DEGREE’S FINAL PROJECT

Degree in Biomedical Engineering

POLYMER NANOPARTICLES FOR THE DELIVERY OF MICRORNA THERAPEUTICS IN REGENERATIVE MEDICINE AND DISEASE TREATMENT: CURRENT STATE OF THE ART AND FUTURE PERSPECTIVES

Report and annexes

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Abstract

microRNAs (miRNAs) are small non-coding RNA molecules, that have been proved to be key regulators of many diseases, such as cancer. This thesis deals with the current state of art of miRNA based therapeutics, with a special attention to the delivery systems employed for their encapsulation and delivery.

Discovered in 1993, it was not until 2002 that the aberrantly expression of microRNAs in cancer was proven. First studies on miRNAs were performed by Victor R. Ambros, Gary Bruce, Lee RC and Feinbaum RL in the Department of Cellular and Developmental Biology at Harvard University. This research was carried out in Caenorhabditis elegans roundworms. In particular, lin-4 and let-7 were the first discovered miRNAs. Such miRNAs not only regulated the behaviour of diverse animal cells but also of human cells.

Regarding the medical applications of miRNAs, diagnostic and therapeutic uses should be outlined. On one hand, the detection of such endogenous molecules as diagnosis biomarkers for diseases holds great potential. On the other hand, miRNA mimic and miRNA inhibitor agents have been used for therapeutic purposes. Particularly, they have been studied to restore miRNA tumour suppressor functions (miRNA mimics) and to inhibit the actions of oncogenic miRNAs (miRNA inhibitors). In particular, main attention has been paid to cancer treatment.

Moreover, miRNAs have been studied as potential inflammation modulators. The ability to regulate inflammation remains essential to treat both tumour and autoimmune diseases, such as multiple sclerosis. On the other hand, the ability to modulate cell activity turns miRNAs into promising tools for tissue regeneration.

In order to encapsulate and deliver miRNAs, two types of vectors are employed: viral-based delivery systems and non-viral based ones. Showing high transfection levels, the viral-based vectors are commonly formed by retroviruses, adeno and adeno-associated viruses or lentiviruses. The selection of these viruses is made according to characteristics such as target cells, packaging capacity, inflammatory potential and vector genoma forms. For instance, adenoviruses are commonly selected for their high packaging capacity; additionally they have broad tropism, and may induce high inflammatory reaction.

On the other hand, non-viral based vectors are also used in miRNA delivery. Special attention has been paid to nanoparticles (NPs). In particular, lipid- and polymer-based NPs and NPs based on inorganic materials have been used for miRNA mimic/inhibitor delivery. Regarding lipid-based vectors, many of them have been clinically tested and even commercialised. As for polymers, Poly Lactic-co-Glycolic Acid
(PLGA), Polyethylenimine (PEI) and Chitosan (CS) have been widely used for the preparation of NPs in miRNA-based therapeutics. Despite their numerous advantages, such as safety or biocompatibility, polymers do not usually provide the NPs with enough transfection levels. Thus, NPs frequently are surface functionalised to enhance transfection ability.

The preparation of the NPs can be completed following different procedures. Single and double emulsification-evaporations methods, as well as nanoprecipitation are the most commonly used techniques to prepare the NPs. On the other hand, different characterisation methods are used to obtain both physicochemical and biological information about the NPs. Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) are some of the methods providing physiochemical parameters such as NP size, shape and zeta potential. The biological information, however, is provided by either cytotoxicity assays, cellular uptake and transfection ones.

The state of the art research was finalised to find out nanoformulations to for the management of brain metastasis in non-small cell lung cancer (NSCLC) within a collaborative project between Prof. Roger Kamm at MIT and Prof. Valeria Chiono, Prof. Gianluca Ciardelli and Dr. Clara Mattu at Politecnico di Torino. One miRNA mimic agent was selected for the purpose: miR-29b mimic. To improve delivery efficiency and avoid off target, a delivery system was proposed based on PLGA/chitosan to have long-term release (PLGA) and high encapsulation ability (chitosan). The particles will be prepared by nanoprecipitation and will be coated with a pegylated-lipid shell functionalised with ligands for blood brain barrier crossing (e.g. transferrin) and cancer targeting (cetuximab). A rough estimation of the needed budget for 6 months activity spent to prepare the nanoparticles was also proposed.

Overall, miRNAs hold great potential for disease treatment: improvements in the delivery systems employed for their encapsulation, as well as the discovery of new aberrantly expressed miRNAs, will enable the complete transition of miRNA-based therapeutics from bench to bedside. The consolidation of miRNA-based therapeutics will definitely change the efforts to fight diseases such as cancer.
Acknowledgement

This thesis could not have been developed without the support, help and advice of many people.

I would like to start by expressing my gratitude to my thesis advisor Prof. Valeria Chiono of the Department of Mechanical and Aerospace Engineering at Politecnico di Torino. Without her guidance and support this research thesis could not have been developed.

I would also like to express my thankfulness to Marco Campisi, ph. D from Polytechnic of Turin and visiting student at Dana-Farber Cancer Institute (Harvard Medical School) and also MIT, for the invaluable help provided in the last steps of the research.

Finally, last but by no means least, I would to thank my parents for their unconditional support from Barcelona throughout the development of this thesis and all my years of study.
Glossary

AAV: adeno-associated virus

AFM: atomic force microscopy

Ago2: Argonaute 2

ASO: antisense oligonucleotide

AuNPs: gold nanoparticles

CFA: Freund’s complete adjuvant-induced

CGT: cancer gene therapy

CLL: chronic lymphocytic leukemia

CSD: critical-sized defect

DC: dendritic cells

DE: double emulsion

DGCR8: DiGeorge syndrome critical region gene 8

DLS: dynamic light scattering

DNMT: DNA methyltransferase

DRG: dorsal root ganglion

dsRNA: double-stranded ribonucleic acid

ECM: extracellular matrix

ELS: electrophoretic light scattering

EM: electron microscopy

FCS: fluorescence correlation spectroscopy

FDA: Food and Drug Administration

HCC: hepatocellular carcinoma
HCV: Hepatitis C Virus
IgG: immunoglobulin G
IND: investigational new drug
iNOPs: interfering nanoparticles
ISH: in situ hybridization
LA: lactobionic acid
LDH: lactate dehydrogenase
LMW-PEI: low molecular weight polyethylenimine
LNA: locked nucleic acid
miRNA: micro ribonucleic acid
mPEG: monomethoxy polyethylene glycol
mRNA: messenger ribonucleic acid
MS: multiple sclerosis
MSC: mesenchymal stem cell
MSN: mesoporous silica-based nanoparticles
MUC1: mucin1-aptamer
NaCl: sodium chloride
NGS: next-generation sequencing
NP: nanoparticle
NSCLC: non-small cell lung cancer
o/w/o: oil-water-oil
OBA: Office of Biotechnology Activities
OCTGT: Office of Cellular, Tissue and Gene Therapies
PDI: polydispersity index

PEG: polyethylene glycol

PLGA: poly lactic-co-glycolic acid

PLL: poly L-lysine

PLL: poly-L-Lysine

pre-miRNA: premature micro ribonucleic acid

pri-miRNA: primary micro ribonucleic acid

PU: polyurethane

qRT-PCR: quantitative real-time polymerase chain reaction

RA: rheumatoid arthritis

RISC complex: RNA-induced silencing complex

RNA: ribonucleic acid

RNAi: ribonucleic acid interference

RS: Raman scattering

SE: single emulsion

DE: double emulsion

SEM: scanning electron microscopy

SERS: surface enhanced Raman scattering

SFM: scanning force microscope

siRNA: silencing ribonucleic acid

SLE: systemic lupus erythematosus

TEM: transmission electron microscopy

TLR: toll-like receptor

TRBP: transactivation response RNA binding protein

Tz: trastuzumab
VEGFab: vascular endothelial growth factor antibody

w/o/w: water-oil-water
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1. Introduction

1.1. Objective of the thesis

Regenerative medicine is progressively advancing thanks to healthcare technology progress, with the development of new-emerging technologies and improvement in the existing ones. One of such technologies is drug delivery. Such approach enables the encapsulation and delivery of pharmaceutical agents with therapeutic purposes in the human body. One emerging class of therapeutic agents is represented by mimic and inhibitory agents of endogenous microRNAs (miRNAs), that are small non-coding RNA molecules that regulate gene expression post-transcriptionally. Whereas the delivery of miRNA mimics and inhibitors has a therapeutic role, detection of endogenous miRNAs in body fluids may be used for diagnostic purposes.

Knowledge on microRNA may enable considerable improvements in different medical fields, such as the treatment and diagnosis of cancer. On the other hand, progress in polymeric delivery systems used as nanocarriers may allow an efficient therapeutic application of miRNA-based agents. The selection of proper materials and techniques for nanocarrier preparation are key tools for biomedical engineers to obtain an efficient delivery of the agents avoiding off-target effects.

Knowing that the delivery of miRNA mimics and inhibitors can lead to promising results in cancer treatment, the main goal of this thesis was to study the state-of-the-art delivery systems, as to find out the most promising materials that can be employed for miRNA agents delivery focusing on the polymer-based ones.

In detail, the following list collects all the objectives of the work:

1. **Origin and biogenesis of the miRNAs:**

   To comprehend how the miRNA biogenesis process takes place and how dysregulations of such molecules lead to diseases such as cancer.

2. **Medical applications of miRNAs**

   To present the different applications on which miRNA agents can be implemented: therapeutics and diagnosis. Indeed, the processes in which miRNA are involved, such as cancer development, tissue regeneration and inflammation, are described.

3. **MiRNA delivery systems**
To analyse the two different vectors that can be employed as miRNA carriers (viral and non-viral), with special focus on polymeric carriers.

To study the advantages and disadvantages of such delivery systems

To describe the procedures required for the preparation of the miRNA agents delivery systems, with attention to nanoparticles.

To introduce the different physicochemical and biological methods available for the characterisation of nanoparticles encapsulating miRNA agents.

To understand the European and American regulations for the implementation of nanoparticles as drug delivery tools, focusing on the procedures to obtain the FDA approval.

To focus on a specific application using nanoparticles to deliver miRNAs with therapeutic purposes

4. **MiRNA therapeutic perspectives**
   
   To make an overview of the current state of art of miRNA delivery systems for cancer treatment as well as future treatment perspectives.

5. **Proposal of polymer-based NPs**
   
   To choose a miRNA agent for the treatment of non-small cell lung cancer (NSCLC) based on the knowledge gained in the thesis. Selection of the materials for NP development, including a protocol explaining the steps for NP preparation. Moreover, introduction of the proposed methods for physicochemical and biological characterisations.

6. **Budget and economic survey**
   
   To make a brief economic evaluation of the polymer-based NPs proposed in the previous chapter.

This thesis work collects information deriving from scientific literature. However, it also discusses the selection of materials and preparation techniques for polymeric nanoparticles designed to encapsulate and deliver a specific miRNA mimic for the treatment of NSCLC cancer type.

Figures, tables and data extracted from references were always reproduced and adapted with permission.
2. Origins and biogenesis of microRNA

2.1. MicroRNA discovery and its history

MicroRNA (miRNA) are small non-coding RNA molecules that regulate gene expression post-transcriptionally.

They were first discovered in 1993. First researches in this field were performed within Victor R. Ambros, Gary Bruce, Lee RC and Feinbaum RL in the Department of Cellular and Developmental Biology of the Harvard University. Despite numerous researchers some studies attribute the discovery of miRNAs to Lee [1]. Notwithstanding these discoveries, first publications on miRNAs in Science Magazine appeared in 2001 [2].

First studies were all performed in a non-parasitic and transparent roundworm (Caenorhabditis elegans). It was revealed that a protein called lin-14 was responsible for the C. elegans larval development [1][3]. In particular, first stages of larval development (L1-L2) were influenced by the downregulation of lin-14. On the other hand, it was detected that the expression of lin-14 could be regulated by lin-4. This modulation took place in the 3' UTR region of lin-14 messenger RNA (mRNA) [1][3]. Thereby, lin-4 was capable of repressing lin-14 during larval development of C. elegans.

Seven years later, the second miRNA gene, called lethal-7 (let-7), was discovered. This C. elegans miRNA gene was responsible for larval development, in detail for the transition between L4 and adult phase. The return of larval cell fates in the course of adult phase was provoked by a decrease in let-7. The excess of let-7 caused premature appearance of adult phase [1][3]. One of the main reasons why the discovery of let-7 represented a further step in miRNA field is that it has been conserved across species from flies to humans. In humans, for instance, let-7 was detected in several tissues such as the brain and the liver. Sharing the same sequence and 5' ends, the family of let-7 encompasses 12 miRNAs, some of which were found in C. elegans [1].

The discovery of both lin-4 and let-7 was revolutionary. Furthermore, findings in miRNAs demonstrated their implication in different physiological processes, which led to a better understanding of cellular biology and disease development at molecular scale. [4] Moreover, in 2002 several miRNAs were found to be abnormally expressed in cancer [5]. Thereby, the discovery of miRNAs involvement in tumorigenesis [6], in addition to all previous discoveries, allowed their implementation in clinical applications such as therapeutic and diagnostic agents in cancer. Much more progress in the field is anyway required.
2.2. Biogenesis of microRNAs

In order to understand properly the process of formation of miRNAs [1], an explanation of their biogenesis is shown below. Several studies stated that miRNA length is comprised between 21 and 23, or even between 21-22 nucleotides [1][3][5]. All studies agreed in the fact that miRNAs are composed of a low number of nucleotides, on average 21 nucleotides.

miRNAs are able to follow two different biogenesis pathways: canonical and non-canonical [7]. During the canonical biogenesis (Figure 2.1), miRNA is transcribed by RNA polymerase II, forming a long primary miRNA of hundreds of nucleotides. These pri-miRNAs, that can be either coding or non coding, are then blinded by a multiprotein complex. This complex, called multiprocessor, is formed by Drosha enzyme and DGCR8. Drosha, that is a III RNase acting in coordination with the DGR8 cofactor, is responsible for the pri-miRNA transformation into a premature miRNA (pre-miRNA). Meanwhile, in the non-canonical pathway a splicing system is responsible for pre-miRNA formation.

These pre-miRNAs, that are made of approximately 70-120 nucleotides [1] are exported from the nucleus to the cytoplasm thanks to Exportin 5 and RAN•GTP complex activity. It is precisely in the cytoplasm that pre-miRNA is transformed into mature structures with a length of 18-23 nucleotides. This process of division of the pre-miRNA, that is accomplished by a III RNase called Dicer and some Transactivation response RNA binding proteins (TRBP) leads to the formation of a short double-stranded miRNA: miRNA-duplex.

At that point, miRNA duplex separation takes place and, after a strand selection, the miRNA chosen strand (guided strand) is incorporated to RNA-induced silencing complex (RISC). In RISC the guided strand forms a complex with different Argonaute family and associated proteins. Moreover, the mechanism of strand selection depends on different factors, such as base pairing stability at the 5’ end. The miRNA strand with less stability is the one acting as a guide strand, the other strand undergoes degradation [1]. After the miRNA incorporation to the RISC, the targeting of the messenger RNA (mRNA) takes place. The biogenesis of miRNAs ends with either a translational repression or mRNA degradation [1][3][4].

The inhibition mechanism of mRNA by miRNAs is determined by the level of base complementarity. mRNA undergoes degradation in case of perfect base complementarity. On the other hand, in case mRNA target and miRNA present sequence mismatches, mRNA translational repression is followed [1][7].
Figure 2.1 Biogenesis of miRNAs. (Adapted from [1][4][6])
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Type</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drosha</td>
<td>Nuclear RNase III</td>
<td>It forms the Microprocessor complex (pre-miRNA)</td>
</tr>
<tr>
<td>DiGeorge syndrome critical region gene 8 (DGCR8)</td>
<td>Double-stranded RNA (dsRNA)</td>
<td>Its acts in coordination with Drosha for the pre-miRNA formation</td>
</tr>
<tr>
<td>Exportin 5 (Exp-5)</td>
<td>Nuclear transport receptor protein depending on RanGTP</td>
<td>It transports pre-miRNA from the nucleus to the cytoplasm</td>
</tr>
<tr>
<td>Dicer-1</td>
<td>Nuclear RNase III</td>
<td>It transforms pre-miRNA into mature miRNA duplex</td>
</tr>
<tr>
<td>TRBP</td>
<td>Transactivation response RNA binding protein</td>
<td>It facilitates the miRNA duplex entry to the RISC complex</td>
</tr>
<tr>
<td>Argonaute 2 (Ago2)</td>
<td>Argonaute catalytic family proteins</td>
<td>It binds and separates miRNA duplex strand</td>
</tr>
</tbody>
</table>

Table 2.1 collects the main molecules involved in miRNAs biogenesis. (Source: [1], [3])

As it is mentioned in [4], in many cancers such as neuroblastoma or ovarian cancer, several mutations in enzymes enrolled in miRNA biogenesis have been detected. Dicer, Drosha, Exp-5 and Ago2 are examples of these enzymes.

A proper understanding of miRNA biogenesis can help in the preparation of delivery systems incorporating miRNAs.

2.3. MicroRNAs nomenclature

The huge quantity of miRNAs expressed in the human gene could lead to non-uniformity and confusion during the process of miRNA cataloguing. In order to prevent and overcome this undesirable situation, a nomenclature system has been established.

The standard nomenclature is formed by a prefix “miR”, followed by a dash and the number of the miRNA. This number is directly related to the order in which the miRNA was discovered and, thus,
enrolled in confirmation experiments. Moreover, whereas the R in capital letters found in the prefix makes reference to mature miRNA, r in lowercase is related either mRNA genes or pre-miRNA [1].

On the other hand, differentiation between mature miRNAs is accomplished by adding a suffix number at the end of the nomenclature. miR-219-1 and miR-219-2 is a case in which this differentiation is required. Similarly, a suffix letter is included at the termination of the term when both sequences can only be distinguished by just one or two nucleotides. An example of that situation is the differentiation between two types of miR-130 miR-130a and miR-130b [1].

Furthermore, in some cases, a prefix of three letters can be added as a means to indicate the specie. For instance, the prefix -hsa makes reference to Homo Sapiens. Hsa-miR-219-1 is an example in which a reference to the specie has been made [1]. Nevertheless, this prefix is not commonly used, specifically when studies are focused on humans, as it is the case of this thesis. Thereby, in this work the mentioned prefix will not be used when referring to a particular miRNA.

2.4. MicroRNA functional targeting

The miRNAs ability to target messenger RNAs (mRNA) have been above-mentioned in the miRNA processing chapter. However, miRNAs can silence hundreds of mRNAs [4]. Thereby, miRNA-based therapeutics (miRNA mimics) can be used in diseases in which mRNA alterations caused the disorder manifestation. On the other hand, miRNAs can also be used as targets of therapeutic agents called miRNA inhibitors. In this case, miRNAs lead to disease, hence their inhibition is needed.
3. Medical applications of microRNA

3.1. MicroRNA in therapeutics

This chapter presents an overview of the roles of miRNAs in different cancer types, as well as their application in cancer treatment.

3.1.1. Role of miRNAs in cancer

Since 2002, when it was first demonstrated the implication of miRNAs in both cancer initiation and development processes [7], many research efforts have been performed to progress in this field.

Before considering miRNAs as therapeutics, it is necessary to introduce the different RNA interference (RNAi) ways of gene silencing. The endogenous miRNA mediated mechanism silences hundreds of genes, as previously studied in the Par. 2.2. On the other hand, double stranded synthetic silencing RNAs (siRNAs) have been introduced. They have a length of 21-22 nucleotides. siRNAs are incorporated into mammalian cells through an exogenous procedure [4]. However, siRNAs are processed by the same enzymes, such as Dicer, and end processing in the RISC complex. However, siRNAs target a specific mRNA so they silence one gene. Conversely, as it has been explained in Par. 2.2, miRNAs are able to target several mRNAs, following an imperfect matching process. This ability to target multiple mRNAs, makes miRNAs powerful as therapeutics [8]. Due to the similarity between siRNAs and miRNA, advances in siRNAs delivery can lead to simultaneous progress in miRNA therapeutics research. [4] Despite the similarities between both miRNAs and siRNAs, this work will focus on the miRNAs.

Numerous studies have demonstrated the presence of miRNAs in genomic regions associated to cancer [1][9][10]. In cancer, miRNAs can function as oncogenic activators or tumour suppressors. [9] Oncogenic miRNAs, alternatively called oncomiRs, are responsible for tumorigenesis. In this case, their overexpression is the main cause of cancer. The mechanism by which oncomiRs provoke such a severe action is tumour suppressors down-regulation [10]. A great example of an oncogenic miRNA is miR-21, whose over-expression has been associated with disorders in multiple body parts such as pancreas, breast, lung, liver and many others. The mir-21 mechanism facilitates the proliferation of tumour cells and is based on the inhibition of the programmed cell death protein PDCD4 [9]. On the other hand, tumour-suppressive miRNAs activity focus on the interruption of tumour cells proliferation [6]. Thereby, the down-regulation of these miRNAs, or even loss of expression, is a relevant cause of several cancers. An example of a miRNA performing this tumour-suppressive role is miR-15a/16-1. Its low level or loss of expression has been associated with different diseases such as Chronic Lymphocytic Leukemia (CLL). Myeloma and prostate cancers are also human disorders in which miR-15a and miR 16-1 implications have been discovered [9].
However, despite substantial differences between oncogenic and tumour suppressive miRNAs, the classification is not so strict. Thus, some miRNAs can have different roles depending on cancer [9]. In addition, the regulation of both tumour suppressive and oncogenic genes can be performed at the same time by a single miRNA agent [6].

Overall, the characteristics of both oncomiRs and tumour suppressors build a basis for new-emerging miRNA-based cancer therapies. These techniques will be discussed in the next par. 3.1.1.1.

3.1.1.1. miRNA mimics and inhibitors in cancer

Taking advantage of the miRNA characteristics introduced in the previous chapter, two different miRNA-based techniques have been developed: miRNA mimics and inhibitors [4][6][7]. The goal of this methodologies is to threat diseases such as cancer, that have been widely demonstrated to be caused by either miRNA over and down regulation. Thereby, is by the restoration or inhibition of miRNA function that these two techniques are capable of overcoming, for instance, cancer development.

Concerning miRNA mimics, its principle is based on the restoration of tumour suppressor miRNAs levels. Being synthetically created, they are double-stranded miRNA oligonucleotides whose function is to copy either endogenous miRNAs or premiRNA performance [7]. These molecules are capable of restoring miRNA expression [4].

Conversely, miRNA inhibitors are synthetically developed to inhibit miRNA functions. Trough binding to miRNA mature complementary sequence, miR inhibitors are able to accomplish their goal [4]. Another role performed by these single-stranded RNA molecules is the interruption of the RISC complex assembly process [8]. These mechanisms are extraordinary ways to boost the expression of tumour suppressor genes, turning miRNA inhibitors into a powerful tool in cancer therapeutics [12]. Figure 3.1 shows the most effective tools that enable oncomiRs suppression: antisense oligonucleotides (ASOs), locked nucleic acids (LNAs) and antagomiRs [4]. Whereas ASOs are first-generation antisense oligonucleotides, LNAs are locked nucleic acids. AntimiRs could be either based on ASOs or LNAs. On the other hand, antimiRs with perfect complementary sequences and 2′-O-methoxyethyl modification are known as antagomiRs [4][7].
3.1.1.2. Cancer targeting

Some studies revealed the implication of miRNAs in initiation, progression and metastasis of tumours [4][5][6][7][8][13]. One of the main roles of miRNAs is that of therapeutics for cancer treatment. Hence, there are two different approaches to exploit miRNAs for therapeutic interventions [2][8]: to suppress oncogene expression by administering miRNA mimics or to avoid endogenous miRNA action by their inhibition with miRNA inhibitors (Par. 3.1.1.1)

Let-7 family is an example of miRNA mimics used in cancer treatment. These miRNAs, whose discovery is described on Par. 2.1, have tumour suppressor function in different cancers. [4] Thereby, their down-regulation results in cancer progression. A powerful tool to overcome such dysregulation consists of a miRNA mimics-based therapy [4]. For instance, mir-34a and let-7 mimics have been tested in the treatment of non-small cell lung cancer. (NSCLC)

On the other hand, an example of the effective use of antagomiRs is represented by miR-21 inhibitor. Some studies have found a relation between miR-21 overexpression and decrease of tumour suppressor genes in many cancers. MAPK is a family of proteins that enables communication between the surface and the nucleus of the cells. On the other hand, the main function of AKR is signal transduction. In breast cancer, the inhibition of both MAPK and AKT pathways performed by miR-21 antagonists results in angiogenesis blocking [8].

Other therapies based on miRNA mimics and inhibitors for cancer treatment are collected in the following table. (Table 3.1)
Despite the numerous applications of miRNAs in cancer treatment, their effective implementation still has many challenges. For instance, further research to achieve more specific and safer delivery systems in vivo is required [8].

### 3.1.2. Inflammation modulation

Overwhelming evidence issued by different studies has demonstrated the ability of miRNA mimics and inhibitors to modulate inflammation [4][8][9][14][15][16][17].

Initial studies on inflammation have shown that the route described by inflammatory cells initiates in the dilated blood vessels and finishes in tissues [14]. These cells have not only an important role in pathologies, but also in homeostasis and tissue maintenance. They also provide defence against pathogens [14].

<table>
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<th>Cancer type</th>
<th>Outcome</th>
<th>Source</th>
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<td>miR-21</td>
<td>Inhibitor (antagomiR)</td>
<td>Breast cancer</td>
<td>Reduction of tumour progress and induction of apoptosis</td>
<td>[7][8]</td>
</tr>
<tr>
<td>miR-34a</td>
<td>Mimics</td>
<td>Lung cancer</td>
<td>Interruption of tumour growth and apoptosis induction</td>
<td>[8]</td>
</tr>
<tr>
<td>miR-221</td>
<td>Inhibitor</td>
<td>Hepatocellular carcinoma (HCC)</td>
<td>Reduction of tumour growth</td>
<td>[7]</td>
</tr>
<tr>
<td>miR-145</td>
<td>Mimics</td>
<td>Pancreatic cancer</td>
<td>Supression of tumour function</td>
<td>[4]</td>
</tr>
<tr>
<td>miR-155</td>
<td>Inhibitor</td>
<td>Lymphoma</td>
<td>Reduction of tumour growth and induction of apoptosis</td>
<td>[4][8]</td>
</tr>
</tbody>
</table>

Table 3.1. miRNA roles in therapeutics. (Sources: [4][7][8])
Other studies have shown the relationship between miRNAs and inflammation diseases. [14] Tumour-based diseases are examples of disorders in which miRNAs modulate inflammation. In some cases, miRNAs facilitate tumour development. In this case, genes involved in inflammation responses represent the target to modulate inflammation. Nuclear factor-κB and SHIP1 are examples of these genes [4].

The impact of miRNAs on certain inflammatory pathologies have also been reported. Pathologies that result in inflammation are commonly caused by immune system dysregulations [14][15]. The immune system follows two different mechanisms of response [15]. The first one is known as innate immune system and is the first body line of defence. This mechanism provides a non-specific response. On the other hand, the adaptive immune system responds specifically to certain stimuli through antigens action. Hence, the main difference between the two immune systems is the specificity of the adaptive immune system. This system is also able to base its response on an immunological memory [15]. However, the immune system mistakes in the recognition of antigens can result in autoimmunity diseases. These diseases, that can be also caused by miRNA dysregulations, can lead to chronic autoimmune inflammation [14][15][16].

The following figure collects some of the most common autoimmune diseases. (Figure 3.2)

![Figure 3.2. Autoimmune disorders caused by miRNA dysregulations. (Sources: [15], [16])](image)

Additionally, the available information suggests that different miRNAs can play opposite roles in inflammatory pathologies. For instance, this has been identified in dorsal root ganglia (DRG). In these studies, that used Freund's complete adjuvant-induced (CFA) inflammatory hyperalgesia, two miRNA dysregulations have shown the same effect: miR-134 down-regulation and miR-1 upregulation [17].
The following table collects miRNA roles in numerous inflammatory pathologies. The main goal of this overview table is to demonstrate the huge quantity of disorders in which miRNAs are involved.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Disease</th>
<th>Dysregulation</th>
<th>Cell target/location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-155</td>
<td>Rheumatoid arthritis (RA)</td>
<td>Up-regulation</td>
<td>B cell and Th17, MMP-1, MMP-3, PBMC, RASF</td>
<td>[14], [15], [16]</td>
</tr>
<tr>
<td>miR-155</td>
<td>Multiple sclerosis (MS)</td>
<td>Down-regulation</td>
<td>Th1 and Th17, CD47, CD47</td>
<td></td>
</tr>
<tr>
<td>miR-155</td>
<td>Systemic lupus erythematosus (SLE)</td>
<td>Up-regulation</td>
<td>T cells, B cells</td>
<td>[14], [16]</td>
</tr>
<tr>
<td>miR-326</td>
<td>Multiple sclerosis (MS)</td>
<td>Down-regulation</td>
<td>Th17, CD47</td>
<td>[14], [15]</td>
</tr>
<tr>
<td>miR-34a</td>
<td>Multiple sclerosis (MS)</td>
<td>Down-regulation</td>
<td>CD47</td>
<td>[15]</td>
</tr>
<tr>
<td>miR-146a</td>
<td>Rheumatoid arthritis (RA)</td>
<td>Up-regulation</td>
<td>T cells and Macs, CRP, ESR, IRAK1, TRAF6</td>
<td>[14], [15]</td>
</tr>
<tr>
<td>miR-182</td>
<td>Rheumatoid arthritis (RA)</td>
<td>Up-regulation</td>
<td>T cells</td>
<td>[14]</td>
</tr>
<tr>
<td>miR-182</td>
<td>Rheumatoid arthritis (RA)</td>
<td>Up-regulation</td>
<td>T cells</td>
<td>[14]</td>
</tr>
<tr>
<td>miR-203</td>
<td>Psoriasis</td>
<td>Up-regulation</td>
<td>Skin cells</td>
<td>[3], [16]</td>
</tr>
<tr>
<td>miR-21</td>
<td>Psoriasis</td>
<td>Up-regulation</td>
<td>Skin cells</td>
<td>[16]</td>
</tr>
</tbody>
</table>

Table 3.2 miRNA implications in different inflammatory diseases, type of dysregulation and cells involved. Code abbreviations: Type 1 and 17 T helpers: Th1 and Th17; Matrix metalloproteinase-1 and 3: MMP-1 and MMP-3; peripheral blood
The data yielded by Table 3.2 provide convincing evidence that miRNAs have a key role in many undesirable inflammatory responses. Nevertheless, miRNA multi-functionality and multiple-targeting faculties make them hard to implement in therapeutics [16]. Hence, although many miRNA mimics and inhibitors have been successfully employed in the regulation of animal inflammatory response, more research is required to achieve such results in humans [17].

Future perspectives in that field will depend on the improvement in miRNA therapeutic techniques (Par. 3.1.1.1) and enhancement in delivery systems (Par. 4).

3.1.3. Tissue regeneration

According to further research, miRNAs are not only employed in targeting and inflammatory processes but also in other medical fields such as tissue engineering. This biomedical research area aims at tissue regeneration and may exploit miRNAs as they regulate cell gene expression [18][19][20].

During last decades, many studies have reported miRNA implications in tissues undergoing regeneration processes. In this regard, miRNAs have been found to be fundamental factors in both tissue development and healing, as well as in homeostasis phenomenon [19]. Therapeutics based on miRNA mimics and inhibitors have gained importance among new-emerging techniques applied in tissue engineering [18].

Becoming a key event for the regenerative medicine progress, the discovery of stem cells led to considerable progress. Different cell mechanisms that enable tissue regeneration have been studied. Cell therapies by locally injecting stem cells as well as the activation of resident progenitor cells are examples of such approaches [18]. The following figure collects main roles of miRNAs in guiding stem cell behaviour. (Figure 3.3)
The importance of the roles described in Figure 3.3 lies on the fact that miRNAs are able to modulate cell activity, specially stem cell one. This fact makes miRNAs powerful tools for tissue regeneration [18][20]. Hence, these molecules have been used for instance as regenerative medicine targets in bone, liver, muscle, Kidney regeneration and wound healing [19]. As an example, with regard to bone regeneration, critical-sized defects (CSDs) healing are commonly treated with osteoconductive and osteoinductive transplants. However, they suffer from drawbacks such as high risk of infections, pain and low rates of supply. This is the reason why other approaches based on tissue scaffolds and protein delivery have been developed [19]. On the other hand, miRNAs have been implemented as modulators of multiple growth factors [19]. Moreover, the relation between angiogenesis and osteogenesis mechanisms is worth mentioning because it has led to satisfactory results in tissue regeneration. In addition, miRNAs hold tremendous potential in the use of mesenchymal stem cell (MSC) for regenerative purposes. For instance, it is possible to increase the osteogenic potential of bone mesenchymal stem cells by the administration of miR-31 inhibitors [19].

Regarding wound healing, miRNAs regulate a cascade of biochemical events (Figure 3.4) that precede scar healing [18][19]. On one hand, some up and down-regulated miRNAs lead to successful scar healing. On the other hand, other miRNA dysregulations are the cause non-healing wounds. Anyway, temporal-control over miRNAs are required for successful wound healing [18].
The following table outlines different roles of miR-29 in tissue engineering. Provides confirmatory evidence that a same miRNA can perform several functions in human body taking miR-29 as an example. The ability of miR-29 family to modulate various gene functions makes it a powerful tool in different applications. However, it is also underlined that, in order to impart an effect on specific cells, targeted release of miRNA mimics/inhibitors is needed.

Concluding evidence supporting the role of miRNAs as a target in tissue regeneration strategies have been summarised in this section. Nevertheless, further studies on miRNAs role in tissue regeneration are required.

### 3.2. MicroRNA in diagnosis

Acting as either oncogenic or tumour suppressors (Par. 3.1.1) microRNAs are responsible for many diseases manifestation. Hence, another miRNA application is their use as diagnostic biomarkers.

Biomarkers allow the diagnosis of either physiological or pathological processes [21]. miRNAs can be identified with high rates of specificity and sensitivity. Hence, their use as biomarkers has many interesting applications. Diagnostics or prognostics are examples [21][22].

The aberrantly expression of miRNAs in numerous diseases such as muscular, nervous and cardiovascular diseases and diabetes has received much attention over last years [22]. An additional disorder in which miRNA signatures become fundamental is cancer. In this illness, the importance of early detection has been underlined [21]. Thus, employing miRNA signatures as early diagnosis...
Biomarkers may improve previous prognosis methods. Moreover, not only these molecules can enhance prognosis, but also prediction of drug and therapy response [21].

On the other hand, there is a specific type of miRNAs providing a powerful tool for diagnosis: circulating miRNAs. These miRNAs, also called secretory miRNAs, have the ability to remain stable in numerous fluids and environment conditions [23]. These molecules have also potential to maintain stability under high pH conditions and room temperature storages. In addition, their structure remains stable in several fluids such as plasma, serum and blood milk.

Diagnostic quality depends on the procedures and techniques that enable the detection of miRNAs. Intrinsic characteristics of miRNAs such as their decreased size, low levels and tissue specificity in expressions have a challenging role in the field of miRNAs detection [21]. The available techniques that for miRNA detection are quantitative real-time (qRT-PCR), next-generation sequencing (NGS), in situ hybridization (ISH), microarray profiling, enzymatic luminescence miRNA assay and northern blotting and nanopore technology [21][22]. Notwithstanding the numerous accessible techniques mentioned, due to high rates of sensibility and specificity, qRT-PCR is considered the gold standard. This technique is not only used for microRNAs detection but also for all RNAs [21].

The following table collects some miRNAs involved in cancer diagnosis.

<table>
<thead>
<tr>
<th>miR</th>
<th>Cancer Type</th>
<th>Clinical significance</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21, miR-155, miR-16 and miR-17</td>
<td>Lung</td>
<td>Diagnosis of lung cancer</td>
<td>[21]</td>
</tr>
<tr>
<td>miR-128</td>
<td>Lung</td>
<td>Lung cancer prediction</td>
<td>[23]</td>
</tr>
<tr>
<td>miR-21</td>
<td>Lymphoma</td>
<td>Relapse-free survival improvement</td>
<td>[21]</td>
</tr>
<tr>
<td>miR-21, miR-155 and Let-7a</td>
<td>Gastric</td>
<td>Overexpression of this miR indicates gastric cancer expression</td>
<td>[21][23]</td>
</tr>
<tr>
<td>miR-92 and miR-17-3p</td>
<td>Colorectal and gastric</td>
<td>Colorectal and gastric cancer expression</td>
<td>[23]</td>
</tr>
<tr>
<td>miR-21 and miR-221</td>
<td>Prostate</td>
<td>Metastatic prostate patients</td>
<td>[21][23]</td>
</tr>
<tr>
<td>miRNA(s)</td>
<td>Tissue</td>
<td>Description</td>
<td>Reference(s)</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>miR-141</td>
<td>Prostate</td>
<td>Local advanced prostate patients (prognostic)</td>
<td>[21][23]</td>
</tr>
<tr>
<td>miR-195</td>
<td>Breast</td>
<td>Overexpression exclusive for breast cancer</td>
<td>[23]</td>
</tr>
<tr>
<td>miR-210</td>
<td>Pancreatic</td>
<td>Overexpression of this miRNA indicates pancreatic cancer manifestation (prognostic)</td>
<td>[21][23]</td>
</tr>
<tr>
<td>miR-155, miR-127</td>
<td>Ovarian</td>
<td>Underexpression of these miRs is related to ovarian cancer (diagnostic)</td>
<td>[21][23]</td>
</tr>
<tr>
<td>miR-21, miR-92,</td>
<td>Ovarian</td>
<td>Overexpression of these miRNAs lead to ovarian cancer (diagnostic)</td>
<td>[21][23]</td>
</tr>
<tr>
<td>miR-93, miR-126</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-29a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-21, miR-22,</td>
<td>Liver</td>
<td>These miRNAs act as a prognostic tool</td>
<td>[21]</td>
</tr>
<tr>
<td>miR-26 and miR-29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3. miRNAs used as diagnosis, prognosis and prediction biomarkers. (Sources: [21][23])

MiRNAs have multiple uses in diagnosis, prognosis and prediction of several cancers types. miR-21 is an example of a such molecule with numerous applications. Whereas miR-21 acts as a diagnostic biomarker in ovarian cancer, it has a key role in the prognosis of liver cancer. (Table 3.3)
All things considered, incredible hope have been placed in microRNAs as either diagnostic, prognostic and prediction biomarkers. Some studies on miRNAs have revealed a better stability of these molecules compared to the rest of mRNAs [24]. Nevertheless, challenging improvements on miRNAs are required (Figure 3.5) in order to transfer results from bench to bedside.
4. MicroRNA delivery systems

In their use as therapeutics, the main goal of the delivery systems is the encapsulation of nucleic acids. Such systems are used in either miRNA mimics and inhibition approaches [25].

MiRNA use as therapeutic tools is strictly dependent on the delivery systems. The characteristics of the vector employed determine the levels of entrance and delivery of the encapsulated nucleic acid. Other uses of microRNAs therapeutics in medicine are also dependent on the features of these agents. Modulation of inflammation (Par.3.1.2) and tissue regeneration (Par.3.1.3) are two examples [17][18].

This chapter presents the different systems that can be used for delivering miRNA mimics and inhibitors. An overview of the available delivery systems, as well as the advantages and drawbacks of each one will be discussed. Most attention will be addressed to polymer-based delivery systems.

![miRNA Delivery Systems Diagram]

*Table 4.1. Classification of mechanisms used for miRNA mimics/inhibitors delivery systems. (Sources: [2], [8], [10], [25], [27]-[28])*

Apart from the classification made in Table 4.1, some studies classifies delivery systems into ex vivo, *in vivo* and *in situ* delivery [25]. This classification focuses on the procedure followed to transfer the agent.
4.1. Viral systems

Viruses are infection agents that do not have their own metabolism. Thus, replication of these agents requires the infection of cells [25][26].

Due to replication, viruses have the power of penetrating into cells in which their genetic material is expressed. These high transfection levels turn viruses into potential tools as miRNA carriers. Thus, viral systems were the first delivery systems to be used and are currently the most employed vectors for miRNA delivery [25].

On the other, the main drawback of viruses compared to non-viral agents is their low safety levels. The replication of these infection agents makes them unsafe. Thereby, some parts of the viral genome are deleted. Viral vectors can be also covered with lipids to deal with safety concerns. Moreover, the genetic material of the virus can be also protected using a protein coat. [26][30]

The following table collects the most remarkable differences between viral vectors. A comparison between strengths and weaknesses of each technique is also included. (Table 4.2)

<table>
<thead>
<tr>
<th>Viral vector</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Transfection capacity</th>
<th>Genetic material</th>
<th>Sources</th>
</tr>
</thead>
</table>
| Retroviral vectors | · Long-term gene expression in dividing cells  
· Ability to penetrate nuclear pores -> in situ delivery  
· Capacity of being integrated into host cell genome -> ex vivo delivery  
· Efficient role in miRNA inhibition | · Inability to be efficiently implemented in in vivo delivery  
· Immunogenicity  
· It can only transduce dividing cells  
· Possible oncogenesis | RNA | 8KB | [25], [26] |
| Lentiviruses | · Capacity to transflect either | · Failure in large gene transfection | RNA | 8KB | [25], [26] |
### Adenoviruses
- Ability to be used for large gene transfections
- Capacity to transfer either dividing and non-dividing cells
- High transfection effectiveness
- Low immunogenicity
- High long-term stability of miRNA carried

<table>
<thead>
<tr>
<th>· Possible oncogenesis</th>
<th>8KB</th>
<th>dsDNA</th>
<th>[25], [26]</th>
</tr>
</thead>
</table>

| · Inability to incorporate genetic sequences into host cells | 30KB |
| · Short-term gene expression | <5KB |
| · Capside mediates high inflammatory response | ssDNA |

### Adeno-associated viruses (AAVs)
- Better miRNAs transfection than adenoviruses
- Long-term expression *in vivo*
- Capacity to transfer either dividing and non-dividing cells
- Non-inflammatory and non-pathogenic

| · Failure in large gene packing | [25], [26] |
| · Complexity of vector production | |

Table 4.2 Overview of some of the most employed viral-based systems and relevant advantages and disadvantages of each one. (Sources: [25][26])
Some studies have shown the effectiveness of viral systems for the delivery of either miRNA mimics or inhibitors [8][25][26]. Regarding inhibitors, it has been found that both miR-15a and miR-16 inhibitors can be carried into lentiviral vectors to treat breast cancer. Likewise, adeno-associated viruses (AAVs) have been used for the delivery of miR-26a in hepatocellular carcinoma [8].

Regarding the barriers in the use of viral systems, some factors should be considered. The first is their low safety levels. An additional drawback of viruses is their low stability. Modification of the viral agents are made in order to provide them with safety and stability. Stable viruses, in particular, lead to better predictability and reproducibility results. Adjustments in viral systems to improve cell specificity are also common [8][25].

Despite being lower efficient delivery agents, non-viral vectors are safer than viral ones. [8] Hence, non-viral delivery systems have turned into an attractive alternative to viral ones.

4.2. Non-viral systems

Despite their lower levels of transfection, non-viral delivery systems have several advantages compared to viral vectors [26]. Low levels of toxicity and immunogenicity make these delivery systems safer than the ones based on viruses. Another strength of these agents is based on their lower cost production and higher scalability compared to viruses. Regarding the loading capacity of nanoparticles, non-viral systems have shown better results [26][27]. All these factors make non-viral systems promising tools for miRNA delivery for biomedical applications.

There are two different non-viral delivery approaches: physical and chemical methods. Physical approaches increase cell membrane permeability to naked DNA, improving gene delivery. Gene gun, electroporation, the use of hydrodynamic, ultrasound, and laser-based energy are examples of physical approaches [25][26]. Despite being the most used physical technique, gene gun has low efficiency.

On the other hand, chemical approaches focus on the use of nanoparticles as delivery tools. Such nanoparticles are based on lipids, polymers or inorganic materials [26]. In this section, attention will be addressed to chemical systems, especially to polymer-based ones.

Chemical systems are frequently employed in miRNA delivery. They often consist of nanocomplexes formed by negatively charged nucleic acids and cationic polymers [25]. The use of nanoparticles in the formation of the delivery system nanocomplex have numerous pros. Some of them are collected in Figure 4.1.
4.2.1. Lipid-based nanovectors

The implementation of lipid-based nanocarriers has become a groundbreaking alternative to viral-based delivery systems [26][27][28][29].

Polar lipids are amphiphilic materials that consist of a hydrophilic polar head group and two hydrophobic non-polar tails. Polar lipids are the main components of the cell membrane. Hence, lipid-based nanocarriers may easily interact with such carriers [29].

The main classes of lipid-based nanovectors are liposomes and cationic lipids. [29] Liposomes are spherical vesicles composed of one or more phospholipid bilayers. In the central part, there is the an aqueous compartment called aqueous core (Figure 4.2) [29]. Liposomes are powerful vectors for the encapsulation of both hydrophilic and hydrophobic drugs. Whereas hydrophilic drugs are normally placed on the aqueous core, hydrophobic ones are engaged into the bilayer [25][29].
Regarding liposomes, considerable progress has been made. The following figure summarises it.

According to their charge, liposomes can be cationic, anionic and neutral. Cationic liposomes have some advantages compared to neutral and anionic ones. Some of these strengths are their simple production, low immunogenicity and high affinity with the cell membrane [26]. Another significant feature of cationic lipids is their ability to incorporate both hydrophilic and hydrophobic drugs [25]. Due to all mentioned advantages, particular attention will be paid to cationic lipids. Such lipids can bind electrostatically to nucleic acids, resulting in the formation of structures called lipoplexes [25][29].

Some barriers regarding the use of cationic lipid-based systems have been reported. Their potential toxicity and short nanoparticle half-life are examples of such difficulties [27]. This last factor has been associated with proneness of these lipids to bind non-specific serum proteins [26][27].

The delivery systems based on lipids have undergone numerous modifications. For instance, cationic lipids have been used in the core of the liposomes for better binding and condensing nucleic acid drugs [27]. Likewise, low molecular weight polyethylenimines (LMW-PEIs) have also been placed on the nanovector core. In this case, the LMW-PEI, covered with anionic or neutral lipid led to enhanced biocompatibility [27].

Another alteration of cationic lipid-based delivery systems has been reported [25]. This approach was based on the vector surface modification using polyethylene glycol (PEG). Such lipids are known as stealth liposomes, and are included on Figure 4.3 [25].

Further approaches have been applied to load one drug in the nanoparticle surface and another one in the core section: e.g. miRNA agents were coated on the nanoparticle surface while lipophilic drugs were encapsulated in the core of the nanoparticle [29].
Apart from liposomes and cationic lipids, lipid nanoemulsions have also been employed as lipid-based delivery systems. These systems, based on the mixture of water and oil to form a single phase, have shown some advantages: ease of preparation and the miRNA loading capacity [29].

4.2.2. Polymer-based nanovectors

Compelling evidence revealed the efficiency of polymers as miRNA carriers. [2][5][8][26][27][28] As stated in the lipid-based nanovectors chapter (Par. 4.2.1), nanoparticles, in particular, are potential tools for miRNA delivery. Specifically, research tends to focus on the development of polymer-based nanoparticles [8]. These nanostructures include both nanospheres and nanocapsules [30]. For instance, the composition of nanocapsules consists of a liquid core (Figure 4.4 B) and a polymeric membrane (Figure 4.4 A). An active substance is generally incorporated on the inner cavity. However, it can also be leaded on the nanocapsule surface or in the polymeric membrane [30].

Both natural and synthetic polymers have been tested as delivery systems. In particular, the addition of these polymers to the surface of nanoparticles have led to enhanced delivery [28][31].

The DNA polyanionic condition at body pH facilitates its interaction with cationic polymers. As it happens with cationic lipids, these interactions result in the formation of a nanocomplex. In this case, however, the nanocomplex is called polyplex. The internalisation of these structures, that protect the leaded material, is due to endocytosis [25].

This chapter will focus on the implementation of Poly Lactic-co-Glycolic Acid (PLGA), Polyethylenimine (PEI) and chitosan. Furthermore, various modifications of materials as well as combination of them will be also introduced.
4.2.2.1. Poly Lactic-co-Glycolic Acid (PLGA)

Being part of the polyester family, polylactic-co-glycolic acid (PLGA) is a copolymer widely used in drug delivery [26][32]. The obtainment of the FDA approval turned these copolymers into promising tools for drug delivery [26].

![Chemical structure of the PLGA. X makes reference to the number of lactic acids, while Y refers to the number of glycolic ones. (Source: [33])]({})

Some studies stated that PLGA-based nanoparticles can result in enhanced therapies. [8][26][31] Such nanoparticles have shown ability to escape from the endolysosomal compartment and successful release of their encapsulated material [8][31]. The long duration of these releases is an advantage of PLGA-based vectors over lipid-based ones. Such delivery periods have gone from weeks to months [31][32].

Another remarkable feature of PLGA nanoparticles is their ability to encapsulate both hydrophobic and hydrophilic drugs [32]. Thereby, these nanoparticles may deliver multiple therapeutic agents [31]. However, this work seeks to address miRNAs use in therapeutics. Techniques used to load miRNAs on nanoparticles will be presented in Par. 4.3.

On the other hand, other features of PLGA nanoparticles turn these copolymers into a promising material for delivery. Their safety, biocompatibility and biodegradability are examples of these characteristics [8].
Despite the numerous advantages of PLGA-based nanoparticles (Figure 4.6), future research should address some vital issues. Difficulties in the reproducible formation of PLGA-based nanoparticles and the considerable method variability are examples of these challenging issues [32]. Moreover, such nanoparticles have shown low levels of miRNA transfection as well as non-specificity in interactions with target cells [26][27].

Some modifications of the PLGA-based nanoparticles have been usually made to overcome these drawbacks. For instance, modified PLGA- has been used for the delivery of miRNA in hepatocellular carcinoma (HCC) treatment. [34] The addition of different components to PLGA nanoparticles led to better delivery responses. (Table 4.3). The resulting delivery system, called mPEG-PLGA-PLL-LA/VEGFab, has been tested for the encapsulation of miR-99a. The release of this miRNA in cancer sites resulted in an effective application for HCC treatment [34].

The following figures show the formation process of this PLGA-based dual delivery system as well as some TEM images.
Figure 4.7 Formation process of mPEG-PLGA-PLL-LA/VEGFab nanoparticles the delivery of miR-99a in HCC treatment. (Reproduced with permission from [34])

Figure 4.8 TEM images of the formation process of the mPEG-PLGA-PLL-LA/VEGFab NPs. A: mPEG-PLGA-PLL-LA, B: PEAL-LA/VEGFab, C: miR-99a-PEAL-LA/VEGFab. The scale bar is 100nm. (Reproduced with permission from [34])

<table>
<thead>
<tr>
<th>Modification/Addition</th>
<th>Benefit</th>
</tr>
</thead>
</table>
| Monomethoxy polyethylene glycol (mPEG) modification | It gives more stability to nanoparticles (NPs)  
It prolongs the circulation half-lives of NPs |
| Poly L-lysine (PLL) modification | It shows low toxicity levels  
It has the ability to absorb negatively charged miRNAs |
| Lactobionic acid (LA) addition | It enables the binding to the membrane proteins on the surface of HCC cells: asialoglycoprotein receptors |
| Anti-vascular endothelial growth factor antibody (VEGFab) addition | It enables the binding to endothelial cell-specific marker: VEGF (common in tumours) |

Table 4.3 Modifications in PLGA-based nanoparticles for the delivery of miR-99a in HCC treatment. (Source:[34])
4.2.2.2. Polyethylenimine (PEI)

Polyethylenimine (PEI) is a family of polymers with functional amino groups. These polymers can be either linear or branched (Figure 4.9). PEIs show elevated transfection levels, especially in vitro, due to their condition of high cationic dense polymers. Significant interactions between PEIs and the anionic molecules of the cell membranes, especially when using modified PEI-based systems, have resulted in effective transfection [2][25].

![Chemical formula of the branched PEI](Source: [35])

PEIs can form non-covalent complexes with both plasmid DNAs and siRNAs. Several studies have considered PEI as the primary cationic polymer for gene delivery [2][25][31].

PEIs can be implemented in delivery systems. Thus, their features can alter the effectiveness of the PEI-based vectors. On the one hand, the levels of effectiveness in gene transfection may depend on the PEI chain length. Studies have exposed that long PEI chain results in better transfection levels. However, shorter ones show less cytotoxicity [31]. On the other hand, the molecular weight of the PEI also has an impact on the delivery response. The cell response to low molecular weight PEI is less cytotoxic than the one received by high molecular weight PEIs [27]. Moreover, linear form of PEI shows higher efficiency and lower toxicity than the branched ones [25].

A low molecular weight PEI has been tested for the delivery of both miR-33a mimics and miR-145. The release of these miRNAs in mice resulted in successful colon cancer treatment, thus causing tumour growth suppression [26]. Regarding the drawbacks of such approach, low levels of transfection and biodegradability have been found. On the other hand, PEI has been used as a miR-145 carrier in lung cancer therapies. This PEI-based delivery system incorporating polyurethane (PU) was an efficient delivery tool for miRNA mimics [2].

Another worth mentioning application of PEI uses these polymers in PLGA/PEI nanoparticles. These delivery systems allow the release of target genes for different disease treatment. Liver cancer is an
example [31]. The size of the nanoparticle has been demonstrated to have a key role in gene delivery efficiency. For that reason, the size distribution of PLGA, PLGA/PEI and PLGA/PEI/pDNA have been studied [31].

![Figure 4.10 TEM images of PLGA (A), PLGA/PEI (B) and PLGA/PEI/pDNA (C). The scales bar are 100, 50 and 200nm respectively. (Extracted from: [31])]

The following table collects the diameters of the nanoparticle/DNA complexes showed in the figure above.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Diameter (nm)</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA</td>
<td>50</td>
<td>±6</td>
</tr>
<tr>
<td>PLGA/PEI</td>
<td>57</td>
<td>±7</td>
</tr>
<tr>
<td>PLGA/PEI/pDNA</td>
<td>60</td>
<td>±7</td>
</tr>
</tbody>
</table>

Table 4.4 Diameter of different nanoparticle complexes. (Extracted from: [31])

The electrostatic interaction between positive charged PEI polymers and anionic PLGA shows promising results. Thus, the placement of PEI on the shell of the PLGA nanoparticle leads to enhancement. For instance, PLGA/PEI-based delivery system was used for the delivery of miR-26a in liver cancer therapy [31]. The release of that miRNA in liver cancer cells (HepG2) had several advantages compared to other commercial liposome-based vectors. (Figure 4.11)
Some studies have tested the ability of PEI-based systems to binding miRNAs. PEI showed higher levels of binding to miRNAs compared to siRNAs. Moreover, other assays revealed a miRNA inability to join siRNA-PEI polyplexes. It was due to the miRNA incapacity to break interactions between siRNA and PEI [36].

4.2.2.3. Chitosan

Apart from the use of PLGA and PEI polymers as non-viral vectors, the employment of chitosan for gene delivery should be taken into consideration.

Chitosan is a linear polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine [37][38]. The following figure presents its formulation. (Figure 4.12)
As it similarly occurs with PEI (Par. 4.2.2.2), the positive charge of chitosan results in a good interaction between such polymer and the anionic molecules. Thus, chitosan interaction with molecules such as RNAs, and hence miRNAs, becomes also promising. Moreover, polyplexes based on this polymer have also been tested [37].

Regarding the strengths of chitosan, its high biocompatibility and low immunogenicity should be highlighted. Another feature of this biodegradable polymer that turns it into a potential delivery biomaterial is its non-toxicity. However, some chitosan-based systems have been modified to ensure suitable transfection levels [37][38][39].

![Figure 4.13 Parameters of chitosan-based complex that can be modulated to enhance transfection. (Sources: [37][38][39])](image)

For instance, a chitosan polyplex have been tested in the delivery of miRNA-124. It has been found that the optimal molecular weight and N:P ratio were 150kDa and 50, respectively [37].

There are other examples of therapies in which chitosan-based systems have been employed as miRNA carriers. Therapeutics based on miR-141 and miR-200c delivery in breast cancer cells (MCF-7) are examples of such applications. These miRNAs were carried on chitosan-based vectors due to their good delivery performance. In this study, the diameter of chitosan/miR-141 polyplex ranged from 296 to 355nm and the one with miR-200c from 294 to 380nm. These nanoparticle sizes depend on the N:P ratio [38].

Taking advantage of both PLGA and chitosan characteristics, PLGA/chitosan-based nanoplexes have been utilized for the delivery of miR-34a in multiple myeloma treatment. These nanoplexes not only provided protection to the encapsulated genetic material but also allowed its effective delivery [39].
This delivery system based on PLGA/chitosan nanoparticles showed a decrease in their mean size when loaded with miR-34a. The following table summarises such decrease.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MicroRNA load (µg)</th>
<th>Mean size (nm)</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>176.6±1.9</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>178.0±2.2</td>
<td>95.1±2.7</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>170.3±0.4</td>
<td>93.2±3.1</td>
</tr>
<tr>
<td>4</td>
<td>300</td>
<td>165.2±1.5</td>
<td>85.5±3.3</td>
</tr>
</tbody>
</table>
From results presented in Table 4.5, it has been found 160nm approximately as the optimal diameter of the PLGA/chitosan-based nanoplex.

### 4.2.3. Inorganic materials-based nanovectors

Inorganic nanoparticles could be also implemented as nanocarriers for the delivery of numerous bio-active molecules into cells. DNA, peptides and proteins are examples of biomolecules that can be encapsulated in nanoparticles based on inorganic materials. The similarity between these molecules and microRNAs turns inorganic nanoparticles miRNA potential carriers. However, these interactions are weaker than the ones obtained using organic materials [26]. For this reason, some chemical and biological modifications of these nanoparticles are required to ensure good transfection levels and biocompatibility [40].

In this chapter, careful attention will be paid to gold nanoparticles (AuNPs) and oxide-based nanoparticles.

#### 4.2.3.1. Gold nanoparticles (AuNPs)

Metal-based nanoparticles are one of the most potential carriers based on inorganic materials. In particular, gold-based nanoparticles (AuNPs) have a key role as a miRNA vector. The following figure collects the main features of gold-based nanoparticles [40].

<table>
<thead>
<tr>
<th>5</th>
<th>400</th>
<th>154.3±2.5</th>
<th>62.3±4.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>500</td>
<td>150.7±1.8</td>
<td>49.1±2.1</td>
</tr>
</tbody>
</table>

*Table 4.5 Mean size of PLGA/chitosan-based nanoparticles prepared in absence and presence of miR-34a. Entrapment efficiency is also collected. Each value results from three different experiments. It also includes the standard deviations.*
Apart from the advantageous characteristics of AuNPs summarised in Figure 4.16, high stability in vivo and good resistance to microbial attacks should be highlighted [26].

On the other hand, weak interaction between gold-based nanoparticles and nucleic acids is the major drawback of these metals [26]. In order to overcome this disadvantage, gold-based nanoparticles are combined with other organic materials. For instance, AuNPs have been attached to Polyethylene glycol (PEG) in order to enhance transfection efficiency and reduce toxicity. Combining organic and inorganic materials, this hybrid delivery system has been employed for the delivery of miR-1 in cancer therapy [26].

Another example of an organic and inorganic hybrid system is the AuNPs conjugation with Polyethylenimine (PEI) [26][40]. The transfection levels showed by this AU/PEI-based delivery system were six times higher than the one obtained by the regular PEI-based vector [40].

Moreover, gold-based systems can undergo modifications based on thiol groups. [40] Thiol groups are organosulfur compounds formed by sulfur atoms bound to hydrogen ones. The addition of thiol groups to AuNPs leads to enhanced delivery conditions. [40] For instance, this modification has been made for miRNA delivery. After the encapsulation of the miRNA in AuNPs, they were modified with thiol-polyethylene glycol. This modification resulted in enhanced loaded capacity, lower toxicity, longer half-lives and better transfection levels than other gold-based systems [27].

Furthermore, gold NPs have been functionalised with antibodies. Such surface functionalization resulted in enhanced NP targeting efficiency. In particular, a monoclonal antibody type called trastuzumab (Tz) was added to the gold NP surface. Being a good recognizer a breast cancer growth factor (HER2), the addition of such antibody improved its targeting efficiency [41].
4.2.3.2. Oxide-based nanoparticles

On the other hand, apart from the use of gold nanoparticles in miRNA delivery, other inorganic materials can be employed for the same medical purpose [40]. The following figure collects main properties of oxides that make possible their implementation as delivery nanoparticles. (Figure 4.17)

![Figure 4.17 Physicochemical properties of oxides. (Source: [40])](image)

This chapter will focus on the oxide-based nanoparticles showing better potential in miRNA delivery: silica-based and magnetic-based nanoparticles.

4.2.3.3. Silica-based nanoparticles

The use of silica-based nanoparticles for miRNA delivery has been widely addressed. [27][40][42] Due to their clear advantages over other inorganic materials, mesoporous silica-based nanoparticles (MSNs) should be highlighted [42]. One of the strengths of these nanoparticles is based on the large volume of their pores. It allows the encapsulation of considerable quantities of drug. On the other hand, it has been stated that these nanoparticles have the ability to escape from the endolysosomal compartment to efficiently release the drug in the cytoplasm [42].

As all inorganic materials, MSNs usually require being in combination with other organic materials. For instance, the tumour inhibition activity of MNS-based systems can be enhanced with the addition of a copolymer. MSNs conjugated with polyethyleneimine–polyethylene glycol (PEI-PEG) copolymer experienced an increase of 80% in its activity [42]. Another polymer employed for the functionalization of MSN surface is Poly-L-Lysine (PLL) [40]. Moreover, surface modification of silica-based NPs can also
be based on sodium chloride (NaCl) addition. The conjugation of this ionic compound to the NP surface led to increased transfection levels of SNPs [40].

Regarding the use of silica-based nanoparticles as miRNA carriers, these NPs have been employed for the delivery of miR-34 in neuroblastoma cancer. In this type of cancer, the overexpression of GD2 cells remains fairly common. Thus, the release of miR-34 in these cells promoted the inhibition of the tumour progression and increased apoptosis [27].

4.2.3.4. Fe$_3$O$_4$-based nanoparticles

Taking advantage of their magnetic properties, magnetic-based nanoparticles (MNPs) have been considered alternative vectors for miRNA delivery. Fe$_3$O$_4$ is an example of these nanoparticles that, conjugated with biomolecules, are efficiently released in specific body sites. Facilitated by an external magnetic field, the delivery of such miRNA results in effective disease treatment. However, supermagnetic-based nanoparticles usually require modifications to enhance their performance [40].

MNPs have been employed in conjunction with PEI for the delivery of miRNAs in mesenchymal stem cells. Such release took place with an external high-gradient magnetic field. This MNP/PEI-based delivery system showed good transfection levels in over 60% of the cells. Moreover, this hybrid system has been effectively used as an anti-miR-10b carrier in cancer treatment. The delivery of the anti-miR-10b led to the inhibition of breast cancer metastasis [27].

As it happened with gold nanoparticles, the addition of Polyethylene glycol (PEG) to MNPs lead to improvements in cellular absorption levels. For instance, the cellular uptake levels of breast cancer cells (BT20) have been demonstrated to increase significantly with the use of these magnetic modified nanoparticles [40].

All the inorganic materials on which the miRNA delivery system can be based have different characteristics. The following table collects distinctive features of the inorganic compounds that have been presented:

<table>
<thead>
<tr>
<th>Inorganic material</th>
<th>Cytotoxicity (mg/mL)</th>
<th>Nanoparticle shape</th>
<th>Nanoparticle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gold</td>
<td>&gt;0.05</td>
<td>Rod or spherical</td>
<td>1-100</td>
</tr>
<tr>
<td>SiO$_2$</td>
<td>&gt;1</td>
<td>Spherical</td>
<td>5-100</td>
</tr>
<tr>
<td>Fe$_3$O$_4$</td>
<td>&gt;0.5-2</td>
<td>Spherical</td>
<td>1-50</td>
</tr>
</tbody>
</table>

Table 4.6 Properties of some inorganic materials used for miRNA delivery. (Adapted from: [40])
4.2.4. Comparison of non-viral systems

This section has given an account of the different non-viral carriers employed for miRNA delivery. The good levels of safety have been found to be their main advantage. However, the main downside regarding such approaches is its low levels of transfection. The following table summarises the different non-viral systems that have been introduced, as well as their main strengths and drawbacks.

<table>
<thead>
<tr>
<th>Delivery system</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Sources</th>
</tr>
</thead>
</table>
| Lipid-based vectors (Par. 4.2.1) | -Scalability and simple production  
- High loading capacity  
- Low immunogenicity  
- High affinity with the cell membrane  
- Versatility | - Low transfection levels  
- They can undergo degradation in endosomes  
- Instability under physiological conditions  
- Short nanoparticle half-life | Yang, N et al. |
| Polymer-based vectors (Par. 4.2.2) | - Safety  
- Biocompatibility  
- Biodegradability  
- Long duration in release | - Low transfection levels  
- Difficulties in the reproducible formation | Yang, N et al. |
| Inorganic materials-based vectors (Par. 4.2.3) | - Their size and surface can be easily modified  
- Low toxicity  
- Biocompatibility  
- Easy synthesis | - Low transfection levels  
- Weak interactions with nucleic acids | Yang, N et al. |

Table 4.7 Advantages and disadvantages of non-viral systems.

In order to address the non-viral system weaknesses presented in Table 4.7 modifications of the delivery are required. The majority of these delivery system alterations are based on the combination
of different non-viral vectors. The selection of such vectors is made according to their advantageous features. For instance, polymers are commonly placed on the vector core while lipids used to cover the nanovector [28][31].

4.3. Preparation techniques

The use of polymer-based nanoparticles as drug delivery systems has spread in the scientific community. The effectiveness of these systems depends on their design which is affected by the techniques employed to prepare the nanoparticles.

Various methods can be employed to prepare nanoparticles. These techniques can be classified into two different groups. The first group collects all the techniques based on in situ polymerization into nanoparticles whereas the second one uses preformed polymers [43][44]. Physicochemical characteristics of the polymer and the drug have a strong influence on the selection of the preparation technique. In some cases, the presence of unreacted monomers and free radicals from polymerization-based methods can result in toxicity from residues. For this reason, much more attention is addressed to techniques using preformed polymers [43].

Regarding techniques based on preformed polymers, there are two methods which have been frequently used. Firstly, emulsion-solvent evaporation is a powerful preparation technique. This methodology, that can be either simple or double, enables the preparation of both nanospheres and nanocapsules. On the other hand, nanoprecipitation is another technique that is used for the preparation of nanoparticles in drug delivery [43][44].

The following figure summarizes techniques employed for the preparation of nanoparticles.

![Figure 4.18 Summary of the techniques used to prepare nanoparticles. (Sources: [43][44])](image-url)
4.3.1. Emulsification: single and double emulsion-solvent evaporation

With regards to the preparation methods based on preformed polymers, most attention is paid to emulsion-solvent evaporation. Allowing the encapsulation of both hydrophobic and hydrophilic drugs, the nanoparticles obtained by emulsion-solvent evaporation show considerable potential as delivery vectors [43][44].

In particular, single emulsion-solvent (SE) evaporation consists of the emulsion formation from two different immiscible phases: organic and aqueous phase [43]. In the first step, the polymer and the drug are dissolved in an organic solvent (in case the drug is hydrophobic). This phase, called organic or oil phase, usually uses ethyl acetate as an organic solvent. The volatility and low toxicity of such organic compound justify its employment [44]. Then, the addition of water and a stabiliser to the organic phase results in an oil-in-water emulsion. An energy source is used to boost the formation of nano-drops. For instance, ultrasonic probes are employed for such purpose. Once the emulsion is completed, the constant agitation of the organic solvent leads to its evaporation. This stirring process takes place at either room temperature or low pressure [43][44]. Then, the polymer precipitates as nanospheres encapsulating the drug. After that, distilled water and centrifugation are used to wash the nanospheres. Likewise, the excess of water is removed using lyophilisation. This technique, also called freeze-dying, is an effective dehydration process [44]. Single emulsion-solvent evaporation is a consolidated technique. Moreover, the adjustment of some factors of the method can lead to enhanced results. The following figure collects some of them.

Figure 4.19 Experimental factors of emulsion-solvent evaporation method. (Sources: [43][44])
Some studies have revealed that the management of these factors may alter both size and drug encapsulation efficiency of nanoparticles. These changes can result in the obtainment of improved nanoparticles [44].

The availability of numerous solvents for single emulsion-solvent evaporation has contributed to the effective implementation of this technique. Moreover, other factors such as its high reproducibility and scalability have also been decisive [44]. Nevertheless, nanoparticles obtained by single emulsification cannot encapsulate efficiently hydrophilic drugs. Thus, the obtainment of nanoparticles encapsulating hydrophilic drugs is accomplished by double emulsion-solvent evaporation [43]. This method, also known as “emulsion of emulsion”, enables the encapsulation of both hydrophilic and hydrophobic agents. Two different types of double emulsion (DE) should be distinguished: water-oil-water (w/o/w) and oil-water-oil (o/w/o) emulsion [43][45]. The selection of the method depends on the hydrophilicity/hydrophobicity of the drug. Water-oil-water emulsion (Figure 4.20) is widely employed for hydrophilic drugs encapsulation. [45] This method is performed in two steps. The first one consists of the hydrophilic drug incorporation into the aqueous phase. Then a lipophilic polymer is solubilised into an oil phase. The oil and water phases are homogenised ending up as an emulsion [43][45]. After that, the primary emulsion system is added drop-by-drop to a water phase carrying a stabilise under vigorous stirring, resulting in the formation of the DE. Then, the evaporation of the organic solvent provides leaves nanoparticles in the water phase. The encapsulation levels of oligonucleotide agents can be improved by adding calcium phosphate to the inner aqueous phase of DE. For instance, the encapsulation of siRNAs has been risen a 37% after this modification [45]. Two different mechanisms are employed to evaporate the organic solvent. The use of a rotary evaporator acting at reducing pressure is one option. Another method consists of stirring at room temperature. Magnetic stirring is a commonly used approach [45]. Similarly, two different surfactants are used to stabilise the emulsion by reducing its surface tension. A more hydrophobic surfactant is used to provide stability to the internal w/o emulsion. On the contrary, a hydrophilic surfactant is employed to stabilise the external w/o/w emulsion. The type and concentration of these surfactants have influence on the nanoparticle size [43][45]. The following figure illustrates the process of nanoparticles preparation using w/o/w.
4.3.2. Nanoprecipitation

Beside single and double emulsion, nanoprecipitation is another method used for nanoparticle preparation. This technique requires two different phases: solvent and non-solvent phase [43]. These phases can be alternatively called organic and aqueous phases. The organic or solvent phase consists of a solvent for the polymer, the active substance (drug), the polymer and a surfactant. On the other hand, a non-solvent for polymer and particular surfactants constitute the non-solvent phase. This phase is commonly an aqueous solution, so it is called aqueous phase. However, the solvent and non-solvent phases are not always present. Some studies have implemented two organics or two aqueous phases. Such combinations, however, should be subjected to solubility conditions [43]. Nanoprecipitation consists of the solvent phase addition to the non-solvent one. The organic phase is added drop by drop under continuous agitation. The following figure describes this process.

*Figure 4.20 Process of formation of nanoparticles by water-oil-water (w/o/w) emulsification. (Reproduced with permission from: [43])*
Apart from emulsification-evaporation and nanoprecipitation other techniques can be employed in nanoparticle preparation. An example of these alternative methods is emulsion-diffusion [43][45]. This approach, based on the emulsification of the organic phase in the aqueous one, is accomplished by vigorous agitation. Then, the following addition of water leads to the solvent diffusion. This process results in the formation of the nanoparticles [43]. The main similarity between such method and nanoprecipitation is that neither of both requires toxic solvents [45].

The following table makes a comparison between some of the most commonly used methods for nanoparticle preparation.

<table>
<thead>
<tr>
<th>Method</th>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single emulsion-solvent</td>
<td>It provides the efficient encapsulation of lipophilic drugs.</td>
<td>The encapsulation of hydrophilic agents is not</td>
</tr>
<tr>
<td>evaporation</td>
<td></td>
<td>possible.</td>
</tr>
<tr>
<td></td>
<td>The modulation of factors such as the phase viscosity leads to particle</td>
<td>Difficulties in scaling up.</td>
</tr>
<tr>
<td></td>
<td>size changes.</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.21 Nanoprecipitation for the preparation of nanoparticles. (Adapted from: [43])
<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double emulsion-solvent evaporation</td>
<td>It provides the encapsulation of hydrophilic and hydrophobic drugs.</td>
<td>The resulting nanoparticles are of various sizes. (polydispersity)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It is a two-step method</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It is difficult to scale up.</td>
</tr>
<tr>
<td>Emulsion-diffusion</td>
<td>· It provides the encapsulation of thermosensitive and lipophilic drugs.</td>
<td>· The encapsulation of hydrophilic agents is deficient.</td>
</tr>
<tr>
<td></td>
<td>· It reduces the particle dispersion and their mean size.</td>
<td>· It requires a long-time agitation.</td>
</tr>
<tr>
<td></td>
<td>· It does not use high-toxic solvents.</td>
<td>· It requires a large volume of water.</td>
</tr>
<tr>
<td></td>
<td>· Good scalability and reproducibility.</td>
<td>· It can lead to organic solvent residues in the final formation.</td>
</tr>
<tr>
<td>Nanoprecipitation</td>
<td>· It is an easy and fast method.</td>
<td>· The encapsulation of hydrophilic agents is deficient.</td>
</tr>
<tr>
<td></td>
<td>· It does not use high-toxic solvents.</td>
<td>· Polymer concentration has influence on the particle size.</td>
</tr>
<tr>
<td></td>
<td>· It provides monodispersed particles.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>· It has high reproducibility.</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.8 Advantages and disadvantages of the nanoparticle preparation methods. (Adapted from: [43][45])

4.4. Characterisation techniques

Numerous delivery systems based on nanoparticles have been tested as miRNA carriers. Likewise, several techniques are employed in order to characterise such nanoparticles (NPs) [46][47][50]. These methods, that can be either in vitro or in vivo, lead to useful comparisons between NPs as well as enhanced production results. Moreover, characterisation techniques allow the appraisal of the NP efficiency when used as nanovectors.
The characterisation methods can be classified into two types according to the assessed properties. Thus, physicochemical characterisation and biological characterisation can be distinguished [46].

### 4.4.1. Physicochemical characterisation

Regarding physicochemical properties, nanoparticle (NP) size is considered the most relevant parameter. Both delivery and entrapment of nanoparticles depend on such parameter. Polydispersity index (PDI) is another widely measured parameter that provides physicochemical information of the nanoparticle [46].

On the other hand, the surface charge has also a crucial role in nanoparticle effectiveness. Expressed as zeta potential, this parameter gives information about the electrostatic interactions between the nanoparticles and the environment. Furthermore, drug release kinetics is also considered as a parameter of interest. It evaluates over a period of time the NP capacity to deliver its encapsulated drug, as well as the ultimate outcomes. [46].

In this section, the most significant characterisation techniques are presented. Such methods are all employed in the assessment of NP physicochemical properties.

#### 4.4.1.1. Electron Microscopy (EM) techniques: TEM and SEM

Electron microscopy (EM) methods consist of accelerated electron beams and electrostatic/electromagnetic lenses. The use of such elements enables the obtainment of high resolution images [47].

The two main EM methods are transmission electron microscopy (TEM) and scanning electron microscopy (SEM). In this section both methodologies, as well as their advantages and drawbacks, will be introduced.

With resolutions under the nanometer, **transmission electron microscopy (TEM)** images provide physical information about nanoparticles. This technique is based on the transmission of an incident electron beam. The interaction of these electrons with the specimen turns them into unscattered, elastically scattered or inelastically scattered electrons. [47] Then, the electromagnetic lenses focus the scattered or unscattered electrons and project them on a screen. Such projection result in the generation of different types of images: electron diffraction, amplitude-contrast, phase-contrast or shadow images. In this last group of images, darkness levels are based on the density of the unscattered electrons [47].
Numerous physicochemical characteristics of nanoparticles can be measured using TEM. Size, shape heterogeneity, degree of aggregation and dispersion are examples of such parameters [47]. Numerous miRNA delivery systems have been characterised using TEM images [31][34][39].

The main advantage of TEM is its high resolution level. Such strength makes TEM the most widely used EM technique [47][50]. Furthermore, there is the possibility to combine TEM with other methods such as energy dispersive X-ray spectroscopy and chemical analyses of electron energy loss spectroscopy. Such combination leads to quantitative analysis of both chemical composition and electronic structure of the NPs [47].

On the other hand, **scanning electron microscopy (SEM)** is an alternative technique based on electron microscopy. SEM is based on the scanning of the incident electron beam across the sample. These incident electrons lead to the emission of other different electrons: elastic scattering of electrons, inelastic scattering of electrons and characteristic X-ray light known as cathodoluminescence. However, most attention is paid to the inelastic ones, that are also called low-energy secondary electrons. The detection of such electrons allows the generation of signals reaching resolutions smaller than the nanometer [47].

As it happens with TEM, SEM methods provide significant physicochemical information of nanoparticles. Nanoparticle size and its distribution, as well as the NP shape, can be obtained using SEM [47][50]. Using such technique levels of nanoparticle aggregation and dispersion can also be measured [47]. SEM has been used to characterise PLGA nanoparticles [32].
With regard to EM weaknesses, certain drawbacks should be also mentioned. [46][47][50] On the one hand, thin section of the samples is required for proper electron beam penetration. Thus, complex processes of sample preparation are needed to meet with such requirement. It usually leads to alternations in the sample structure and creation of artifacts [46][47]. Moreover, the elevated cost of the equipment employed and the damage or even destruction of the samples are also significant EM drawbacks. In the case of SEM, nanoparticles have to be coated using an ultrathin layer of electrically conducting material. Thus, the NP size is evaluated in non-physiological conditions. An exceptional method whose NPs are imaged in their natural state is environmental SEM (ESEM). The main weakness of such method, however, is its reduced resolution [47].

4.4.1.2. Atomic Force Microscopy (AFM)

The atomic force microscopy (AFM) is a type of scanning force microscope (SFM) that consists of a silicon or silicon nitride micro-machined cantilever. At the end of such cantilever, a sharp tip detects its deflection. Moreover, the attraction between atoms at the end of the cantilever and the ones on the material surface is measured. Then, scan over specimen surface by the cantilever results in a high-resolution image of approximately 0.5nm [47].

Figure 4.22 SEM images and respective size distribution of PLGA-based nanoparticles. (Reproduced from [32] under Creative Commons Attribution License)
The physicochemical properties obtained through AFM are similar to the ones provided by TEM and SEM. Thus, this characterisation method provides information on nanoparticle size, distribution, shape, dispersion and aggregation [47][48].

AFM technique can be classified into three types according to the type of its scanning: noncontact, contact and intermittent sample contact mode. These modes are also called static, dynamic and tapping mode, respectively [47].

AFM technique provides 3D surface images of the samples with sub-nanoscaled topographic resolution. The measurements of samples are made under either ambient, dry and aqueous conditions. Thus AFM not only provides nanoparticle size and shape information under physiological conditions but also in other biological ones. For instance, AFM is used to observe in real time the interactions of nanoparticles that include lipid bilayers. It represents a significant advantage of such technique compared to TEM and SEM ones. Moreover, AFM method does not cause appreciable damage to the sample surface. It is one of the main reasons why assiduous attention have been paid to such characterisation technique [47].

AFM has been employed, for instance, in the physicochemical characterisation of cationic acrylate nanoparticles [48]. In this study, such technique was used to determine the NP shape. The following figure shows an image obtained by AFM.

---

Figure 4.23 Atomic force microscopy (AFM) image of the cationic acrylate-based NPs. Image obtained using the AFM tapping mode. The lyophilisation of the colloidal particle suspension on mica has been employed to prepare the samples. The AFM included rectangular silicon cantilevers with an integrated tip. Values of nominal spring constant of 42 N/m and a resonance frequency of 200 – 400 kHz have been used to make the measurements. (Source: Reproduced with permission from [48])
However, some drawbacks of this method should be mentioned. The main one is its adverse overestimation of the lateral sample dimensions. The larger size of the cantilever tip compared to the sample one is a chief cause of such mistake. In the same way, as EM methods, AFM is a time-consuming method. Furthermore, the information provided by this technique is usually limited to the sample surface [47].

4.4.1.3. Dynamic Light Scattering (DLS).

The dynamic light scattering (DLS) is one of the most used methods for the physicochemical characterisation of nanoparticles. The equipment employed for this characterisation method is collected in Figure 4.24.

![Dynamic Light Scattering (DLS) test setup](image)

*Figure 4.24 Setup for dynamic light scattering (DLS) test. (Reproduced from [49] under Creative Commons Attribution License)*

This method is based on a time-dependent measurement of the visible light intensity. Such intensity, called scattering intensity, fluctuates due to the nanoparticles Brownian motion. This motion leads to changes in the NP relative position as well as variations in the scattering intensity. The velocity of these variations depends on the particle size. (Figure 4.25) On the one hand, the quick movement of small particles results in accelerated scattering intensities. On the other hand, slow variations in this intensity are linked to bigger particles [47][50]. Apart from the time-dependent intensity function, the autocorrelation one is applied in order to characterise NP size. [49] The following figure represents the differences between small and large particles. Such differences are based on the intensity and autocorrelation variations. These parameters provide considerable insight into both sample size and polydispersity [50].
Overall, DLS provides relevant nanoparticle information such as its size, shape and structure. Moreover, the NP aggregation state and its biomolecular conformation can be evaluated using DLS. Being a non-invasive method, DLS has many other strengths. The DLS short duration, good reproducibility and low cost are advantages derived from its use [47].

DLS, as well as TEM and AFM, has been employed in the measurement of nanoparticle size. However, the working range of these three techniques showed differences. The following table collects such dissimilarities.

<table>
<thead>
<tr>
<th>Characterisation technique</th>
<th>Approximated working range of size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmission electron microscopy (TEM)</td>
<td>0.5 nm - 1 µm</td>
</tr>
<tr>
<td>Atomic force microscopy (AFM)</td>
<td>1 nm - 1 µm</td>
</tr>
<tr>
<td>Dynamic light scattering (DLS)</td>
<td>1 nm - 5 µm</td>
</tr>
</tbody>
</table>

Table 4.9 Working size range of different characterisation techniques. (Adapted from [49] under Creative Common Attribution License)

As can be seen from Table 4.9, the selection of the characterisation technique can be made according to the working size range. For instance, DLS is chosen to measure size of NPs under the µm.

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Figure 4.25 DLS measurement of intensity and autocorrelation variations in both large (A) and small (B) particles. (Reproduced from [49] under Creative Commons Attribution License)
Despite the numerous advantages of DLS, such method also has some drawbacks. Interferences in the scattering intensity due to dust particles and particles in biological samples is a significant disadvantage. It becomes more noteworthy between organic samples, whose refractive index is similar to the surrounding bio-matter one [50]. For this reason, DLS shows limitations when used for the characterisation of heterogeneous-sized particles. [47][50] Another weakness of this method is its incapacity to characterise non-spherical particles [47].

4.4.1.4. Fluorescence Correlation Spectroscopy (FCS)

As is the case with DLS, fluorescence correlation spectroscopy (FCS) uses an autocorrelation analysis to provide quantitative information of nanoparticles. However, FCS focuses on the temporal analysis of the fluorescent variation. The results of this analysis are fitted to a suitable model describing the system reaction and diffusion characteristics [47]. Thus, FCS provides significant information on nanoparticles. The NP hydrodynamic dimension, as well as the kinetic chemical reaction rates, are the main physicochemical characteristics obtained through FCS. Furthermore, the molecular diffusion coefficient and the concentration effects can be measured using such approach [47].

Regarding the strengths of FCS, its high spatial and temporal resolution should be underlined. On the other hand, the requirement of low levels of sample is an advantage of this characterisation method over the DLS one. Moreover, unlike DLS, FCS has the ability to monitors specifically the probe nanoparticles and avoid interferences from the medium.

FCS has also been employed for the determination of the oligonucleotide (ON) loading rate in cationic acrylate nanoparticles [48]. The following figure summarises the evolution of such parameter.
However, all these FCS advantageous characteristics are subjected to the selection of the fluorescent chemical compound. Such compound called fluorophore needs to possess some particular characteristics. Some of them are the high quantum yield and the low irreversible destruction (photobleaching). [47] These strict requirements lead to limitations in the fluorophore selection. On the other hand, the limited number of available models is also a challenging factor associated to FCS implementation [47].

4.4.1.5. Raman Scattering (RS)

Raman scattering (RS) is a characterisation method based on the photon inelastic scattering measurement. RS focuses on the differences in frequency between the photons undergoing inelastic scattering and the incident ones. In the Raman spectrum, two different lines can be distinguished according to the frequencies: Stokes and Anti-Stokes lines. When the frequency of the scattered photons is lower than the one of the incident photons, lines are called Stokes. Unlikely, when this frequency is higher, lines are named Anti-Stokes [47].

RS is a long-established technique used for the characterisation of both nanomaterials and nanostructures, such as nanoparticles. This technique does not need sample preparation. It is an advantageous factor in comparison with other methodologies that turn RS into an effective in situ application [47]. Another strength of RS is its capacity to characterise biological specimens in aqueous solutions. It makes RS a powerful tool for tissue analysis. The assessment of proteins in solution as well as their structure in NP aggregates has been also performed through RS [50]. On the other hand, the
conventional methods based on RS provide physicochemical information such as nanomaterial size and size distribution. These measurements, however, are obtained indirectly from the analysis of both shift and broadening of the spectral line [47][50].

Nevertheless, its low spatial resolution compared to other methods is a significant RS drawback. Likewise, proper RS signal requires large amounts of samples, becoming another drawback. Such situation is due to fluorescence interferences and remarkably small cross-sections. Surface-enhanced Raman scattering (SERS) has been introduced to overcome such difficulty. This technique has been tested on gold nanoparticles, leading to enhanced RS signals and improved spatial resolution [47]. Furthermore, SERS has been employed for the rapid detection and identification of different miRNAs. In this case, SERS have used an innovative substrate called hollow Au nanoflowers (HAuNFs) [51]. The following figure collects the normalised mean SERS spectra of three different miRNAs.

![Figure 4.27 Normalised mean SERS spectra of three miRNAs: miR-10a-5p, miR-125a-5p and miR-196a-5p. The SERS spectra were obtained in the range of 600-1700cm making an average of 10 different points. The SERS spectrum intensity has been normalised to obtain the relative one. (Reproduced from [51] under Creative Commons Attribution License)](image)

4.4.1.6. Electrophoretic light scattering (ELS)

Regarding characterisation techniques employed for zeta potential (ZP) measurement, electrophoretic light scattering (ELS) has a significant role. The zeta potential is a parameter that refers to the electric potential of the nanoparticle in the shear surface. The velocity measurement of the charged particles
towards an electrode leads to ZP determination. This procedure is followed under the influence of an external electric field [47].

The value of the ZP fluctuates between ±30 mV. ZP values over 30mV are associated with nanoparticle instability and aggregation. On the other hand, when the ZP is more than 30mV, stable conditions are ensured [47].

Regarding ELS, the use of such method enables the simultaneous velocity measurement of different charged particles. This ELS feature makes this technique one of the most-used ones for ZP measurement [47]. However, this characterisation method also has some disadvantages. On the one hand, the electro-osmotic effect has a negative impact on the ELS effectiveness. This phenomenon leads to lower levels of measurement precision and reproducibility. On the other hand, despite the enhanced light penetration of diluted solutions, ZP alterations in such solutions are also worth mentioning. Such changes depend on environmental conditions such as the ionic strength and the pH. Consequently, the results obtained by diluted solutions differ from the measurements provided by concentrated ones [47].

4.4.2. Biological characterisation

Physicochemical characteristics are determinant in the delivery effectiveness of nanoparticles. The success of such nanovectors also depends on their biological features. This section introduces the biological characteristics of NP as well as the different assays available to assess such features.

4.4.2.1. Cytotoxicity

Before the employment of a nanoparticle-based system for the delivery of miRNAs or drugs, some biological characteristics are analysed. An example of such features is the NP cytotoxicity. This undesired capacity of NP to be toxic, depends on many physicochemical properties: NP size, shape, surface modifications, solubility and chemical composition. Thus NP cytotoxicity has been studied according to these characteristics [52].

Despite the assiduous attention paid on cytotoxicity assays, none of them has been established as the standard one. However, the majority of the tests have performed in vitro due to their remarkable ease in implementation. Precise control over in vitro assay conditions enables to mimic the in vivo behaviour of the NPs [52].

One of the decisions to be made before the cytotoxicity assay is the NP dose. This parameter, commonly expressed as mass per unit volume, is based on the NP exposure dose. Another factor to be considered is the NP delivery in multiple cells type. Thus, cytotoxicity assays require the combination of different cells: epithelial, endothelial and macrophages [52][53].
Regarding the physicochemical characteristics of NPs, NP size is the one with more impact on biological features. This strong influence is due to the indirect relationship between NP size and surface area. Thus, particle size decrease not only leads to an increase in surface area but also in the molecules expressed on it. Hence, cytotoxicity levels have been proved to increase as the NP size decreases [52].

Other factors that can result in high toxicity are impurities within the NP formulation and its delocalisation. This last factor can lead to either inflammation or immune response. (Par. 3.1.2).

On the other hand, the selection of the cytotoxicity assay is crucially important for the accurate assessment of such parameter. Some of these tests are the identification of cytokine/chemokine production assay, lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For instance, MTT assay consists of the mitochondrial activity measurement. Conversely, lactate dehydrogenase is an enzyme present in many living cells. LDH tests enable the cytotoxicity assessment by measuring such lactate levels [53]. The chief goal of all these assays is to avoid false-negative and false-positive tests. However, the single application of one of these test becomes insufficient for an accurate cytotoxicity assessment. For such reason, the combination of different assays has become extremely common among biological characterisation tests [52].

For instance, a powerful combination of different cytotoxicity assays has been employed in iron oxide nanoparticles. Such approach focused on the viability and cytotoxicity assessment. First, these parameters were studied using MTT and LDH wet transfer methods. (Figure 4.28) From such test, it was found that cell death increased as NP concentration did. However, lower cytotoxicity levels and smaller viability were associated with a 0.06 mg Fe/ml concentration [53].

Second, cytotoxicity of dry transfer method was tested using same assays. (Figure 4.29) This time, however, LDH assay proved levels of cytotoxicity lower than 10%. Likewise, MTT assay confirmed a
drop in viability levels over 50%. This lack of dose-dependent response was due to NP interference with MTT assay. Thus, a third experiment was carried out [53].

A third test was carried out again using resazurin assay instead of MTT. Resazurin is a redox indicator used for the assessment of cell viability and cytotoxicity [53]. In this case, the results of the experiment confirmed that cell viability depends on NP dose.

4.4.2.2. Cellular uptake

The number of nanoparticles (NPs) that interact with targeted cells (e.g., cancer cells) is becoming increasingly important. Specifically, the cellular uptake measures the number of NPs that are taken up by such cells. Furthermore, the biological characterisation of NPs using such parameter results in proper NP dose selection. Regarding safety and regulatory concerns, cellular uptake is also crucial. It is because NP not only can be in contact with targeted cells but also with untargeted ones [54].
As it happens with cytotoxicity, cellular uptake levels are highly dependent on the physicochemical characteristics of NPs. Thus, features such as NP shape, size and the material used for the core have an impact on cellular uptake levels. Furthermore, surface modifications of the NP also influence such levels [54].

Regarding the techniques employed for the quantification of intracellular NPs, different methods stand out among the rest. The selection of such techniques depends on NPs physicochemical features and their spectroscopy properties [54].

Techniques based on fluorescence are important methods employed for NP quantification. Such techniques, however, only allow the identification of NPs with fluorescence signals. Such signals can be either produced by the NPs or using a fluorescence tag [54][55]. Such fluorescent signals are identified and measured by different techniques: flow cytometry, fluorescence spectroscopy or fluorescence imaging techniques. The low resolution of these techniques does not allow the single quantification of NPs. Thus, the number of loaded NPs in cells is indirectly expressed. The quantification of the signal events or intensity enables the measurement of the loaded NP [54].

In flow cytometry, single cells suspended in a steady stream pass through a laser detection unit. The collection and analysis of the integrated fluorescent signals allow the differentiation between NPs free cells and NPs loading cells [54].

Flow cytometry has been used, for instance, to quantify the cellular uptake levels of conjugated polymers (CP). [55] Showing extremely bright fluorescence, such materials are commonly used as fluorescent probes. Cellular uptake levels of poly(fluorene-alt-benzothiadiazole (PFBT) nanoparticles have been assessed using flow cytometry [55].

On the other hand, in fluorescence microscopy, the event quantification is based on digital images. Such quantification is performed manually or using an automated analysis. However, fluorescence microscopy-based techniques neither provide NP mass nor NP number. Such microscopy signals are compared with control samples leading to semi-quantitative results. [54].

Cell uptake studies on PLGA/chitosan-based NPs have been undertaken using fluorescence microscopy. Due to its cell-adhesion features, RGD peptide (GRGDSP) has been conjugated to the NPs via emulsion-solvent evaporation (Par. 4.3.1). The enhancement caused by such modification has been tested in different non-small-cell lung cancer cells (NSCLC) [56]. The following figures collects the fluorescence images and graphical representations of the fluorescence intensity that prove such enhancement.
In the first place, results from Figure 4.31 showed that FluTax-NP complex had better cellular uptake levels than the free FluTax. On the other hand, the modified FluTax-NP-RGD complex presented even higher uptake levels than other two structures. Regarding NSCLC cells, H1975 were the ones showing the highest uptake levels.

This study used both flow cytometry analysis and western blotting. This last method allowed the specific examination of proteins. In this case, western blot analysis was employed for integrin αv β3 detection [56].

The quantification of the NP absolute number per cell of requires high resolution levels. Thus, EM images (Par. 4.4.1.1) are extensively employed for such parameter measurement.

4.4.2.3. Oligonucleotide delivery

One significant feature of NP is its delivery capacity. Such parameter has a strong influence on both efficiency and viability of the NP delivery complex. Thus, many tests to assess the NP delivery levels are commonly undertaken [34][56][57][58][59]. This chapter of the thesis will focus on the most used methodologies for miRNA identification: RNA sequencing (RNA-seq) and quantitative real-time PCR (qRT-PCR). Likewise, some attention will also be paid to western blot analysis. Despite not being a direct analysis of miRNA, such method is a potential tool to investigate the protein expression caused by miRNA.
Regarding methods based on polymerase chain reaction (PCR), quantitative real-time PCR (real-time PCR) and reverse transcription PCR (RT-PCR) should be stated. Real-time PCR, also called quantitative PCR (qPCR), consists of the monitoring of the DNA amplification during the PCR process. On the other hand, the RT-PCR is based on the RNA transformation into its complementary DNA (cDNA), thanks to reverse transcriptases. Such cDNA molecule is then amplified through standard PCR [57].

With regards to blotting assays, northern and western blot should be highlighted. Whereas the first one focuses on RNA detection, western blot's goal is to recognise proteins [34][56][58].

Real-time PCR, for instance, has been employed to evaluate the delivery efficiency of new-emerging vectors called interfering nanoparticles (iNOPs). Such technique was used to quantify the endogenous miRNA levels delivered by iNOPs [57]. Likewise, real-time PCR and western blot have been used to assess the delivery efficiency of PLGA-based dual targeted nanoparticles. These NPs have been tested in hepatocellular carcinoma (HCC) treatment. The chief purpose of such miRNA delivery was to reduce the expression of both miR-99a targeted genes: mTOR and Ago2. This process resulted in tumour suppression and thus efficient carcinoma treatment [34].

The following figure collects both real-time PCR and western blot assays of mTOR and Ago2 expression.

![Figure 4.32: Real-time PCR images of mTOR expression (A) and western blot images of Ago2 expression (B) in HepG2 carcinoma cells. These cells have been tested in negative control conditions (PBS), TRizol Reagent (Lip2000) and using the PLGA-based nanocomplex. (Reproduced from [34] under Creative Commons Attribution License)](image)

The Figure 4.32 above shows that both targeted genes expression decreased when using the whole PLGA-based NP complex. Hence, such delivery system was proved to be a more efficient miR-99a delivery compared to other mechanisms [34].
Apart from q-PCR, northern and eastern blotting, RNA sequencing (RNA-seq) has been employed for gene delivery assessment. This method is based on the fact that the organism response to abiotic conditions depends on its genome changes. In particular, these changes are expressed in sets of all RNA molecules called transcriptomes. Thus, RNA-seq consists of the measurement of such transcriptome changes to provide information of their levels in particular physiological conditions. The RNA-seq accuracy and precision levels are similar to the ones obtained by real-time PCR [59].

4.4.3. Safety and security

The clinical implementation of NP as miRNA delivery vectors is subjected to some regulatory considerations. However, there is no specific regulation for such delivery systems. Thus, general nanomaterials and medicine regulation should be considered [60][61].

On the one hand the European Union’s (EU) regulatory framework regulates nanomaterials. Such organism is formed by both horizontal and vertical legislations. The second legislation group includes the rules applicable to a specific field. These regulations pay careful attention to nano-scale medical products that are potentially released in human’s body. Such materials, as it is the case of polymer-based NPs and the other ones presented in Par. 4.2, are considered as class III medical devices [60][61].

The employment of such delivery vectors in America is under the supervision of two different agencies: the Food and Drug Administration (FDA) and the Office of Biotechnology Activities (OBA) [60][62].

The major part of the commercial medical devices are developed following FDA steps. For this reason, most attention will be paid to the rules provided by the FDA administration. According to this organisation, the drug development process may be divided into five steps. The following figure collects such procedure [62].
Although the steps summarised in Figure 4.33 make reference to drugs, they can be also applied to all the delivery systems employed for drug delivery. Thus, the polymer-based NP steps to receive the FDA approval are the same.

In the first step, researchers leading their efforts to research and development of the drug. The information gathering allows the development of the product. Parameters such as the optimal drug dosage, its delivery levels and its effectiveness in comparison with similar products are examples of such gathered information [62].

Second, the preclinical research is compulsory before the clinical one. Such investigation has to prove acceptable levels of toxicity and dosage levels [62]. Once this step has been completed, the drug is tested in humans in the clinical research. This third step enables the evaluation of the interaction between the drug and the human body. On the other hand, the medical product developers need to submit an investigational new drug (IND) application to the administration [61][62]. The following table collects the information that must be included in such protocols.
Regarding cancer gene therapy (CGT) products, a specific FDA division focus on their evaluation: Office of Cellular, Tissue and Gene Therapies (OCTGT). OCTGT reviews the different CGT products presented in this research field. Thus, the safety evaluation of both viral (Par. 4.1) and non-viral vectors (Par. 4.2) can be made by such Office [60].

On the other hand, pre-IND procedures are advisable before the submission of the IND (Table 4.10). Such previous actions consist of the communication with OCTGT/FDA to avoid a clinical hold. Pre-IND application is made then to review the guidance documents provided by the administration. Likewise, the drug developers or sponsors ask for advice on their research [60][62].

In case FDA approves the IND application, clinical research begins. This research is divided into four phases according to the study participants and length. In phase 1, for instance, not more than a hundred participants are involved in a several-months study. Conversely, up to 3000 humans are immersed in phase 3 studies. The length of such varies from 1 year to 4. [62] Moreover, the FDA purpose in these phases varies. Thus, FDA objective is to ensure both safety and subject rights the first investigation phase [60][62]. In phases 2 and 3, however, the agency focuses on the quality of the drug evaluation. For this reason, such scientific evaluation must be convincing enough to guarantee both effectiveness and safety of the product [60].

Once the developer or sponsor has gathered compelling evidence on drug’s safety and efficacy, he proceeds to step 4. In this step, the developer should submit a new drug application (FDA) including all documents and research that demonstrates both drug efficacy and safety. If this step is successfully completed and validated, the product receives the FDA approval. After the drug’s placement on the market, FDA performs a post-market drug safety monitoring. One of the Administration mechanisms to ensure the post-market product safety and effectiveness is the review of reporting problems [62].
4.4.4. Characterisation techniques in a specific application

This chapter presents a concrete biomedical engineering application that uses polymer NPs in miRNA-based cancer therapeutics. The selection of such application has been made considering some factors: First, because it uses a polymer-based NP, which is of our special interest. Second, because it clearly exemplifies the surface functionalization of NP to enhance targeting. Moreover, because some of the methods employed to prepare and characterise these NPs have been introduced in Par. 4.3 and Par. 4.4.

4.4.4.1. Functionalised hybrid-nanoparticles for the miR-29b delivery in cancer cells.

This approach employed miRNA delivery for the treatment the most common types of lung cancer: non-small cell lung cancer (NSCLC). Such study was based on the ability of DNA methyltransferases (DNMTs) to inhibit the cell proliferation and apoptosis of NSCLC cells. On the other hand, the capacity of miR-29b to target DNMTs was also exploited. For these reasons, such miRNA was selected to enhance DNMTs targeting efficiency in NSCLC treatment [63].

Furthermore, miR-29b has been also associated with enhanced NSCLC sensitivity to some chemotherapeutic agents [63]. In addition, this miRNA has been proved to regulate other processes. For instance, miR-29 regulated the dendritic cells (DCs) during complement activation of T cells in multiple myeloma [64].

Researchers had to face some challenges in order to implement miR-29b in NSCLC therapy. The poor cellular uptake levels, the immune response stimulation and the off-target effects were some of the difficulties experienced by the scientists. In order to overcome such adversities, they developed hybrid NPs made of human immunoglobulin G (human IgG) and poloxamer-188 (polyoxyethylene–polyoxypropylene block copolymer). These NPs were know as MAFMILHNs [63].

Such NPs were surface-functionalised with a transmembrane protein called mucin1-aptamer (MUC1). Being overexpressed in 80% of lung cancers, such protein is associated with NSCLC tumour formation. Thus, the addition of this protein to the NP surface enabled the miR-29b targeting to MUC1-expressing cancer cells [63].

The following figure summarises the process of MAFMILHNs’ preparation.
Regarding the methodologies employed for NP characterisation, some of the ones introduced in Par. 4.4 were used. On the first hand, the morphology of such NPs was obtained by SEM images (Par. 4.4.1.1). Spherical shape and high dispersion levels were obtained. The loading capacity and encapsulation efficiency were also assessed in order to determine the NP effectiveness. Values of 8.6 ± 0.1% and 98.8 ± 0.4% were obtained, respectively [63].

The most relevant physicochemical parameters of the NPs varied with the the conjugation of MUC1 to their surface. The following table collects such changes.

<table>
<thead>
<tr>
<th>Physicochemical property</th>
<th>Non-functionalised NPs</th>
<th>Functionalised NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP size</td>
<td>236 nm</td>
<td>595 nm</td>
</tr>
<tr>
<td>Polydispersity index (PDI)</td>
<td>0.242</td>
<td>0.554</td>
</tr>
<tr>
<td>Zeta Potential (ZP)</td>
<td>-2.1</td>
<td>+4.1</td>
</tr>
</tbody>
</table>

*Table 4.11 Variation of the NP size, PDI and ZP after the conjugation of MUC1 to their surface. (Adapted with permission from [63])*

Another parameter assessed was the cellular uptake. Hyperspectral microscopy was employed for the evaluation of such parameter. The images obtained by this technique showed that all MAFMILHN, functionalised and non-functionalised, were spherical. Such consistency between hyperspectral microscopy images and SEM ones proved that the first method was valid for cellular uptake appraisal [63].
On the other hand, the main goal of this approach was to inhibit lung cancer progression, that was caused by DNMT3B. Thus, MAFMILHNs were implemented to downregulate such oncoprotein both in vivo and at the cellular level. The measurement of DNMT3B decrease A549 adenocarcinoma cells was carried out using Western-Blot Analysis (Par. 4.4.2.3). The following figure collects such experiment.

![Western blot analysis](image)

*Figure 4.35 Western-blot analysis to evaluate the downregulation of DNMT3B and β-tubulin in A549 cells. (Reproduced with permission from [63])*

From the Western Blot analysis (Figure 4.35), it was observed a DNMT3B downregulation in A549 cells undergoing MAFMILHN’s treatment. The delivery of miR-29 through lipofectamine and MUC1-aptamer-functionalised hybrid NPs was not as efficient as the MAFMILHN’s one [63].
5. **MicroRNA therapeutics perspectives**

This chapter presents an overview of the miRNA preclinical and clinical studies conducted over the last years. Moreover, the future perspectives of miRNA therapeutics are also analysed.

5.1. **Current state of the art**

Since the discovery of miRNAs (Par. 1), a considerable number of miRNAs have been under preclinical and clinical trials [4]. The main purpose of such trials is the demonstration of both safety and efficiency of the medical device or drug. In this case, miRNA-based therapeutics.

A 15-nucleotide antisense RNA oligo with complementarity to miR-122 (LNA miravirsen) was one of the first employed miRNA-based molecules. Tested in HCV (Hepatitis C Virus) treatment, such miRNA-based therapeutics took part in phase I clinical trials in 2009. Adverse reactions were not detected in non-human phase I trials, so phase II ones were initiated. In the initial steps of this phase, patients experimented a dose-dependent decrease in HCV load levels. In additional phase II studies, with longer follow-up and larger number of patients, some mutations took place. Although these alterations were not proved to produce therapy resistance, further analysis on this issue was required [4].

Regarding miRNA-based cancer therapeutics, a miR-34 mimic (MRX34) has been the foremost compound. Being encapsulated in lipid carriers called NOV40, such NP has the ability to adhere properly to tumour cells. This is because of the NP’s ability to become positively charged in low pH tumour conditions [4].

It was not until 2013 that these NPs entered phase I trials. Patients suffering from different types of cancer were enrolled in such trials. As of June 2016, almost hundred HCC, NSCLC or pancreatic cancer had been enrolled to the research. In some of these studies, immune-related adverse events took place, even involving the patient deaths. The cause of these events was unknown and future preclinical trials will require re-design, paying special attention to the immune-related toxicities [4].

The following tables collect some of the miRNA-based therapeutics that are under preclinical and clinical trials.
<table>
<thead>
<tr>
<th>miRNAs with tumour suppressive function (miRNA mimics as therapeutics)</th>
<th>miRNAs</th>
<th>Diseases</th>
<th>Important mRNA targets</th>
<th>Preclinical models</th>
<th>In vivo delivery systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7 family</td>
<td>• Solid tumours (e.g. breast, colon, ovarian, lung, liver and glioma)</td>
<td>MYC, BCLXL, pan-RAS, EZH2, HMGA2, FAS, P21, PGRMC1 and DicerI</td>
<td>• Lung cancer (orthotopic)[157]</td>
<td>• Krus[155] GEM[156]</td>
<td>Neutral lipid emulsions</td>
</tr>
<tr>
<td>• B-cell lymphoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mir-34a</td>
<td>• Solid tumours (e.g. lungs, liver, colon, brain, prostate, pancreatic, bladder and cervical)</td>
<td>BCL2, MET, MYC, CD46, CD44, SRC, EZF1, JAG1, FOXP1, PDGFRα, PDLD1 and SIRT1</td>
<td>• Lung cancer (xenograft and orthotopic)[87]</td>
<td>• Krus[155] GEM[156]</td>
<td>• Lipid nanoparticles</td>
</tr>
<tr>
<td>• Myeloma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• B-cell lymphoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>* mir-143</td>
<td>• Solid tumours (e.g. bladder, lung, breast, colon, pancreas, cervical, and head and neck)</td>
<td>KRAS, ERK5, VEGF, NFkB1, MYC, MMP9, PLK1, CDH2 and EGFR</td>
<td>• Colon cancer (orthotopic)[90]</td>
<td>• Pancreatic cancer (orthotopic)[91]</td>
<td>• Prostate cancer (orthotopic)[92]</td>
</tr>
<tr>
<td>* mir-145</td>
<td>• Solid tumours (e.g. breast, colon, ovarian, lung, and cervical)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mir-200 family</td>
<td>Solid tumours (e.g. breast, ovarian and lung)</td>
<td>ZEB1, ZEB2, BMI1, SUZ12, JAG1, SOX2, SP1, CDM1 and KRAS</td>
<td>• Lung cancer (orthotopic)[93,94]</td>
<td>• Ovarian cancer (orthotopic)[95]</td>
<td>• Breast cancer (orthotopic)[96]</td>
</tr>
</tbody>
</table>

**OncomiRs (antimiRs as therapeutic agents)**

| miR-10b | Solid tumours (e.g. breast and glioma) | NF1, CDH1, EZF1, PK23CA, ZEB1 and HNDD1 | • Glioblastoma (orthotopic)[97] | • Breast cancer (orthotopic)[98] | Locked nucleic acid anti-miRs |
| mir-155 | • Solid tumours (e.g. liver, lung, kidney, glioma and pancreas) | SHH, SP1, HDAC4, RHOA, SOCS5, BCL2, JMD2A, SOX6, SMAD2, SMAD3 and TP53N1 | | | | |
| • B-cell lymphoma | | | | | | |
| • Lymphoid leukemia | | | | | | |

**mir-155** overexpressing GEM[133,134]

**pHLIP-conjugated anti-miR**

| * miR-211 | • Solid tumours (e.g. liver, pancreas and lung) | CDKN1B, CDKN1C, BWF1, RB1, WEE1, APAF1, ANXA1 and CTCF | Liver cancer (HCC xenograft)[117] | | Cholesterol-conjugated anti-miR |

**Other**

| miR-122 | HCV infection and related liver diseases | HCV 5’ site, CAT1, CD320, ALDOA and PPARγ | HCV mouse model[134,135] | | Phosphorothioate DNA-locked nucleic acid anti-miR |
| miR-33 | Atherosclerosis | SREBF2, ABCA1, CROT, CPT1A, HADHB and PRKAA1 | HFD mouse[136,137] | | 2-Fluoro or MOE phosphorothioate DNA anti-miR |
| miR-208 | • Cardiac disease | MED13, SOX6 and MYH7B | | Dahl hypertensive rat[138] | Locked nucleic acid anti-miR |
| • Cardiac stress | | | | | |
| • Myocardial infarction | | | | | |
| miR-21 | • Kidney fibrosis | PTEN, PDCD4, SMAD7, SPRY and PPAR | • Pressure overload model of heart disease[139] | • Kidney injury mouse model[140] | Locked nucleic acid anti-miR |
| • Cardiac fibrosis | | | | | |
| miR-192 | Diabetes-related kidney complications | Type I collagens, ZEB1 and ZEB2 | Streptozotocin-induced type 1 diabetes mouse[141] | | Locked nucleic acid miRNA mimic |
| miR-29c | Diabetes-related kidney complications | HDAC4 and MMPs | db/db mouse[142] | | Naked antagoniR |
| * miR-103 | Diabetes | CAV1 | • ob/db mouse[143] | • HFD mouse | Locked nucleic acid anti-miR |
| * miR-107 | | | | | |
| miR-15 | Myocardial infarction | CHEK1 | Ichaemia–reperfusion injury mouse[144] | | Locked nucleic acid anti-miR |

Table 5.1 Selected miRNAs for cancer and other diseases’ treatment, its targets, preclinical model as well as the delivery system employed. Abbreviations: 2-Fluoro, 2-fluoro; db/db, spontaneous diabetes due to a mutation in the leptin receptor gene; ob/ob, spontaneous diabetes due to a mutation in the leptin gene; DOPC, 1,2-dioleoyl-sn-glycerol-3-phosphatidylcholine; GEM, genetically engineered mouse; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HFD, high-fat diet; PEI, polyethylenimine; miRNA, miR, microRNA; MMPs, matrix metalloproteinases; MOE, 2-O-methoxymethyl; pHLIP, pH-lowering peptide. (Source: Reproduced with permission from [4])
Table 5.2 Selected miRNA therapeutics under clinical trial. Abbreviations: DOPC, 1,2 dioleoyl-sn glycerol-3 phosphatidylcholine; eIF, eukaryotic initiation factor; GalNAc, N-acetyl-galactosamine; HBV, hepatitis B virus; LNA, locked nucleic acid; LNPs, lipid nanoparticles; miRNA, microRNA; PEI, polyethylenimine; RSV, respiratory syncytial virus. (Source: Reproduced with permission from [4])

One of the most attractive miRNA-based therapeutics was developed by EnGeneIC in collaboration with Asbestos Diseases Research Institute: MesomiR-1. (Table 5.2) Such miR-16-based therapeutics entered phase I trials in both NSCLC and malignant pleural mesothelioma patients. The miR-16 was encapsulated in EDV nanocells. Then, these cells were surface-functionalised with epidermal growth factor receptor (EGFR) antibodies. Such functionalization lead to improvements in the targeting to tumour sites [4].

This growing body of literature on miRNA therapeutics entering preclinical assays proves that such molecules hold limitless potential.

5.2. Future perspectives

Despite the numerous miRNA therapeutics that have been conducted in preclinical trials, thus far, just a few of such therapeutics have moved towards clinical studies. It is because there are still many challenges and questions that need to be addressed.
Firstly, the identification and selection of the best miRNA candidate and the miRNA targets according to the type of disease is essential [3][4][27]. The heterogeneity of the miRNA expression is a chief obstacle for the proper identification of the target miRNA. In order to encounter such barrier, multiple biopsies are taken at different times. These medical tests enable the identification of the common regulatory miRNAs [4]. Furthermore, the focus on a single miRNA target proves insufficient. It is because of the multiple genes dysregulation in cancer. Thus, some approaches have focused on the multiespecificity of the miRNAs rather than on their target specificity [2].

Moreover, the enhanced identification of chief miRNA targets will depend on the growth of the genomic and proteomic data in human biology. Thereby, the combination of databases such as CLIP-seq with predication tools will result in elucidated miRNA target predictions [4].

Another miRNA therapeutics challenge is the selection and implementation of effective delivery systems [2][27]. Thus far, no ideal miRNA carrier has been found in vivo or in vitro. The miRNA vector specificity as well as its toxicity levels are the main issues concerning delivery systems. Improvements in such issues would accelerate the miRNA-based therapeutics’ transition from bench to bedside [27].

Overall, the growing body of literature on human biology and the comprehensive preclinical analysis provided by novel miRNA carriers remains essential. If such advances are made, miRNA therapeutics will not only be potential tools but also a long-term clinical reality [3][4]. Apart from cancer therapeutics, miRNAs are expected to be employed in combination with cytostatic compounds. It will allow the modulation of oncogenic features such as metastasis and chemoresistance [2].
6. Proposal of polymer-based NPs

In this chapter of the thesis, one miRNA agent (miR-29b mimic) was proposed according to the knowledge gained in previous chapters.

Polymeric nanoparticles, based on PLGA and chitosan, could be employed for the delivery of miR-29b in NSCLC treatment. Moreover, ligands could be added to the nanoparticle surface in order to enhance the transfection levels and delivery efficiency.

The steps to prepare the NP-based delivery complexes, the different methods used to characterise them, as well as the cytotoxicity assays were collected in this section. A section including materials for release was also included.

6.1. Protocol for NP preparation

On the first hand, the method chosen for NP preparation will be nanoprecipitation (Par. 4.3.2). The preparation steps are the following.

1. Dissolve 6 mg of PLGA in 2ml of acetone at room temperature.
2. Add the miR-29b to the aqueous phase. Different concentrations of miRNA should be added in order to estimate the one providing highest entrapment levels.
3. Add previous solution to 5 ml of an acidified aqueous phase made of MilliQ water (acetic acid 0.5% v/v). It must contain 0.3mg of chitosan and poloxamer 188 (1% w/v). Importantly, filtrate the aqueous phase to avoid chitosan aggregation. Use a 0.45μm polyamide filter to complete such process.
4. Add the system to solution prepared at step 1.
5. Initiate the homogenisation of the aqueous phase using an ultracentrifuge. Make this process for 1 minute at 24000 rpm. Then, get the phase mechanically stirred at 600 rpm during 3 hours, leading to the organic component evaporation
6. Add the pegylated phospholipid (PEG lipid) to the NP to coated them. By using functional PEG-lipid it is possible to graft ligands after the coating step.
7. Increase the pH of the NP solution to approximate 8.5 using potassium carbonate.
8. Add 100 μg of EGFR Antibody C225 Cetuximab slowly to the pH-adjusted NP solution. Stir the solution and incubate it at ambient temperature for 1 h.
9. Use bovine serum albumin (BSA) to perform the blocking treatment at a concentration of 1% (w/w).
10. Remove the excess of antibodies through centrifugation at 10000 RPM for 40 minutes.
6.2. NP characterisation

Once the NPs have been formed, characterisation procedures are mandatory to ensure the viability of the vectors. The advisable methods to characterise the NP are the following:

Regarding physicochemical properties, transmission electron microscopy (TEM) is proposed to evaluate the NP morphology and size. First, coat the TEM stub with a double-sided carbon tape. Then, use a spatula to spread the NPs across the tape surface and dry the sample. Use uranyl acetate to allow contrast. It should be done for 2 minutes. Finally, use either distilled water or compressed air to remove loose NPs.

On the other hand, dynamic light scattering (DLS) is proposed as a characterisation technique for zeta potential estimation.

In order to assess cytotoxicity, MTT assay is suggested. To undertake such assay, plate the NSCLC cells in culture dishes. Using the MTT assay kit, follow the next protocol:

1. Grow the NSCLC cells and treat as desired.
2. Discard media and add serum-free media as well as the MTT reagent.
3. Incubate the NSCLC at 37º for 3 hours.
4. Add the MTT solvent
5. Shake it on an orbital shaker or similar for 15 minutes.
6. Quantify the absorbance at OD590nm. Such parameter is proportional to the number of viable cells.
7. Determine change as percentage of control after background subtraction.

The viable cells with active metabolism will transform MTT into formazan. Conversely, dead cells loose such ability and provide no signal.

Furthermore, flow cytometry analysis is suggested to measure the cellular uptake levels of miR-29b. Briefly, seed the NSCLC cells in dishes and incubate them overnight. For instance, 5 × 105 cells/well and 60mm-dishes can be used. Then, incubate NPs with different miR-29b concentrations for 4 hours. Moreover, PBS-treated cells are suggested as negative control. Finally, measure the cellular uptake levels with a flow cytometer.

6.3. Materials for NP preparation

- Poly(D,L-lactide-co-glycolide) (PLGA)
- Chitosan
- Poloxamer 188
- hsa-miR-29b-2
- Pegylated phospholipid
- Cetuximab antibody (2259C)
- Acetone (2 ml)
- Polyamide filter 0.45μm
- Acetic acid 0.5% v/v.
- Potassium carbonate
7. **Budget and economic survey**

The economic parameters are relevant when carrying out a project or an investigation. Furthermore, the economic viability is essential when comparing with other possible project approaches. This chapter will include an estimation of the budget as well as the economic survey of the polymer-based NPs proposed in Par. 6.

7.1. **Budget estimation**

The budget estimation is divided into two parts, according to the expenses’ origin.

7.1.1. **Staff**

The following table collects the salaries involved in the project. It has been established an approximate price per hour.

<table>
<thead>
<tr>
<th>Staff description</th>
<th>Price/hour (€)</th>
<th>Working hours</th>
<th>Total price (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project’s directors</td>
<td>35</td>
<td>30</td>
<td>1050</td>
</tr>
<tr>
<td>Biomedical engineer</td>
<td>15</td>
<td>300</td>
<td>4500</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td></td>
<td></td>
<td><strong>5550€</strong></td>
</tr>
</tbody>
</table>

*Table 7.1 Staff associated costs*

7.1.2. **Products for NP preparation**

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
<th>Quantity</th>
<th>Price (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA</td>
<td>Sigma-Aldrich®</td>
<td>808482-5G</td>
<td>5g</td>
<td>280,00</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Sigma-Aldrich®</td>
<td>448869-50G</td>
<td>50g</td>
<td>94,50</td>
</tr>
<tr>
<td>Poloxamer 188</td>
<td>Sigma-Aldrich®</td>
<td>P2164009</td>
<td>50mg</td>
<td>125,00</td>
</tr>
<tr>
<td>hsa-miR-29b-2</td>
<td>Mission®</td>
<td>HMI0437-5NMOL</td>
<td></td>
<td>209,00</td>
</tr>
<tr>
<td>Pegylated lipid (PEG phospholipid)</td>
<td>Creativepegworks®</td>
<td>PLS-2044</td>
<td>1g</td>
<td>300,00</td>
</tr>
</tbody>
</table>
### 7.1.3. Equipment

The next gathers the costs of the materials used to develop the whole nanoparticle complex.

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
<th>Quantity</th>
<th>Price (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T 25 digital ultraturrax</td>
<td>IKA®</td>
<td>0003725000</td>
<td>1</td>
<td>1.749,00</td>
</tr>
<tr>
<td>Optima Max ultracentrifuge</td>
<td>Beckman®</td>
<td>393315</td>
<td>1</td>
<td>12.497,00</td>
</tr>
<tr>
<td>Transmission electron microscope</td>
<td>Hitachi®</td>
<td>*</td>
<td>1</td>
<td>100.000,00</td>
</tr>
<tr>
<td>Dynamic light scattering system (zetasizer)</td>
<td>Malvern®</td>
<td>*</td>
<td>1</td>
<td>30.000,00</td>
</tr>
<tr>
<td>MTT assay kit (cell proliferation)</td>
<td>Abcam®</td>
<td>ab211091</td>
<td>1 (1.000 tests)</td>
<td>285,00</td>
</tr>
</tbody>
</table>
7.2. Economic viability

As it can be observed in Par. 7.1.3, the majority of the costs are associated with the equipment. In particular, costs arising from assays’ instruments amount to 154.008,10€. This quantity is more than the 95% of the total project expenses.

Nevertheless, the majority of the equipment required for NP characterisation, except for TEM and RT-PCR, is available in our institution. Such previous acquirements would considerably rise the project investment. In addition, some alternative methodologies and instruments could be used- For instance, SEM could be used instead of TEM. On the other hand, fluorescence activated cell sorting (FACS) could be employed instead of flow cytometer, as its provided information is more accurate.

Be that as it may, the total cost of the project is relatively high, thus alternative economic approaches might be considered. For instance, characterisation assays could be undertaken by an external company or institution. Then, the expected direct costs would drop to 6764,5€, as only the expenses
of NP materials and staff would be considered. However, costs arising from assay services should be added to such amount. This alternative approach would probably lead to a substantial improvement in short-term costs, instrument acquisition would definitely worth in the long term.

Overall, due to the magnitude of the expected costs, such project would require external funding. For instance, this investment could be provided be Horizon 2020. Such programm, is a powerful financial instrument for those innovate projects that, like ours, wish to break down barriers between research and market placement. The outcomes of the miRNA-based therapeutics implementation in NSCLC patients would definitely outweigh its costs.
8. Conclusions

This last chapter includes a discussion on the objectives established in the first sections as well as some future recommendations concerning the use of miRNAs therapeutics in disease treatment.

Here, the main objectives of the thesis (Par. 1.1) are discussed in light of the thesis work:

1. **Origin and biogenesis of the miRNAs**: the historical key events that had more influence on miRNA history have been outlined. On the other hand, the process of miRNA formation, from the RNA transcription to miRNA translational repression or degradation, has been explained in detail. Furthermore, the dysregulation of miRNAs in diseases such as cancer has been discussed. The possibility to employ miRNAs in either therapeutics (miRNA mimics) or as targets of therapeutic agents (miRNA inhibitors) have been introduced.

2. **Medical applications of miRNAs**: the possibility to employ miRNAs in cancer treatment has been studied, providing numerous examples of both mimics and inhibitors-based therapies. On the other hand, the role of miRNAs as inflammation modulators has been demonstrated. However, the wide majority of such researches has been undertaken in animals. Moreover, it has been provided concluding evidence that miRNAs may also have a role in tissue regeneration. In particular, the multiple roles of miR-29 in tissue repair has been analysed. Apart from these applications, the potentiality of miRNAs in diagnosis biomarkers has been proved. The ability of some miRNAs, such as miR-21, to act as diagnostic biomarker in different cancer types has been discussed.

3. **MicroRNA delivery systems**: firstly, the two existing types of miRNA vectors (viral and non-viral) have been compared. Despite no ideal delivery system for the delivery of miRNAs exist, both advantages and disadvantages of each type have been presented. Whereas high transfection levels have been proved to be the main strength of viral vectors, low safety levels have been stated as their major drawback. On the other hand, lower transfection levels have been reported for non-viral systems. These delivery vectors have shown many advantages compared to viral ones: lower toxicity and immunogenicity levels, lower cost of production and better scalability. In particular, careful attention has been addressed to nanoparticles and nanospheres based on polymers, such as PLGA, chitosan and PEI. The nanoparticles have normally combined more than one of these polymers. PLGA/PEI and PLGA/chitosan-based nanoparticles are examples of such combination. Furthermore, other polymers such as PEG have been added to the surface of lipid and gold-based nanoparticles, leading to enhanced delivery. Thus, it has been found that combining different materials according to their strengths is the most convenient way to develop a nanoparticle. Thereby, the different techniques employed for nanoparticle preparation have been described. Emulsification-evaporation and nanoprecipitation have been stated as the main characterisation methods.
Likewise, the available methods that provide either physicochemical or biological information of nanoparticles have been presented. Furthermore, regarding nanoparticles’ safety and security, both European and American regulatory frameworks have been introduced. In addition, the procedures to obtain the FDA approval have been defined. Finally, it has been presented a specific application of miRNA-based therapeutics for cancer treatment.

4. **MicroRNA therapeutics perspectives**: The most relevant miRNA-based therapeutics approaches entering preclinical trials have been stated. Moreover, studies that have moved on to clinical trials have been also identified. Regarding future perspectives, improvement in delivery systems and proper miRNA selections have been stated as the major therapeutics challenges.

5. **Proposal of polymer-based NPs**: miRNA-based therapeutic for NSCLC treatment was proposed, relying on the literature information presented in previous chapters. Such materials, such as PLGA and chitosan, may be used for miR-29 encapsulation and delivery in target NSCLC cells. In order to increase the delivery levels, a NP surface functionalization has been suggested. In particular, the addition of ligands (e.g. antibody fragments) the NP surface has been advised. Moreover, it has been defined a brief protocol for NP preparation. Likewise, key methods to obtain both physicochemical and biological properties of NPs have been proposed.

6. **Budget and economic survey**: an evaluation of the budget and economic viability of the proposed polymer-based NP has been prepared. Thus, costs arising from staff, materials and inventory have been estimated. It has been found that the vast majority of the costs are associated with the characterisation assays.
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