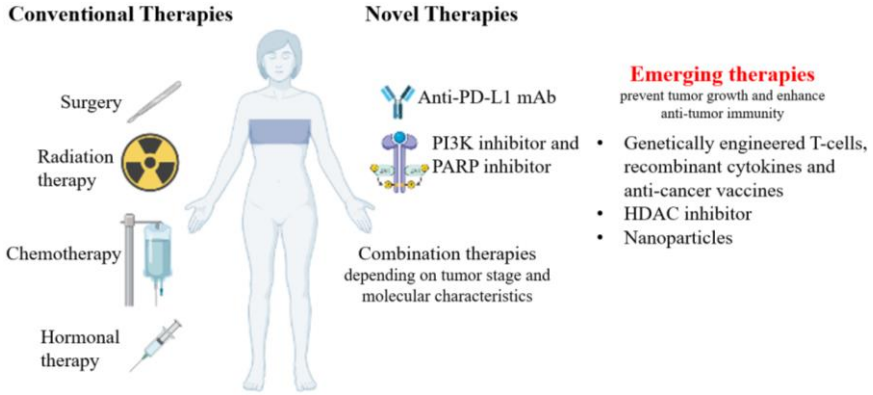


Figure 2. Breast cancer therapies. Adapted from the Qatar Biomedical Research Institute.[19]

In-depth knowledge of the link between cancer cells and the immune system has led to the formulation of biopharmaceuticals through specific targets, such as epigenetically and genetically abnormal molecules, for a more efficient strategy in the treatment of cancer. Among current therapies, biopharmaceuticals such as monoclonal antibodies (mAbs) and non-antibody proteins, coupled with small molecule pharmaceuticals, stand out, allowing an efficient control of the progression of various cancers and significantly improving the overall survival of patients.[17] Biopharmaceuticals have emerged as an alternative treatment being successfully applied in disease control, prevention, and diagnosis, such as the case of vaccination and gene therapy[20], addressed below in section “1.2 Biopharmaceuticals”.

More accurate strategies are linked to specific cancer cell biomarkers for enhanced detection and early classification of breast cancer, which hold great promise in the development of targeted therapies. Some biomarkers have already been discovered as targets for novel drugs, like estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor 2 (HER2) receptor, vascular endothelial growth factor (VEGF) and Ki-67.[18] Traditional biopharmaceuticals have been successfully applied in treating breast cancer, although they do not consider the heterogeneity of individual patient responses. An individualized treatment, such as personalized medicine, would be the most appropriate to overcome certain adverse effects.[5,17] Furthermore, the quality of these drugs requires fundamental clinical efficiency to combat the needs of each treatment, from their manufacture to formulation and delivery.[17]

1.2 Biopharmaceuticals

Biopharmaceuticals are a class of biologic medicinal products, such as proteins or nucleic acids, obtained from biological sources, such as animals (including humans), plants or microorganisms, often involving the technology of recombinant DNA.[20] Unlike chemically synthesized drugs, biopharmaceuticals are structurally complex macromolecules, which can pose challenges in their manufacturing processes. Nonetheless, they are generally regarded as effective therapeutic agents due to their high selectivity, specificity, and low nonspecific toxicity.[20,21]

The first biopharmaceutical ever introduced in the market was human insulin in 1982, with the Humulin® trade name, for therapeutic use in *diabetes mellitus*. This drug was genetically engineered through the expression host *Escherichia coli* (*E. coli*), which is recognized as the most widely used microbial species in the biotechnology industry for large-scale production of proteins, especially non-glycosylated proteins.[22] Later in 1998, the first monoclonal antibody biopharmaceutical, trastuzumab for the treatment of HER2-positive metastatic breast cancer, was approved by the Food and Drug Administration (FDA). This milestone is intimately linked to the discovery of more specific cancer cell biomarkers, whereby enhanced approaches knowing the occurrence, development and prognosis of cancer will originate more effective treatments.[18] Recently, in 2020, the Nobel Prize in Chemistry was awarded to Emmanuelle Charpentier and Jennifer Doudna who discovered the CRISPR/Cas9 genetic scissors for genome editing. This revolutionizing idea starts by cutting a specific part of the DNA sequence with a programmable engineering guide RNA, allowing advances in cellular engineering, personalized medicine and promising clinical therapeutics.[23] Another great achievement was the development of prophylactic messenger RNA vaccines against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which were the first to receive emergency use authorization to combat the COVID-19 pandemic.[24]

Figure 3 represents the evolution of biopharmaceuticals approvals profile since 1989 until 2018, in the European Union (EU) and United States of America (USA). Even though the biopharmaceutical industry is currently dominated by protein-based biopharmaceuticals, products based on nucleic acids (gene therapies, DNA or RNA vaccines, antisense oligonucleotide (ASOs), small interfering RNAs (siRNA), aptamers and modified RNA molecules) are being increasingly used, showing that they can have a high impact and prominent roles in the biopharmaceutical industry.[25]

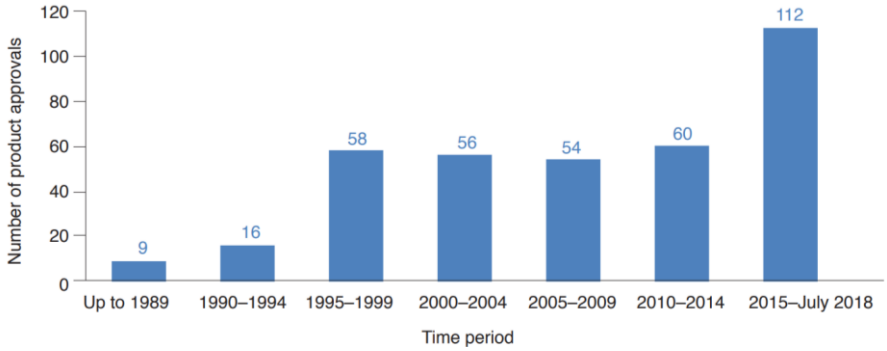


Figure 3. Number of biopharmaceutical approvals in USA and EU region over the indicated periods.[25]

Biopharmaceutical innovation continues to rise in different categories, not being an exception the breakthrough in genetic therapy, which despite has not progressed as much as other classes in the approvals in USA and EU, there is a major interest in their manufacture. Several examples of products based on nucleic acids, its therapeutic indications as well as the approval date are presented in Table 2.[25]

Table 2. Trade name, product and therapeutic indication of nucleic-acid-based biopharmaceuticals and gene therapy products approved by regulatory agencies in EU and USA by the end of July 2018. Information taken from Walsh.[25]

Trade Name (Product)	Description	Therapeutic indication	Approval date/Location
Tegsedi® (inotersen)	A 20-nucleotide single-stranded oligonucleotide manufactured by direct chemical synthesis	Hereditary transthyretin amyloidosis	2018 (EU)
Luxturna® (voretigene neparvovec-rzyl)	A live, nonreplicating adeno-associated virus genetically modified to express the human RPE65 gene	Retinal dystrophy	2017 (USA)
Spinraza® (nusinersen sodium)	An 18-nucleotide antisense oligonucleotide manufactured by direct chemical synthesis	Spinal muscular atrophy	2017 (EU) 2016 (USA)
Exondys 51® (eteplirsen)	Chemically synthesized antisense oligonucleotide	Duchenne muscular dystrophy	2016 (USA)
Imlygic® (talimogene laherparepvec)	Engineered herpes simplex virus type 1 capable of producing GM-CSF	Melanoma	2015 (EU & USA)
Kynamro® (mipomersen sodium)	Chemically synthesized antisense oligonucleotide	Familial hypercholesterolemia	2013 (USA)
Macugen®	Synthetic PEGylated	Neovascular, age-	2006 (EU)

(pegaptanib sodium injection)	oligonucleotide that specifically binds VEGF	related macular degeneration	2004 (USA)

To overcome the cost related to the application of these biopharmaceuticals in the marketplace, an emerging trend is the application of biosimilars. These biopharmaceuticals must accomplish the same clinical significance in terms of safety, purity, and potency of the original products whose patent has expired, being thus obtained at a lower cost.[20] To reach the sales market, this type of medicines must undergo several tests that guarantee their therapeutic equivalence to the brand name drug, becoming an officially approved version.[21] Hence, the major benefit of biosimilars include the increased availability of the therapy that can balance the healthcare expenditure.[26] These therapeutic drugs received an abbreviated drug review and a licensing route in 2010, which was part of the Patient Protection and Accessible Assistance Act. As a result, it is expected that the number of the approval of these blockbusters will increase in the next years, considering that these drugs started to receive regulatory approval by the European Medicines Agency (EMA) in Europe since 2006.[27]

Notably, the breakthroughs of technologies have facilitated the market of biotechnology products that encompass their manufacturing, formulation, and delivery. It is a multibillion-dollar industry due to innovative biopharmaceuticals frequently launched targeting a wide range of diseases.[21,26] Between the years 1989 and 2012, these biotechnology products grew worldwide in sales, coming up with US$163 billion.[21] This can be reflected on the biopharmaceutical registrations and approvals in the USA and the EU, with a total of 212 by the end of 2014.[26] In 2017, only the ten best-selling biopharmaceutical products could generate sales of over $80 billion, representing 44% of the total biopharmaceutical product revenues.[25]

1.2.1 Plasmid DNA-mediated gene therapy

Biopharmaceuticals based on plasmid DNA (pDNA) are considered a useful molecular tool that support gene therapy and DNA vaccination interventions.[28] Gene therapy consists in the alteration of gene expression of the gene of interest in the form of pDNA, allowing the down-regulation or replacement of mutated genes.[1] A plasmid, schematically represented in Figure 4, is a part of the circular DNA (cDNA) that reproduces mainly in bacterial species, and it is helpful in cloning genes, rearranging them,[29] and in the process of producing a great amount of DNA or proteins in the laboratory. When the desired gene is inserted into the plasmid, it can express the gene product in animal cells without further manipulation.[30] Plasmids can occur in different isoforms, namely the supercoiled (sc), open circular (oc) and linear (ln). Normally, plasmids are supercoiled, characterized by automatically occurring in DNA induced by helical stresses, forming twists into the double helix. Both DNA strands are intact and continuous in the sc isoform, but relaxation of the double helix achieved by a nick or break in one or in both strands will originate, respectively, the oc and ln pDNA isoforms. The most biologically active morphologic isoform is the sc pDNA, often preferred for therapeutic applications due to its highest transfection efficiency.[31] The other types of isoforms are seen as impurities since they are less efficient in inducing gene expression than sc plasmids.[32]

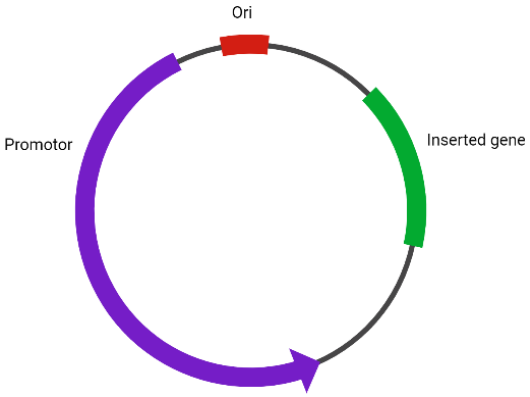


Figure 4. Schematic representation of a pDNA constituted by promotor, ori sequence and the inserted gene of interest.

The beneficial effects of pDNA in therapeutics were first reported in the 1990s, following the discovery that it led to a protective and specific cytotoxic T lymphocyte (CTL) response.[33] Considering the involved mechanism, this technology can be further employed in a wide range of applications, such as infectious diseases and cancer.[34] The implementation of pDNA in human therapeutics foreseeing the production of defective proteins involves different steps, starting with the identification of the gene or genes related to the disease, followed by manufacturing and formulation of recombinant pDNA, ultimately allowing the administration of the therapeutic gene of interest.[35] If we increase the medicinal range of gene therapy applications, it is possible to formulate a valid vaccine. Consequently, the clinical applications of a DNA vaccine can be well structured in order to become viable, and not just by the qualities of the demonstrations in animal models.[30]

Through the application of a pDNA, in the form of a vaccine, it is possible to code antigenic proteins that activate both humoral and cellular immune response with the help of CTL, helper T cells and antibodies.[36] In turn, the dendritic cells, antigen-presenting cells, are efficiently activated by harbouring T cells that recognize the antigens expressed in the tumour. Therefore, the induction of T cell immunity leads to the possibility of developing vaccines against cancer.[37] Also, this type of vaccine will express the tumour-specific antigens and after a long-term period, it will generate memory cells in the immune system. Therefore, DNA vaccines are a reliable and valuable form of antigen-specific immunotherapy, being safer, stable and easily produced, than other types such as protein-based vaccines.[36,38] Furthermore, FDA confirms that DNA vaccines are purified plasmid of bacterial preparations able to fight a pathogen, through an immune response.[35] The overall mechanism behind pDNA vaccination is shown in Figure 5.[34]

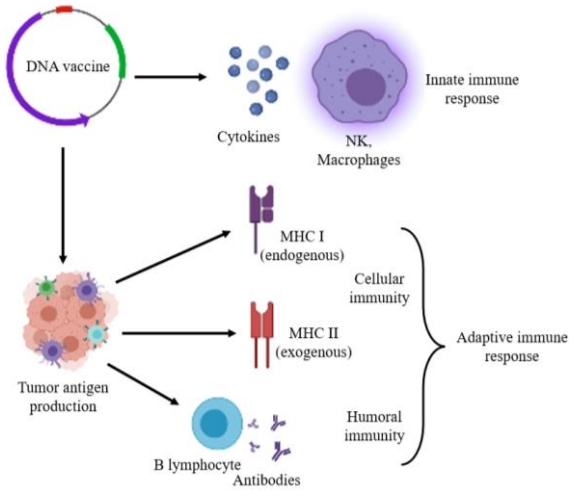


Figure 5. Immune system activation induced by DNA vaccines. Adapted from Lopes et al.[34]

DNA vaccines encode the antigen of interest that whenever expressed in host cells, will stimulate cell-mediated immunity improving the protective effect.[34,39] These biotechnology-based vaccines have brought up new opportunities to prevent many non-infectious diseases, such as cancer, being successfully exemplified by the case of the human papilloma virus (HPV) vaccine which is currently applied in preventing cervical cancers.[20] These noteworthy strategies of genetic engineering contribute to an improved therapeutic potential, and such effectiveness relies on a prolonged duration of action with high targeting, making it more biologically localized.[20,40] Therefore, it is necessary to choose an adequate procedure for efficient purification to preclude the generation of unnecessary variants of pDNA.[32]

Foreseeing the commercialization of these products, they must receive regulatory approval from regulatory agencies, such as the FDA in the USA and EMA in Europe. Alternative procedures established individually on product and process development are subject to an open discussion and approval by the referred entities. To guarantee that the quality of pDNA products meet their specifications, it is necessary to implement analytical methodologies capable of describing the product in its fullness until the end of its processing.[41]

To minimize the impact of the immunogenicity of the vaccine while guaranteeing their efficacy, regulatory agencies have established acceptable levels regarding impurities derived from pDNA manufacturing bioprocesses. Given the guidance of the FDA, it is important to ensure that in the development of vaccine manufacturing there are certain quality criteria to produce a product that is consistently safe, pure, effective, and potent. Specifically, the purity proportion of sc pDNA isoform must be higher than 80%, requiring a final purity of 97% of the final pDNA product.[42,43] A pDNA purification process must be able to isolate the most biologically active isoform (sc pDNA) by eliminating unwanted cellular components that lack the ability to induce an immune and biological response. Hence, as shown in Table 3, established acceptable levels for major contaminants include: levels of genomic DNA (gDNA) lower than 10 ng of gDNA/µg of pDNA; endotoxins should not exceed 0.1 endotoxin unit (EU/µg) of pDNA; host cell proteins and RNA must be undetectable. It is necessary to keep those guidelines in mind, particularly regarding

endotoxins, as they can produce symptoms of toxic shock syndrome, decrease transfection efficiency and exhibit cytotoxic effects.[44]

Table 3. Criteria for prophylactic DNA vaccines given by FDA. Information taken from Sousa et al.[44]

Product	Recommendation
sc pDNA	> 80%
gDNA	< 10 ng of gDNA/µg of pDNA
Endotoxins	< 0.1 EU (endotoxin unit)/µg of pDNA
Host cell proteins and RNA	Undetectable
Final	97%

According to the World Health Organization (WHO) guidelines, pDNA is regarded as a promising molecular strategy for which no serious adverse reactions or major concerns have been reported after administration.[45] In addition, the use of toxic solvents (e.g. chloroform, phenol) as well as molecular biology materials used in plasmid isolation (enzymes derived from animals such as ribonuclease or lysozyme and process leachables), should be avoided or maintained according to the permitted daily exposure (PDE) in pharmaceutical products.[41] That said, several features affecting the efficacy must be considered when designing a DNA vaccine.[38] However, additional information has been accounted for the manufacture, activity and safety of the DNA vaccine in order to enhance the expertise in DNA-based products. The importance of this guidance by the FDA is to ensure consistency in product regulation, making the manufacturing of these products more safe, pure and potent in experimental approaches.[42]

These improved vaccines need a special attention in developing countries, where traditional vaccines are not affordable for everyone.[35] The DNA-based vaccine technology offers many advantages such as high stability; are easier to handle; have a relatively low-cost; are non-infectious; lead to the expression of antigens in their native form and efficiently generate immune cells; provide immunization and long-term immunity, thereby reducing the number of doses of vaccines required; raise both cellular and humoral immunity; are safer, and thus cause less side-effects; and are easier to reproduce for similar systems used for a broad spectrum of diseases.[35,38]

1.2.2 Manufacturing technologies of plasmid DNA biopharmaceuticals

Pharmaceutical companies need to guarantee the conduct for a safely and effective production through the guidance from the International Organization for Standardization (ISO) and from the International Society of Pharmaceutical Engineers (ISPE).[46] The general manufacturing process of biopharmaceuticals, sketched in Figure 6 for pDNA, comprises two main stages, the upstream and downstream processes.[47]

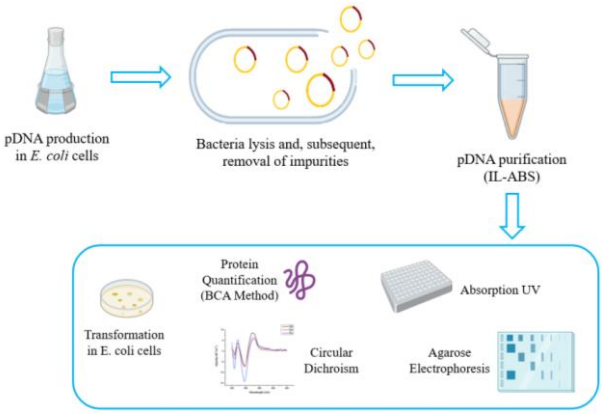


Figure 6. Schematic representation of the experimental procedures performed in this thesis.

The manufacturing of biopharmaceuticals can be divided in the upstream process, involving the fermentation of a microorganism to produce pDNA, followed by the downstream process that comprises the recovery of the plasmid and its subsequent separation from host impurities through purification techniques.[46]

The upstream processing requires the growth of a microbial host, usually the bacterium *E. coli*, and depends on several parameters such as the bacterial strain, culture media, and growth parameters. Reaching ideally established and well-controlled conditions, it is possible to obtain the desired biopharmaceutical by large-scale bioreactors.[48] Depending on the needs of a given product, bioreactor systems are used for bulk production in different hosts. After the successful production of biopharmaceuticals using bioreactors by fermentative processes, the recovery of the cells or the supernatant, depending on the location of the products of interest, can be achieved through centrifugation or membrane filtration.[47] To ensure minimal contaminants while maximizing pDNA purification, it is necessary to consider certain factors such as the type of process (batch, fed batch, perfusion or continuous culture), temperature, pH, oxygen supply control, sterilization of materials and equipment used.

The second stage is the downstream processing, entailing purification procedures often involving highly selective chromatographic techniques. This stage combines several steps to remove all types of impurities, whether they are related to the host cell (host cell proteins, gDNA, among others), to the product itself (aggregates, fragments, cut pDNA species) or due to the type of process used (e.g., buffers, leached ligands, antifoam). Three main stages are usually considered, namely the initial recovery that separates cells from the supernatant, the main purification stage in which removal of most of contaminants is accomplished, and the polishing step that removes the remaining contaminants, ultimately allowing to obtain a highly pure bioproduct.[48]

1.2.2.1 Upstream stage

The upstream process starts by identifying the gene of interest, followed by preparation of recombinant pDNA, and its introduction to the selected host for production. This step implies several considerations about what type of microorganism is most suitable and which culture

conditions are able to achieve the optimal production of pDNA.[48] The selection of the host organism depends on the type and the intended use of the product of interest. The value of microbial systems has been increasing, becoming a well-known and established workhorse organism in the laboratory for biopharmaceuticals production.[49,50] Advances in molecular biology tools and in the recombinant DNA field allowed the heterologous expression using bacterial systems, like *E. coli*, and has become the most used fermentation system. The main advantages of *E. coli* encompass its simpleness, easiness of use, cost-effectiveness, and ability to grow to high cell densities, revealing however some drawbacks related to the presence of endotoxins,[47,48] shown in Table 4.

Table 4. The advantages and disadvantages of *Escherichia coli* as a host for biopharmaceuticals production. Information taken from Baeshen et al.[22] and Walsh.[51]

Advantages	Disadvantages
High DNA yields	Aggregations of heterologous proteins intracellularly
High-density cultures	Lack to perform post-translational modifications (particularly glycosylation) of proteins
Fast growth kinetics	Lipopolysaccharide surface (generation of endotoxins)
Cost-effectiveness	
Easy scale-up process	

During the fermentation process, it is important to ensure a balance between environment and integrity of the biosynthesized pDNA, achieved by uncovering new types of fermentation protocols and media formulation, optimized plasmid backbones and rationally engineered *E. coli* strains. Such modifications can impact the final plasmid quality since operational parameters may compromise its stability and copy number.[52] It was demonstrated by Silva et al.[53] that despite the higher pDNA yields by fermentation, the cell physiology and plasmid stability were compromised. This study points out to the importance of ensuring a proper balance between yield and stability of produced pDNA.[53]

A typical fermentation experiment to produce pDNA was shown by Gonçalves et al.[52] After the transformation of the *E. coli* strain with the desired plasmid, a pre-inoculum culture is prepared on a rich medium supplemented with the appropriated antibiotic (kanamycin). Cells are grown until reaching the early exponential phase – OD_{600} (optical density at 600 nm) ~ 1.5. In batch fermentation, the complex medium is autoclaved in the bioreactor adding glucose and kanamycin separately. Then, the reactor is inoculated with the pre-inoculum envisaging an initial OD_{600} of 0.1. The air vessel provides a flow rate of 1 VVM (volume of gas/volume of medium per min) and dissolved oxygen set-point is controlled at 30 % using an agitation cascade. Fed-batch fermentation starts with the complex medium and glucose. Upon exhaustion of glucose, this operation continues by adding exponentially more nutrient feed. After that, it is possible to calculate the feed rate and, within 24 hours, an increase of the temperature up to 42 ºC is observed. In all fermentations, parameters like pH, necessity of antifoam, the carbon source levels, among others, are controlled.[52] The general pDNA upstream processes is represented in Figure 7.

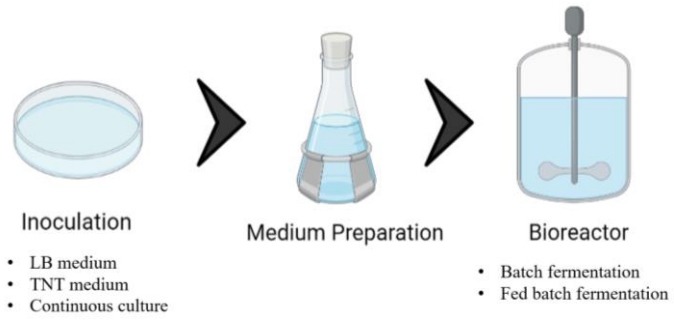


Figure 7. Schematic representation of pDNA production in *E. coli.*

E. coli has become the most popular choice in the field of biopharmaceuticals since its clinical success in the 1980's for manufacturing and commercialization of insulin. In addition to its prominent role for the production of protein-based biopharmaceuticals (more than 60% of approved products are obtained from *E. coli*), this microorganism can be successfully applied in producing bacterial pDNA for application in gene therapy and DNA vaccination.[54] Although *E. coli* provides an important role in the biopharmaceutical industry for host cell engineering, it is necessary to assign the best design of all technological processes to attain high production levels of pDNA of superior quality.[54,55] Therefore, to achieve high amounts of pDNA, it is required to improve the final cell concentration and the average number of copies of the plasmid. For that, it is necessary to obtain a low percentage of the major contaminants in the *E. coli* lysate like RNA, denatured gDNA, proteins, endotoxin (lipopolysaccharide associated with Gram-negative bacteria), and bacterial proteins, among other forms of DNA, that are less effective in purification processes.[41,56,57] Considering the information mentioned above, an optimized upstream process toward high yields of pDNA with minimal amount of impurities will have an impact in subsequent downstream processes, allowing more economical, reliable and simpler purification methods to be developed.[56,57]

1.2.2.2 Downstream stage

The downstream processing of pDNA is initiated after the growth of plasmid-containing cells by bacterial fermentation and comprises all the steps required for recovery and purification of the product of interest. The disintegration of the cell membrane allows the release of all the intracellular components, including pDNA,[51,58] and is usually performed by chemicals, originating a lysate that will need further processing to eliminate contaminants such as cell debris (cell walls, organelles, among others), host cell proteins, gDNA and RNA.[58,59] A schematic representation of different steps of downstream processing of pDNA, as well as techniques commonly used to assess purity and quality of pDNA, is shown in Figure 8.

First described by Birnboim and Doly in 1979,[60] alkaline lysis of plasmid-containing cells is achieved with lysozyme solution and an alkaline sodium dodecyl sulfate (SDS) solution (0.2 N NaOH, 1% SDS). Afterwards, to precipitate chromosomal DNA without interfering with the plasmid, a high salt solution (3 M sodium acetate, pH 4.8) is added, leading to the formation of an insoluble network. The mixture is then subjected to a centrifugation or filtration step that allows the

separation of pDNA from the precipitate enriched in *E. coli* gDNA, host cell proteins and lipid contaminants. Lastly, it is possible to recover the plasmid-containing supernatant that will be further treated with ethanol precipitation envisaging the removal of water-soluble contaminants.[60] Other works by Prazeres et al.[40] and Li et al.[59] have reported additional processing steps using precipitation methods before chromatography. These pre-purification steps employ an initial nucleic acids precipitation step with isopropanol, followed by centrifugation (10,000 × g, during 20 min at 4 ºC); then, the nucleic acids-enriched pellets are treated with 10 mM Tris–HCl (pH 8.0) and ammonium sulfate up to a concentration of 2.5 M, thereby allowing the removal of some RNA impurities and proteins.[40,59] In addition to alcohols (isopropanol and ethanol), the removal of impurities can also be achieved by ammonium acetate, polyethylene glycol or calcium chloride, making size exclusion chromatography and anion-exchange chromatography proper only as a polishing step, being preceded by other high-performance chromatographic step.[62] Ultimately, it is important to maintain a uniform distribution of pH to prevent the denaturation of the plasmid. Also, care should be taken to avoid chromosomal DNA and RNA fragmentation before the generation of the cleared lysate; otherwise, the plasmid would be significantly contaminated.[40,61]

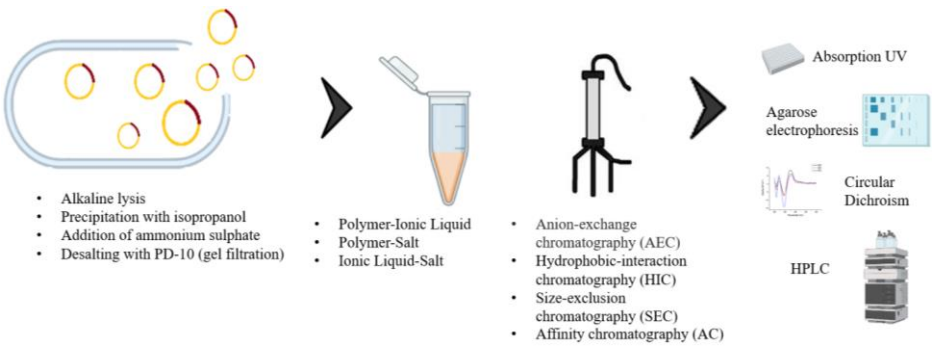


Figure 8. Downstream processing of pDNA encompassing cell disintegration, pDNA recovery processes, capture and polishing purification steps, as well as quality control techniques generally applied to assess pDNA integrity, purity and yield.

The desired plasmid represents only less than 3% (w/w) of the bacterial extract, and after lysis it is surrounded by biomolecules with similar physicochemical properties and structural characteristics. Therefore, the following procedures for eliminating these impurities are considered one of the main issues faced in the purification of pDNA. Over the years, several techniques have been employed in the purification of pDNA, such as chromatography and liquid-liquid extraction (LLE) using aqueous biphasic systems (ABS).[58,62]

Chromatographic purification processes are commonly applied due to their superior selectivity, leading to high purity levels and high yields. The most used techniques are affinity chromatography (AC), ion exchange chromatography (IEX), hydrophobic interaction chromatography (HIC) and gel filtration, also known as size-exclusion chromatography (SEC).[47] Some of those approaches have been studied for the purification of DNA with promising results, either as single-step strategies or included in multi-step purification procedures, listed in Table 5.

Table 5. Overview of chromatographic strategies for DNA purification (NR – Not Reported).

Chromatography types	Biomolecule	Purity	Yield
Anion-exchange chromatography[63]	pDNA	NR	72%
Anion-exchange chromatography[64]	pDNA	97.1%	47%
Hydrophobic interaction chromatography[65] (Two different processes)	pDNA	13.4% -------- 98.8%	91.1% ---------- 68.5%
Hydrophobic interaction chromatography[66]	pDNA	100%	95%
Size-exclusion chromatography[67]	mcDNA	98.1%	66.7%
Triple-helix affinity chromatography[68]	pDNA	NR	50%
Phenyl-boronic acid chromatography[69]	pDNA	2.5 (Purification factor)	93-96%

Anion-exchange chromatography (AEC) uses chemical properties to separate the negatively charged species from the positive charges. Since DNA is a polyanionic molecule negatively charged due to phosphate groups, it binds to resins with positively charged functional groups.[63] Thus, it is expected that the molecules initially eluted are of lower surface charge density, followed by molecules that have high net charge.[70] It is a technique that is less time-consuming, requires sanitization with sodium hydroxide and has a wide option of industrial media. However, a drawback involves the costs associated with this type of chromatography and, most relevantly, the inability of large molecules to penetrate the porous beads.[63] Successful purifications of pDNA was achieved using AEC,[64] in which DNA was displaced from the column by increasing the salt concentration in the elution buffer.[63,70]

Another strategy used for DNA separation is HIC, where the sample normally binds to the column with a high salt limit that will interact with the water molecules, reducing the solvation of the solution, forming hydrophobic regions that, consequently, are absorbed by the HIC column. The sample in the column is eluted by decreasing the salt gradient, because the less salt the more hydrophobic the molecule will be, and as a result, molecules will be eluted by increasing order of hydrophobicity.[71] HIC takes advantage of the high hydrophobicity character of single-stranded pDNA and other nucleic acids impurities with high content in single strands.[66] Separation by HIC includes an equilibration step that prepares the stationary phase by using a salt buffer, usually ammonium sulfate that helps to reduce protein and endotoxin content. Then, after the sample is applied and binding to the column, elution occurs by decreasing the salt concentration for separating the pDNA isoforms. Finally, the regeneration of the stationary phase with NaOH is required for the removal of strongly bound molecules. The absorbance of the plasmid-containing fractions can be measured at 260 nm.[66,72] This strategy has been used for successful purification of DNA vaccines against rabies.[66]

Size-exclusion chromatography (SEC) is a technique that requires column calibration and depends on the macromolecule size or hydrodynamic volume in relation to the average size of the packaging pores.[73] In a SEC column the molecules that elute first are the ones with high molecular weight, followed by those with lower molecular weight over time.[74] Translating to the separation of the pDNA, the larger plasmid is eluted faster than a plasmid with low molecular weight that is

more retained in the column.[31] For this separation, an appropriate stationary phase should be considered for the elution of nucleic acids. Sephacryl S1000 has been reported to be a simple, reproducible and inexpensive stationary phase for obtaining pure pDNA from *E. coli* lysates.[75]

Affinity chromatography (AC) separates different components of the sample according to their biological interactions or individual chemical structure.[62,76] By eliminating additional steps, this method is highly selective, allowing improvements in the yield, and thus assuring quality in therapeutic biomolecules.[62] The preparation of the column and the conditions to be used depend on the molecular and physicochemical properties of the target biomolecule, as well as the thermodynamic nature of interactions. The elution process can be accomplished specifically and non-specifically, respectively, due to addition of a competitive ligand or by changing the composition (ionic strength, pH, among others) of the mobile phase. Noteworthy, modern approaches have enable the use of selective ligands of non-biological origin, which combine a stronger selectivity with high capacity and durability of synthetic systems.[62,76] Strategies involving AC have been described for purifying pDNA, namely based on triple-helix affinity chromatography (THAC)[68] when an immobilized oligonucleotide recognizes specific sequences present on DNA; or protein-DNA[77] where a protein immobilized on the matrix specifically recognizes a DNA motif.[62]

For biopharmaceutical applications, the predominant strategy for purification relies on liquid chromatography, which is a competent high-resolution analysis method.[58] Nevertheless, the main difficulty at this stage is the fact that pDNA shares some features with the contaminants from lysate extracts, and also because of the available chromatographic matrices that have low selectivity towards pDNA. Over the years, several attempts were made to improve the diffusion of these molecules or the ability for binding to chromatographic supports. These complications arise from the size, shape and conformation of the biomolecules, making it difficult to efficiently achieve their separation.[78] That said, non-chromatographic operations, such as membrane-based separations, aqueous biphasic systems (ABS) and precipitation, are being studied as an alternative to the chromatographic operations, given that they are generally simpler and more cost-effective technologies. ABS is an attractive alternative that allows clarification and partial purification in a single stage.[47,79] It is considerably important that these processes consider the balance of regulatory agencies guidance on purity, potency, safety and efficacy.[59]

1.3 Aqueous Biphasic Systems

ABS are a type of LLE, which is formed by mixing two aqueous solutions of different components above certain concentrations. Almost 50 years after being described for the first time (1896),[80] Per-Åke Albertsson in the mid-1950s[81] proposed their use as an alternative technique for the separation of biological macromolecules. After that, several combinations to create ABS, namely polymer-polymer, polymer-salt and ionic liquid (IL)-salt, were described.[82,83] From this point further, several studies demonstrated the potential of ABS for the separation and purification of proteins,[84] antibodies[85] and nucleic acids (pDNA).[86]

ABS possess advantages over conventional water-organic solvent systems for purification,[87] namely the low interfacial tension that contributes to a rapid and efficient mass transfer. The application of ABS for the large-scale recovery of biomolecules offers promising

opportunities in the field of biotechnology because it is a scalable technology, energy-efficient, characterized by simple operations, requires simple apparatus and can hold high biomass load.[83,87] Moreover, it can combine the continuous operation mode with the biocompatibility and environment-friendly features due to the ABS high content of water, being thus particularly suitable for the separation of labile molecules such as nucleic acids. This technique includes other advantages such as the easiness in process integration and low toxicity afforded by the phase-forming components if properly designed.[88]

The phenomenon underlying phase separation relies on the thermodynamic equilibrium and conformation of the compounds, such as polymers and salts, as well as their surroundings causing the interactions in the two phases. The basis of the biomolecule migration is in the selective distribution of substances caused by their intrinsic (*e.g.*, hydrophobicity/hydrophilicity, conformational characteristics) and extrinsic properties (*e.g.*, ionic strength, molecular weight, pH). The manipulation of these parameters makes these systems very powerful in contrast to other separation techniques. Partitioning can be manipulated so that a certain interaction becomes predominant to isolate the target compound in one of the phases, while promoting migration of impurities to the opposite phase. However, the partitioning mechanism is complex and, particularly for large molecules, it is difficult to predict. Currently, there is still no pre-optimized model with the conditions already determined for the purification of a known biomolecule.[89] The most important aspects for this purification method are low interfacial tension and high-water content that preserves the biological activity of biomolecules converting it to a mild method.[83,87,88]

In order to determine the separation of the phases, a phase diagram is constructed, providing information on the volume and composition of each phase. This diagram comprises a curve, the binodal curve, above which a biphasic system is obtained, whereby below this curve a monophasic system is obtained. The general composition is represented on the axes of the respective phase diagram. In the biphasic region, the composition of the mixture is given by the overlapping values in the binodal curve and in the equilibrium tie line, that is, the line that connect the composition values of the two phases in equilibrium. A representation of a phase diagram of ABS is provided in Figure 9.[88] The tie-line length (TLL) provides information on the difference in composition between the coexisting phases, useful to appraise the performance of the system. This parameter also allows to work on the phases' volume to allow the bioproduct concentration.[83]

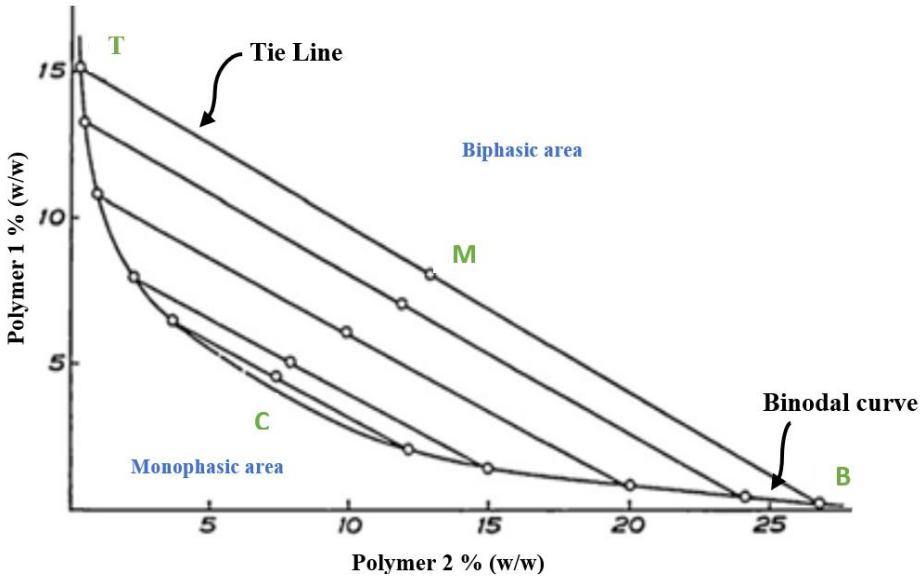


Figure 9. Representation of a hypothetical phase diagram of a system consisting of two polymers. Tie line is described by the composition values of two phases in equilibrium (top phase, T, and bot phase, B). The critical point (C) corresponds to the identical compositions of the two phases. The overall system composition is represented by the M point. Adapted from Asenjo et al.[88]

The most popular system is composed of poly(ethylene glycol) (PEG) and dextran (Dex), allowing and ABS that almost does not depend on pH, salt content and temperature.[90] PEG-Dex systems are composed of two polymers that differ in physical and chemical properties, and therefore define the partitioning of the desired component to the phase to which they have a higher affinity. Although the recurrent success of these polymers, the high-cost associated to dextran is a relevant drawback, which has led to the application of other polymers such as ethylene oxide/propylene oxide copolymers (EOPO),[91] poly(propylene glycol) (PPG),[92] poly(ethylene oxide sulphide) (PEOS)[93], and even biopolymers.[94] Other alternatives include the use of salts, like citrate,[95] phosphate[96] and sulfate[97] in polymer-salt systems. These systems generally exhibit lower costs and lower phase viscosities, making them easier to manipulate on a larger scale with a shorter time for phase separation. The main drawback of these systems has been their low selectivity in the partition of nucleic acids.[87,98] These systems are constituted by a more polar salt-rich phase, the bottom phase, and a less polar (hydrophobic) phase that generally is majorly enriched in PEG, the top phase. The isolation of a bioproduct in one of these phases is achieved by manipulating the type of ions and the consequent ionic strength in the salt phase, by changing the average molecular weight of the polymers or by introducing an additional salt into the system, such as NaCl.[88]

The possibility of recycling and reusing polymers in new stages of purification will allow decreasing costs and the environmental footprint of ABS. To support recycling and the use of low-cost agents, PEG-salt systems were employed due to their low viscosity and, consequently, shorter time for phase separation. The PEG can be reused directly from the final phase to another system with an extension of 50-90%, depending on the nature of the feed. However, to obtain the PEG-phase without impurities, processes such as deproteinization, desalination by ultrafiltration and extraction of PEG from the phase with an organic solvent, followed by evaporation, have been used.[87] In addition to polymers, phosphate and sulfate salts increase the load in wastewater

treatment,[99] which is not advantageous. Therefore, it will be important to use only salts without these associated problems.

ABS can also be applied as a pre-purification step before chromatography, being able to reduce the volume of the process stream as well as the load of contaminants by clarifying, concentrating, and purifying the target biomolecules directly from the crude material. As represented in Table 6, some studies have reported the application of ABS for the partial purification and enrichment of pDNA.[89] It is noteworthy that the highest yields, ranging between 78 % to 100 %, belongs to systems composed of PEG and salt. However, despite the remarkable yields, we cannot conclude about the performance of the systems since most of these works do not address pDNA purity.

Table 6. Representative examples of the application of ABS for the isolation and purification of pDNA (NR – Not reported).

	ABS composition	**Purity**	**Yield**
pDNA[89]	24% (w/w) PEG 400 - 18.5% (w/w) sodium citrate	NR	99%
	26.5% (w/w) PEG 600 - 18% (w/w) sodium citrate		78%
pDNA[98]	15% (w/w) PEG 300, 22% (w/w) dipotassium hydrogen phosphate (25 ºC)	NR	80%
pDNA[100]	35% (w/w) PEG 600 and 6% (w/w) $(NH_4)_2SO_4$	NR	100%
pDNA[86]	75% (w/w) sodium citrate and 25% (w/w) ammonium sulfate	17.2%	91.1%
pDNA[101]	4.5% (w/w) $EO_{50}PO_{50}$, 4.5% (w/w) Dextran T500 and 50 mM Na_2HPO_4 or 2.5% (w/w) $EO_{50}PO_{50}$, 9% (w/w) Dextran T500 and 50 mM Na_2HPO_4	NR	69%

ABS is a well-developed extraction technique capable of promoting the integration of clarification, concentration and partial or total purification of pDNA in a single step. Thus, there is the need to identify more promising and cost-effective ABS for such a purpose.

1.3.1 Ionic liquids

By definition, ionic liquids (ILs) are organic salts with melting temperatures below 100 °C, composed of a large organic cation and a smaller inorganic or organic anions conferring them an asymmetrical ionic structure and lower temperatures of crystallization.[102,103] Figure 10 shows some cations and anions usually found in ILs.

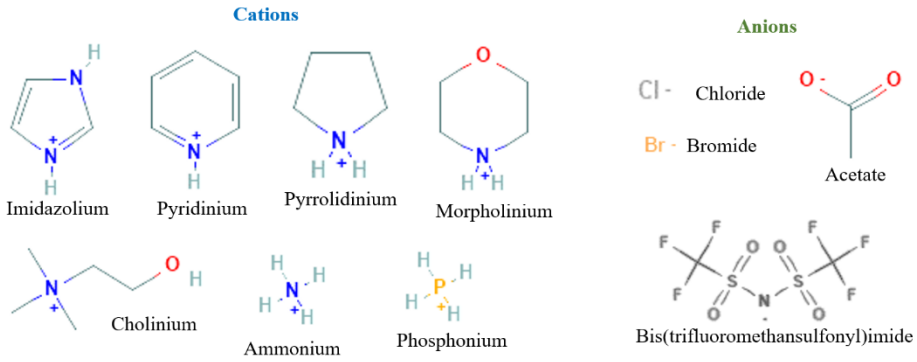


Figure 10. Most common cations and anions that constitute ILs.[104]

Due to their ionic nature, ILs present negligible vapor pressure and non-flammability, being also characterized by high chemical and thermal stabilities. However, one of the most interesting properties of ILs is their tunable character, achieved by the possibility to combine several anions and cations. As a result, physico-chemical properties of ILs can be adjusted to meet the requirements of specific applications.[103] Also, aqueous solutions of ILs exhibit improved solvation performance, being responsible for advanced extractions and separations.[105] As phase-forming components of ABS and if properly engineered, ILs present the ability to tailor the phases' polarities, allowing to overcome the limited polarity range of polymer-based ABS.[103] Furthermore, ILs display a stabilizing role over pDNA,[106] which justifies their application in various methods for the purification of nucleic acids. Several approaches for pDNA extraction include liquid-liquid extraction with magnetic ionic liquids (MILs),[107] buffered ionic liquids (BILs)[108] or hydrophobic ionic liquids (HILs).[109] Solid supports chemically modified with ILs, named supported ionic liquids (SILs), were recently investigated as chromatographic stationary phases for the purification of nucleic acids.[110,111] Nevertheless and although the potential of ABS as a purification tool,[106] IL-based ABS have been scarcely studied for the purification of nucleic acids,[112] and thus will be the target of study in this work.

The application of ILs as phase-forming components of ABS was first reported by Gutowski et al.[113] using 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl) and potassium phosphate (K_3PO_4).[113] The possibility of using ILs in ABS formation was then investigated in purification applications, which is mainly due to their advantages over polymer-polymer-based systems, including low viscosity, quick phase separation and tailored extraction efficiency.[114,115] Up to date, IL-based ABS have been successfully applied for the extraction of a wide range of compounds, including proteins,[116,117] pharmaceuticals[118,119] and nucleic acids.[112]

The chemical structure of ILs exerts an important role with respect to their effect on separations. It is also possible to control the polarities and phase affinities by changing the ions in ABS, whereby ILs act as adjuvants.[120] Due to the low quantity of ILs required in these cases, these processes tend to be more sustainable, and ABS formation is generally easier.[120,121] All these systems require however an optimization of the processes in order to improve the IL-based ABS purification technique.[115] Despite their powerful effects on separation processes, some concerns on IL-salt ABS have been highlighted, particularly by the high concentrations of inorganic salts that may damage the biomolecules being purified, not being a more acceptable option for the environment either.[122] Salts often used are based on phosphates, sulfates and carbonate anions[123],

raising environmental issues. Therefore, less toxic and biodegradable organic salts such as citrate, tartrate and acetate[124,125] have been introduced in IL-based ABS, among others compounds like carbohydrates[126,127] or amino acids.[128,129]

Although the number of publications dealing with IL-based ABS is constantly evolving, the number of reports addressing the reuse of ILs is still low. This shortage of studies on reusability is mainly due to economic and environmental aspects that limit its potential.[130] However and considering the applicability of ILs at an industrial scale, it is necessary to take these aspects into consideration and to guarantee that they are ''green solvents''. Although ILs do not pollute the atmosphere due to their lack of volatility, they manage to contaminate the aquatic environment as most of them are miscible with water. Thus, improved resources are needed for its economically accessible recovery without damaging the environment, solving problems such as biodegradation and toxicity.[131,132] These impacts underscore the importance of recovering, regenerating, removing, and reusing these salts and other phase-forming components, especially the IL as it is usually the most expensive one, requiring additional steps that provide new directions in the formation of ABS with ILs.[130] Over the years, there has been a growing interest in the wide applicability of ILs due to the development of biocompatible ILs, as well as in the development of suitable purification techniques.[133] However, when facing the IL biocompatibility challenge with biomolecules, the specific target application must be considered, leading the environmental requirements by ecotoxicological tests and understanding the potential health hazards complying with certain requirements.[133,134]

In 2012, the work by Huang and Huang[135] claimed a relatively good extraction performance on yeast DNA using the ABS formed by [Bmim][BF_4]-KH_2PO_4, as judging by the improvements achieved in the absorbance ratios at 260 and 280 nm (A_{260}/A_{280}). More recently, Freire's group[112] addressed the extraction of RNA and DNA from a bacterial lysate using biocompatible phase-forming components, namely PPG with a molecular weight of approximately 400 g/mol (PPG 400) and ILs formed by the cholinium cation and different acidic amino acids as anions, namely aspartate, glutamate, arginine, and lysine. The presence of aspartate and glutamate anions revealed to be of critical importance for preserving the integrity of RNA and enhancing their thermal stability. As RNA is preferentially partitioned towards the IL-rich phase in ABS composed of cholinium-based ILs with PPG 400 (20-20% w/w), these systems were proposed by the authors as integrated extraction-preservation methodologies for bacterial RNAs. To reinforce the sustainability of this strategy, it was additionally demonstrated that both phase-forming components could be reused up to three times without negative impacts in the extractive and stabilization performance. Despite the remarkable stabilization and extraction of RNA, its purity is not as high as it should be for some applications.[112] Thereby, further studies on IL-ABS will allow improvements in the purification performance of such systems in terms of different phase-forming components, pH or mixture points.

1.4 Scope and objectives

Cancer has been a rising burden worldwide, being ranked as a leading cause of death and an important barrier in life expectancy. Estimates made by GLOBOCAN 2020 indicated 19.3 million of new cases and 9.9 million deaths caused by cancer worldwide. The most commonly diagnosed cancer is female breast cancer, with 11.7 % of incidence of all cancer occurrences and

6.9 % of mortality.[4] In order to combat these oncological diseases, several strategies using mAbs, antisense oligonucleotides, gene therapy and vaccines have been introduced in the biopharmaceutical field to improve human health.[136]

Mammaglobin-A (MAM-A) is a specific breast protein that is highly overexpressed in breast cancer cell lines and primary breast tumours. This change in expression is restricted to the cells of the mammary epithelium, showing that it may represent an useful and relevant clinical approach marker of breast cancer specific immunotherapy.[14,15] Considering that this protein is overexpressed in 40 % to 80 % of primary breast cancers, it is suggested that the development of MAM-A-based vaccine therapies targeting the immune system may be relevant for BC prevention.[12–14] Indeed, the evolution of pharmaceutical biotechnology has made possible to clone relevant genes in pDNA and express them in human cells. Thus, pDNA has become widely applicable in DNA vaccination and gene therapy strategies in humans. However, considering the envisaged application in human therapeutics, these biopharmaceuticals must be of pharmaceutical-grade and hence obtained free of contaminants.[35]

The adversity of an effective and safe vaccine lies in recognizing an economic method that is capable of meeting the appropriate guidelines of regulatory authorities and that is reproducible on a large scale.[58] The manufacture of pDNA encompasses the upstream and downstream processes, although the main bottlenecks are encountered in the last stage. Due to their high selectivity, chromatography is generally used to separate pDNA from structurally related impurities, demonstrating however some limitations in terms of the low stability of resins, high cost of ligands and time demands, negatively affecting pDNA production.[137,138] There is thus a need for developing innovative purification technologies, within the best quality possible, for the unsettled bottleneck of the pDNA purification improvement. LLE based on ABS, and particularly comprising ILs due to their tailoring ability, may be considered as an alternative to purify pDNA. IL-based ABS have great potential and versatility, complementing the downstream process with a single operation, although they have not been widely studied for nucleic acids purification.

The main objective of this work consists in the development of novel IL-based ABS for the purification of pVAX-GFP pDNA directly from bacterial lysates, aiming to upgrade pDNA yields and purities in a single-purification step. To accomplish this goal, intermediate aims were designed: i) Optimization of the recovery of pDNA from *E. coli* cells using the alkaline lysis method to define the number of processing steps that are required; ii) development and characterization of IL-based ABS for the purification of pDNA from bacterial lysates; iii) assessment of the structural stability and functionality of pDNA purified using the most promising ABS. It should be remarked that although the model pVAX-GFP will be subject of study in this thesis, the developed methodology are planned to be technologically flexible and readily applicable to other targets. Ultimately, the developed technology will be applied to pING-MAM, a pDNA vaccine prototype against mammaglobin-A with potential application in primary breast cancers gene therapy.

2. Materials and Methods

2.1 Materials

Different reagents were used throughout the laboratory experiments: Tris(hydroxymethyl)aminomethane (Tris) was acquired from from Alfa Aesar (Massachusetts, USA), HCl from Acros Organics (Geel, Belgium), agarose and Xpert Green DNA STAIN from Grisp (Porto, Portugal), loading buffer solution from Takara (Shiga, Japan), DNA ladder marker (NZYDNA Ladder III) from NZYTech (Lisbon, Portugal), D-glucose from AnalaR (Poole, England), (Ethylenedinitrilo)tetraacetic acid (EDTA) and potassium acetate from Sigma-Aldrich (Missouri, USA), Sodium Dodecyl Sulfate (SDS) and ammonium sulfate from Panreac (Barcelona, Spain), and acetic acid and isopropanol from Fisher Chemical (New Hampshire, USA).

E. coli cultivation media were prepared using tryptone and yeast extract, both from Thermo Fisher Scientific (Waltham, USA), NaCl and NaOH from Fisher (New Hampshire, USA), agar from Grisp (Porto, Portugal) and kanamycin from Sigma-Aldrich (Missouri, USA).

Polymers, ionic liquids, sugars, and salts were investigated as agents for ABS formation. PEG polymer with molecular weight (MW) of approximately 600 $g \cdot mol^{-1}$ was obtained from Alfa Aesar (Massachusetts, USA). The ILs tetrabutylphosphonium bromide ($[P_{4444}]Br$, 95 % purity), 1-butyl-3-methylimidazolium bromide ($[C_4mim]Br$, 98 % purity), 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$, 99 % purity), and 1-hexyl-3-methylimidazolium chloride ($[C_6mim]Cl$, 98 % purity) were obtained from Iolitec (Heilbronn, Germany). Tetrabutylammonium bromide ($[N_{4444}]Br$, 98 % purity) was obtained from Fluka (Munich, Germany). The chemical structures of ILs investigated in this work are presented in Figure 11.

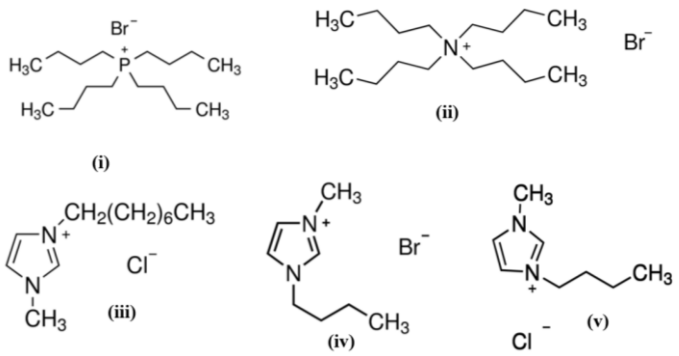


Figure 11. Chemical structures of the ILs investigated in this work: (i) $[P_{4444}]Br$; (ii) $[N_{4444}]Br$; (iii) $[C_6mim]Cl$; (iv) $[C_4mim]Br$; (v) $[C_4mim]Cl$.

The salts used to form ABS were tripotassium phosphate (K_3PO_4, 97 % purity) from Alfa Aesar (Massachusetts, USA) and tri-potassium citrate monohydrate ($K_3C_6H_5O_7.H_2O$, 99 % purity) from Acros Organics (Geel, Belgium). The sugars studied were sorbitol (91.0-100.5 % purity) and D(-)-fructose (98 % purity) obtained, respectively, from Fisher Bioreagents (Leicestershire, UK) and Panreac (Barcelona, Spain).

2.2 Production of pDNA by *E. coli* cultivation

E. coli cultivation was performed in Luria Bertani (LB) medium (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, pH adjusted to 7.0 with 1 N NaOH). After autoclaving the mixture for 25 min at 120 °C, *E. coli* cells were grown in 100 mL shake flasks containing 30 mL LB with 30 µg/ml of antibiotic for about 6 hrs at 37 ºC and 250 rpm in an orbital shaker, until at least an optical density at 600 nm (OD_{600}) of 1 was attained. The cell culture volume required to inoculate 250 mL of LB medium with an initial OD_{600} of 0.2 was determined resorting to equation (1).

$$OD_{600PI} \times V_{PI} = (V_{PI} + V_{Ferm}) \times \text{Initial}OD_{600Ferm} \quad \text{Eq. (1)}$$

where OD_{600PI} and "Initial $OD_{600Ferm}$ stand for the optical density of the pre-inoculum (1) and the optical density at which we want to start fermenting (0.2), while V_{PI} and V_{Ferm} stand for the volume to be removed from pre-inoculum to start fermentation (30 mL) and the volume of fermentation (250 mL), respectively.

The required volume of the pre-inoculum was placed in sterile 15 mL tubes and centrifuged (4200 rpm, 10 min, room temperature). The pellet was added to the 250 mL LB supplemented with 30 µg/ml kanamycin, allowing the cells to grow overnight (15 hours) until the stationary phase ($OD_{600} \approx 3.0$). Finally, the cells were harvested by centrifugation at 6000 rpm (15 min, 4 ºC) with an SLA 3000 rotor in a Sorvall RC 6 centrifuge (Milford, MA, USA) and preserved in a - 20 ºC freezer until use.

2.3 Production of the pDNA lysate by alkaline lysis

The alkaline pDNA lysate was obtained resorting to the usage of 3 stock solutions, namely, P1 (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH adjusted to 8.0 with HCl 1 M), P2 (0.2 N NaOH, 1 % (m/v) SDS) and P3 solution (5 M potassium acetate, 5.75 mL of acetic acid and distilled water to make up 50 mL). The protocol for producing the alkaline lysate includes successive steps intended to promote cell lysis and removal of some impurities, as reported by Diogo et al.[139] Along the entire protocol, different samples named M1-M4, respectively with an increasing number of processing steps were obtained. The first one (M1) only includes an alkaline lysis step; in addition to the alkaline lysis step, sample M2 includes an additional buffer exchange step for 10 mM Tris at pH 8; the third one (M3) includes the alkaline lysis and an isopropanol/ethanol precipitation step; finally, sample M4 includes the alkaline lysis, precipitation with isopropanol/ethanol and ammonium sulfate and a desalting step with PD-10 desalting columns (GE Healthcare, Uppsala, Sweden). These samples (M1 to M4) were analysed by agarose gel electrophoresis, and the most promising samples were then tested in ABS purification assays. These assays allowed to identify how many processing steps are required, and consequently, what is the requirement in terms of purity of the initial lysate sample suitable for the downstream purification by ABS.

The pellet resulting from 250 mL of cells was resuspended in 8 mL of P1 solution by vortex mixing. The total volume was divided into smaller centrifuge tubes (2 mL tubes). In each tube, 400 µL of P2 solution (a total of 8 mL) was added to perform alkaline lysis followed by

gentle homogenization. After an incubation at room temperature for 10 min, 400 µL of P3 solution was added in each tube to stop lysis. Following a gentle homogenization and an incubation of 10 min on ice, the neutralized alkaline lysate was centrifuged in an SS-34 rotor at 13000 rpm and 4ºC for 30 min to remove cell debris and part of genomic DNA and host cell proteins. The supernatant was placed into new tubes and centrifuged again in the same conditions. Until further processing, the resulting pDNA-containing lysate (≈ 24 mL with pH 5.0) must be stored at - 20 ºC. Sample M1 ends in this step, being conserved at – 80 ºC until use. Sample M2 additionally undergoes a buffer exchange step using Vivaspin concentrators (10000 MWCO, Sartorius, Gottingen, Germany), in other words, the DNA lysis buffer is replaced by 10 mM Tris-HCl pH 8.

The pDNA lysates were transferred into a conical tube, 8.4 mL of isopropanol (100% v/v) were added, and the mixture was carefully homogenized. For the precipitation of all nucleic acids (pDNA, RNA and traces of gDNA), the tubes were left to rest in ice for 2 h. The mixture was divided into smaller tubes and centrifuged at 13000 rpm for 30 min at 4 ºC. The supernatant was discharged, the pellet washed with 100 µL of ethanol 70 % (v/v) and centrifuged again in the same conditions. After removing all traces of ethanol, the plasmid containing pellet was resuspended in 100 µL of Tris-HCl (10 mM, pH 8.0) in each microcentrifuge tube, being this sample termed M3. Finally, further processing of the sample included the addition of 2.5 M solid ammonium sulfate ($(NH_4)_2SO_4$). After 15 min of incubation on ice, precipitated proteins and RNA were removed by centrifugation in the conditions described above. The pDNA-containing supernatant was then placed into a new microcentrifuge tube (≈ 1 mL). The sample was subsequently desalted to remove ammonium sulfate resorting to a PD-10 desalting column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions, and using 10 mM Tris-HCl buffer (pH 8.0) as the elution buffer. Briefly, the column was initially washed with 15 mL of distilled water and equilibrated with 20 mL of 10 mM Tris-HCl at pH 8. Afterwards, 1 mL of the sample was injected and then, 10 mL of Tris-HCl was added to the column. Fractions of 1 mL were collected (10 mL in total) and pooled according to the elution order. Lastly, the column was washed with 30 mL of distilled water and conserved in ethanol 20 % (v/v). The sample obtained after desalting is named M4. All samples M1-M4 were stored at - 80 °C until analysis by agarose electrophoresis.

For comparison purposes, pDNA was isolated using a commercial kit (NzyMiniprep, Nzytech, Lisbon, Portugal) according to the manufacturer's instructions.

2.4. Aqueous Biphasic Systems

2.4.1 Phase Diagrams

The binodal curve of each phase diagram was determined through the cloud point titration method at 25 °C (± 1 °C) and atmospheric pressure. The repetitive drop-by-drop addition of the aqueous salt or carbohydrate solution to each polymer or IL solution was carried out until the detection of a cloudy and biphasic solution, followed by the drop-by-drop addition of distilled water until the detection of a monophasic region.[103] In this way, the binodal curve is obtained with the fractions read from the weight mass at the cloud point, being, subsequently, identified the monophasic and biphasic regions.

2.4.2 ABS preparation

The systems carried out in this work were established by using mixtures of polymer-salt and IL-salt aqueous solutions. Each ABS was formulated with specific mixture points in the biphasic region of the phase diagrams and prepared after recovering the pDNA from the bacteria. The systems were prepared by mixing suitable amounts of 10 mM Tris-HCl at pH 8, PEG (different MW at different concentrations) or IL, and salt or sugar in 1.5 mL microcentrifuge tubes, followed by the addition of sample containing pDNA to a total weight of 1 g in the system.

Unless otherwise stated, all ABS components as well as the respective samples were weighed, and in order to achieve equilibrium and guaranteeing the complete separation of the phases, the systems were incubated for 5 min by tube inversion at room temperature (circa 22 °C). Then, the systems were centrifuged at 5000 × g for another 5 min at room temperature to accelerate phase separation. The phases were properly separated using a 2 mL syringe, and the resulting top phase, bottom phase and precipitate phase were weighed. The top phase corresponding to the IL-rich phase or polymer-rich phase, in IL-salt or polymer-salt systems, respectively, was submitted to precipitation with ethanol 96 % (ratio 1:4, incubation at - 20 °C for at least 2 h). Afterwards, the samples were centrifuged at 13 000 rpm for 30 min at 4 ºC and re-suspended with 50 µL of 10 mM Tris-HCl. The bottom phase corresponding to the salt phase in both IL-salt and polymer-salt systems, was desalted and concentrated using Vivaspin concentrators (10000 MWCO, Sartorius, Gottingen, Germany). The sample was concentrated to the volume around 50 - 100 µL using Tris 10 mM pH 8. In case of three-phase partitioning, the precipitate was isolated after removal of top and bottom phases and resuspended in 50 µL of 10 mM of Tris-HCl (pH 8). Finally, the top, bottom and precipitated phases were stored at - 80 ºC for further analysis by agarose gel electrophoresis.

2.4.3 PEG-based systems

PEG with MW of 600 $g \cdot mol^{-1}$(PEG 600) was investigated for ABS formation. Along with the use of PEG, the studied ABS were established by using tripotassium phosphate (K_3PO_4, 97 % purity) and tri-potassium citrate monohydrate ($K_3C_6H_5O_7.H_2O$, 99 % purity). These components were added according to the proportions demonstrated in Table 7.

Table 7. Types of PEG-salt systems investigated in this work and the corresponding concentration of each phase-forming component.

Polymer (wt%)	Salt (wt%)
25% PEG 600	15% K_3PO_4
30% PEG 600	15% $K_3C_6H_5O_7.H_2O$

2.4.4 IL-based systems

For the preparation of the IL-based ABS, the ILs studied were [P_{4444}]Br (95 % purity), [C_4mim]Br (98 % purity), [C_4mim]Cl (99 % purity), [C_6mim]Cl (98 % purity), and [N_{4444}]Br (98 %

purity). The purity was given by the suppliers and their chemical structures are shown in Figure 12. IL-based ABS were formed in the presence of salts such as K_3PO_4 (97 % purity) and $K_3C_6H_5O_7.H_2O$ (99 % purity) and/or sugars like sorbitol (91.0-100.5 % purity) and D(-)-fructose (98 % purity). The concentration in IL-salt systems was 25 % for the IL and 15 % for salts, while with sugars it was 30 % for IL and 40 % for sugars, as summarized in Table 8.

Table 8. Types of IL-salt/sugar systems investigated in this work and the corresponding concentration of each phase-forming component.

Ionic Liquid (wt%)	Salt/Sugar (wt%)
25% $[P_{4444}]Br$	15% $K_3C_6H_5O_7.H_2O$
30% $[P_{4444}]Br$	40% Frutose
30% $[P_{4444}]Br$	40% Sorbitol
25% $[C_4mim]Br$	15% K_3PO_4
25% $[C_4mim]Cl$	15% K_3PO_4
25% $[C_6mim]Cl$	15% K_3PO_4
25% $[N_{4444}]Br$	15% K_3PO_4

2.5 Quality control of nucleic acids

2.5.1 Agarose Gel Electrophoresis

The integrity, identification and purity of nucleic acids were evaluated by horizontal gel electrophoresis in 1 % agarose gels in the presence of DNA ladder marker (NZYDNA Ladder III, Nzytech, Lisbon, Portugal). Gels were run at 120 V for 45 min with 1x TAE buffer in the presence of 1.4 µL of Xpert Green DNA STAIN. 10x TAE stock solution was prepared using 2 M Tris base, 0.5 M EDTA, 1M glacial acetic acid at a pH of 8.0. Samples were mixed with a DNA 10x loading buffer solution with a ratio of 10:1, for better visualization of the run.

2.5.2 DNA Quantification and Purity

After identifying the nucleic acids on an agarose gel, the quantity and purity of DNA were evaluated, respectively, using Equations (2) and (3).[140] Absorbance was measured in a 96-well microplate reader (Synergy HT, Biotek, USA) at different wavelengths in the ultraviolet region of the spectra.

$$[DNA] = (A_{260} - A_{320}) \times F_{dil} \times 50\ (\mu g/mL) \quad \text{Eq. (2)}$$

$$\text{Purity ratio} = (A_{260} - A_{320}) / (A_{280} - A_{320}) \quad \text{Eq. (3)}$$

where "A_{260}", "A_{280}" and "A_{320}" refers to the absorbance at 260 nm, 280 nm and 320 nm, respectively. "F_{dil}" represents the dilution factor associated to the sample analysed. According to Gallagher and Desjardins[141] the purity ratio within 1.8 to 2.0 indicates that the DNA solutions have high purity. Absorbance at 260 nm is quantitative for where the DNA absorbs light most strongly

and at 280 nm indicates the peak absorption of proteins. Absorbance at 320 nm reflects other types of contaminants, particularly associated with dirty cuvettes or particles in solution.[141]

2.5.3 Total Protein Quantification

Considering that the agarose electrophoretic analysis can only verify the presence of nucleic acids, the content of the total proteins was determined resorting to the BCA (bicinchonic acid) assay from ThermoFisher Scientific (Waltham, USA). Using bovine serum albumin (BSA) as the standard, a calibration curve was prepared following the kit protocol recommendations and within the working range of 5 - 250 µg/mL. A total of 100 µL of each standard or sample were added to 2.0 mL of BCA reagent and incubated for 30 min at 60 °C. Afterwards, samples were cooled down for 10 min and then the absorbance was measured at 562 nm.

2.5.4 Circular Dichroism

After developing a purification method, it is important to determine if it affects the stability and integrity of the target biomolecule. In order to evaluate the conformational structure and stability of pDNA, circular dichroism (CD) analysis were performed as previously described.[106] Briefly, the samples were measured at a constant temperature of 25 °C using a scanning speed of 100 $nm \cdot min^{-1}$, with a response time of 4 s over wavelengths ranging from 210 to 300 nm. The recording bandwidth was of 1 nm with a step size of 0.5 nm using a quartz cell with an optical path length of 1 mm. Three scans were averaged per spectrum to improve the signal-to-noise ratio. Measurements were performed under a constant nitrogen flow, which was used to purge the ozone generated by the light source of the instrument.

2.5.5 Determination of *E. coli* transformation efficiency using purified pDNA

After the purification process, transformation efficiency of purified pDNA was determined to evaluate if the purified pDNA maintained its integrity and structural stability, and hence, if it can confer antibiotic resistance to bacterial cells. pDNA purified using the most promising strategy was used to transform *E. coli* cells (Nzy5α, Nzytech, Lisbon, Portugal) by the heat-shock method. To this end, purified plasmid (0.005 µg) was added to 100 µL of chemically competent cells, the suspension was left on ice for 20 min, and the heat shock was performed at 42 °C during 45 s. After the heat shock, 800 µL of LB medium was added to the transformation mixture and it was incubated at 30 °C, 250 rpm for 2 h under aerobic-dark conditions, to allow the expression of antibiotic resistance gene. Different volumes of this culture were then plated on LB agar plates containing kanamycin (30 µg/mL) and incubated aerobically at 37 °C for 24 h until the appearance of colonies.[142] A control experiment using pDNA isolated using a commercial kit was also performed. The transformation efficiency (TE) was calculated by the following Equation (4).[143]

$$TE = \text{Colony number} / \text{Transformed DNA} / \text{Dilution} \qquad \text{Eq. (4)}$$

where colony number refers to the number of colonies counted in the agar plates, the "transformed DNA" represents the amount of DNA transformed expressed in µg and "Dilution" is the total dilution of the DNA before plating.

3. Results and Discussion

Considering that the downstream processing is the main limiting step in biopharmaceuticals manufacturing, the main goal of this MSc thesis was the development of more sustainable and cost-effective pDNA purification methods. To accomplish this goal, ABS were implemented with the purpose of improving the purity and decreasing the costs behind the purification processes. Overall, pDNA was produced by *E. coli* fermentation, and then subjected to alkaline lysis using different processing steps aiming its recovery from the microorganism. The isolation and purification of pDNA from the resulting bacterial lysate was then investigated by ABS, where different phase-forming components were studied. Lastly, quality control of pDNA purified using the most promising strategy was carried out through CD, by the determination of *E. coli* transformation efficiency, and the assessment of the total protein content.

3.1 Production and recovery of pDNA

The production of pDNA was successfully achieved by fermentation of *E. coli* cells in LB medium supplemented with kanamycin in shake-flasks. Subsequently, the next steps of this work progressed towards recovering the plasmid by the alkaline lysis method. Thus, one of the initial objectives of this work was to achieve an efficient procedure for the extraction of pDNA from bacterial cells. First described by Bimboim and Doly,[60] the alkaline lysis method has been proved to be highly efficient in the recovery of pDNA, and was therefore selected in this work. This method consists of a selective denaturation of high MW chromosomal DNA (cDNA) at alkaline pH while scDNA remains double-stranded. Moreover, it is quite simple and allows the recovery of pDNA without denaturation within a pH range of about 12.0 - 12.5, and some low MW of RNA.

As described above (see section "2.3 Production of pDNA lysate by alkaline lysis"), four different samples (M1-M4) with different processing steps were evaluated in the first stage of the downstream process of pDNA, namely by the recovery of pDNA. Sample M1 was obtained by processing *E. coli* cells containing pDNA. These cells are completely disrupted through the introduction of an alkaline solution to denature and renature the mass of cDNA to form an insoluble network. Simultaneously, the three majors contaminating macromolecules were co-precipitated and removed by centrifugation, creating a supernatant with the pDNA and residual low MW RNA. As shown in the agarose gel electrophoresis given in Figure 12, the typical profile of the bacterial lysate sample obtained from *E. coli* includes two well-defined bands, a high MW band (circa 3000 bp) that corresponds to pVAX-GFP, and a low MW band (below 400 bp) corresponding to low MW (LMW) RNA. Specifically, it demonstrates that with the M1 sample, a faint band corresponding to pDNA was obtained while the band corresponding to LMW RNA was not well-defined. This pattern could be caused due to interferences caused by the lysis buffer and other intrinsic contaminants on the electrophoretic run. To avoid the existing problems observed with the M1 sample, particularly with the influence of sample buffer components on electrophoresis, sample M1 was subjected to a buffer exchange step to 10 mM Tris-HCl pH 8, obtaining the M2 sample. However, as shown in Figure 12, these problems persisted as the amount of both pDNA and LMW RNA are low, and the definition of RNA band was not improved. As such, new strategies were formulated which involved further processing steps, resulting in samples

denominated by M3 and M4. All these samples were also analysed by agarose gel electrophoresis, given in Figure 12.

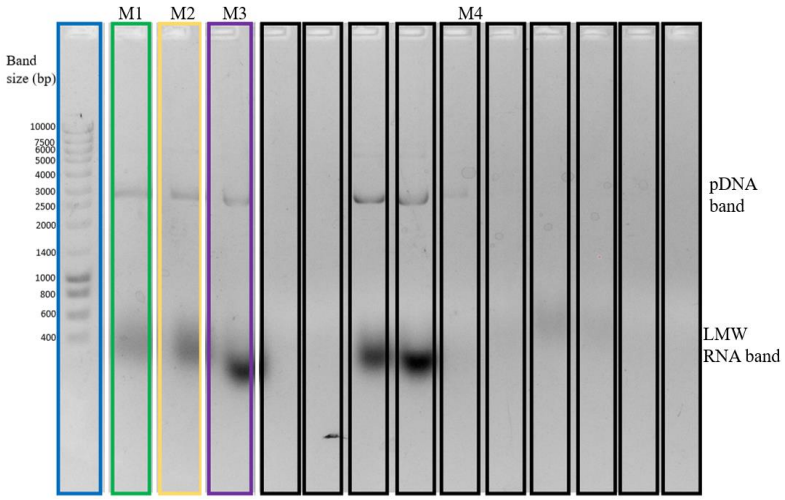


Figure 12. Agarose gel electrophoresis of representative samples (M1-M4) of the alkaline lysis method with different processing steps. Blue: DNA ladder III marker; Green: Alkaline lysis (M1); Yellow: Alkaline lysis with buffer exchange to 10 mM Tris pH 8 buffer (M2); Purple: Alkaline lysis and isopropanol/ethanol precipitation (M3); Black: Alkaline lysis, alcohol precipitation, ammonium sulfate precipitation and desalting, resulting in 10 fractions from the column PD-10 (M4).

Despite no significant results being found when changing the buffer (M2, yellow), an improvement of the intensity of the bands was observed in M3 (purple). This result indicates that the addition of isopropanol/ethanol seems to improve the sample quality by removing some impurities. Nevertheless, the observed results can be further improved in the sample M4 (black), achieved by preforming a precipitation with both isopropanol/ethanol and ammonium sulfate, followed by elution in the PD-10 column (lane 7 and 8) for ammonium sulfate removal. For every 10 mL eluted in PD-10 column it was possible to confirm that nucleic acids appear from the third milliliter onwards. After elution of different fractions from the PD-10 column, these are analyzed and pooled together, concentrating them to be treated as a single sample. With the increment of the elution volume, the pDNA concentration of the aliquots starts to diminish. According to the molecular weight marker it can be stated that the pDNA band stops running between 2500 bp and 3000 bp and the RNA band around 400 bp. In this way, the bands of pDNA and RNA appear better when subjected to the precipitation and washing steps with alcohols, that is, in sample M4.

The result from a pDNA-containing lysate volume of 50 mL followed the standard of lanes in black in Figure 12. In addition to this volume, we had another working volume of larger calibre bacterial lysate (250 mL). Despite the differences in the starting volume of cell culture, they displayed the same elution profile, obtaining similar results (see Annex A).

Beyond electrophoresis, DNA concentrations and purity of all samples present in Figure 12 were evaluated through absorbance measurements, presented in Figure 13, providing insight of the sample quality and its suitability. When the A_{260}/A_{280} ratio range is between 1.8 to 2.0 it is

indicative of a pure dsDNA sample (which is the case with pDNA). The reduction of the ratio means that the sample is contaminated by proteins (detection peak at 280 nm) and an increased ratio is due to RNA contamination (2.1 means pure RNA ratio).

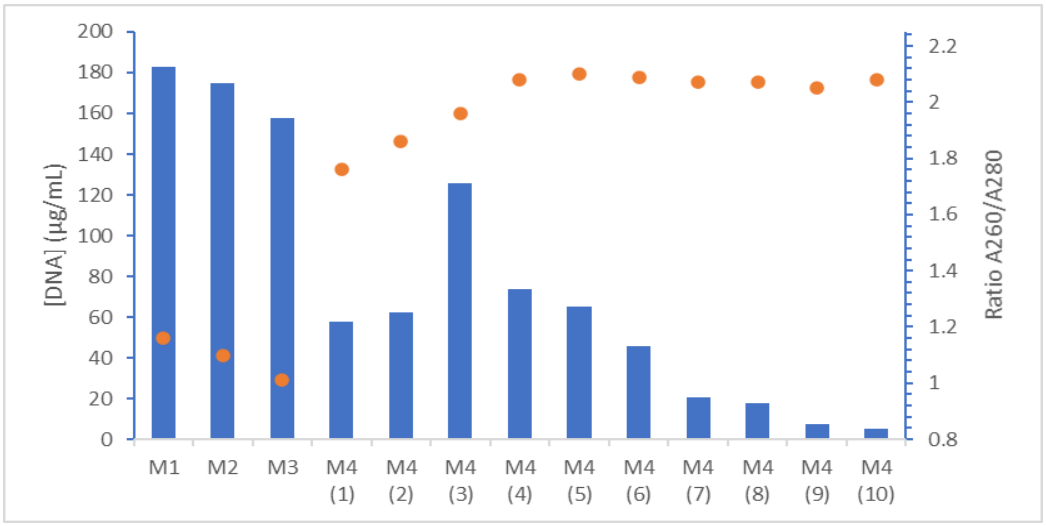


Figure 13. DNA quantification (bars, blue) and purity (dots, orange) of samples resulting from the application of alkaline lysis with different processing steps (M1, M2, M3 and M4) to the cell pellet from a 50 mL culture volume of *E. coli*.

Even though the A_{260}/A_{280} ratios should be interpreted with caution and complemented with additional measurements (qualitative such as agarose electrophoresis, or quantitative such as analytical chromatography[144]), they provide insights regarding the type of nucleic acid (dsDNA or RNA) present as well as a rough indication of purity. Considering the data provided in Figure 13, the samples M1, M2 and M3 possess a high concentration of DNA but present a very low purity ratio which possibly indicates a set of contaminants, mainly proteins. Nevertheless, the purity ratio in the sample M4, namely in samples 3 and 4 with higher content in nucleic acids, was improved and can be explained by the extra step with ammonium sulfate precipitation, allowing the removal of many proteins. Nonetheless, it can be seen some RNA contamination in the respective samples in Figure 12. As for the identification of alcohols (ethanol and isopropanol), buffer components (Tris) and other contaminants (such as lipopolysaccharides), they are not significantly detectable by the UV-Vis light of the spectrophotometer.[145]

As mentioned above, molecular therapies require the use of large amounts of pDNA, so it is necessary to have an efficient large-scale purification process.[40] The information inherent to improve DNA isolation can be met using processes with two-phase systems. According to the nucleic acid profile in agarose electrophoresis and data given in Figure 12, it was decided to select M3 and M4 samples for ABS partition studies.

3.2 Partition of pDNA in ABS

The mandatory guidelines for biological product intended for human usage requires the removal of impurities while maintaining the biological activity of the target product. Meanwhile, the number of overall process steps should also be kept to a minimum to reducing the operation costs.[40] Considering this information, the following steps involved the application of ABS using polymers, ILs, salts and sugars for the selective partition of pDNA.

3.2.1 Screening of ABS phase-forming agents for pDNA purification

In an initial stage, the ABS explored were composed of IL-salt since they have been proved to be useful in separation process for biotherapeutics, like nucleic acids.[112] Depending on the ABS, these systems may be considered a novel and sustainable way to purify biomolecules for the described unique properties in this thesis, benefiting from the economical and environment aspects.[131,132] ILs, due to their tunable character, can comprise a wide variety of different cations/anions and have their physicochemical properties adjusted to improve separations.[102,103] As a result and due to the ability to control such characteristics of ILs, numerous options for pDNA isolation can be developed.

To evaluate the effect of the bacterial lysate sample on the ABS purification performance, samples M3 and M4 were investigated. To accelerate phase separation of the systems, a centrifugation step (5 min at 5 000 g, room temperature) was performed. Then, the top phase microcentrifuge tube (1.5 mL) was filled with ethanol 96 %, incubated at least 2 h at - 20 ºC, and the pellets were obtained by centrifugation at 10 000 rpm for 15 min at 4 ºC and re-suspended with 50 µL of 10 mM Tris-HCl. The bottom phase was concentrated in Vivaspin concentrators, that needed a washing step first and then introduced the bottom phase together with 5 mL of 10 mM of Tris-HCl. The sample was removed when the volume is around 100 µL and, finally, washed again. The precipitated phase was resuspended in 50 µL of 10 mM of Tris-HCl. For these two samples studied (M3 and M4), two types of systems were evaluated, one consisting of IL-salt (25 wt% - 15 wt%, respectively) and the other of IL-sugar (30 wt% - 40 wt%, respectively). Such systems were prepared to demonstrate the partition of DNA in ABS with different phase-forming components with highly different salting-out ability, which will help us to understand which of the ABS will be the most advantageous for the proposed goal. The combinations studied were $[P_{4444}]Br$ – $K_3C_6H_5O_7$; $[C_6mim]Cl$ – K_3PO_4; $[C_4mim]Cl$ – K_3PO_4; $[C_4mim]Br$ – K_3PO_4, and $[P_{4444}]Br$ – Fructose; and $[P_{4444}]Br$ – Sorbitol. The partition behaviour of DNA in such ABS is represented in Figure 14 [A] for the sample M3 and Figure 14 [B] for the sample M4.

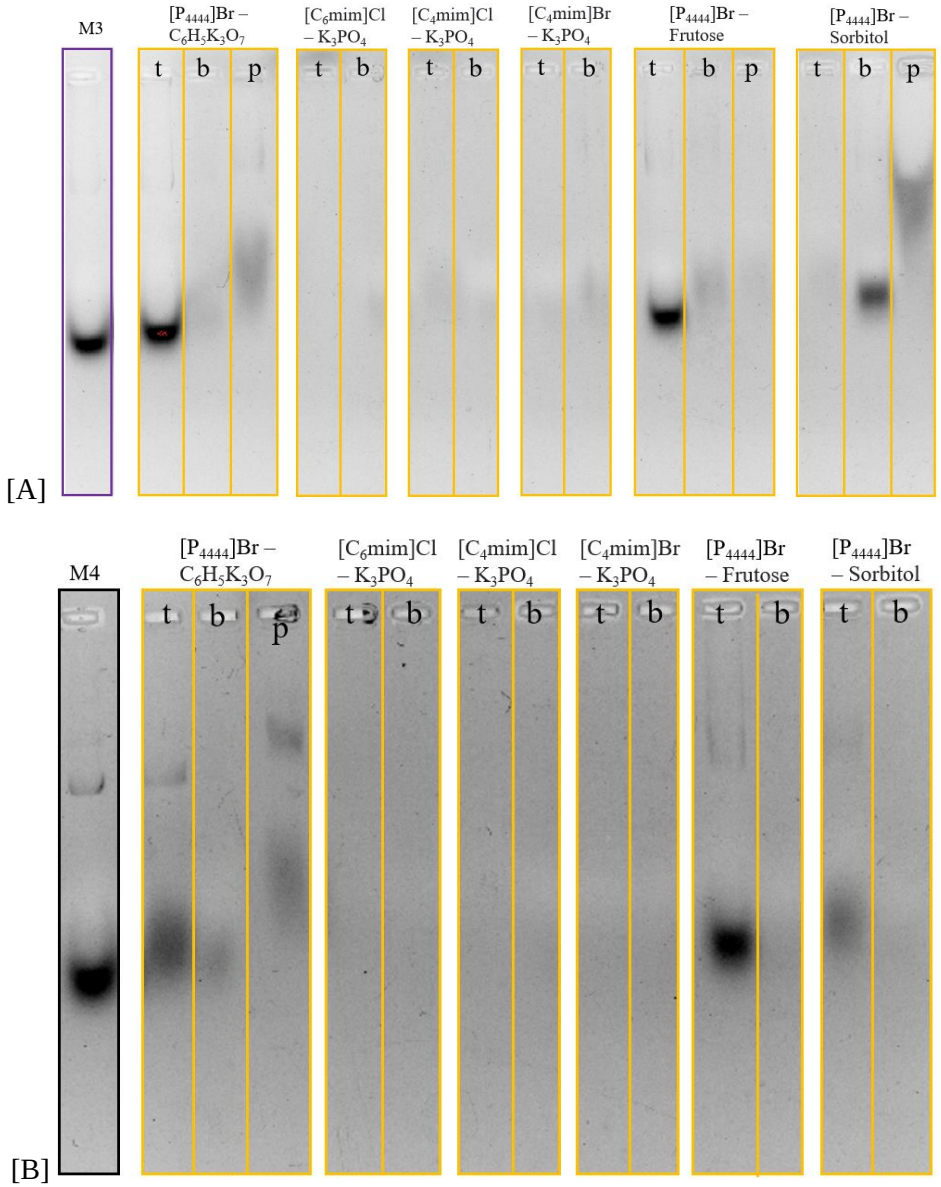


Figure 14. Agarose gel electrophoresis of nucleic acids in the different ABS with M3 ([A]) and M4 ([B]). 20 µL of each sample were loaded in the corresponding well. Top and bottom phases and the precipitate are represented as "t", "b" and "p", respectively.

Analysing the electrophoresis results of the phases of the systems performed with the M3 sample, a lack of reproducibility is noted, which may be due to interference caused by proteins, being hard to observe the pDNA. On the other hand, the pDNA obtained in the $[P_{4444}]Br – K_3C_6H_5O_7$ system with sample M4 (Figure 14 [B]) is better identified, suggesting that the use of ammonium sulfate allowed the removal of some proteins which were eliminated in the chromatography step. Hence, the method which comprehends the adequate processes for an efficient purification is M4, despite the additional step in the column PD-10.

Towards the analysed systems (orange), it was not possible to properly detect the pDNA and RNA bands. Probably, either the treatment of the samples was not the most adequate

(precipitation with ethanol and desalting/change of buffer) or maybe the components used to formulate the systems in question denature the nucleic acids.

Beyond IL-salt systems, much effort was taken to investigate the IL - sugar systems (see Annex B), since they are promising IL-ABS. However, due to the relatively low solubility of some sugars and the fact that it is easier to manipulate the phases containing salt, it was decided to continue the following studies with the $[P_{4444}]Br$ – $K_3C_6H_5O_7$ ABS, further optimized in the next chapter.

3.2.2 Optimization of pDNA purification using $[P_{4444}]Br$ – $C_6H_5K_3O_7$

Preliminary trials identified $[P_{4444}]Br$-$K_3C_6H_5O_7$ as the most promising ABS, and therefore it was selected to optimize the pDNA extraction process. With that in mind, the next assays were developed for the M4 sample containing both pDNA and RNA. In the present system, the IL-rich phase corresponds to the top phase (top), while the bottom phase (bot) is mainly composed of salt. This type of system makes use of an IL and an organic salt, which, in general, presents a "greener" separation as it works with biodegradable and non-toxic compounds, being friendly to the environment.[125] Additionally, a system composed of polymer-salt reported in the literature[89] was studied in order to compare the selectivity of pDNA, namely by using PEG 400 and $C_6H_5Na_3O_7.2H_2O$.[89]

Initially, the phase diagram of the system under study was determined, allowing the study of an array of different concentrations of the main components. The phase diagram was determined to evaluate the concentrations of each of the components that lead to the formation of a two-phase system, shown in Figure 15. As a result, aqueous solutions of $K_3C_6H_5O_7$•H_2O at ≈ 62 wt% and $[P_{4444}]Br$ at 80 wt% were individually prepared for the cloud point titration data previously described forming the binodal curve.

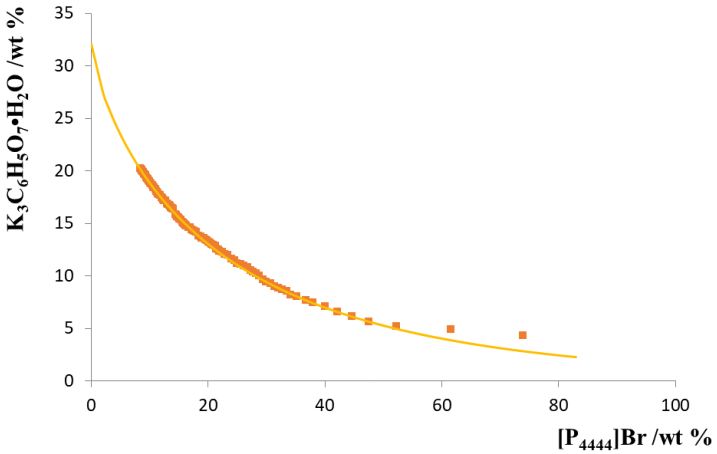


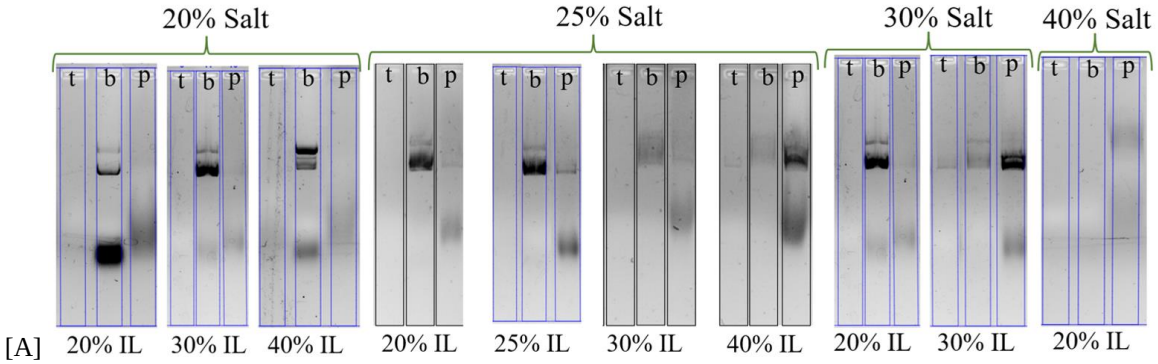
Figure 15. Phase diagram of $[P_{4444}]Br/K_3C_6H_5O_7$ system at room temperature (± 22 °C).

According to several researchers[125,146] the IL $[P_{4444}]Br$ contains an aliphatic cation with four butyl chains with a high ability to form ABS with a wide range of salts. Table 9 gives the mixture points used in the IL-salt system to determine the best concentrations for pDNA partition. Under all tested conditions, the sample was always maintained at 15 %, and Tris-HCl was added to make up 1 g, the total mass of the system.

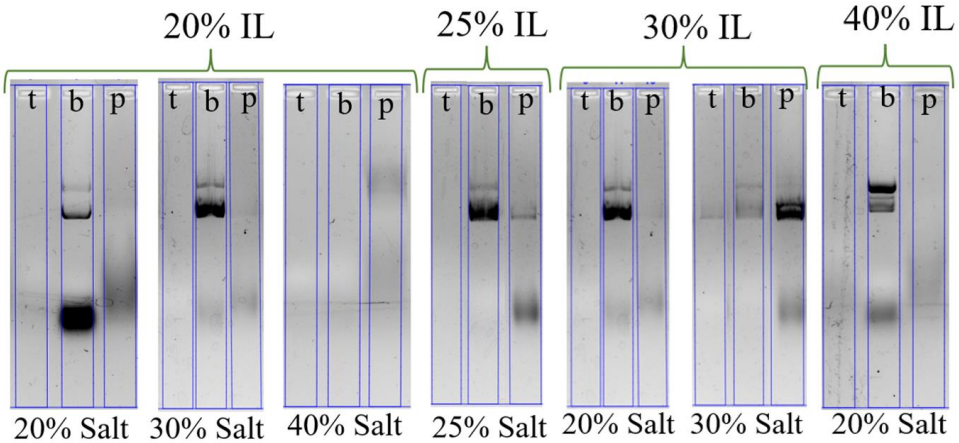
Table 9. Mixture points with the system $[P_{4444}]Br$-$K_3C_6H_5O_7$ studied in order to isolate pDNA.

$[P_{4444}]Br$ (wt%)	20%	30%	40%	20%	25%	30%	40%	20%	30%	20%
$K_3C_6H_5O_7$ (wt%)	20%			25%				30%		40%

For a more direct perspective onto the effect of the concentration of each ABS component on the selective partitioning of pDNA, the ABS described in Table 9 were prepared and each phase was analysed by agarose gel electrophoresis, whose results are grouped in Figure 16 according to the effect of IL and salt concentrations, respectively Fig. 16 [A] and 16 [B].



[A]



[B]

Figure 16. Agarose gel electrophoresis of nucleic acids in ABS composed of $[P_{4444}]Br$-$K_3C_6H_5O_7$, at different concentrations, with 15 % of pDNA lysate. [A] and [B] represent, respectively, the effect of IL and salt concentrations. 20 µL of each sample were loaded in the corresponding well. Top and bottom phases and the precipitate are represented as "t", "b" and "p", respectively.

In general, most of the mixture points studied allow the recovery of pDNA in its sc isoform, which is highly advantageous considering that this isoform is the most biologically active for potential therapeutic applications. From the analysis of Figure 16 [A], as the IL concentration increases (20 % salt), scDNA band intensity decreases and ocDNA band increases, meaning that there is a change in the isoforms of DNA. From the analysis of Figure 16, two general conclusions can be drawn, which are related to the overall trend of nucleic acids partitioning as function of the concentration of each ABS component: i) for lower concentrations of $[P_{4444}]Br$-$K_3C_6H_5O_7$, both pDNA and RNA tend to migrate to the bottom, salt-rich phase; ii) for higher concentrations of both ABS components, either pDNA as RNA exhibit an increased tendency to precipitate at the interface.

According to this information, it can be stated that the increase in the concentration of salt causes the nucleic acids to move from the citrate-phase towards the precipitate phase, although the pDNA precipitation is less extent that with RNA, which may be explained by a higher hydrophobicity associated to RNA. Therefore, there is selectivity in these systems, where ideally RNA is completely precipitated and pDNA migrates to the ABS bottom phase.

By finely tuning the concentration of IL while maintaining constant the concentration of salt, it was observed that RNA precipitates at the interface and pDNA is recovered in the bottom phase. Given the high purity of the plasmid, it can be stated that the proteins move towards the opposite phase of pDNA or precipitate at the interphase. This result shows an improvement of the selectivity of the system for the mixture point 25 wt% of $[P_{4444}]Br$ and 25 wt% of $K_3C_6H_5O_7$ given in Figure 16.

The biphasic system chosen to compare against the $[P_{4444}]Br$-$K_3C_6H_5O_7$ ABS performance (this work) was with PEG 400 at a concentration of 24 wt%, $C_6H_5Na_3O_7.2H_2O$ at a concentration of 18.5 wt% and 10 wt% of lysate load, as reported by Rahimpour et al.[89] According to the investigators, these concentrations were the ideal conditions for maximum plasmid recovery (around 99 %), with up to 68 % of RNA removal and a total protein removal (100 %).[89] Figure 17 shows the agarose gel electrophoresis results for the phases of the ABS composed of PEG 400 and $C_6H_5Na_3O_7.2H_2O$.

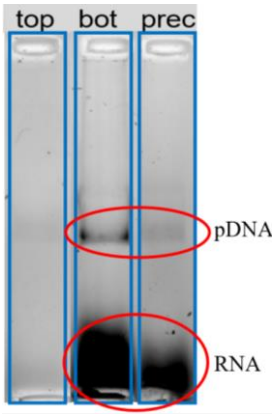


Figure 17. Agarose gel electrophoresis of nucleic acids in ABS composed of PEG 400 (24 wt%), $C_6H_5Na_3O_7$ (18.5 wt%), 10 wt% of pDNA lysate and 47.5 wt% of 10 mM Tris-HCl. 20 µL of each phase were loaded in the corresponding well.

The pattern of nucleic acids performed in our laboratory demonstrate to be in accordance with the work of Rahimpour et al,[89] in which pDNA is preferentially partitioned towards the bottom phase, with some traces encountered in the precipitated phase. On the other hand, RNA is found in the bottom phase and in the precipitate. Even though the bacterial lysate sample used in this work and that reported from Rahimpour et al.[89] are different, the qualitative electrophoretic analysis of the ABS developed in this work (25 wt% $[P_{4444}]Br$ and 25 wt% $K_3C_6H_5O_7$) and that from the literature (24 wt% PEG 400 and 18.5 wt% $C_6H_5Na_3O_7.2H_2O$) seem to indicate that the IL-based system display an improved performance to promote the recovery of pDNA with higher yield and purity.

In both systems presented (PEG-salt and IL-salt), pDNA prefers the bottom phase enriched in the citrate salt. However, in the PEG-salt system, there is a higher precipitation of pDNA which may be due to the collapse of nucleic acids in aqueous solutions of PEG, having a lower solvent ability for these biomolecules than ILs. In addition, DNA and RNA have a strong attractive strength between them, and when DNA compacts, it segregates from the polymer molecules resulting in the partition to the bottom phase and interface.[89] In the IL-salt system, the IL cation has a higher hydrophobicity which reduces this phase affinity for water, thus leading to the exclusion of RNA and its preferential precipitation at the interphase.

According to the electrophoresis results, IL-based systems, when compared to the literature, do not present RNA contamination at the chosen point. Therefore, the best results are displayed by the ABS composed of $[P_{4444}]Br$ and $K_3C_6H_5O_7$ due to a more selective precipitation of RNA, with a more effective isolation of pDNA in the bottom phase.

3.3 Quality control

The pDNA recovered in the bottom phase of the system formed by $[P_{4444}]Br$ and $K_3C_6H_5O_7$, at the mixture point of 25 wt% IL and 25 wt% salt, was analysed in terms of the total

protein content, and conformation of pDNA and its transformation efficiency to assess whether it maintains integrity and is functional activity. It was also analysed the pDNA sample obtained from the lysis step, defined as M4, and the sample obtained with the Nyztech extraction kit.

3.3.1 Protein Quantification

The total protein quantification was measured through the bicinchoninic acid (BCA) colorimetric method detecting a purple-colored reaction (562 nm) of the cuprous cation (Cu^{+1}) that is nearly linear with the increase in protein concentration. For that purpose, a series of dilutions of a model protein (BSA) was prepared in order to construct a standard curve with a working range of 5 - 250 µg/mL.[147] Thus, it was possible to determine the protein concentrations of our pDNA samples, which were initially unknown, through the obtained absorbance (see in Annex C). It was analysed the pDNA sample from lysis (sample M4) and the bottom phase of the ABS (pDNA bot) depicted in Table 10.

Table 10. Absorbance at 562 nm of pDNA samples and respective protein concentration.

	Abs (nm)	**[BSA] (µg/mL)**	**Mass (µg)**	**% Protein removal**
Sample M4	0.228	43.69	0.0066	0
pDNA bot	0.207	37.86	0,0062	13.35

Through this method it was able to understand which phase of ABS was contaminated with protein. Such data showed some contaminations in the pDNA-rich phase, only capable of removing 13.4 % of protein, which is still a concern about its availability for pharmaceutical applications. In this sense, optimization of IL-based ABS to reduce proteins content is still needed.

3.3.2 Circular Dichroism

According to regulatory agencies, the most required plasmid isoform is supercoiled (sc), which provides a perfect example of the combination of energy, structure, and function in biomolecules. This preferential structure must be predominant over other unwanted conformations. However, changes in the secondary structure of DNA can compromise the tertiary curvature causing different degrees of supercoiling. Consequently, it can affect the quality of the final product, narrowing all the processes inherent to its use.

Circular dichroism (CD) measurements allow the detection of small changes in DNA structure and differentiating the different specific DNA arrangements. This technique differentiates the absorption of circularly polarized light to the right from the left. Therefore, CD bands will be affected by interactions between the planar bases, as well as between the base and the furanose ring of the DNA molecule. The CD spectrum of pDNA is comparable to the typical spectrum of B-

DNA (DNA with double helix), with positive bands at 220 and 270 nm and a negative band at 245 nm.[148] CD spectroscopic experiments were performed with the objective of monitoring the pDNA conformational structure to determine its stability.

The pDNA samples results provided in Figure 18 were obtained from the extraction using a Nyztech kit (pDNA Kit), from the lysis (sample M4) and the pDNA resulting from the bottom phase of the studied ABS (pDNA bot).

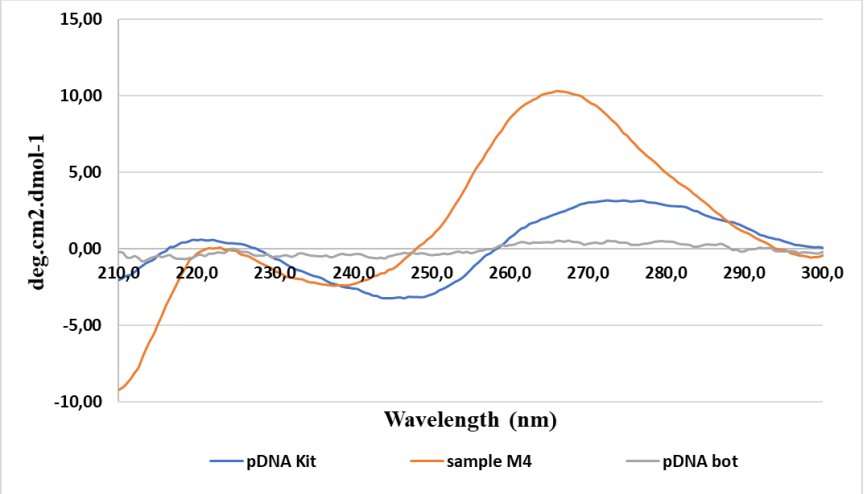


Figure 18. Circular dichroism spectra of pDNA samples resulting from: the isolation using a commercial kit (blue); the alkaline lysis method (M4, orange); the bottom phase of the ABS formed by $[P_{4444}]Br$ (25 wt%)/$K_3C_6H_5O_7$ (25 wt%) system (grey).

The pDNA samples from lysis (both from the kit and from M4) demonstrate the expected pDNA ellipticity, showing the two positive bands at 220 and 270 nm and one negative band at 245 nm. As for the bottom phase recovered from the $[P_{4444}]Br$ (25 wt%) / $K_3C_6H_5O_7$ (25 wt%) system, it did not show any significant signal in its spectrum, possibly due to the low concentration of pDNA, determined as 10.65 µL/mL in comparison with 58.60 µL/mL from the pDNA kit. Despite not concluding about the bot phase results, it was possible to ensure the conformation of the pDNA structure resulting from the M4 lysis that was used in the described system. Future studies dealing with higher concentrations of pDNA are needed to prove its stability after the ABS extraction step.

3.3.3 Determination of *E. coli* transformation efficiency

The determination of *E. coli* transformation efficiency using pDNA recovered from the bottom phase was also evaluated to assess if pDNA was obtained in a functional active form. The pDNA uptake by cells have the advantage of being a simple and cheap process, have high transformation efficiency and enables the transfer of relatively large segments of DNA. However, the bacterial transformation efficiency can be influenced by certain parameters, such as the

incubation time of bacteria with the DNA, the pDNA concentration for the transformation mixture, the temperature of incubation and the growth phase.[142]

After the pDNA uptake of the *E. coli* competent cells, the number of colonies was counted after growth on solid medium to provide the transformation efficiency (TE), which reflects pDNA viability. Table 11 demonstrates the number of colonies translated in TE for the pDNA from the extraction kit (pDNA Kit) and the bottom phase of the $[P_{4444}]Br$ (25 wt%) - $K_3C_6H_5O_7$ (25 wt%) system (pDNA bot).

Table 11. Transformation efficiency of pDNA, respectively, from the bottom phase of the ABS composed of $[P_{4444}]Br$ (25 wt%) and $K_3C_6H_5O_7$ (25 wt%) and that from the application of a commercial kit.

	pDNA bot	**pDNA kit**
colonies	3	6
TE (cfu/µg)	120 000	240 000

The data provided refer to a total dilution of 0.005 µL of LB medium before plating. It shows that the sample resulting from the extraction kit achieved twice as many colonies compared to the ABS phase sample. This variation may be due to the dilution carried out or also to the interference associated with the conformational form of pDNA in the salt-rich phase (Figure 18). Nevertheless, the pDNA recovered from the ABS phase has proven its integrity by being structurally effective and functional in conveying resistance to *E. coli* cells and allowing their growth in medium containing antibiotic.

3.4. Conclusions and future perspectives

In this work, a novel downstream process using IL-based ABS was investigated to purify pDNA from *E. coli* lysates. The first stage of this work dealt with the optimization of the number of alkaline lysis processing steps able to obtain a sample suitable for application in ABS purification experiments. As expected, the higher the number of processing steps, the higher amount of impurities removed. It was observed that a sample comprising the alkaline lysis method, followed by two successive washing steps with isopropanol and ethanol, and an ammonium sulfate precipitation step followed by desalting with PD-10 gel filtration column (sample M4), was the most suitable, and hence selected for ABS purification assays.

Among the components evaluated, the ABS constituted by $[P_{4444}]Br$-$K_3C_6H_5O_7$ demonstrated promising results in initial experiments and after successive optimizations, it was found that pDNA and RNA preferentially partitioned to the bottom, salt-rich phase, at low concentrations of both components. On the other hand, for higher concentrations of salt and IL, both classes of nucleic acids precipitated at the interface of the system. However, by properly engineering IL and salt concentrations, at the mixture point 25-25 wt% of $[P_{4444}]Br$-$K_3C_6H_5O_7$, it was found that RNA was selectively precipitated while pDNA was recovered with high integrity

and purity in the bottom phase. Interestingly, pDNA is generally recovered in its sc isoform, the most biologically active isoform. Based on these results, future work to optimize the ABS performance should comprise systems composed of $[P_{4444}]Br$ and $(NH_4)_2SO_4$, the salt used in the PD-10 gel filtration step, ideally avoiding the need of this step.

Partitioning of pDNA and RNA was generally evaluated by agarose gel electrophoresis and complemented by spectrophotometric analysis. However, future investigations by analytical chromatography will allow to accurately determine the yield and purities of purified sc pDNA. As analysed by agarose gel electrophoresis, the integrity of purified pDNA seems to be maintained during the ABS purification process, although it was not possible to conclude about its structural stability by CD. The low concentration of pDNA used for CD experiments may account for the absence of signal. To overcome this problem, future experiments increasing the scale at which the ABS is performed, and hence increasing the amount of purified pDNA will allow to draw more rigorous conclusions by CD. Nevertheless, it should be remarked that the purified pDNA was able to confer resistance to *E. coli* cells after transformation, supporting the fact that it is purified with high integrity and is structurally stable.

In comparison with the initial bacterial lysate sample, it was observed that the total protein content was reduced by 13 %. Future work comprising additional number of replicates are required to confirm these values, and if required additional investigations must take place to reduce the protein content in the purified pDNA. Aiming to certify that pDNA fulfils the acceptable parameters for the regulatory agencies envisaging therapeutic applications, quantification of endotoxin levels needs to be performed. The analysis of transfection efficiency and biological activity in cell culture experiments of purified pDNA will contribute to ascertain the suitability of the IL-based ABS strategy herein developed to manufacture pharmaceutically-grade pDNA for gene therapy.

Overall, a promising IL-based technology for the purification of pDNA from bacterial lysates was demonstrated in this work, in which the presence of IL seems to be responsible for upgrading pDNA purity over other conventional ABS. However, the purification of pDNA was not completely achieved, for which further investigation is still required. The next step should involve the reduction of lysis processes involving ABS that are more effective in pDNA partitioning. In addition, a stage of recovery of the salt-rich phase should be addressed, as well as the testing of recovering the ABS phases. Moreover, additional investigations must take place to reduce the protein content in ABS phases, while certifying the conformational structure of the biomolecule and considering the acceptable parameters for the regulatory agencies.

4. Bibliography

1. Teo PY, Cheng W, Hedrick JL, Yang YY. Co-delivery of drugs and plasmid DNA for cancer therapy. *Adv Drug Deliv Rev*. 2016;98:41-63. doi:10.1016/j.addr.2015.10.014

2. Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab*. 2016;23(1):27-47. doi:10.1016/j.cmet.2015.12.006

3. You W, Henneberg M. Cancer incidence increasing globally: The role of relaxed natural selection. *Evol Appl*. 2018;11(2):140-152. doi:10.1111/eva.12523

4. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin*. 2021;71(3):209-249. doi:10.3322/caac.21660

5. Davis JD. DNA damage and breast cancer. *World J Clin Oncol*. 2011;2(9):329. doi:10.5306/wjco.v2.i9.329

6. Ghoncheh M, Pournamdar Z, Salehiniya H. Incidence and mortality and epidemiology of breast cancer in the world. *Asian Pacific J Cancer Prev*. 2016;17:43-46. doi:10.7314/APJCP.2016.17.S3.43

7. Breast Cancer RISK FACTORS AND PREVENTION. Accessed October 17, 2021. http://www.jbcp.jo/understandingbreastcancer/35

8. Tong CWS, Wu M, Cho WCS, To KKW. Recent advances in the treatment of breast cancer. *Front Oncol*. 2018;8(JUN). doi:10.3389/fonc.2018.00227

9. Feng Y, Spezia M, Huang S, et al. Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. *Genes Dis*. 2018;5(2):77-106. doi:10.1016/j.gendis.2018.05.001

10. Hortobagyi GN. Treatment of Breast Cancer. Wood AJJ, ed. *N Engl J Med*. 1998;339(14):974-984. doi:10.1056/NEJM199810013391407

11. Law AMK, Lim E, Ormandy CJ, Gallego-Ortega D. The innate and adaptive infiltrating immune systems as targets for breast cancer immunotherapy. *Endocr Relat Cancer*. 2017;24(4):R123-R144. doi:10.1530/ERC-16-0404

12. Li L, Goedegebuure SP, Fleming TP, Gillanders WE. Developing a clinical development paradigm for translation of a mammaglobin-A DNA vaccine. *Immunotherapy*. 2015;7(7):709-711. doi:10.2217/imt.15.40

13. Tiriveedhi V, Tucker N, Herndon J, et al. Safety and preliminary evidence of biologic efficacy of a mammaglobin-A DNA vaccine in patients with stable metastatic breast cancer. *Clin Cancer Res*. 2014;20(23):5964-5975. doi:10.1158/1078-0432.CCR-14-0059

14. Jaramillo A, Majumder K, Manna PP, et al. Identification of HLA-A3-restricted CD8+ T cell epitopes derived from mammaglobin-A, a tumor-associated antigen of human breast cancer. *Int J Cancer*. 2002;102(5):499-506. doi:10.1002/ijc.10736

15. Watson MA, Fleming TP. Mammaglobin, a mammary-specific member of the uteroglobin gene family, is overexpressed in human breast cancer. *Cancer Res*. 1996;56(4):860-865.

16. Kim SW, Goedegebuure P, Gillanders WE. Mammaglobin-A is a target for breast cancer vaccination. *Oncoimmunology*. 2016;5(2):1-3. doi:10.1080/2162402X.2015.1069940

17. Zhou L, Xu N, Sun Y, Liu XM. Targeted biopharmaceuticals for cancer treatment. *Cancer Lett*. 2014;352(2):145-151. doi:10.1016/j.canlet.2014.06.020

18. Liu M, Li Z, Yang J, et al. Cell-specific biomarkers and targeted biopharmaceuticals for breast cancer treatment. *Cell Prolif*. 2016;49(4):409-420. doi:10.1111/cpr.12266

19. Lenka DG, Nair DVS, Saleh DR, Toor SM. Pink October - The Fight Against Breast Cancer. Accessed October 17, 2021. https://www.hbku.edu.qa/en/news/fight-breast-cancer

20. Chen Y-C, Yeh M-K. Biopharmaceuticals. *Intechopen*. Published online 2018:1-11. doi:http://dx.doi.org/10.5772/intechopen.79194

21. Mitragotri S, Burke PA, Langer R. Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. *Nat Rev Drug Discov*. 2014;13(9):655-672. doi:10.1038/nrd4363

22. Baeshen MN, Al-Hejin AM, Bora RS, et al. Production of biopharmaceuticals in E. Coli: Current scenario and future perspectives. *J Microbiol Biotechnol*. 2015;25(7):953-962. doi:10.4014/jmb.1412.12079

23. Uyhazi KE, Bennett J. A CRISPR view of the 2020 nobel prize in chemistry. *J Clin Invest*. 2021;131(1). doi:10.1172/JCI145214

24. Zhang NN, Li XF, Deng YQ, et al. A Thermostable mRNA Vaccine against COVID-19. *Cell*. 2020;182(5):1271-1283.e16. doi:10.1016/j.cell.2020.07.024

25. Walsh G. Biopharmaceutical benchmarks 2018. *Nat Biotechnol*. 2018;36(12):1136-1145. doi:10.1038/nbt.4305

26. Kesik-Brodacka M. Progress in biopharmaceutical development. *Biotechnol Appl Biochem*. 2018;65(3):306-322. doi:10.1002/bab.1617

27. Sauna ZE, Lagassé HAD, Alexaki A, et al. Recent advances in (therapeutic protein) drug development. *F1000Research*. 2017;6. doi:10.12688/f1000research.9970.1

28. Prazeres DMF, Monteiro GA. Plasmid Biopharmaceuticals. *Microbiol Spectr*. 2014;2(6):1-18. doi:10.1128/microbiolspec.plas-0022-2014

29. Greco A, Mariani C, Miranda C, et al. The DNA rearrangement that generates the TRK-T3 oncogene involves a novel gene on chromosome 3 whose product has a potential coiled-coil domain. *Mol Cell Biol*. 1995;15(11):6118-6127. doi:10.1128/mcb.15.11.6118

30. McDonnell WM, Askari FK. DNA Vaccines. *N Engl J Med*. 1996;334(1):42-45. doi:10.1056/NEJM199601043340110

31. Latulippe DR, Zydney AL. Size exclusion chromatography of plasmid DNA isoforms. *J Chromatogr A*. 2009;1216(35):6295-6302. doi:10.1016/j.chroma.2009.07.009

32. Sousa F, Prazeres DMF, Queiroz JA. Improvement of transfection efficiency by using supercoiled plasmid DNA purified with arginine affinity chromatography. *J Gene Med*. 2009;11(1):79-88. doi:10.1002/jgm.1272

33. Yankauckas MA, Morrow JE, Parker SE, et al. Long-Term Anti-Nucleoprotein Cellular and Humoral Immunity Is Induced by Intramuscular Injection of Plasmid DNA Containing NP Gene. *DNA Cell Biol*. 1993;12(9):771-776. doi:10.1089/dna.1993.12.771

34. Lopes A, Vandermeulen G, Préat V. Cancer DNA vaccines: current preclinical and clinical developments and future perspectives. *J Exp Clin Cancer Res*. 2019;38(1):1-24. doi:10.1186/s13046-019-1154-7

35. Iology MOB, Walker JM. *Recombinant Gene Expression*. Vol 824. Third. (Lorence A, ed.). Humana Press; 2012. doi:10.1007/978-1-61779-433-9

36. Ghasemi-Dehkordi P, Doosti A, Jami MS. The concurrent effects of azurin and Mammaglobin-A genes in inhibition of breast cancer progression and immune system stimulation in cancerous BALB/c mice. *3 Biotech*. 2019;9(7):1-15. doi:10.1007/s13205-019-1804-7

37. Farkona S, Diamandis EP, Blasutig IM. Cancer immunotherapy: The beginning of the end of cancer? *BMC Med*. 2016;14(1):1-18. doi:10.1186/s12916-016-0623-5

38. Khan KH. DNA vaccines: Roles against diseases. *Germs*. 2013;3(1):26-35. doi:10.11599/germs.2013.1034

39. Coban C, Koyama S, Takeshita F, Akira & Ken S, Ishii JJ. Molecular and cellular mechanisms of DNA vaccines. *Hum Vaccin*. 2008;4(6):453-457. doi:10.4161/hv.4.6.6200

40. Prazeres DMF, Ferreira GNM. Design of flowsheets for the recovery and purification of plasmids for gene therapy and DNA vaccination. *Chem Eng Process Process Intensif*. 2004;43(5):609-624. doi:10.1016/j.cep.2003.02.002

41. Prazeres DM., Monteiro G., Ferreira GN., Diogo M., Ribeiro S., Cabral JM. Purification of plasmids for gene therapy and DNA vaccination. In: Vol 7. ; 2001:1-30. doi:10.1016/S1387-2656(01)07031-4

42. Klinman DM, Klaschik S, Tross D, Shirota H, Steinhagen F. FDA guidance on prophylactic DNA vaccines: Analysis and recommendations. *Vaccine*. 2010;28(16):2801-2805. doi:10.1016/j.vaccine.2009.11.025

43. Stadler J, Lemmens R, Nyhammar T. Plasmid DNA purification. *J Gene Med*. 2004;6(SUPPL. 1):54-66. doi:10.1002/jgm.512

44. Sousa F, Freitas S, Azzoni AR, Prazeres DMF, Queiroz J. Selective purification of supercoiled plasmid DNA from clarified cell lysates with a single histidine–agarose chromatography step. *Biotechnol Appl Biochem*. 2006;45(3):131. doi:10.1042/ba20060082

45. Mark Saltzman W, Shen H, L. Brandsma J, eds. *DNA Vaccines : Methods and Protocols*. second. Humana Press Inc; 2006. file:///Users/alex.neumann/Documents/Mendeley Desktop/Edited by Edited by/World/[Darren_Swanson]_Creating_Adaptive_Policies_A_Gui(BookSee.org).pdf

46. Guiochon G, Beaver LA. Separation science is the key to successful biopharmaceuticals. *J Chromatogr A*. 2011;1218(49):8836-8858. doi:10.1016/j.chroma.2011.09.008

47. Tripathi NK, Shrivastava A. *Scale up of Biopharmaceuticals Production*. Elsevier Inc.; 2018. doi:10.1016/B978-0-12-813629-4.00004-8

48. Jozala AF, Geraldes DC, Tundisi LL, et al. Biopharmaceuticals from microorganisms: from production to purification. *Brazilian J Microbiol*. 2016;47:51-63. doi:10.1016/j.bjm.2016.10.007

49. Graumann K, Premstaller A. Manufacturing of recombinant therapeutic proteins in microbial systems. *Biotechnol J*. 2006;1(2):164-186. doi:10.1002/biot.200500051

50. Terpe K. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol*. 2006;72(2):211-222. doi:10.1007/s00253-006-0465-8

51. Walsh G. *Biopharmaceuticals: Biochemistry and Biotechnology*. second.; 2004.

52. Gonçalves GAL, Prather KLJ, Monteiro GA, Carnes AE, Prazeres DMF. Plasmid DNA production with Escherichia coli GALG20, a pgi-gene knockout strain: Fermentation strategies and impact on downstream processing. *J Biotechnol*. 2014;186:119-127. doi:10.1016/j.jbiotec.2014.06.008

53. Silva F, Queiroz JA, Domingues FC. Plasmid DNA fermentation strategies: influence on plasmid stability and cell physiology. *Appl Microbiol Biotechnol*. 2012;93(6):2571-2580. doi:10.1007/s00253-011-3668-6

54. Castiñeiras TS, Williams SG, Hitchcock AG, Smith DC. E. coli strain engineering for the production of advanced biopharmaceutical products. *FEMS Microbiol Lett*. 2018;365(15):1-10. doi:10.1093/femsle/fny162

55. Gonçalves GAL, Bower DM, Prazeres DMF, Monteiro GA, Prather KLJ. Rational engineering of Escherichia coli strains for plasmid biopharmaceutical manufacturing. *Biotechnol J*. 2012;7(2):251-261. doi:10.1002/biot.201100062

56. Carnes AE, Hodgson CP, Williams JA. Inducible Escherichia coli fermentation for increased plasmid DNA production. *Biotechnol Appl Biochem*. 2006;45(3):155. doi:10.1042/ba20050223

57. Domingues FC. Influence of Growth Conditions on Plasmid DNA Production. *J Microbiol Biotechnol*. 2009;19(May 2014). doi:10.4014/jmb.0805.329

58. Urthaler J, Buchinger W, Necina R. Improved downstream process for the production of plasmid DNA for gene therapy. *Acta Biochim Pol*. 2005;52(3):703-711. doi:10.18388/abp.2005_3434

59. Li LZ, Liu Y, Sun MS, Shao YM. Effect of salt on purification of plasmid DNA using size-exclusion chromatography. *J Chromatogr A*. 2007;1139(2):228-235. doi:10.1016/j.chroma.2006.11.027

60. Bimboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. 1979;7(6):1513-1524.

61. Durland RH, Eastman EM. Manufacturing and quality control of plasmid-based gene expression systems. *Adv Drug Deliv Rev*. 1998;30(1-3):33-48. doi:10.1016/S0169-409X(97)00105-1

62. Sousa F, Prazeres DMF, Queiroz JA. Affinity chromatography approaches to overcome the challenges of purifying plasmid DNA. *Trends Biotechnol*. 2008;26(9):518-525. doi:10.1016/j.tibtech.2008.05.005

63. Eon-duval A, Burke G. Purification of pharmaceutical-grade plasmid DNA by anion-exchange chromatography in an RNase-free process. 2004;804:327-335. doi:10.1016/j.jchromb.2004.01.033

64. Bicho D, Caramelo-Nunes C, Sousa A, Sousa F, Queiroz JA, Tomaz CT. Purification of influenza deoxyribonucleic acid-based vaccine using agmatine monolith. *J Chromatogr B Anal Technol Biomed Life Sci*. 2016;1012-1013:153-161. doi:10.1016/j.jchromb.2015.12.036

65. Bonturi N, Radke VSCO, Bueno SMA, Freitas S, Azzoni AR, Miranda EA. Sodium citrate and potassium phosphate as alternative adsorption buffers in hydrophobic and aromatic thiophilic chromatographic purification of plasmid DNA from neutralized lysate. *J Chromatogr B Anal Technol Biomed Life Sci*. 2013;919-920:67-74.

doi:10.1016/j.jchromb.2013.01.010

66. Diogo MM, Ribeiro SC, Queiroz JA, et al. Production, purification and analysis of an experimental DNA vaccine against rabies. *J Gene Med*. 2001;3(6):577-584. doi:10.1002/jgm.218

67. Almeida AM, Eusébio D, Queiroz JA, Sousa F, Sousa A. The use of size-exclusion chromatography in the isolation of supercoiled minicircle DNA from Escherichia coli lysate. *J Chromatogr A*. 2020;1609. doi:10.1016/j.chroma.2019.460444

68. Wils P, Escriou V, Warnery A, et al. Efficient purification of plasmid DNA for gene transfer using triple-helix affinity chromatography. *Gene Ther*. 1997;4(4):323-330. doi:10.1038/sj.gt.3300388

69. Gomes AG, Azevedo AM, Aires-Barros MR, Prazeres DMF. Validation and scale-up of plasmid DNA purification by phenyl-boronic acid chromatography. *J Sep Sci*. 2012;35(22):3190-3196. doi:10.1002/jssc.201200225

70. Prazeres DMF, Schluep T, Cooney C. Preparative purification of supercoiled plasmid DNA using anion- exchange chromatography. *J Chromatogr A*. 1998;806(1):31-45. doi:10.1016/S0021-9673(97)01254-5

71. Rinderknecht EH, Gerardo A. Zapata. LOW PH HYDROPHOBIC INTERACTION CHROMATOGRAPHY FOR ANTIBODY PURIFICATION. *US Pat*. Published online 1997:12-25.

72. Abdulrahman A, Ghanem A. Recent advances in chromatographic purification of plasmid DNA for gene therapy and DNA vaccines: A review. *Anal Chim Acta*. 2018;1025:41-57. doi:10.1016/j.aca.2018.04.001

73. Barth HG, Jackson C, Boyes BE. Size Exclusion Chromatography. *Anal Chem*. 1994;66(12):595-620. doi:10.1021/ac00084a022

74. Mori S, Barth HG. *Size Exclusion Chromatography*. Springer Berlin Heidelberg; 1999. doi:10.1007/978-3-662-03910-6

75. Diogo MM, Queiroz JA, Prazeres DMF. Chromatography of plasmid DNA. *J Chromatogr A*. 2005;1069(1):3-22. doi:10.1016/j.chroma.2004.09.050

76. Hage DS. Affinity chromatography: A review of clinical applications. *Clin Chem*. 1999;45(5):593-615. doi:10.1093/clinchem/45.5.593

77. Darby RAJ, Forde GM, Slater NKH, Hine A V. Affinity purification of plasmid DNA directly from crude bacterial cell lysates. *Biotechnol Bioeng*. 2007;98(5):1103-1108. doi:10.1002/bit.21492

78. Valente JFA, Queiroz JA, Sousa F. Dilemma on plasmid DNA purification: binding capacity vs selectivity. *J Chromatogr A*. 2021;1637:461848. doi:10.1016/j.chroma.2020.461848

79. Rathore AS, Agarwal H, Sharma AK, Pathak M, Muthukumar S. Continuous Processing for Production of Biopharmaceuticals. *Prep Biochem Biotechnol*. 2015;45(8):836-849. doi:10.1080/10826068.2014.985834

80. Van Berlo M, Luyben KC, Wielen van der. Poly(ethylene glycol)-salt aqueous two-phase systems with easily recyclable volatile salts. *J Chromatogr B*. 1998;711:61-68.

81. Albertsson P-Å. History of Aqueous Polymer Two-Phase Partition. In: Walter H, E. Brooks

D, Fisher D, eds. *Partitioning in Aqueous Two-Phase System: Theory, Methods, Uses, and Applications to Biotechnology*. Academic P. ; 1985:1-10. doi:10.1016/b978-0-12-733860-6.50008-6

82. Freire MG, Cláudio AFM, Araújo JMM, et al. Aqueous biphasic systems: A boost brought about by using ionic liquids. *Chem Soc Rev*. 2012;41(14):4966-4995. doi:10.1039/c2cs35151j

83. Grilo AL, Aires-Barros MR, Azevedo AM. Partitioning in Aqueous Two-Phase Systems: Fundamentals, Applications and Trends. *Sep Purif Rev*. 2016;45(1):68-80. doi:10.1080/15422119.2014.983128

84. Baskir JN, Hatton TA, Suter UW. Protein partitioning in two-phase aqueous polymer systems. *Biotechnol Bioeng*. 1989;34(4):541-558. doi:10.1002/bit.260340414

85. Azevedo AM, Rosa PAJ, Ferreira IF, Aires-Barros MR. Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing. *Trends Biotechnol*. 2009;27(4):240-247. doi:10.1016/j.tibtech.2009.01.004

86. Gomes GA, Azevedo AM, Aires-Barros MR, Prazeres DMF. Purification of plasmid DNA with aqueous two phase systems of PEG 600 and sodium citrate/ammonium sulfate. *Sep Purif Technol*. 2009;65(1):22-30. doi:10.1016/j.seppur.2008.01.026

87. Hatti-kaul R. Aqueous Two-Phase Systems. *Mol Biotechnol*. 2001;19(3):269-277.

88. Asenjo JA, Andrews BA. Aqueous two-phase systems for protein separation: A perspective. *J Chromatogr A*. 2011;1218(49):8826-8835. doi:10.1016/j.chroma.2011.06.051

89. Farshad Rahimpour, Farzaneh Feyzi, Saeid Maghsoudi RH-K, Rahimpour F, Feyzi F, Maghsoudi S, Hatti-Kaul R. Purification of Plasmid DNA with Polymer-Salt Aqueous Two-Phase System: Optimization Using Response Surface Methodology. *Biotechnol Bioeng*. 2006;95(4):627-637. doi:10.1002/bit

90. Johansson HO, Matos T, Luz JS, et al. Plasmid DNA partitioning and separation using poly(ethylene glycol)/poly(acrylate)/salt aqueous two-phase systems. *J Chromatogr A*. 2012;1233:30-35. doi:10.1016/j.chroma.2012.02.028

91. Johansson HO, Karlström G, Tjerneld F. Temperature-induced phase partitioning of peptides in water solutions of ethylene oxide and propylene oxide random copolymers. *Biochim Biophys Acta - Gen Subj*. 1997;1335(3):315-325. doi:10.1016/S0304-4165(96)00150-X

92. Salabat A, Abnosi MH, Bahar AR. Amino acids partitioning in aqueous two-phase system of polypropylene glycol and magnesium sulfate. *J Chromatogr B Anal Technol Biomed Life Sci*. 2007;858(1-2):234-238. doi:10.1016/j.jchromb.2007.08.039

93. Hernandez-Mireles T, Rito-Palomares M. New aqueous two-phase systems based on poly(ethylene oxide sulfide) (PEOS) and potassium phosphate for the potential recovery of proteins. *J Chem Technol Biotechnol*. 2006;81(6):997-1002. doi:10.1002/jctb.1506

94. Simeone M, Alfani A, Guido S. Phase diagram, rheology and interfacial tension of aqueous mixtures of Na-caseinate and Na-alginate. *Food Hydrocoll*. 2004;18(3):463-470. doi:10.1016/j.foodhyd.2003.08.004

95. Tubio G, Picó GA, Nerli BB. Extraction of trypsin from bovine pancreas by applying polyethyleneglycol/sodium citrate aqueous two-phase systems. *J Chromatogr B Anal Technol Biomed Life Sci*. 2009;877(3):115-120. doi:10.1016/j.jchromb.2008.11.016

96. Ferreira JF, Bresolin LRP, Silveira E, Tambourgi EB. Purification of bromelain from ananas comosus by PEG/phosphate ATPS. *Chem Eng Trans*. 2011;24:931-936. doi:10.3303/CET1124156

97. Antov M, Pericin D, Trbojevic B. The effect of sulphates on partitioning of pectinases in aqueous two-phase systems. *Acta Period Technol*. 2004;280(35):179-186. doi:10.2298/apt0435179a

98. Luechau F, Ling TC, Lyddiatt A. Partition of plasmid DNA in polymer-salt aqueous two-phase systems. *Sep Purif Technol*. 2009;66(2):397-404. doi:10.1016/j.seppur.2008.12.003

99. Vernau J, Kula MR. Extraction of proteins from biological raw material using aqueous polyethylene glycol-citrate phase systems. *Biotechnol Appl Biochem*. 1990;12(4):397-404.

100. Trindade IP, Diogo MM, Prazeres DMF, Marcos JC. Purification of plasmid DNA vectors by aqueous two-phase extraction and hydrophobic interaction chromatography. *J Chromatogr A*. 2005;1082(2):176-184. doi:10.1016/j.chroma.2005.05.079

101. Kepka C, Lemmens R, Vasi J, Nyhammar T, Gustavsson PE. Integrated process for purification of plasmid DNA using aqueous two-phase systems combined with membrane filtration and lid bead chromatography. *J Chromatogr A*. 2004;1057(1-2):115-124. doi:10.1016/j.chroma.2004.09.060

102. Ventura SPM, Neves CMSS, Freire MG, Marrucho IM, Oliveira J, Coutinho JAP. Evaluation of anion influence on the formation and extraction capacity of ionic-liquid-based aqueous biphasic systems. *J Phys Chem B*. 2009;113(27):9304-9310. doi:10.1021/jp903286d

103. Ventura SPM, Sousa SG, Serafim LS, Lima ÁS, Freire MG, Coutinho JAP. Ionic liquid based aqueous biphasic systems with controlled pH: The ionic liquid cation effect. *J Chem Eng Data*. 2011;56(11):4253-4260. doi:10.1021/je200714h

104. Kim S, Chen J, Cheng T, et al. PubChem in 2021: new data content and improved web interfaces. *Nucleic Acids Res*. 2021;49(D1):D1388-D1395. doi:10.1093/nar/gkaa971

105. Ventura SPM, E Silva FA, Quental M V., Mondal D, Freire MG, Coutinho JAP. Ionic-Liquid-Mediated Extraction and Separation Processes for Bioactive Compounds: Past, Present, and Future Trends. *Chem Rev*. 2017;117(10):6984-7052. doi:10.1021/acs.chemrev.6b00550

106. Dinis TB V., Sousa F, Freire MG. Insights on the DNA Stability in Aqueous Solutions of Ionic Liquids. *Bioeng Biotechnol*. 2020;8(October). doi:10.3389/fbioe.2020.547857

107. Clark KD, Sorensen M, Nacham O, Anderson JL. Preservation of DNA in nuclease-rich samples using magnetic ionic liquids. *RSC Adv*. 2016;6(46):39846-39851. doi:10.1039/c6ra05932e

108. Mazid RR, Cooper A, Zhang Y, et al. Enhanced enzymatic degradation resistance of plasmid DNA in ionic liquids. *RSC Adv*. 2015;5(54):43839-43844. doi:10.1039/c5ra05518k

109. Martzy R, Bica-Schröder K, Pálvölgyi ÁM, et al. Simple lysis of bacterial cells for DNA-based diagnostics using hydrophilic ionic liquids. *Sci Rep*. 2019;9(1):1-10. doi:10.1038/s41598-019-50246-5

110. Neves MC, Pereira P, Pedro AQ, et al. Improved ionic-liquid-functionalized macroporous supports able to purify nucleic acids in one step. *Mater Today Bio*. 2020;8(November):100086. doi:10.1016/j.mtbio.2020.100086

111. Pereira P, Pedro AQ, Neves MC, et al. Efficient Isolation of Bacterial RNAs Using Silica-Based Materials Modified with Ionic Liquids. *Life*. 2021;11(10):1090. doi:10.3390/life11101090

112. Quental M V., Pedro AQ, Pereira P, et al. Integrated Extraction-Preservation Strategies for RNA Using Biobased Ionic Liquids. *ACS Sustain Chem Eng*. 2019;7(10):9439-9448. doi:10.1021/acssuschemeng.9b00688

113. Gutowski KE, Broker GA, Willauer HD, et al. Controlling the aqueous miscibility of ionic liquids: Aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. *J Am Chem Soc*. 2003;125(22):6632-6633. doi:10.1021/ja0351802

114. Pei Y, Li L, Li Z, Wu C, Wang J. Partitioning Behavior of Wastewater Proteins in Some Ionic Liquids-Based Aqueous Two-Phase Systems. *Sep Sci Technol*. 2012;47(2):277-283. doi:10.1080/01496395.2011.609241

115. Yu C, Han J, Wang Y, et al. Liquid-liquid equilibrium composed of imidazolium tetrafluoroborate ionic liquids + sodium carbonate aqueous two-phase systems and correlation at (288.15, 298.15, and 308.15) K. *Thermochim Acta*. 2011;523(1-2):221-226. doi:10.1016/j.tca.2011.05.028

116. Du Z, Yu YL, Wang JH. Extraction of proteins from biological fluids by use of an ionic liquid/aqueous two-phase system. *Chem - A Eur J*. 2007;13(7):2130-2137. doi:10.1002/chem.200601234

117. Li Z, Liu X, Pei Y, Wang J, He M. Design of environmentally friendly ionic liquid aqueous two-phase systems for the efficient and high activity extraction of proteins. *Green Chem*. 2012;14(10):2941-2950. doi:10.1039/c2gc35890e

118. Li CX, Han J, Wang Y, Yan YS, Xu XH, Pan JM. Extraction and mechanism investigation of trace roxithromycin in real water samples by use of ionic liquid-salt aqueous two-phase system. *Anal Chim Acta*. 2009;653(2):178-183. doi:10.1016/j.aca.2009.09.011

119. Jiang Y, Xia H, Guo C, Mahmood I, Liu H. Phenomena and mechanism for separation and recovery of penicillin in ionic liquids aqueous solution. *Ind Eng Chem Res*. 2007;46(19):6303-6312. doi:10.1021/ie070325p

120. Ferreira AM, Faustino VFM, Mondal D, Coutinho JAP, Freire MG. Improving the extraction and purification of immunoglobulin G by the use of ionic liquids as adjuvants in aqueous biphasic systems. *J Biotechnol*. 2016;236:166-175. doi:10.1016/j.jbiotec.2016.08.015

121. Neves CMSS, de Cássia S. Sousa R, Pereira MM, Freire MG, Coutinho JAP. Understanding the effect of ionic liquids as adjuvants in the partition of biomolecules in aqueous two-phase systems formed by polymers and weak salting-out agents. *Biochem Eng J*. 2019;141(August 2018):239-246. doi:10.1016/j.bej.2018.10.022

122. Quental M V., Passos H, Kurnia KA, Coutinho JAP, Freire MG. Aqueous Biphasic Systems Composed of Ionic Liquids and Acetate-Based Salts: Phase Diagrams, Densities, and Viscosities. *J Chem Eng Data*. 2015;60(6):1674-1682. doi:10.1021/je501044u

123. Bridges NJ, Gutowski KE, Rogers RD. Investigation of aqueous biphasic systems formed from solutions of chaotropic salts with kosmotropic salts (salt–salt ABS). *Green Chem*. 2007;9(2):177-18. doi:10.1039/b611628k

124. Han J, Yu C, Wang Y, et al. Liquid-liquid equilibria of ionic liquid 1-butyl-3-

methylimidazolium tetrafluoroborate and sodium citrate/tartrate/acetate aqueous two-phase systems at 298.15 K: Experiment and correlation. *Fluid Phase Equilib.* 2010;295(1):98-103. doi:10.1016/j.fluid.2010.03.044

125. Passos H, Ferreira AR, Cláudio AFM, Coutinho JAP, Freire MG. Characterization of aqueous biphasic systems composed of ionic liquids and a citrate-based biodegradable salt. *Biochem Eng J*. 2012;67:68-76. doi:10.1016/j.bej.2012.05.004

126. Freire MG, Louros CLS, Rebelo LPN, Coutinho JAP. Aqueous biphasic systems composed of a water-stable ionic liquid + carbohydrates and their applications. *Green Chem*. 2011;13(6):1536-1545. doi:10.1039/c1gc15110j

127. Wu B, Zhang Y, Wang H. Phase Behavior for Ternary Systems Composed of Ionic Liquid + Saccharides + Water. *J Phys Chem B*. 2008;112(20):6426-6429. doi:10.1021/jp8005684

128. Domínguez-Pérez M, Tomé LIN, Freire MG, Marrucho IM, Cabeza O, Coutinho JAP. (Extraction of biomolecules using) aqueous biphasic systems formed by ionic liquids and aminoacids. *Sep Purif Technol*. 2010;72(1):85-91. doi:10.1016/j.seppur.2010.01.008

129. Freire MG, Neves CMSS, Marrucho IM, Canongia Lopes JN, Rebelo LPN, Coutinho JAP. High-performance extraction of alkaloids using aqueous two-phase systems with ionic liquids. *Green Chem*. 2010;12(10):1715-1718. doi:10.1039/c0gc00179a

130. Editor MGF. *Ionic-Liquid- Based Aqueous Biphasic Systems Fundamentals and Applications.*; 2016. doi:10.1007/978-3-662-52875-4

131. Wu B, Liu WW, Zhang YM, Wang HP. Do we understand the recyclability of ionic liquids? *Chem - A Eur J*. 2009;15(8):1804-1810. doi:10.1002/chem.200801509

132. Mai NL, Ahn K, Koo YM. Methods for recovery of ionic liquids - A review. *Process Biochem*. 2014;49(5):872-881. doi:10.1016/j.procbio.2014.01.016

133. Sivapragasam M, Moniruzzaman M, Goto M. Recent advances in exploiting ionic liquids for biomolecules: Solubility, stability and applications. *Biotechnol J*. 2016;11(8):1000-1013. doi:10.1002/biot.201500603

134. Wood N, Stephens G. Accelerating the discovery of biocompatible ionic liquids. *Phys Chem Chem Phys*. 2010;12(8):1670-1674. doi:10.1039/b923429b

135. Huang D, Huang D. The research for the extraction of yeast's nucleic acid with [BMIM] BF 4-H 2O-KH 2PO 4 ionic liquid aqueous two-phase system. *Adv Mater Res*. 2012;455-456:477-482. doi:10.4028/www.scientific.net/AMR.455-456.477

136. Werner RG. Economic aspects of commercial manufacture of biopharmaceuticals. *J Biotechnol*. 2004;113(1-3):171-182. doi:10.1016/j.jbiotec.2004.04.036

137. Voß C. Downstream processing of plasmid DNA for gene therapy and genetic vaccination. *Chem Eng Technol*. 2008;31(6):858-863. doi:10.1002/ceat.200800069

138. Rosa PAJ, Ferreira IF, Azevedo AM, Aires-Barros MR. Aqueous two-phase systems: A viable platform in the manufacturing of biopharmaceuticals. *J Chromatogr A*. 2010;1217(16):2296-2305. doi:10.1016/j.chroma.2009.11.034

139. Diogo MM, Queiroz JA, Prazeres DMF. Purification of Plasmid DNA Vectors Produced in Escherichia coli for Gene Therapy and DNA Vaccination Applications. In: Barredo JL, ed. *Methods in Biotechnology 18: Microbial Processes and Products*. Humana Press; 2005:165-178. doi:10.1385/1-59259-847-1:165

140. How do I determine the concentration, yield and purity of a DNA sample. Published 2021. Accessed November 3, 2021. https://worldwide.promega.com/resources/pubhub/enotes/how-do-i-determine-the-concentration-yield-and-purity-of-a-dna-sample/

141. Gallagher SR, Desjardins PR. Quantitation of DNA and RNA with Absorption and Fluorescence. Published online 2006:1-21. doi:10.1002/0471142727.mba03ds76

142. Pereira P, Pedro AQ, Tomás J, et al. Advances in time course extracellular production of human pre-miR-29b from Rhodovulum sulfidophilum. *Appl Microbiol Biotechnol*. 2016;100(8):3723-3734. doi:10.1007/s00253-016-7350-x

143. How should I calculate the transformation efficiency (C2992)? | NEB. Accessed October 22, 2021. https://www.neb.com/faqs/0001/01/01/how-should-i-calculate-the-transformation-efficiency-c2992

144. Diogo MM, Queiroz JA, Prazeres DMF. Assessment of purity and quantification of plasmid DNA in process solutions using high-performance hydrophobic interaction chromatography. *J Chromatogr A*. 2003;998(1-2):109-117. doi:10.1016/S0021-9673(03)00618-6

145. Koetsier G, Cantor E. A Practical Guide to Analyzing Nucleic Acid Concentration and Purity with Microvolume Spectrophotometers. *New Engl Biolabs*. 2019;1(1):1-8. https://www.neb.com/-/media/catalog/application-notes/mvs_analysis_of_na_concentration_and_purity.pdf?rev=be7c8e19f4d34e558527496ea51623dc

146. Sintra TE, Cruz R, Ventura SPM, Coutinho JAP. Phase diagrams of ionic liquids-based aqueous biphasic systems as a platform for extraction processes. *J Chem Thermodyn*. 2014;77:206-213. doi:10.1016/j.jct.2013.10.024

147. Pierce ™ BCA Protein Assay Kit. *Thermoscientific*. Published online 2020:0-4.

148. Sousa F, Prazeres DMF, Queiroz JA. Circular dichroism investigation of the effect of plasmid DNA structure on retention in histidine chromatography. *Arch Biochem Biophys*. 2007;467(2):154-162. doi:10.1016/j.abb.2007.08.027

5. Annexes

Annex A

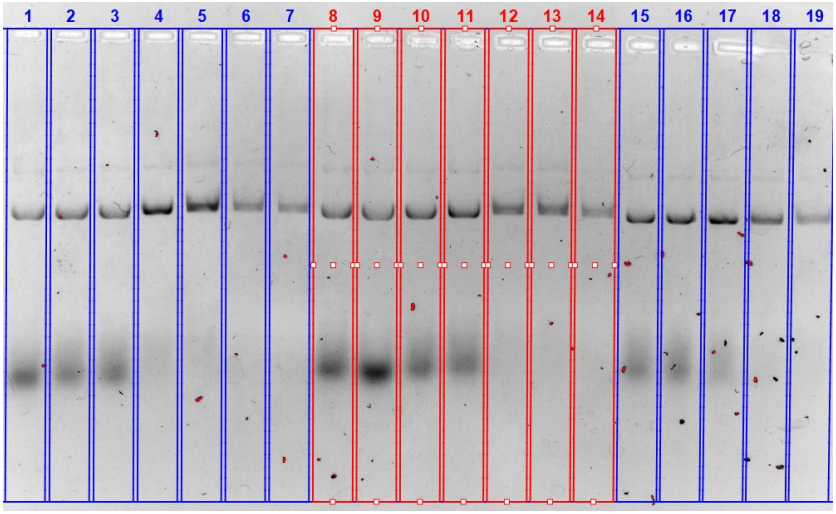


Figure 1A. Agarose gel electrophoresis of a pDNA resultant from an alkaline lysis from a 250 mL of bacterial cell lysate, alcohol precipitation, ammonium sulfate precipitation and desalting, resulting in 10 fractions from the column PD10 (M4).

Annex B

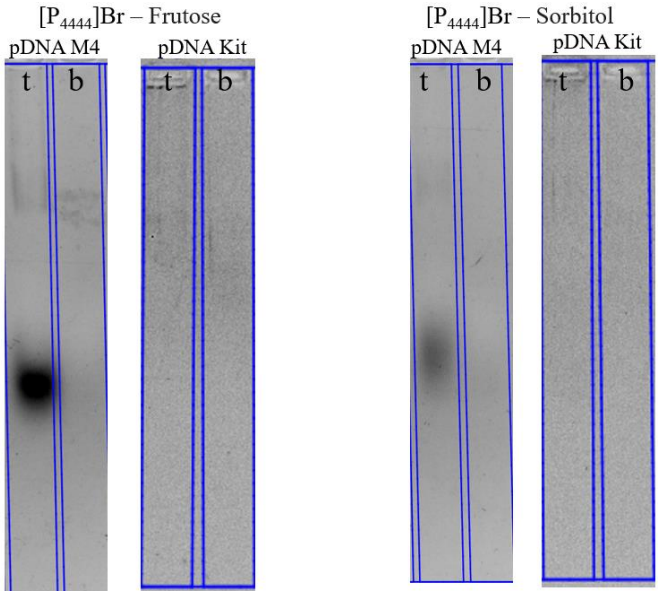


Figure 1B. Agarose gel electrophoresis of the pDNA sample M4 (pDNA M4) and from the extraction kit (pDNA kit), in IL – sugar systems, namely $[P_{4444}]$Br/Fructose and $[P_{4444}]$Br/Sorbitol.

Annex C

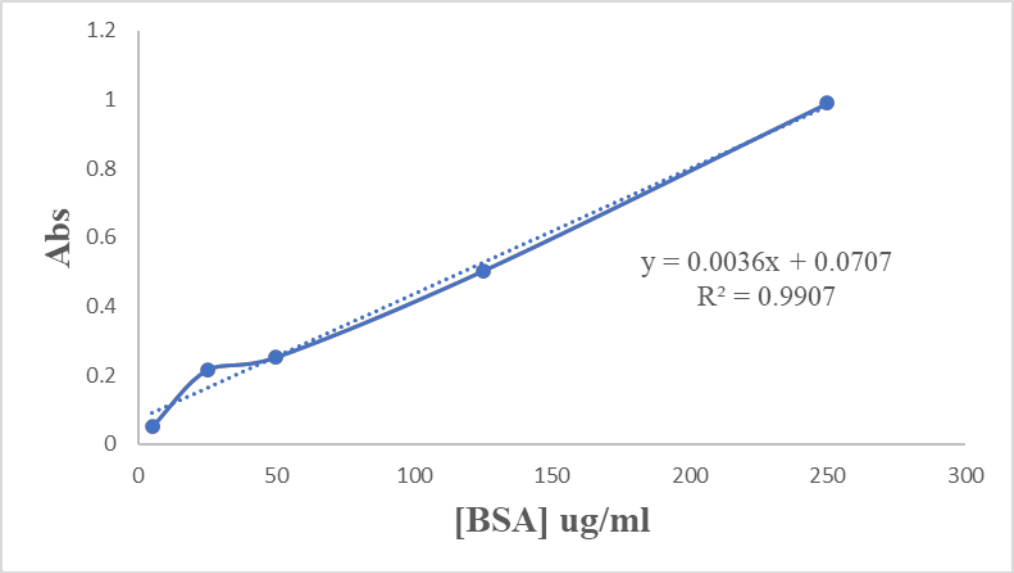


Figure 1C. Calibration curve of total protein concentration by the BCA method.