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Monolith and cryogel supports as new approaches to nucleic acids purification

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I'm grateful to my parents for all their sacrifices, patience and support throughout all these years.

Resumo

Nos últimos anos, avanços no estudo do genoma e proteoma humano têm fornecido informações essenciais em relação ao funcionamento correto dos genes e ao impacto das mutações desses genes na saúde humana. A terapia genética tem-se mostrado como uma das ferramentas mais promissora para o tratamento de doenças genéticas ou adquiridas. Apesar de os vetores virais serem os mais utilizados atualmente, o ácido desoxirribonucleico plasmídico (pDNA) tem-se mostrado uma forte alternativa para o tratamento destas doenças. Para obter pDNA na forma superenrolada, é necessário a purificação de extratos complexos, em que algumas moléculas partilham características e propriedades semelhantes. Para além disso, podem causar reações adversas no organismo humano, sendo imperativo a sua remoção para que o pDNA possa ser utilizado em aplicações terapêuticas. A remoção destas impurezas, por vezes, envolve processos dispendiosos e potencialmente nocivos para o ambiente devido ao uso de solventes orgânicos e sais.

Para tornar a purificação de ácidos nucleicos mais sustentável, têm-se explorado técnicas alternativas, tais como a utilização de monólitos e de criogéis como suportes cromatográficos. Monólitos consistem numa peça única de uma rede de canais altamente interconectada, tornando-os diferentes de outros suportes, contendo mesoporos e macroporos. Os criogéis são matrizes de gel preparadas a temperaturas de congelamento, caracterizadas pelo sistema de poros grandes interconectados. Ambos os tipos de suporte apresentam vantagens relativamente aos suportes convencionais particulados, nomeadamente uma maior capacidade e reprodutibilidade, elevadas taxas de fluxo e facilidade de "scale-up".

Esta dissertação consiste em três capítulos que incluem uma introdução, um artigo de revisão, e as conclusões e perspetivas futuras. No primeiro capítulo é apresentada uma pequena introdução sobre a aplicação de ácidos nucleicos para o tratamento de doenças, os seus vetores, formas de produção e purificação. O segundo capítulo, consiste num artigo de revisão que aborda novas abordagens na utilização de criogéis e monólitos como alternativa aos métodos cromatográficos tradicionais para a purificação de ácidos nucleicos. No terceiro capítulo, apresentam-se as conclusões desta dissertação e as perspetivas futuras sobre o tema abordado.

Palavras-chave

Cromatografia; criogéis; DNA; DNA plasmídico; terapia genética; monólitos; RNA; DNA plasmídico superenrolado

Abstract

In recent years, advances in the study of the human genome and proteome have provided essential information regarding the correct functioning of genes, as well as the impact of gene mutations on human health. Gene therapy has been shown to be one of the most promising tools for the treatment of genetic or acquired diseases. Although viral vectors are currently the most used, plasmid DNA has been shown to be a strong alternative for the treatment of these diseases. To obtain plasmid DNA (pDNA) in supercoiled form, it is necessary to purify complex extracts, in which some molecules share similar characteristics and properties, which in addition can cause adverse reactions in the human body. The removal of these impurities involves costly processes and potentially harmful to the environment due to the use of organic solvents and salts.

To make the purification of nucleic acids more sustainable, alternative techniques such as the use of monoliths and cryogels have been explored. Monoliths are based on organic and inorganic polymers and consist of a single piece of a highly interconnected network of channels, with mesopores and macropores, making them different from other supports. Cryogels are gel matrices prepared at freezing temperatures, characterized by a system of large and interconnected pores. Both types of supporst have advantages over conventional particulate supports, such as greater capacity and reproducibility, high flow rates and ease of scale-up.

This dissertation consists of three chapters that include an introduction, a review article, and the conclusions and future perspectives. The first chapter presents a short introduction on the application of nucleic acids for the treatment of diseases, their vectors, forms of production and purification. The second chapter consists of a review article that addresses new approaches to the use of cryogels and monoliths, as an alternative to traditional chromatographic methods for the purification of nucleic acids. The third chapter presents the conclusions of this dissertation and future perspectives on the topic discussed.

Keywords

Chromatography; cryogels; DNA; gene therapy; monolith; plasmid DNA; RNA; supercoiled pDNA

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asRNA	Antisense RNA
AT	Adenine-thymine
Bp	base pairs
DNA	Deoxyribonucleic acid
EMA	European Medicines Agency
FDA	Food and Drug Administration
gDNA	genomic deoxyribonucleic acid
GC	Guanine-cytosine
HIC	Hydrophobic interaction chromatography
IVT	*in vitro* transcription
Kbp	Kilo-base pair
Ln	Linear
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
oc	open circular
ori	origin
pDNA	Plasmid deoxyribonucleic acid
polyA	polyadenylation
RNA	Ribonucleic acid
RNAses	RNA-degrading enzymes
sc	supercoiled
siRNA	small interfering ribonucleic acid
SELEX	Systematic evolution of ligands by exponential enrichment
SDS	Sodium dodecyl sulphate

Objectives

The aims of this dissertation were:

-to review the therapeutic application of nucleic acids, their vectors, forms of production and purification.

- to describe and discuss the recent advances in the use of monolithic and cryogenic supports to purify nucleic acids as an alternative to traditional chromatographic methods.

Chapter 1

1. Introduction

1.1. Overview of molecular therapies

The foundations for what are now called molecular therapies were laid in the late 70s and early 80s when techniques for subcloning mammalian genes into prokaryotic plasmids emerged [1]. In 1989, the first human gene therapy clinical trial was made by Rosenberg et al to treat advanced melanoma [2]. By November 2017, the number of trials has reached 2597, and by late 2020 this figure has grown to 3180 [3]. The human genome project, that ended in 2003, greatly improved trials, due to the increased knowledge of human genetics and the role they play in genetic diseases.

The human genome contains between 20000 and 25000 protein encoding genes that drive biological processes and most rare diseases involve dysfunctional genes [4]. The increase in knowledge of the human genome and proteome has supplied valuable information in relation to the role and action of normal genes [5], as well as the impact of their mutations on human health. Molecular therapies aim to develop and explore molecular and cellular therapeutics to correct genetic and acquired diseases. Usually, molecular therapies consist of treating and preventing diseases by transferring genes, thus eliminating the cause of the disease rather than to treat the symptoms [6,7].

Diseases of genetic origin can now in theory be treated by the administration of healthy copies of the gene responsible for the illness or via stimulating a protecting immune response by administering genes encoding for specific antigens. Figure 1 represents clinical trials by country. The USA represents over 63% of all trials conducted so far.

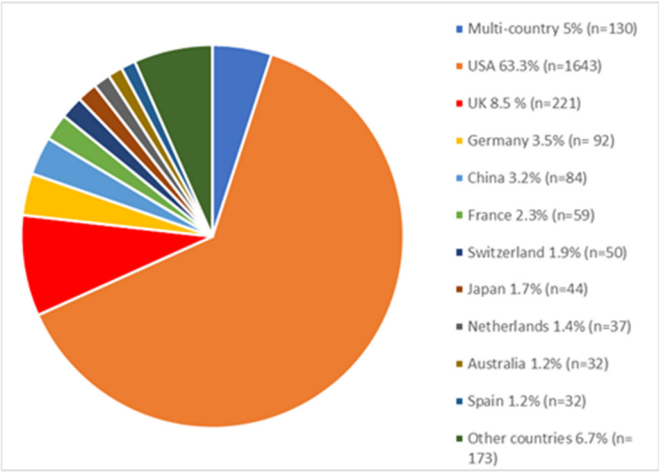

Figure 1 – Clinical trials by country (adapted from [8]).

1.2. Gene therapy

Gene therapy is a molecular therapy that consists of the therapeutic delivery of nucleic acids into patient's cells for treatment of genetic diseases.

Genetic diseases are usually the result of a mutation on specific genes, which can affect cellular mechanisms, resulting in the suppression, uncorrected or uncontrolled production of proteins. Gene therapy aims to revolutionize medicine by treating the causes of disease rather than the symptoms [7] and can be defined as the transfer of genetical material into a patient to treat an illness, which can be both *in vivo* or *ex vivo* [4,9].

The application of gene therapy involves three independent steps [10]: administration, delivery, and expression. Administration refers to the introduction of deoxyribonucleic acid (DNA) into the body. Delivery encompasses the translocation of genetic material from the administration site to the target cell nucleus. Delivery of gene therapy is affected by the bioavailability of the gene to the target cell, gene uptake, and intracellular trafficking. Finally, expression impacts the production of the therapeutic gene product in the cell. Successful gene therapy involves combining an appropriate disease target with an appropriate gene delivery system to produce long-term therapeutic results with little or no toxicity [10]. Gene therapy allows to choose a transgene for production, which can be either a protein, which due to genetic abnormality is deficient or it can be a protein with therapeutic effect [11].

The European Medicines Agency (EMA) states that a gene therapy product must fulfil the following requirements: "(a) contains an active substance which have or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence; (b) its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of the sequence" [12]. United States Food and Drug Administration (FDA) defines gene therapy as products "that mediate their effects by transcription and/or translation of transferred genetic material and/or by integrating into the host genome and that are administered as nucleic acids, viruses, or genetically engineered microorganisms. The products may be used to modify cells in vivo or transferred to cells ex vivo prior to administration to the recipient" [13]. Figure 2 shows the clinical phases of gene therapy clinical trials.

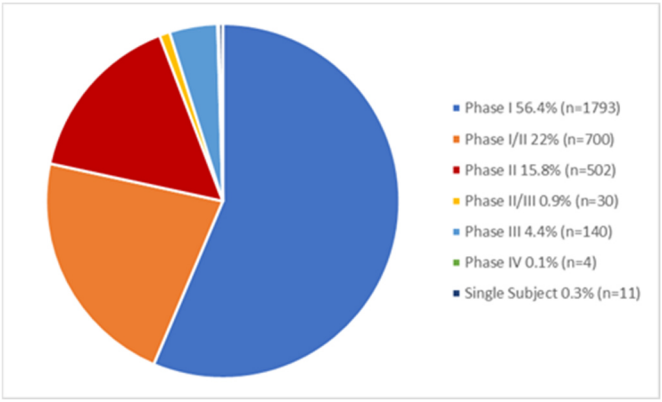

Figure 2 – Clinical phases of gene therapy trials (adapted from [8]).

Independently of the vector chosen, gene therapies are becoming an important alternative for classic treatments [14], in order for this to happen successfully, the role of the therapeutic gene in disease pathophysiology must be clearly understood [1]. By 2020, according to the Journal of Gene Medicine [8], there were 3180 trials worldwide, of these the majority (67,4%) were addressed to cancer diseases as shown in figure 3.

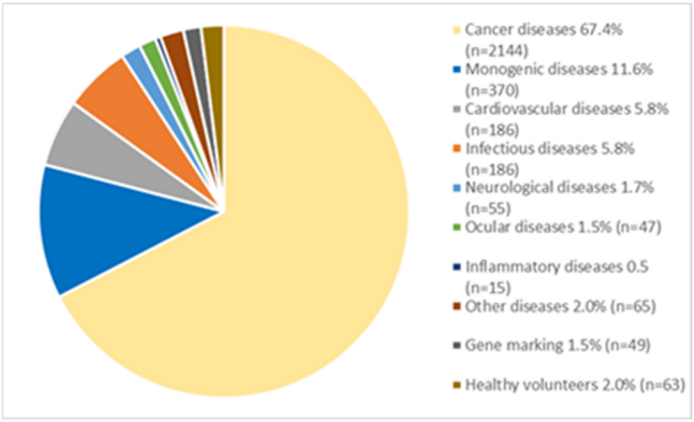

Figure 3– Clinical trials by disease type (adapted from [8]).

1.3. Approaches and strategies for gene therapy

Both somatic and germ-line cells can be used for gene therapy, with the somatic cells being commonly used and germlines being controversial, with ethical questions being posed [15]. The introduction and expression of recombinant genes in somatic cells has the goal of treating an inherited or acquired illness. DNA won't be passed on to next generations [16], unlike germ line cells, which modify the reproductive cells, thus affecting every descendant [17].

According to Gothelf and Gehl [11] there are three different approaches, distinguished by where the final product will act: the intracrine method, also known as single cell method in which every single cell in a population must be transfected with the plasmid to benefit from the transgene. The paracrine approach consists of the transfection of some cells in a population with the plasmid and then the produced protein acts locally, by stimulating an immune response. The systemic approach consists of transfecting a few cells in a population with the plasmid. The produced protein is then released into the blood stream, where it can induce a systemic response [11].

The four primary genetic approaches employed in gene therapy are [4]:

(1) Gene addition consists of adding a new gene into desired cells to produce new proteins.

(2) Gene correction involves the use of gene-editing techniques to eliminate repeated or defective elements of a gene or to replace a defective or dysfunctional DNA region.

(3) Gene silencing limits protein translation from the targeted messenger RNA (mRNA).

(4) Cell elimination techniques to destroy cancer cells but have also been employed against large benign tumours (comprising non-cancer cells) [4].

Gene therapies can be further divided into corrective gene therapy and cytotoxic gene therapy. Corrective therapy aims at the correction of a genetic defect in target cells by the transference of genes directly to the cell to correct the genetic defect, while the cytotoxic gene therapy involves the destruction of target cells using a cytotoxic pathway by transferring genes to the target cell which initiate cell destruction [10].

The gene therapy can involve two strategies: *in vivo* and *ex vivo*, as shown in figure 4. The ex vivo approach involves the harvesting and cultivation of cells from patients after surgical biopsy or organ resection. Cells are then cultured in vitro to allow efficient gene transfer, then the cells are reintroduced back into the patient. In contrast, in vivo involves the direct administration of the transgene into the target organ of the patient [10].

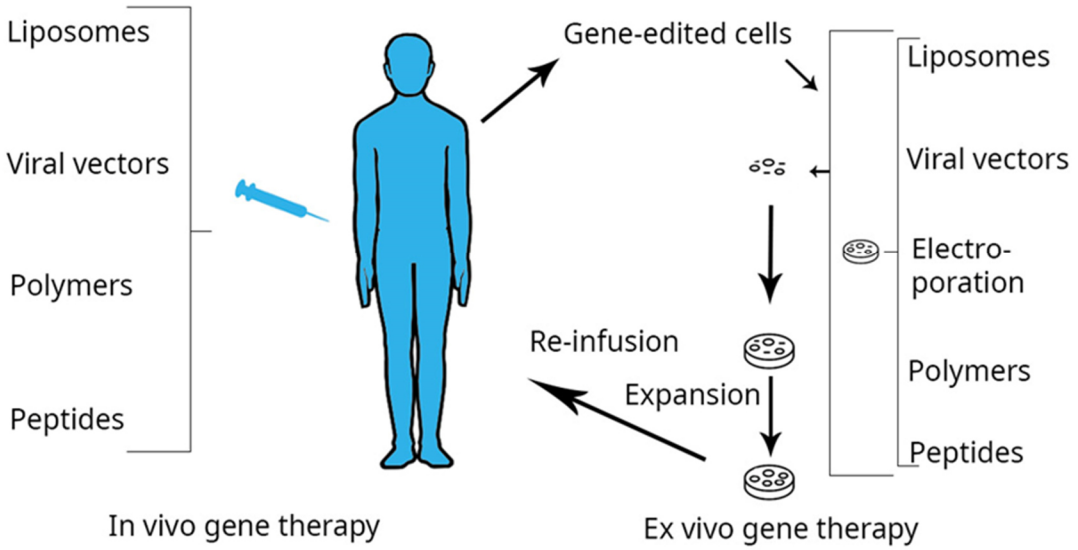

Figure 4 – In vivo gene therapy vs ex vivo gene therapy (adapted from [20]).

Presently, there are different DNA therapies approved for clinical use (table 1) and many others are being tested in various clinical trials in different countries.

Table 1- DNA therapies approved for clinical use.

Tradename	Date of approval	Approving agency	Indication	Manufacturer
Gendicine™	October 2003	State Food and Drug Administration of China	Head and neck squamous cell carcinoma	Shenzhen SiBiono GeneTech (Shenzhen, China)
Oncorine™	November 2005	China	Late-stage refractory nasopharyngeal cancer	Sunway Biotech Co. Ltd (China)
Rexin-G™	December 2007	Philippines	A broad spectrum of intractable cancers	Epeius Biotechnologies (USA)
Neovasculgen ™	December 2011	Russia	Peripheral artery disease, including critical limb ischemia	The Human Stem Cells Institute (Russia)
Glybera™ (alipogene tiparvovec)	November 2012	EMA	Lipoprotein lipase deficiency	UniQure (Netherlands)
Imlygic™	October 2015	FDA	Melanoma	Amgen (USA)

Strimvelis™	June 2016	EMA	Adenosine deaminase deficiency	GlaxoSmithKlin (United Kingdom)
Invossa™ (TissueGene C)	July 2017	Republic of Korea	Osteoarthritis	Kolon TissueGene, Inc. (Republic of Korea)
Kymriah™ (tisagenlecleucel)	August 2017	FDA	Acute lymphoblastic leukaemia	Novartis Pharmaceuticals (Switzerland
Yescarta™ (axicabtagene ciloleucel)	October 2017	FDA	B-cell lymphoma	Kite Pharma, Incorporated (USA)
Luxturna™ (voretigene neparvovec-rzyl)	December 2017	FDA	Retinal dystrophy (biallelicmRPE65 mutation	Spark Therapeutics (USA)
Zolgensma™ (Onasemnogene Abeparvovec)	May 2019	FDA	Spinal muscular atrophy, a severe neuromuscular disorder caused by a mutation in the SMN1 gene	AveXis (USA)
Zynteglo™	May 2019	EMA	Beta thalassemia	Novartis (outside USA) luebird bio (USA)

1.4. Vectors

The success of gene therapy relies on the ability to safely and effectively deliver genetic information to target cells [21]. As a matter of fact, the most relevant challenge to the clinical success of gene therapy is making gene transfer more efficient [21]. Usually, naked nucleic acids are a poor choice due to their poor uptake into the cells due to its properties (charge, size and poor stability). Because of this poor uptake, gene transfer into cells must be aided through biological (viral and non-viral methods), physical or chemical methods.

About seventy percent of clinical trials utilize viruses (e.g., an adeno-associated virus) for gene therapies. Non-viral vectors, such as direct injections of DNA, lipofection, and various other forms, comprise the remaining thirty percent [4] as displayed in figure 5. Gene therapy vectors

have the function of carrying the nucleic acid into the cell, protect the DNA from degradation and to efficiently deliver the genes to the target cells [23].

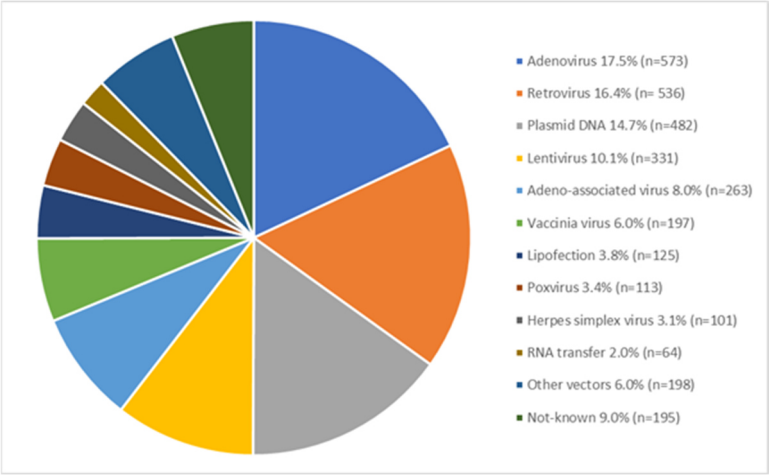

Figure 5 – Types of vectors (adapted from [8]).

Non-viral gene vectors offer versatile, simple, cost effective, and potentially safer alternatives but may lack adequate efficacy for clinical use. Conversely, viral vectors are often more efficient but can be more immunogenic than non-viral delivery systems [4]. Greater transduction effectiveness and long-term gene expression is offered by viral vectors, despite sometimes being associated with toxicity and immunogenicity [24].

1.4.1. Viral based vectors

Viral vectors are the most used vectors in gene therapy since viruses developed a method of encapsulating and delivering genes to human cells in a pathogenic manner [25]. These viruses are unlike wild type viruses and are used to transfer therapeutic genes into target cells. This means that they were engineered by deleting genes that allow replication, assembly, or infection, making them viable for use in gene therapy.

Nonetheless, despite being the most used type of vector, they suffer from some drawbacks, such as marked immunogenicity with induction of inflammatory system that can lead to deterioration of transduced tissue toxin production, insertional mutagenesis, and their limitation in transgenic capacity size [26,27].

According to the Journal of Gene Medicine [8], the most used virus vectors are Adenovirus and Retrovirus, as shown in figure 5. These viruses demonstrated high efficiency at delivering both

DNA and RNA to various cell lines. In table 2 are showed different viral delivery systems and advantages/disadvantages of its application.

Table 2 - Viral delivery systems

Viral system	Genetic material	Advantages	Disadvantages	References
Adenovirus	Double stranded RNA	Can be administrated *in vitro*; infects a wide range of cells, even dividing ones; High expression levels. Large inserts can be accommodated in the vector Cell targeting possible. Very high transfection efficiency ex vivo Very high titers	Not suitable for long-term gene expression due to lack of integration into the hosts genome. Host immune response; complicated vector genome Short duration of expression; insert size of 7,5kb.	[7,28,29,30]
Retrovirus	Enveloped single stranded RNA	Non-immunogenic. Relatively high titers Substantial clinical experience *ex vivo* Wide cellular tropismo	Low stability. Safety concern of insertional mutagenesis Transfects only proliferating cells Cellular targeting difficult to achieve	[7,28]
Lentivirus	Enveloped single stranded RNA	Can infect nondividing cells	Safety concerns from immunodeficiency virus origin	[28]

Adeno- associated viruses	Single stranded DNA	Infects dividing and nondividing cells Can be administered in vivo; integrates into the host genome Non-Immunogenic Long-term expression	Limited capacity for foreign genes High titers difficult to obtain Small transgenes Risk of insertional mutagenesis	[28,40,31]
Poxviruses (Vaccinia)	Double stranded DNA	Large DNA inserts Stable; Possible lyophilisation No integration into the host genome	Host immune Response; possible Lyophilisation Replication-competent vector with many adverse reactions immune response may limit usefulness	[31]
Herpes simplex	Enveloped double stranded DNA	Good persistence in selected cell-types Large expression cassette. Vector particles produced at high titers Natural tropism for neuronal or B lymphoid cells Well suited as oncolytic vector infects non-dividing cells	Toxicity and immunogenicity. High levels of pre-existing immunity Transient expression of the transgene The vector genome does not integrate into the host cell genome Risk of recombination with latently herpes simplex virus-infected cells	[32]
Alphaviruses	Single stranded RNA	High titer production	Transient expression	[33]

		Broad host range; extreme Transgene expression Low immunogenicity		

1.4.2. Non-viral vectors

The term non-viral vectors comprise all the physical and chemical systems, except viral systems. Usually includes either chemical methods, such as cationic liposomes/micelles and polymers with negatively charged nucleic acid, or physical methods, such as gene gun, electroporation, particle bombardment, ultrasound utilization, and magnetofection [35].

The efficiency of these systems is lesser than viral systems for gene transduction, but their cost-effectiveness, availability, and more importantly, low induction of immune system and no limitation in size of transgenic DNA compared with viral system have make them more adequate for gene delivery [34]. In general, the non-viral vectors are directly administered (plasmid DNA/Naked DNA)/ chemical /physical [36]. Non-viral vectors are listed in table 3.

Table 3 – Non-viral delivery systems

Delivery system	Characteristics	Advantages	Disadvantages	References
Naked pDNA	No carrier gene transfer is performed using physical methods such as needle injection (DNA vaccines), gene gun, electroporation, ultrasound and hydrodynamic delivery.	Easy to produce, manipulate and use very safe high efficiency when electroporation or hydrodynamic methods are used	Very short duration of expression Inefficient transfection ex vivo and in vivo Rapid degradation Low efficiency for ultrasound and injection methods	[37-39]
Cationic lipids	Include liposomes micelles, emulsion, and solid lipid nanoparticles.	No capacity imitations Low immunogenicity	Some toxicity; rapidly cleared from plasma Transient expression	[7,39,40]

		efficient ex vivo transfection	Inefficient in vivo transfection	
Cationic polymers	Polymers with protonated groups at physiological ph.	Highly effective ex vivo transfection	Hard to transfect non-dividing cells may induce cytotoxicity and host immune response	[39-41]
Cationic peptides	Redox-sensitive thiols can be incorporated into the carriers.	Generally have low cytotoxicity and immunogenicity; Great biodegradability; endosomal escape	Some peptide choices may induce toxicity with modest transfection	[42,43]

The greatest challenge for non-viral vectors is the delivery to the target cells. Many intra and extracellular barriers hinder administration success and gene delivery of these vectors. Degradation, endothelial barriers, inactivation of pDNA by serum proteins and DNA uptake by the reticuloendothelial system are barriers that vectors face and, even after reaching the target cell, there are also intracellular barriers such as lysosomal degradation of DNA, difficulties in translocation of DNA to the nucleus, endosome escape and cytoplasmatic instability of DNA. Even though many novel strategies are being developed that bring many improvements in the systemic delivery of DNA, in vivo delivery continue to be a big challenge and many efforts are being made to improve the uptake of naked DNA [40 ,44].

1.5. DNA Vaccines

DNA vaccines are based on recombinant DNA technology, which involves transferring a gene into a bacterial plasmid that codes for a protein but having an expression in eukaryotic cells [48]. DNA or nucleic acid vaccination belongs to the fourth generation of vaccines after live attenuated vaccines, inactivated vaccines, and recombinant protein-based vaccines. The first DNA vaccine was conceived in 1990 by Wolff *et al* [46], who used pDNA encoding for bacterial enzyme β - galactosidase. The vector was injected by direct transfer into muscle cells, leading for transcription in the nuclei, and subsequent synthesis of the enzyme [45,46]. Later, Ulmer *et al* were the first to describe on the protective efficacy of DNA vaccination against Influenza A [45,47]. Since then, DNA vaccines have been shown to induce protective immunity in numerous animal models of viral, and bacterial diseases.

As an emerging technology, DNA vaccines have several potential advantages, including ease of preparation, stability, relatively low cost, and safety for immunocompromised patients and a genomic capacity from 2 to 19 kilobases that can be transferred to muscle cells, although larger ones are being tested for multigene vectors [49]. Following the global pandemic of Covid-19, caused by the SARS-CoV-2, DNA vaccines have been in the spotlight because vaccines can be produced more rapidly (in as little as two or three weeks) than conventional vaccines.

The administration of a single dose of pDNA can provide a broad spectrum of immune responses, such as the activation of T-lymphocytes CD8+ and CD4+, which have regulatory function in the production of antibodies and the secretion of cytokines [50].

2. Therapeutic pDNA manufacture

Naked pDNA is the third most used vector with 14,7% of all gene therapy trials, surpassed by Adenovirus and Retrovirus, as represented in figure 5. Since pDNA is an appealing alternative to viral vectors [52], developing better purification and production methods is necessary in order to improve gene therapy success. It is known that manufacturing of pDNA follows a series of steps such as vector construction, transfection into appropriate hosts (Bacteria), cultivation, fermentation, lysis, clarification, purification, and a polishing final step [54].

The presence of proteins in the final pDNA preparations can lead to immune responses and biological reactions due to the presence of cytokines, hormones and/or antibodies and endotoxins, which are the most common fever causing substance (pyrogen) in humans, even in very minor quantities. These endotoxins consist primarily of highly negatively charged lipopolysaccharides from the cell walls of gram-negative bacteria such as *Escherichia coli*. In addition, the presence of genomic DNA (gDNA) may cause the possibility of an oncogene being activated or inactivated in the recipient cells [53]. Figure 6 represents the purification process to obtain pDNA.

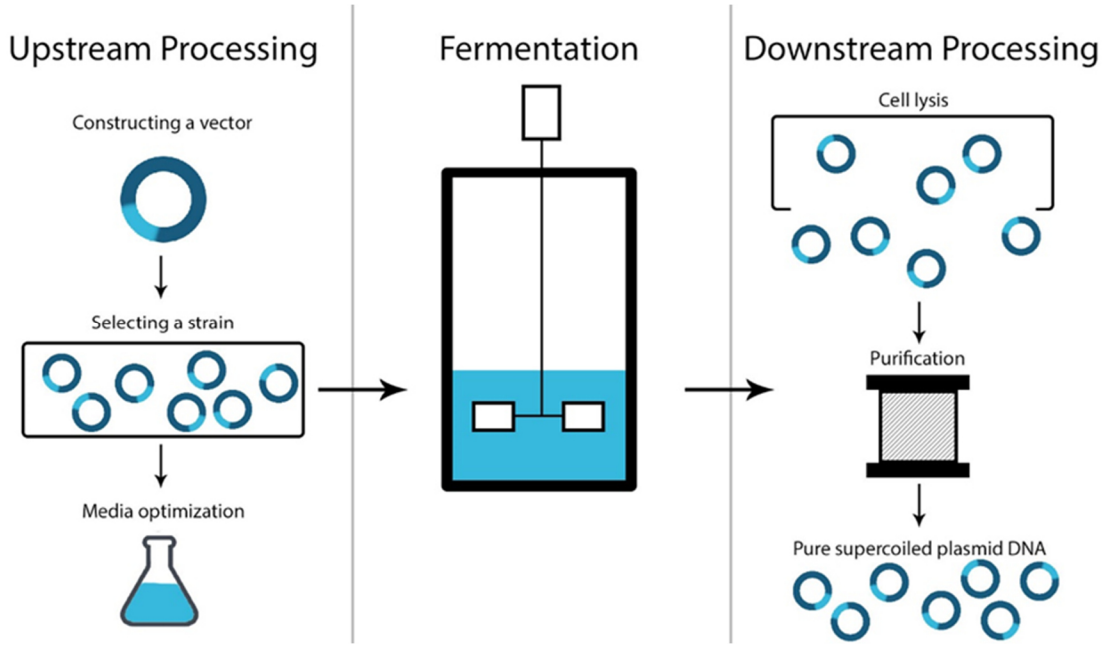

Figure 6 – Plasmid DNA purification process diagram (adapted from [54]).

Plasmid DNA usually makes up around one percent of the total bacterial cell content as does gDNA, endotoxins represent two percent, whereas RNA and proteins are the most widespread lysate components, comprising twenty-one percent of the total lysate [53].

2.1. Plasmid design

A plasmid is a covalently closed, double stranded DNA molecule, with sizes going from one base pair to over 100kbp [55,56], that naturally exists in prokaryotic and some eukaryotic cells and has the ability to self-replicate.

Each pDNA molecule consists of two strands of a linear polymer of deoxyribonucleotides linked through phosphodiester bonds. These phosphate groups are negatively charged at pH > 4. The winding of the two anti-parallel DNA strands around each other and around a common axis, stabilized by Watson-Crick hydrogen bonds between AT and GC base pairs and by stacking forces is what creates the classic right-handed double helix structure [56]. The close packaging of the aromatic bases makes the interior of the double helix highly hydrophobic [56]. The helix axis of pDNA can be coiled in space to form a higher order structure named supercoiled (sc) pDNA resulting in superhelix structures with varied topological conformations [57].

Stress sustained during the manufacturing and the recovery process can disrupt the structural stability of pDNA despite it being a very stable biomolecule [58]. A fraction of pDNA molecules population can also exist in a non-supercoiled form [56]. In these cases, the supercoiling is lost either by a single or double strand break, with the single break originating the open circular (oc) isoform and the double strand break creating the linear isoform. Sc pDNA is more efficient at transmitting gene expression than oc and linear variants [59]. Figure 7 represents the three isoforms.

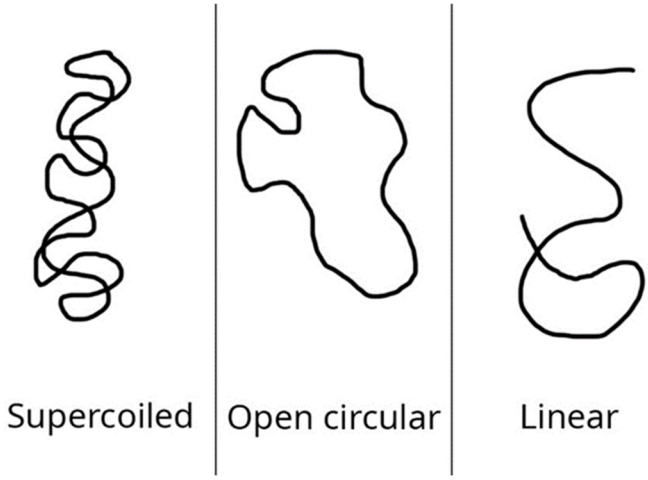

Figure 7 - Different pDNA topologies

The propagation of plasmids occurs in bacteria, so in addition to their therapeutic contents, they contain a bacterial replication origin (*ori*) and a selection marker [60]. The *ori* is a specific DNA sequence consisting of 50-100 bp to which the bacterial host cell enzymes bind, initiate, and regulate replication. This region permits the propagation and maintenance of the pDNA in the host cells during growth. Cells are chosen via selection markers, such as a Kanamycin resistance gene. Despite being a powerful selection mode, the use of antibiotic resistance genes is discouraged by the regulatory agencies since plasmids carrying them can transform the recipient microflora by propagating resistance genes [61].

A typical plasmid contains two distinct halves, as shown in figure 8. The mammalian expression cassette with the mammalian or viral enhancer/promoter sequences for gene expression, a 5' untranslated region, which includes introns, reporter transgene or gene of interest and a polyadenylation (polyA) sequence. The other half, the bacterial backbone includes the *ori* and the selection marker. For ease of manipulation, modular regions would be separated by a unique restriction site [61].

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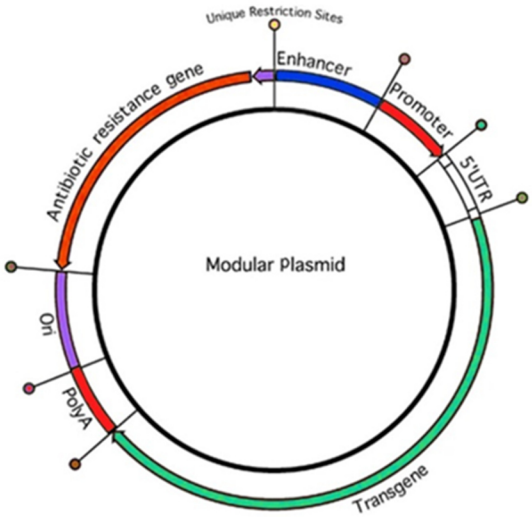

Figure 8 – Diagram of a modular plasmid, adapted from [61].

2.1.1 - Production of pDNA

Despite there are no specific guidelines about the most suitable genotypic or phenotypic characteristics that bacterial strains host should possess to produce the desired pDNA, it is known the choice of host can influence the final pDNA product.

Therefore, the *E. coli* is the bacteria of choice based on its high cellular density, low risk of promoting genetic modifications, its compatibility with subsequent purification steps and its ability to sustain the stability of this biomolecule [62]. The majority of pDNA vectors used in clinical trials are propagated by growing *E. coli* in a bioreactor [63]. However, the biggest challenge in working with vectors is achieve scalable and economical means of producing large quantities of sc pDNA, enabling it to produce the needed quantity and to fulfil FDA and the EMEA requirements [64]. This is due to the low concentration of pDNA, around 3% of the total contents, in the *E. coli* extract. Furthermore, since *E. coli* is a gram-negative bacterium, it contains highly immunogenetic endotoxins in its outer membrane, which can be a problem in

pDNA purification [65]. Other impurities include gDNA, RNA and proteins. These impurities may induce adverse reactions in patients if present in quantities higher than recommended, as shown in table 4.

Table 4: Specifications of *E. coli* lysate cell content and accepted levels of host impurities in the final purified sc pDNA for DNA vaccination (adapted from [64,66].

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

While the main isoform of the pDNA is the sc form, the process of purification and storage can induce cuts on the sc pDNA resulting in oc and linear isoforms. Therefore, FDA requires at least 97% sc pDNA in therapeutic solutions since this isoform has the highest biological activity.

2.1.2. Cell lysis and clarification

The first crucial step in downstream processing is cell lysis [54]. This step aims to release all intracellular components such as pDNA, RNA, gDNA, endotoxins and proteins into the medium. Many methods and technologies can be used for the disruption and lysis of the initial material, followed by removal of proteins and other contaminants. The disruption of the bacterial cells can be achieved using three distinct methodologies: chemical, physical and enzymatic. The chemical methods are the most widely used since physical methods employ ultrasounds which can trigger an increase in the temperature, being aggressive for the pDNA structure. The most prevalent procedure for cell disruption is alkaline lysis. This method aims to disrupt cells at a high pH using sodium hydroxide, in the presence of sodium dodecyl sulphate (SDS) which destabilizes the cell wall integrity and allows for its intracellular components to be released. Subsequently, the addition of sodium acetate precipitates denatured gDNA, proteins and cellular debris [67]. Nevertheless, the lysis step can be critical for pDNA physical stability

because, some sc pDNA unwinds due to hydrogen bond disruption promoted by the alkaline conditions, being irreversibly converted to oc pDNA. Also, gDNA can be fragmented during alkaline lysis, which can make further downstream removal substantially more difficult [67,68].

2.2. Purification of pDNA

The extract obtained after the upstream fermentation processes contain substantial amounts of impurities that need to be removed. The method of choice usually is liquid chromatography for process-scale therapeutic pDNA manufacturing [56]. Liquid chromatography is one of the most used purification methods in biotechnology, not only at analytical and preparative, but also at industrial level. This is due to its simplicity, robustness, versatility, and high reproducibility, meaning it can be utilized in the purification of a range of biomolecules, such as pDNA [69].

The purification step must promote the separation of the sc pDNA from its other topoisomers (oc and linear) together with the removal of the most significant impurities like gDNA, RNA, proteins, and endotoxins [57]. To fully capitalize on the chromatographic process, there must be a good mix of skills, like choosing the proper materials, having a good understanding of liquid–solid interactions [70]. A liquid chromatographic process requires three components: stationary phase, mobile phase, and molecules to separate [71].

The selection of an adequate stationary phase is essential for the success of the pDNA purification. The optimal support should be chemical and physically stable, exhibits low nonspecific adsorption with high binding capacity and mass transfer, and maintaining good flow properties throughout. Moreover, the stationary matrix should be inexpensive, simple to use, incompressible, can be used in several chromatographic runs stable to sanitization by alkaline conditions [69].

The capacity of the adsorbents is another matter to consider. In order to increase the binding capacity and to provide easy access to immobilized ligands inside the pores, it is crucial that the pores be at least five times bigger than the average size of the target biomolecules. The pore size of a matrix is inversely correlated to its surface area, which in turn directly impacts the number of immobilized ligands and therefore the binding capacity [69].

2.2.1 - Chromatographic methods for pDNA purification

As referred above, liquid chromatography is the method of choice for the large-scale purification of sc pDNA due to its simplicity, robustness, versality and high reproducibility [72]. The size and chemical properties of the target nucleic acid molecules (charge and hydrophobicity), the

accessibility of the nucleotide bases to ligands, and the topological constraints imposed by supercoiling are exploited via the interaction of nucleic acids with solid supports, with the objective of selectively isolating and purifying pDNA from impurities [54].

There are multiple chromatographic methodologies used in the purification of pDNA, such as anion exchange, size exclusion, affinity chromatography, hydrophobic interaction chromatography (HIC) and reverse phase chromatography [73].

HIC separates molecules based on their hydrophobicity in the presence of low or medium concentrations of salt. pDNA molecules have their hydrophobic bases packed and shielded inside the double helix, thus avoiding major interaction with the stationary phase, unlike single stranded nucleic acids and endotoxins, which interact with the hydrophobic ligands, allowing for the separation of pDNA from single stranded nucleic acids and other toxins. HIC has been applied in pDNA purification, however exhibits a major drawback due to high salt concentrations used [73,74].

Affinity chromatography is a distinctive method based on a highly specific molecular recognition. It relies on a strong but reversible interaction between the target molecule and the immobilized ligand, like many interactions found in biological systems. The specific recognition includes a mixture of various types of intermolecular forces, such as hydrogen bonding, electrostatic, hydrophobic and van der Waals interactions [75]. This method can be utilized for one-step purification of sc pDNA from bacterial lysate, nevertheless the biological ligands can be unstable and are also associated with a low binding capacity [73,76, 77].

Size-exclusion chromatography explores the distinct hydrodynamic sizes of the plasmids and their impurities to purify the target molecule. It can be used as a main purification step or as a polishing step, however it is not efficient for the removal of gDNA since it elutes near pDNA [73,78].

Anion-exchange chromatography is the most used chromatographic technique for the purification of pDNA. This method is based on the electrostatic interactions between negatively charged pDNA and positively charged stationary phase. It possesses the ability to separate sc from oc isomers, but it also elutes gDNA close to the pDNA [73,79].

3. RNA for therapeutic applications

Recent studies found diverse RNAs as well as noncoding RNAs and their corresponding action mechanisms in the cells. Some of these RNAs regulate various cellular pathways and thus are presumed to be important marks for the treatment of human diseases [80].

The largest barrier to all types of therapeutic RNA is delivery, despite diverse mechanisms of action. The biggest challenge is to bring therapeutic RNA molecules into the target cells efficiently in a safe and reproducible manner. FDA has already approved the first two small interfering RNA (siRNA) therapeutics, patisiran and givosiran [81]. Table 5 shows the approved RNA therapies until 2019.

RNA has varied roles in the body, which led to distinct approaches to exploiting RNA for therapeutic use. RNA therapeutics can be split into different functional classes: Interdiction of gene expression, such as siRNA, microRNA (miRNA) and antisense oligonucleotide (ASO), protein encoding mRNA and protein targeting (RNA aptamers) [82].

Table 5 – RNA approved drugs [80,82].

Drug	Brand name	Date of aproval	Target disease	Target molecule	RNA type
Fomivirsen™	Vitravene	1998	CMV Retinitis	IE2 mRNA	asRNA
Pegaptanib™	Macugen	2004	Age-related macular degeneration	VEGF protein	RNA aptamer
Alicaforsen™		2008	Pouchitis	ICAM-1 mRNA	asRNA
Mipomersen™	Kynamro	2013	Familial hypercholesterolemia	ApoB mRNA	asRNA
Nusinersen™	Spinraza	2016	Spinal muscular atrophy	SMN2 mRNA	asRNA
Eteplirsen™	Exondys 51	2016	Duchenne muscular dystrophy	Dystrophin mRNA	asRNA
Inotersen™	Tegsedi	2018	Transthyretin mRNA	Hereditary transthyretin amyloidosis	asRNA

Patisiran™	Onpattro	2018	Hereditary transthyretin amyloidosis	Transthyretin mRNA	siRNA
Golodirsen™	Vyondys 53	2019	Duchenne muscular dystrophy	Dystrophin mRNA	asRNA
Givosiran™	Givlaari	2019	Acute hepatic porphyria	ALAS1 mRNA	siRNA

3.1.1. Non-coding RNA

Non-coding RNAs (ncRNAs), such as siRNAs and miRNAs, perform crucial roles in gene regulation. They have been discovered over two decades ago and recently they have been used as a new class of therapeutic agents for the therapy of a broad range of disorders such as cancers and infections [84]. siRNA and miRNA have distinct methods of action despite sharing common features, such as being short duplex RNA molecules that utilize gene suppressing effects at the post-transcriptional level by targeting mRNA. In fact the biggest difference between siRNAs and miRNAs is that the former is very specific with only one mRNA target, while the latter has numerous targets. Accordingly, the therapeutic methodologies of siRNAs and miRNAs are thus distinct, as shown in table 6 [83]. RNA interference (RNAi) is a natural form of silencing gene expression via promotion of the degradation of mRNA. siRNA based therapeutic techniques require the introduction of synthetic siRNA into the target cells to obtain RNAi, thus preventing the expression of a mRNA, promoting a gene silencing effect. In relation to miRNA, it possesses two methods for its therapeutic use: miRNA replacement and miRNA inhibition. Using synthetic miRNAS to simulate the function of the endogenic miRNAs corresponds to the replacement procedure in mRNA is degraded/inhibited leading to a gene silencing effect [83].

The inhibition approach consists of using synthetic single stranded RNAs acting as miRNA antagonists to inhibit the action of the endogenous miRNAs. This biomolecule can also be used as biomarker tool that can pinpoint the existence of a pathology and even the stage, progression, or genetic link of a certain pathogenesis [84].

Table 6 -Comparasion between siRNA and miRNA [84]

	siRNA	miRNA
Prior to Dicer processing	Double-stranded RNA that contains 30 to over 100 nucleotides	Precursor miRNA (pre-miRNA) that contains 70-100 nucleotides with interspersed mismatches and hairpin structure
Structure	21-23 nucleotide complex with 2 nucleotides 3'overhang	19-25 nucleotide RNA duplex with 2 nucleotides 3'overhang
mRNA target	One	Multiple (could be 100 at the same time)
Mechanism of gene regulation	Endonucleolytic cleave of mRNA	Translational repression Degradation of mRNA Endonucleolytic cleave of mRNA
Clinical applications	Therapeutic agent	Drug target Therapeutic agent Diagnostic and biomarker tool

3.1.2. mRNA

In the last few decades, the idea of genetic vaccines has been implanted with prominence on DNA vaccines with the intention of obtaining flexible, easy to produce, safe and effective vaccines.

The attention DNA vaccines receive arises from RNA's instability, inefficient *in vivo* delivery, and excessive inflammatory responses. Nevertheless, presently the production of *in vitro*-transcribed mRNA is a relatively straightforward process, creating high quality therapeutic grade mRNA that is highly translatable and does not induce serious inflammation [85].

In order to be used as an alternative to pDNA vaccines, mRNA must have additional safety features, such as no persistence, no integration in the genome and no autoimmune response [86]. Furthermore, mRNAs are produced via *in vitro* transcription (IVT), which enables the production of considerable amounts of mRNA easily and efficiently [86]. Vaccination with mRNA can use various vectors, like pDNA vaccination. Direct injection of naked mRNA, gene gun delivery of mRNA loaded on gold beads, injection of mRNA encapsulated in liposomes or *in vitro* transfection of the mRNA in cells are just some of the vectors used to deliver mRNA [86].

Lately mRNA vaccines have attracted considerable interest as covid-19 vaccines, culminating in the Moderna (mRNA-1273) and Pfizer–BioNTech (BNT162b2) vaccines [87].

3.1.3. Antisense RNA

Antisense RNAs (asRNAs) are little molecules that have a regulative function by recognizing sequences and structural elements that are present in themselves and their respective target mRNAs. mRNA transcription and/or translation inhibition or induction of degradation is mediated via asRNA [88]. These molecules possess considerable advantages as a drug due to its regulatory function since asRNA-mediated regulation inhibits mRNA transcription and/or translation. Moreover, a small amount of asRNA is required to generate an effect. In terms of drug targets, this is an advantageous effect since a low dosage is enough for eliciting a response [89]. There are some asRNA therapeutics already approved by the FDA in the market, such as Fomivirsen™ and Golodirsen™ [82].

3.1.4. RNA aptamers

RNA aptamers consist of oligonucleotides that attach to target ligands with huge specificity and affinity. This type of molecule is produced by *in vitro* selection or by a method called Systematic Evolution of Ligands by EXponential enrichment (SELEX). Aptamers connects to its corresponding target, on a molecular level, by many non-covalent interactions, such as electrostatic interactions, hydrophobic interactions, and induced fitting [90]. RNA aptamers possess biotechnological and therapeutic potential for applications, since they have molecular recognition ability that compete with those of the generally used antibodies. In relation to antibodies, aptamers offer a few advantages such as complete *in vitro* synthesis, ease of production, desirable storage properties and low immunogenicity when used in therapeutic applications [91].

3.2. Production of RNA

With new roles for ncRNAs being discovered, coupled with the increase in research, its required access to considerable amounts of affordable ncRNA agents.

Currently most of the RNA produced is manufactured via synthetic methods, such as IVT and chemical synthesis, although in recent years efforts have been made to create recombinant methods to produce natural RNAs *in vivo*, using prokaryotes hosts [83,92]

3.2.1. *In vitro* transcription

One of the most used enzymatic approach for template directed synthesis of RNA molecules is IVT. This process is based on the creation of a template that contains a bacteriophage promoter sequence (e.g. from the T7 coliphage) anterior of the sequence of interest, followed by transcription using the corresponding RNA polymerase [93]. Even though this method yields RNA in the order of the milligrams and is commercial convenient, it requires a lot of work and is an expensive approach that can lead to RNA products with heterogeneous 3' and 5' ends. Such heterogeneity is caused because of the transcription termination that occurs out of the desired site. For example, a few bases before the 3' end or bases beyond the template length, and might be a deterrent to its future applicability, this method is also hindered by the need for high quantities of expensive RNA polymerases [94].

3.2.2. Chemical synthesis

Chemical synthesis of RNA allows for quick RNA production on a procedure based on phosphoramidite chemistry [95], with the obtention of high quantities of the target compound [96]. This method also permits the addition of chemical alterations in synthetic miRNA molecules, thus reducing their metabolic volatility as well as their degradation by cellular ribonucleases, also minimizing inherent immunostimulatory potential [95].

3.2.3. Recombinant biosynthesis

ncRNAs produced in vivo via fermentation do not carry the artificial but essential post-transcriptional modifications that are important for ncRNA higher-order structure, stability, and biological function. This approach aims to overcome some of the problems of the chemical methods described above [92].

This process seeks to provide substantial amounts of biological ncRNA agents with the correct folding and natural alterations that are important for therapeutic RNA [93]. Moreover, this technique is cheaper and less laborious when compared to synthetic methods. In its design, as successful biosynthesis must have in its design a combination of a few options such as the finest combination in terms of the host, vector, fermentation conditions and purification methods [98, 99]. In relation to RNA biosynthesis, the choice of host microorganism has been narrowed down to two: *E. coli* and *Rhodovulum sulfidophilum* (*R. sulfidophilum*). There are some advantages in using E.coli such as having an unmatched growth rate; can easily archive a high cell density; the growth conditions are straightforward and its easy and fast transformation with exogenous DNA [99].

However, the presence of RNA-degrading enzymes (RNAses) can induce changes on the RNA molecules. So, to obtain RNA, a "camouflage" procedure was established to get over this problem. tRNA scaffolds allow recombinant RNA to be camouflaged as a natural RNA and thus, hijacking the host machinery, evading cellular RNAses [98]. Figure 9 represents the bioengineering of ncRNA in *E.coli*. In the last few years, *R. sulfidophilum* have attracted attention for recombinant RNA production due to its properties, such as the secretion of nucleic acids into the extracellular medium and a non-release of RNAses to the extracellular medium [100].

Recovering RNA directly from *R. sulfidophilum* culture medium avoids time consuming and laborious extraction methods involving hazardous chemicals while simultaneously ensures the target RNA integrity since no host RNAses are secreted. Also using *R. sulfidophilum* may simplify downstream processing due to extracellular release of RNA [101].

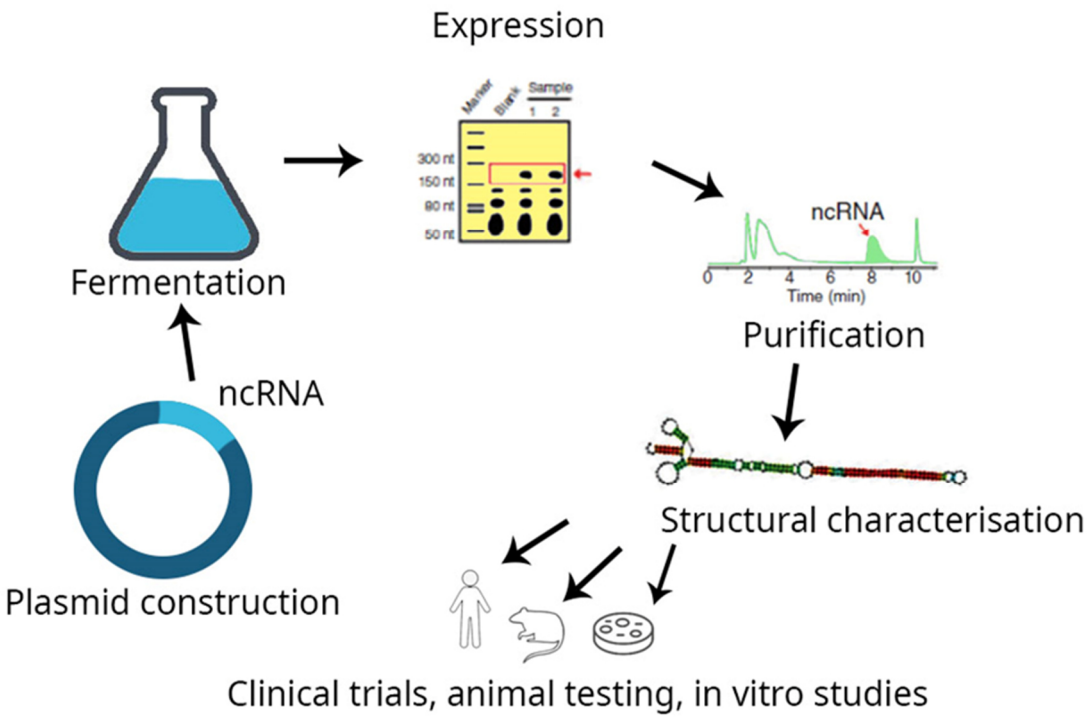

Figure 9 - Bioengineering ncRNA agents for research and development (adapted from [83])

3.3. Purification of RNA

In general, all protocols for RNA purification and isolation have in common the risk of RNA degradation during the process. RNA is an unstable molecule that has a very short half-life after extraction due to the presence of RNases [103]. Hence, in recent years downstream purification strategies based on affinity chromatography seem to be an effective strategy for therapeutic RNA purification.

Boronate affinity chromatography permits the exploration of one of the few chemical differences between RNA and DNA, which is the occurrence of cis-diol groups of ribose sugar at the 3' end of the RNA molecule and the lack of them in the deoxyribose structure of DNA [103].

Another approach is amino acid-based affinity chromatography. This technique uses amino acids, such as L-Arginine, L-Histidine, L-Lysine and O-Phospho-L-Tyrosine. They allow the purification and separation of various types of RNA such as tRNA, total RNA among others using standard particle-based columns like amino acid–agarose matrices [104-107]

A different RNA purification process is the RNA affinity tags. It permits the purification of long structures produced via *in vitro*. This method consists of using oligoribonucleotides sequences functioning as tags for RNA affinity purification (such as RNA aptamers) with the wanted properties and ability to bind specifically and with high affinity to a specific ligand [108].

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Chapter 2

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Review

Continuous beds: a promising tool for chromatographic purification of nucleic acids?

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Abstract: The need of new biopharmaceuticals with a high grade of purity has increased exponentially, creating pressure in the biotechnological industry to develop novel processes to produce and purify these bioproducts. The purification of nucleic acids for gene therapy and vaccines has been a challenge due to its physical and chemical properties. Moreover, the development of such advanced biotherapeutic agents require considerable amounts with high purity and quality to satisfy the demands of regulatory agencies. Conventional chromatography has been widely applied for the purification of nucleic acids due to its versatility, efficiency, and easy manipulation. However, some drawbacks have been described with conventional matrices, such as low flow rates, low binding capacity and high mass transfer resistance for the movement of large biomacromolecules. To overcome these limitations, in recent years, the development and implementation of continuous beds, such as monoliths and cryogel-based systems, have emerged as important alternatives. Thus, the current review describes and discusses the recent advances made in chromatography with the use of monolithic and cryogenic supports to purify nucleic acids.

Keywords: chromatography**;** cryogels; monoliths; pDNA; purification; RNA

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1. Introduction

In present times, due the Covid-19 pandemic, the need of new biopharmaceuticals with a high grade of purity increased exponentially, creating pressure in the biotechnological industry to develop novel ways to produce and purify these pharmaceutical grade bioproducts [1]. In fact, the purification of biomolecules, such as plasmid DNA (pDNA), minicircle DNA (mcDNA), messenger RNA (mRNA), small interfering RNA (siRNA) and microRNA (miRNA), has been a challenge due to the physical and chemical properties these compounds present. These biomolecules have been used to create new and revolutionary gene therapies and vaccines [2]. Moreover, the application of such biopharmaceuticals requires large quantities of these molecules with high purity and quality to meet the demands of regulatory agencies [3]. All these conditions result in a continuous pursuit and methods for purifying nucleic acids.

Currently, nucleic acids can be purified from cell extracts by using numerous specialized procedures, however the most usual involve chromatographic techniques [3]. Amongst these, liquid chromatography is one of the most common utilized at analytical, preparative, and industrial level, due to its versatility, price, robustness, and high reproducibility. As matter of fact, different chromatographic techniques, such as affinity [4], hydrophobic interaction [5], size exclusion [6] and ion exchange chromatography [7] have been used to purify nucleic acids. The mentioned techniques have commonly used particulate matrices such as agarose, dextrose, or silica. While being highly efficient, they have presented some drawbacks such as low flow rates, losses at high pressures,

difficulties in scaling up [8,9] and potential harm to the environment [10]. Moreover, they exhibit low binding capacity and high mass transfer resistance for the movement of large biomacromolecules, such as pDNA [9]. To surpass these limitations, several supermacroporous supports have been developed, allowing the formation of convective channels with minimal mass transfer resistance on the interior of the supports [11]. However, a diffusion transport is still observed in these systems. Consequently, in recent years, the development and implementation of continuous beds, such as monoliths and cryogel-based systems, have emerged as alternatives to solve some of the drawbacks, previously mentioned, characteristic of conventional chromatographic matrices.
Thus, the current review describes and discusses the advances made in chromatographic processes with the use of monolithic and cryogenic supports to purify nucleic acids.

2. Monoliths

Monoliths are considered the fourth generation of chromatographic stationary phases and consist of a single piece of highly interconnected channel networks making them distinct from other support types [12]. Although originally appearing during the 1990's [13], monoliths have been gaining interest as alternatives to conventional packed column [14,15]. In fact, the varied interconnected channels [16] provide a high porosity to the monolith with both mesopores and macropores interconnected in a wide diversity of ways [17,18]. Mesopores grant a high surface area for the molecules to interact with the support, while the macropores act as a flow through channels, allowing for solvent transport [19]. Due to the presented characteristics, monoliths exhibit low resistance to mass transfer and are easily functionalized and prepared [19]. All these advantages improve the recognition and purification efficiency of the target molecules, when compared to the conventional supports [19].

Monoliths possess numerous sub-categories, being originally classified as organic, inorganic, or hybrid.

Organic monoliths, as the name infers, use organic polymers in a free radical polymerization [20]. This reaction can be induced by heat, LED light or ultraviolet [20]. These supports have large pores, suitable for macromolecule separation, and involve the usage of an initiator, crosslinkers, monomers and porogenic solvents [20]. The most common monomers used are methylacrylates [21], acrylamides [22], and styrene [23]. The most used solvents are 1-Propanol and 1,4-butanediol, which allow the components to mix homogenously [20]. The crosslinkers serve as bridges between polymeric chains, to allow the mixture to grow into more than one direction and to regulate porosity. These compounds present one or more than one double covalent bond, allowing the ramification of the carbon chain during polymerization [24]. Usually, organic polymer monoliths are not sensitive to harsh pH conditions, have a good biocompatibility, are easily synthesized and exhibit elevated level of interconnectivities. However, they are prone to swelling, shrinkage and become unstable under temperatures above 200 °C [25].

Inorganic monoliths are mainly composed by different inorganic materials, such as alumina, zirconia, titania, hydroxyapatite, silica, etc, with the last being the most used [26]. These supports are usually prepared via a sol-gel process with several distinct protocols, depending on the inorganic material [27–31]. For example, in the preparation of silica-based monoliths, silanes such as tetramethoxysilane (TMOS) or tetraethoxysilane are used in the sol-gel process. These compounds go through hydrolytic polymerization in an aqueous solution of acetic acid with polyethylene glycol (PEG). The product is then

treated with ammonia to produce mesopores on the silica skeleton [32]. These monolith types do not swell or shrink when in contact with various solvents and the resulting monolithic structure can be stable at very high temperatures, up to 750 °C. Moreover, silica monoliths present a high surface area, up to 648 g/m^2 [33]. However, as all silica supports, they can be easily degraded by phosphate buffers and can only be used in a pH range between 2 and 8 [33].

Hybrid monoliths, also known as hybrid organic-inorganic monoliths, consist of two or more constituents combined at a nanometric or molecular level [34]. These supports present several advantages, such as a remarkable biocompatibility, good mechanical properties, flexibility and a long lifetime [35]. Furthermore, due to the individual advantages of both organic and inorganic monoliths, the hybrid supports have attracted attention due to their improved characteristics, such as high surface area, elevated sensitivity and excellent thermal stability [36]. Hybrid monoliths are divided into two groups based on their chemical composition: hybrid silica-based monoliths (HSM) and hybrid polymer-based monoliths (HPM) [34]. HPM monoliths are prepared via functionalization of the matrix through chemical bonding, being possible to maintain specific structures for the functional groups [34,36]. On the other hand, HSM are prepared by sol-gel process with a silica precursor containing organic moieties [37].

2.1 Monoliths for nucleic acid purification

2.1.1 DNA purification

In 2011, Sousa *et al* demonstrated the efficiency of a carbonyldiimidazole (CDI) monolith to purify supercoiled (sc) pDNA isoform [38], using the same salt and ionic strength conditions already applied with histidine-agarose matrix [39]. In both cases, the ligands have an imidazole group that has been shown to be highly specific for the sc isoform that exhibit a much higher biological activity than the open circular isoform. The chromatographic approach using the CDI achieved a HPLC purity of 100%, with a pDNA yield of 89%, and a step recovery yield of 74,4% for the sc peak. Furthermore, the analysis of impurities was performed, resulting in the agreement values with those established by the FDA [40]. The dynamic binding capacity (DBC) of CDI monolith for pDNA was six to twelve times higher than those achieved using histidine agarose-based supports [41], showing that this monolith has a great potential to be applied in affinity chromatography. Based on these promising results, Černigoj *et al* used a multimodal histamine derivatized CDI monolith for separation of pDNA isoforms and observed a high DBC, between 2.7 mg/mL and 4.0 mg/mL, depending on the experimental conditions [41]. Subsequently, this support was applied for purification of a cell lysate, aiming to obtain sc pDNA with high yields and purity, taking advantage of the specificity of the histamine to sc pDNA and the higher monoliths DBC [41]. Two purification strategies were performed: a simple and a combined strategy. In the simple purification approach, an ascending sodium chloride gradient was used and a purity of 96,66% and a recovery yield of 99,33% were obtained. On the other hand, the combined purification strategy performed with an ascending sodium chloride and then a decreasing ammonium sulfate gradient, resulted in a purity of 78,92% and a recovery yield of 91,55%. The quantification of host impurities in the purified sc pDNA sample showed that both strategies were able to decrease all impurities to safe levels. Nevertheless, the combined strategy was more efficient since it reduced by 128-fold and 39-fold for endotoxins and genomic DNA, respectively, when compared to the simple strategy [41].

In 2013, Soares et al developed a method that combined the high selectivity of arginine affinity ligands with the versality of CIM® epoxy monoliths to purify sc pDNA for a human papillomavirus vaccine. The comparison with a conventional arginine-agarose

matrix showed that the arginine monolith capacity was considerably higher than the conventional one. It was also demonstrated that the DBC of the arginine monolith was, at least, the triple when compared to the arginine-agarose matrix. With this functionalized monolith, a purity > 99% was obtained for the sc pDNA fraction, with a recovery yield of 39,18%. Moreover, the impurity analysis indicates that this support presents the capabilities to reduce host impurities to acceptable levels. Nevertheless, this chromatographic procedure requires additional optimization in order to improve the recovery yield of the sc pDNA fraction [42].

A CIM® polymethacrylate monolith functionalized with ethylenediamine was used by Bicho *et al* (2015) to purify a hemagglutinin DNA Influenza vaccine. With this approach a 97,1% of purity was obtained for sc pDNA fraction, which is in accordance with the established values imposed by the FDA (> 97%). In fact, the analytical impurity analysis demonstrated an efficient capability of this chromatographic support to reduce host impurities to acceptable levels. However, the recovery yield was only 47,0%, indicating that this purification process requires additional improvement [43].

In 2015 Almeida et al performed an optimization of the chromatographic protocol for the purification of a DNA vaccine for the human papillomavirus using design of experiments [44]. This optimization process indicated an optimal point with a recovery of 91,39% and purity of 98,82%. Three chromatographic runs were made, always obtaining a purity of 100% and a recovery yield between 75,83 and 88,76%, being the last a little smaller than anticipated by the software. However, is important to mention that the impurity analysis of the obtained sc pDNA fraction was not made, meaning that was not evaluated the possible changes in the capabilities of this support to remove host impurities [44].

In 2016 Bicho et al used a modified carbonyldiimidazole monolith with agmatine to evaluate the efficacy of purification of an influenza DNA vaccine and also to performa DBC study of this support [45]. This chromatographic procedure was made with two types of elution, an ascending sodium chloride gradient and a decreasing ammonium sulfate gradient. In the first strategy, a purity of 99,6% and a recovery yield of 45,3% were obtained, being necessary two chromatographic runs. With the ammonium sulfate gradient, was observed a recovery yield of 51,8% and a purity of 98,3%. Both strategies provide low recovery yields, being necessary further improvements in the chromatographic protocol, and possible modifications on the support itself. It was also made the purity analysis, confirming the capacity to reduce host impurities to acceptable levels. As previously referred, a DBC study was elaborated, dictating that for this monolithic support, a lower flow rate, higher pDNA concentration and acidic conditions lead to greater DBC [45].

Limonta et al (2017) compared the performance of a CIM® C4-HLD monolith with a Sartobind phenyl membrane column, in the purification of an experimental hepatitis C pDNA vaccine [46]. The monolithic support demonstrated the ability of doubling the sc/open circular (oc) ratio, providing a purity within a range of 92,76 – 99,43% and a recovery yield within the range of 80,9 – 100%. The impurity analysis was performed, confirming the hability of this support to eliminate host impurities to acceptable levels. With this study a scale-up possibility was confirmed, allowing researchers to produce enough pharmaceutical-grade vaccine to begin clinical trials [46].

A CIM® epoxy monolith functionalized with arginine homopeptides was used by Cardoso et al (2018) to purify pDNA. In this case, three distinct monoliths, a monolith with arginine ligands, a monolith with di-arginine ligands and a monolith with tri-arginine ligands were tested. The purity provided by these supports was always < 97%, except for the monolith with tri-arginine ligands which promote a purity above 99%. However, the recovery yield decreased with the size of the ligand, starting in 88,0% and ending with only 52,7%. Furthermore, the impurity analysis of the sc pDNA fraction confirmed the ability of theses monoliths to remove host impurities [47].

The performance of two monolithic supports, one with lysine ligands and another with cadaverine ligands, were evaluated by Almeida et al (2019) for the purification of minicircle DNA. Initially, the lysine functionalized monolith was tested, resulting in an incompatibility between recovery yield and purity, meaning that to achieve good purity, the recovery yield needed to be sacrificed, proving the lack of selectivity for mini circular DNA. The other monolith, with cadaverine ligands proved to be useful for the purification of mcDNA, allowing a recovery yield of 78,6% and a purity of 98,4%. Moreover, it was proved that the monolithic support could reduce impurities to levels within the FDA criteria [48]. In 2020, Almeida et al also tested an epoxy monolith derivatized with arginine ligands with a spacer arm for purifying a human papillomavirus DNA vaccine. This spacer arm improves the access to the ligand, increasing the sensitivity of this support. Despite the purity presented by this monolith was 93,3%, a value inferior to the established by the regulatory agencies, a reasonable recovery yield of 72% was obtained. Furthermore, the impurity analysis showed that this monolithic support could reduce host impurities to acceptable levels. With the information presented, the usage of this support must be optimized to achieve a purity percentage of at least 97% [49]. The monolithic supports described above are presented in Table 1.

Table 1. Monolithic supports used for DNA purification

Ligand	Matrix	Purity (%)	Recovery Yield	Purpose	References
----------	Carbonyldiimidazole	100,00	89,00	Not applicable	[38]
Histamine	Carbonyldiimidazole	78,92 - 96,66	91,55 - 99,33	Not applicable	[42]
Ethylenediamine	CIM®	97,10	47,00	Influenza DNA	[50]
Arginine	CIM® epoxy	> 99,00	39,18	Human	[43]
Arginine	CIM® epoxy	100,00	75,83 - 88,76	Human	[44]
Agmatine	Carbonyldiimidazole	98,30 - 99,60	45,30 - 51,80	Influenza DNA	[45]
C4 HLD	CIM®	92,76 - 99,43	80,90 - 100,00	Hepatitis C DNA	[46]
Arginine	CIM® epoxy	> 97,00	52,70 - 88,00	Not applicable	[47]
Lysine	CIM® epoxy	----------	----------	Not applicable	[48]
Cadaverine	CIM® epoxy	98,40	78,60	Not applicable	
Arginine + Space	Epoxy	93,30	72,00	Human	[49]

2.1.2 RNA purification

In recent years, the enormous increase of several RNA-based therapies, such as cancer and vaccines, has led to a large investment in new approaches for the production and purification of different types of this nucleic acid. Currently, the high demand for mRNA for use in Covid 19 vaccines requires a technology platform and cost-effective

manufacturing process with a well-defined product characterization. The production of these vaccines involves in vitro transcription (IVT) followed by a purification platform with multiple steps that can include DNase digestion, precipitation, and chromatography.

The chromatography is a mainstream purification process widely accepted in the pharmaceutical industry and has been tested in RNA purification using different approaches (Table 2).

In 2014, Pereira *et al* conducted a study by using a CDI monolithic chromatographic support with agmantine ligands to purify pharmaceutical-grade pre-miR-29. Three chromatographic strategies were used to increase efficiency and selectivity: an increasing gradient of sodium chloride, an increasing gradient of arginine and a decreasing gradient of ammonium sulphate. Both increasing gradients improved the final yield and purity of the pre-miR-29, but the sodium chloride gradient originated the best result for the recovery yield, with 97,33%. On the other hand, with the arginine gradient, the best result was achieved for the purity, with 90,11%. Additionally, the impurity analysis was performed on to the fraction with purified pre-miR-29, confirming the capacity of this monolith to isolate the analyte without any significant impurities [51].

RNA molecules were purified by Levanova *et al* (2018) using a CIM-OH monolithic columns based on steric exclusion chromatography. The results showed that this support was able to separate double stranded RNA (dsRNA) from single stranded (ssRNA) but provided a greater resolution for dsRNA with a size higher than 700 bp. Additionally, it was able to purify whole viral ssRNA and dsRNA genomes from other contaminants. At last, this monolith lacked when researchers tried to isolate RNAs with a highsize difference, being only able to isolate short RNA fragments from longer RNAs [52].

Table 2. Monolithic supports used for RNA purification

Target	Ligand	Monolith	Purity (%)	Recovery Yield (%)	Purpose	Reference
RNA	Agmantine	CDI (NaCl gradient)	75,21	97,33	----------	
RNA	Agmantine	CDI (Arginine gradient)	90,11	94,88	----------	[51]
RNA	OH	CIM	----------	----------	----------	[52]

3. Cryogels

Cryogels, are a novel supermacroporous chromatographic supports with a characteristic sponge-like morphology and a pore size controllable network of pathways [53]. These supports were originally reported in the 1970s, intriguing researchers due the unusual properties of these polymeric gels [54]. One major difference between cryogels and traditional chromatographic supports is the type of the mass transfer. Cryogels ensures unrestrictive convectional transport, when the later can only perform mass transport by diffusion [55]. With this convection mass transport, these supports allow an efficient chromatographic separation of nanoparticles, cell organelles and even entire cells [54]. The convective flow is only possible due the macropores present in the cryogels, which have a size range of 10-100 μm [56].

Cryogenic monoliths are good alternatives to traditional protein-binding matrices with many different biomedical applications. Advantages such as high blood-compatibility and water content, non-degradable, no toxicity, and pressure drop properties, allow the use of these supports with biological macromolecules without any diffusion issues [57,58]. Another strong point of cryogels is their unique and tunable properties, which makes them useful in various fields by controlling fabrication parameters (e.g., polymer choice, temperature, solute concentration, and cooling rate) [59]. Nevertheless, cryogels also possess some disadvantages, being the main one the low surface area these supports present [58]. Applications for these chromatographic supports are divided in four categories: capturing biomolecules; biomolecules immobilization; separation of cells; environmental separations, being the first the target of this review [54].

Additionally, cryogels can be derivatized to be used in tissue engineering research. Due to the 3D structures and hydrophilicity these materials present cell affinity and cell proliferation are ideal for these studies [60].

It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.1 Preparation of cryogels

Cryogels are prepared by a process called cryogelation, which occurs between -5 and -20 ºC, depending on the solvent crystallization point [55].

There are three steps as showed in figure 1. The first step involves the preparation of a solution containing monomers and initiators, with the solvent being usually water, and then freezing it. During this stage there are two main parts, the frozen solvent, which creates ice crystals and the unfrozen liquid microphase (UFLMP), where the polymer precursors are located. As the crystallization progresses, the ice crystals link with each other forming an interconnected network. As UFLMP presents only a small portion of the total initial volume, the concentration of gel precursors increases dramatically promoting the gel-formation, forming the gel around the crystals. After the polymerization, the mixture is thawed to room temperature, melting away the crystals, which leaves macropores surrounded by the new formed gel, revealing a macroporous interconnected matrix [61-63].

Cryogels can be synthesized from a variety of monomers such as pre-made vinylic monomers, 2-hydroxyethyl methacrylate (HEMA) [57] or acrylamide (AAm) [64]. In addition, natural or synthetic polymers such as alginate [65], agarose [65], gelatin [66], chitosan [67] or PEG [68], can be functionalized with vinylic groups to cross-link them through a free radical cryo-polymerization. The ammonium persulfate (APS) and

N,N,N,N tetramethylethylenediamine (TEMED) initiator system are the most commonly used for cryogel fabrication [59].

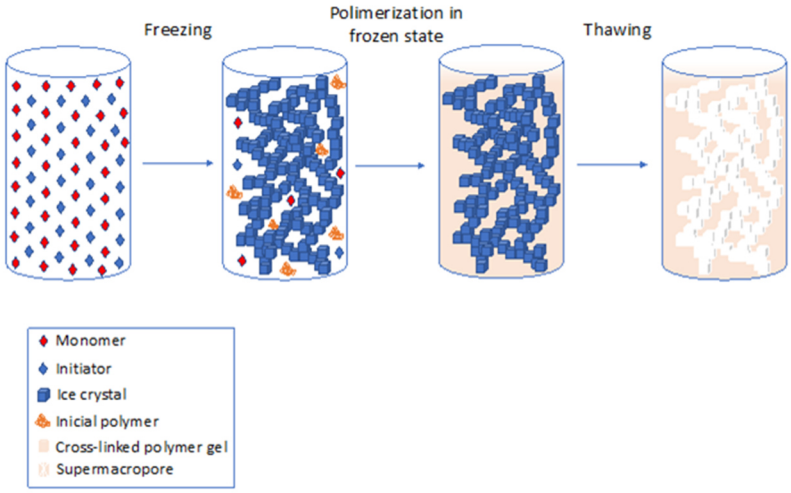

Figure 1. Cryogelation process.

3.2 Cryogels for nucleic acid purification

3.2.1 DNA purification

In 2013, Üzek *et al* performed a study for a novel cryogelation process to incorporate nanospines into the cryogel structure, with the purpose of purify plasmid DNA [69]. Three different cryogels were synthetized, the poly(2-hydoxyethyl methacrylate-*N*-methacryloyl-L-phenylalanine)-freeze dried [p(HEMA- MAPA)-FD], the poly(2-hydoxyethyl methacrylate-*N*-methacryloyl-L-phenylalanine p(HEMA-MAPA) and the poly(2-hydoxyethyl methacrylate)-freeze dried [p(HEMA)-FD]. All three supports were characterized by Fourier-transform infrared spectroscopy (FTIR), scanning electronic microscope (SEM) and nitrogen adsorption/desorption by using Brunauer–Emmett–Teller isotherm to acquire the surface area of the cryogels. DNA adsorption studies were performed by analyzing the effects of pH, temperature, salt type and concentration, and initial DNA concentration. The experimental conditions were optimized and set at a pH between 5 and 6, temperature 40 °C, sodium sulphate 1M and DNA concentration of 4 mg/mL. It was concluded that the [p(HEMA- MAPA)-FD] cryogel provided the greater adsorption capacity, with 45,31 mg DNA/g cryogel, twice as much as the same cryogel preparated with normal procedures and 40 times greater when compared with poly(2-hydroxyethil methacrylate) (pHEMA) cryogels [69].

Two alternative hydrophobic cryogels for isolating genomic DNA were synthetized in 2013 by Çorman *et al*: a poly(2-hydroxyethyl methacrylate-*N*-methacryloyl-L-tryptophan) [p(HEMA-MATrp)] cryogel with MATrp hydrophobic ligands and a p(HEMA) cryogel embedded with p(HEMA-MATrp) monosize particles [p(HEMA-MATrp)/p(HEMA)] [53]. Both cryogels were synthetized by using APS and TEMED as initiators and its characterization was performed via FTIR spectroscopy, swelling studies, SEM, elemental analysis and surface area measurements. pH studies demonstrated that at pH 5.0, DNA was strongly adsorbed by specific binding. Furthermore, with the various salt concentration studies, it was determined that by using sodium sulphate the supports achieve higher levels of DNA adsorption. At last, it was shown that for the p(HEMA-MATrp) cryogel the maximum DNA adsorption was 15 mg/ g polymer and for the p(HEMA-MATrp)/p(HEMA) the maximum DNA adsorption was 38 mg/g polymer [53].

A new pHEMA cryogel with dye affinity ligands for the purification of plasmid DNA was developed in 2015 by Çimen *et al* [70] Initially, the pHEMA support was produced by free radical polymerization, with APS and TEMED as initiators. Posteriorly, the Cibacron Blue F3GA was added, functionalizing the cryogel as the affinity ligands. SEM and FTIR spectroscopy were used to characterize this novel cryogel and the effects of ionic strength, temperature, and DNA concentration on the purification of the samples were studied. The results demonstrated that this chromatographic support behaved ideally at low temperatures, with low ionic strength and high DNA concentration. Moreover, the maximum DNA adsorption for this support was 32,5 mg/g cryogel, being almost 30 times greater when compared to the pHEMA cryogel [70].

In 2018, Santos *et al* performed the purification of an Influenza DNA vaccine using pHEMA cryogel prepared by cryo-polymerization and then characterized by SEM [57]. A study to evaluate the DBC of this support was elaborated to fully characterize this cryogel. The purification of the desired pDNA isoform from a clarified lysate sample was done in a two-step procedure using sodium chloride. The assessment of DNA vaccine allowed to conclude that the level of contaminants such as proteins, genomic DNA, RNA, and endotoxins are in accordance with FDA regulations. With the overall process, researchers obtained a 98.1% of purity and a recovery yield of 69.19%. The novelty of the method is that the matrix itself interacts with the scDNA [57].

The cryogel supports described above are presented in Table 3.

Table 3. Cryogel supports used for DNA purification

Target	Ligand	Monolith	Capacity (mg/g)	Purity (%)	Recovery Yield (%)	Purpose	Reference
DNA	MATrp	p(HEMA-MATrp)	15,0	----------	----------	----------	[53]
DNA	p(HEMA-MATrp) monosize particles	pHEMA	38,0	----------	----------	----------	[53]
pDNA	Cibacron Blue F3GA	pHEMA	32,5	----------	----------	----------	[70]
pDNA	----------	pHEMA	----------	98,10	69,19	Influenza DNA vaccine	[57]

3.2.2 RNA purification

The cryogels have been also applied in RNA purification. In 2012, Srivastava et al used a poly(hydroxyethylmethacrylate-co-vinylphenylboronic acid) [poly(HEMA-co-VPBA)] cryogel for the RNA separation from a crude sample of lysed E.coli, confirming the capability of this support to separate RNA from crude extracts, in one step. Additionally, the binding capacity of this cryogel was determined and a 1,13 mg/mL of column was obtained. Although, it was demonstrated that this support was able to

successfully separate RNA from crude extracts, the analysis of the presence of impurities in the purified RNA samples was not performed [71].

In 2016, an innovative cryogel was developed by Köse et al for separation and purification of RNA [72]. A polymerizable derivative of adenine designated as adenine methacrylate (AdeM) was synthesized through a substitution reaction between adenine and methacryloyl chloride. After preparing the AdeM, 2-hydroxyethyl methacrylate (HEMA)-based cryogels were prepared in partially frozen medium, by the copolymerization of the AdeM and HEMA monomers. Experiments were conducted using the batch system, and different conditions of pH, initial RNA concentration, temperature, and interaction time were tested. Swelling ratios reached 510% and the capacity was 11.86 mg/g, while being able to be reused 5 times [72]. This research group also created a novel cryogel consisting in a guanine-incorporated pHEMA matrix, with the objective of obtaining highly purified RNA based on a real natural interaction between guanine on the polymeric material and cytosine on the ribonucleic acid. This support was synthetized using HEMA and GuaM monomers, with TEMED and APS as initiators of the polymerization reaction. In this study, both FTIR and SEM were used to characterize the cryogel. Experiments were conducted using three different supports: the p(HEMA-GuaM) cryogel, a standard pHEMA cryogel and a commercial kit. Results showed that the novel cryogel presented a significant increase in adsorption capacity (5,59 mg/g) when compared with the other two methods, reaching almost 29 times the adsorption capacity of the standard pHEMA cryogel and 1,6 times the absorption capacity of the commercial kit. Due to the natural interaction, the adsorption of the target molecule onto the ligand was highly selective and occurred in a very short time, without diffusion drawbacks. Moreover, the RNA adsorbed onto this new cryogel was successfully recovered without any denaturation in a fast, low-cost and single-stepRNA purification. Although the capacity for the removal of impurities from crude samples was not demonstrated [73]. Table 4 represents the chromatographic supports used for RNA purification.

Table 4. Cryogel supports used for RNA purification

Target	Ligand	Monolith	Binding capacity (mg/mL)	Capacity (mg/g)	Purpose	Reference
RNA	Boronate	poly(HEMA-co- VPBA)	1,13	----------	Not applicable	[71]
RNA	----------	HEMA-AdeM	----------	11,86	Not applicable	[72]
RNA	Guanine	pHEMA	----------	5,59	Not applicable	[73]

4. Conclusions

The demand for nucleic acids for both gene therapy and vaccines has been rising these past few years and the purification is a key step to obtain nucleic acids that meet the regulatory agencies demands.

Chromatography has been the purification process widely used in the pharmaceutical industry, however the traditional supports have some drawbacks, such as low capacity and difficulties in scale-up. To avoid these constraints and make the purification process more efficient, continuous beds have been hailed as a new alternative to traditional chromatographic methods. These continuous beds can be prepared in a variety of ways with many different materials, allowing them to have custom properties that are attractive for separation and purification of biomolecules such as nucleic acids. Despite being demonstrated that these matrices can be used to purify nucleic acids efficiently and are a truly viable alternative to packed beds at an industrial scale, more improvements must be done for its application in larger scale.

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Chapter 3

Conclusion and future remarks

The continuous growth of the knowledge of human genetics has revealed that some diseases are caused by dysfunctional genes, which means theses kind of illness can in theory be treated by using healthy copies of the gene in question. There are already in the market approved gene therapies and the rise in the number of clinical trials has created immense demand of these molecules

Most clinical trials and gene therapies have been done with the aid of viral vectors, such as adenovirus, however lately there has been a shift to use non-viral vectors such as pDNA and RNA molecules. This leads to an increase in the demand of these molecules. This demand led to the necessity to develop efficient purification strategies in order to obtain these molecules with high purity, integrity, biological activity and that meets the regulatory agencies demands.

The most important step in purification of the target biomolecules is the purification step, traditionally it's performed using liquid chromatography with techniques such as affinity chromatography, anion exchange chromatography, hydrophobic interaction chromatography, among others, using particulate matrices. However, these conventional supports suffer from some disadvantages such as low flux rates, low binding capacity and high mass transfer resistance for the movement of large biomacromolecules, such nucleic acids.

In order to overcome these limitations, alternatives to packed columns have been studied, this article is a review that takes a look at potential alternatives like continuous beds, such as monoliths and cryogel based matrices. These matrices can be made from a variety of materials, allowing them to be fine tuned for the desired project.

In the review, performance of these materials for the purification of nucleic acids has been analysed from adequate literature.

It can be concluded that these materials can offer good efficiency in the purification of nucleic acids, despite the number of studies using nucleic acids being quite low and mainly at a laboratorial scale. In the future, more studies have to be made with these continuous beds in order to be applied as a true alternative to conventional packed columns.