UNIVERSIDADE D
COIMBRA

Alexandro Pereira Azevedo

THERANOSTIC NANOSYSTEMS COMBINING GENE THERAPY AND IMAGING AGENTS FOR CANCER TREATMENT

VOLUME 1

Dissertation of the Master's Degree in Biochemistry, with the orientation of Henrique Manuel dos Santos Faneca, PhD, and Carlos Frederico de Gusmão Campos Geraldes, PhD, and presented to the Department of Life Sciences of the Faculty of Sciences and Technology of the University of Coimbra.

October de 2020

Faculty of Sciences and Technology of the University of Coimbra

THERANOSTIC NANOSYSTEMS COMBINING GENE THERAPY AND IMAGING AGENTS FOR CANCER TREATMENT

Alexandro Pereira Azevedo

VOLUME 1

Dissertation of the Master's Degree in Biochemistry, with the orientation of Henrique Manuel dos Santos Faneca, PhD, and Carlos Frederico de Gusmão Campos Geraldes, PhD, and presented to the Department of Life Sciences of the Faculty of Sciences and Technology of the University of Coimbra.

October de 2020

1 2 9 0

UNIVERSIDADE D COIMBRA

Acknowledgements

Throughout one of the most difficult journeys of my life, I could not fail to thank those who in one way or another supported and contributed for the realization of this thesis.

With this in mind, I would like to thank my supervisors, Dr. Henrique Faneca and Dr. Carlos Geraldes, for all of the support, guidance and knowledge transmitted during the realization of this work.

I would like to thank all the colleagues of the research group, namely Dina Farinha, Rose Cordeiro, Daniela Santo, Flávia Rodrigues, and Rita Martins, for all the help and advice given.

To my group of friends, I would like to acknowledge the importance of their support as well as all the leisure moments experienced during this year.

To Flávio, Jalves and JP for their daily brotherhood and coexistence, in which each lunch became an endless gathering.

Last but not least, to thank my family and my girlfriend who without their support would not be able to finish this stage of my life.

Abstract

Cancer is one of the most prevalent and deadly diseases in the world, to which conventional treatment options, such as chemotherapy and radiotherapy, have been applied to overcome the disease or used in a palliative manner to enhance the life quality of the patient. However, there is an urgent need to develop new preventive and treatment strategies to overcome the limitations of the commonly used approaches. The field of cancer nanomedicine, and more recently the field of nanotheranostics, where imaging and therapeutic agents are combined in a single platform, provide new opportunities for the treatment and the diagnosis of cancer. This combination could bring us closer to a more personalized and cared-for therapy, in opposition to the conventional and standardized approaches. Gene therapy is a promising strategy for the treatment of cancer that requires a transport system to efficiently deliver the genetic material into the target cells. Hence, the main purpose of this work is to review recent findings and developments regarding theranostic nanosystems, with special focus on those that incorporate both gene therapy and imaging agents for cancer treatment.

Keywords: Gene Therapy; Theranostic nanosystems; Cancer; Nanoparticles; Medical Imaging

Resumo

O cancro é uma das doenças mais prevalentes e com maior taxa de mortalidade no mundo, cujas opções de tratamento convencionais, como a quimioterapia e radioterapia, têm sido utilizadas para tratar a doença ou usadas em cuidados paliativos para melhorar a qualidade de vida do paciente. Contudo, há uma necessidade urgente de desenvolver novas estratégias de prevenção e de tratamento para ultrapassar certas limitações provenientes dos tratamentos convencionais. A área da nanomedicina oncológica, e mais recentemente a nanoteranóstica, na qual agentes de imagem e terapêuticos são combinados numa só plataforma, proporcionam novas opções de tratamento e de diagnóstico para o cancro. Esta combinação poderá permitir uma terapia mais personalizada e cuidada, em comparação a outros tratamentos padronizados e convencionais. A terapia génica é uma estratégia promissora para o tratamento oncológico que requer um sistema de transporte que entregue eficientemente o material genético nas células alvo. Assim sendo, o objetivo principal deste trabalho é fazer uma revisão dos desenvolvimentos e descobertas recentes correspondentes aos nanosistemas teranósticos, com especial foco naqueles que incorporem simultaneamente agentes de terapia génica e imagem para o tratamento do cancro.

Palavras-chave: Terapia génica; Nanosistemas teranósticos; Cancro; Nanopartículas; Imagem médica

Table of contents

List of Figures

List of Tables

Abbreviations

AAV	Adeno-associated viruses
ACMF	Alternating current magnetic field
ADA-SCID	Severe combined immunodeficiency due to adenosine deaminase deficiency
ALG	Alginate
ATC	Anaplastic thyroid cancer
BBB	Blood brain barrier
BRAF	V-Raf murine sarcoma viral oncogene homolog B
CA	Cationic amylose
CD/5-FC	Cytosine Deaminase/ 5-Fluorocytosine System
CEUS	Contrast enhanced US
CHI	Chitosan
CPUA	Cationic poly(urethane amide)
CT	Computer tomography
CTL	Cytotoxic T lymphocytes
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DTT	Dithiothreitol
EGF	Endothelial growth factor
EGFR	EGF receptor
EMA	European Medicines Agency
EMF	External magnetic field
EPR	Enhance permeability and retention effect
FA	Folic acid
FDA	US Food and Drug Administration
FUS	Focused ultrasound
GBM	Glioblastoma
GCV	Ganciclovir
GDEPT	Gene-directed enzyme prodrug therapy
GFP	Green fluorescent protein
GMP	Good manufacturing practices
GSH	Glutathione
HA	Hyaluronic acid

HCC	Hepatocellular carcinoma
hMSCs	Human mesenchymal stem cells
HSV-TK	Herpes Simplex Virus-1 Thymidine Kinase
IC50	Half maximal inhibitory concentration
ICG	Indocyanine green
Ils	Interleukins
INFs	Interferons
IR	Infrared
LUV	Large unilamellar vesicles
mAB	Monoclonal antibodies
MBs	Microbubbles
MDR	Multidrug resistance
mRNA	Messenger RNA
miRNA	Micro RNA
MLV	Multilamellar vesicles
MRI	Magnetic resonance imaging
MSNs	Mesoporous silica nanoparticles
NBs	Nanobubbles
NIR	Near-infrared
NLC	Nanostructured lipid carrier
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
OCT	Optical coherence tomography
OI	Optical imaging
PAI	Photoacoustic imaging
PDA	Polydopamine
PD-L1	Programmed death ligand-1
PDT	Photodynamic therapy
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PET	Positron emission tomography
PFC	Perfluorocarbon
PI	Propidium iodide
PLGA	Poly (lactide co-glycolide)

PTT	Photothermal therapy
PTX	Paclitaxel
QDs	Quantum dots
RB	Retinoblastoma
RNA	Ribonucleic acid
RNAi	Interference RNA
ROS	Reactive oxygen species
RVG	Rabies virus glycoprotein
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SLN	Solid-lipid nanoparticles
SO	Singlet oxygen
SPECT	Single photon emission CT
SPIONs	Superparamagnetic iron oxide nanoparticles
SUV	Small lamellar vesicles
TMZ	Temozolomide
TRAIL	Tumour necrosis factor-related apoptosis inducing ligand
US	Ultrasound
USPIOs	Ultrasmall SPIOs nanoparticles
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein
ZIF-8	Zeolitic imidazolate framework-8

1. Challenges in cancer treatment

Cancer is a generic term for a large group of diseases that is characterized by the abnormal growth of malignant cells [1,2]. One of the major concerns related to these cells is their ability to invade other parts of the human body such as vital organs. This process is referred to as metastization and is a concerning factor related to cancer because it generally leads to complications associated with the treatment and therefore severely influences the survival rate of the patients [2,3]. Cancer being a leading cause of death worldwide has become more frequent and more deadly mainly because of the population growth and aging. This human development has led to an increase in risk factors that are related to cancer, such as tobacco and alcohol use, air pollution, obesity and infectious agents [4–7].

In 2018 it was estimated that there would be approximately 18 million new cancer cases from which 9.6 million would perish (Figure 1). It is noteworthy to say that its incidence and mortality varies from region to region, meaning that some are more affected than others. For the same year and for both sexes combined, it was estimated that in Asia there would be almost half of the total cases and more than half of the total cancer deaths, therefore being one of the most affected regions. The European continent would account for almost a quarter (23,4%) of the total cases and for 20% of the cancer deaths followed by the American continent with 21% and 14,4% of the total cases and deaths worldwide, respectively. Among the various regions of the world, Asia and Africa both have higher mortality rates (57.3% and 7.3%) compared to their incidence rates (48.4% and 5.8%, respectively) [4]. These huge numbers demonstrate why cancer is a major worldwide health problem.

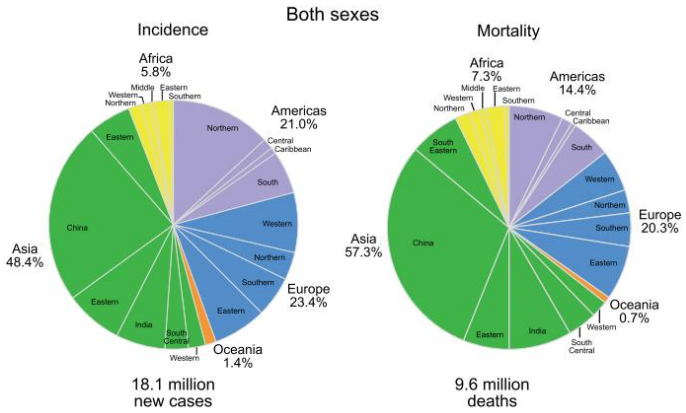

Figure 1-Representative charts of the distribution of the number of cases and deaths worldwide, for both sexes in 2018, adapted from ref 4.

The main cancer treatment strategies include surgery, radiotherapy, and chemotherapy, or a combination of these previous methods [2,7]. All of these strategies are used to hopefully cure the patient, to control the disease progression in the hope of prolonging the patient's life, or to ensure the relief of symptoms and increase his life quality.

Surgery is with no doubt the most traditional and therefore the most used strategy among the conventional approaches. The surgical procedures are intended to physically remove the primary or secondary metastatic tumours in the hope of diminishing the physical constraints caused by them. As with other areas, technological advances have improved postoperative morbidity and mortality with various new methods being developed such as robotic and laparoscopic procedures. Although surgical interventions are widely used to manage cancer it has been shown that these interventions could prompt disease recurrence [8,9].

Radiation therapy or radiotherapy is another treatment strategy that is used to manage cancer, where its main objective is to affect the DNA of cancerous cells with the use of high doses of radiation. This high energy will damage the DNA of the cells and therefore affect their ability to proliferate and potentially result in their death. Unfortunately, healthy cells can also be affected by this therapeutic strategy and therefore lead to some side-effects [10,11].

Chemotherapy, on the other hand, was originally intended to treat microbial infections and is defined as the use of chemical compounds to treat a disease [12].

Chemotherapy drugs primarily act on rapid-growing cancer cells, mainly by interfering with DNA synthesis, replication, transcription and cell division, with various types and mechanisms of action [1,7]. Depending on the way these drugs act, they can be attributed to different groups such as alkylating agents, anti-tumour antibiotics, antimetabolites, topoisomerase inhibitors, metal-based agents and others. Most of these drugs cannot target cancer cells, meaning that high doses are required for the drug to reach them, affecting healthy cells and consequently leading to toxicity (hepatotoxicity, nephrotoxicity, cardiotoxicity, and neurotoxicity)[7]. Multidrug resistance (MDR) is another limitation in chemotherapy since some cancer cells develop or have inherent molecular mechanisms that are able to overcome the potential effects of these drugs. Some of these mechanisms include the overexpression of efflux pumps, that will expel the therapeutic drug, reducing drug uptake, or enhancing drug metabolism and DNA repair mechanisms. Therefore MDR is critical for the survival rate of the patients and could influence their quality of life [1,7,11,13].

1.1 Cancer nanomedicine

In these past decades and ever since the magic bullet concept was envisioned by Paul Ehrlich, we have seen the development of nanocarriers, some authors considering this concept to be one of the major propellers for the growing interest in this area [14]. These were meant for the controlled delivery of therapeutic agents and were also known as nanoparticle-based drug delivery systems or nanotherapeutics. The use of these nanomaterials to deliver these agents was aimed at overcoming the limitations inherent to some of the conventional treatment strategies previously mentioned and therefore improving their overall efficacy while diminishing their side-effects [1,15,16].

Cancer nanomedicine is one of the major areas of nanotechnology, that is characterized by the use of nanoparticles or nanomaterials for cancer-related biomedical applications such as disease treatment, diagnostic and molecular imaging. These nanoparticles are typically characterized by having dimensions in the nanometers range. It is also noteworthy to say that cancer nanomedicine is a multidisciplinary area

comprising knowledge from various disciplines/fields such as chemistry, physics biotechnology, and life sciences [16–20].

Briefly, there are several advantages regarding the use of nanosystems for the treatment of cancer, such as the ability to incorporate several components into the nanosystems due to their superior size compared to conventional therapeutic agents. These components would contribute to an overall increase in the efficacy and safety of the treatment with the introduction of several properties into the nanosystem, such as the ability for a controlled release of the drug through external or internal stimuli, or the capacity to specifically deliver into target cells more than one therapeutic agent leading to a combined and possibly synergistic effect. These features would only add up to the underlying benefits of using nanosystems for the delivery of therapeutic agents, since they can increase the solubility of poorly soluble molecules and reduce their degradation by enzymes for example, thereby improving their bioavailability [18,19,21]. On the other hand, these nanocarriers can take advantage of the tumour properties accumulating in the cancer tissue either in a passive way, owing to the enhanced permeability and retention effect (EPR), or in an active way, by differentiating between pathological and non-pathological tissues due to the unique features of the tumour, such as overexpressed receptors[19]. Finally, these nanosystems have the potential to overcome drug resistance problems since they are not easily recognized by acquired or inherent defence mechanisms [11,15,19,20].

2. Nanoparticles

2.1 Types of nanoparticles

Nanosystems, either for therapeutic or diagnostic purposes, can be developed for oral, topical, local, and systemic administration, with various formulations approved or in clinical trials. Amongst these, the systemic administration has been the most studied one either in pre-clinical or clinical circumstances [22]. In a general overview, these nanocarriers can be divided into two main categories according to their chemical composition: in organic and inorganic nanoformulations [22–25]. However, due to the

versatility and flexibility of the nanocarriers, regarding the various possible functionalizations and combinations, many of these could be included in both categories.

Organic nanoparticles comprise a wide range of nanosystems, such as lipid- or polymer-based nanoparticles, prepared with natural or synthetic compounds [21,23]. These are by far the most studied nanoplatforms for the delivery of therapeutic compounds for cancer treatment. Lipid-based nanosystems include different types of nanoformulations, such as liposomes, solid-lipid nanoparticles (SLN), and nanostructured lipid carriers (NLC). The clinical applications of liposomes are well known and their ability to deliver hydrophobic, hydrophilic, or both therapeutic molecules is one of the reasons why researchers continue to develop new formulations[26]. They are spherical structures formed by one or several lipid bilayers with an aqueous phase inside them. Different types can be prepared, such as multilamellar vesicles (MLV), large unilamellar vesicles (LUV), or small lamellar vesicles (SUV), that essentially vary in size and the number of lipid bilayers. Liposomes are versatile nanosystems, which properties are dependent on the lipids used to prepare them and the method of preparation [26,27]. Likewise, polymer-based nanoparticles have also been employed to improve the overall efficacy of therapeutic molecules either by encapsulating, complexing or adsorbing them. When designing these nanocarriers, several natural, such as chitosan or hyaluronic acid (HA), or synthetic polymers, such as poly (lactide co-glycolide) (PLGA), poly(l-lysine), polyethylenimine (PEI), or polyethylene glycol (PEG), could be selected, depending on the desired properties for the nanosystem. It is worth mentioning that these polymers can be further subdivided into biodegradable or nonbiodegradable polymers. The former have linkages that can be naturally degraded by biological processes within the body either by enzyme or chemical degradation, and therefore used to prevent accumulation and consequently diminish toxicity [24,28,29].

Inorganic nanoparticles include a variety of nanomaterials, such as quantum dots (QDs), silica bases-nanomaterials, magnetic and gold nanoparticles [23,25]. These nanoparticles benefit from the same advantages as organic nanoparticles when it comes to enhancing drug protection and circulation. However, depending on the physico-chemical characteristics of the nanoparticle, they can offer stimuli-responsive properties

or enable tumour imaging. For example, QDs, being semiconducting nanocarriers with a core-shell structure, have been used as optical imaging probes due to their appealing fluorescent properties [1,30]. On the other hand, superparamagnetic iron oxide nanoparticles (SPIONs) and ultrasmall SPIOs nanoparticles (USPIONs) were the first contrast agents used for magnetic resonance imaging (MRI) [22,31].

2.2 Physicochemical properties

In the design and development of nanoparticles for the delivery of therapeutic agents, there are several parameters that will influence its pharmacokinetics and pharmacodynamics and therefore it is noteworthy to understand how these physicochemical properties affect the efficiency of the treatment, from its administration route to its clearance and degradation (Figure 2) [1,16,19,21,32]. The most common administration route, as mentioned earlier, is the systemic delivery, and therefore the physicochemical properties will be discussed having this administration route in mind [20].

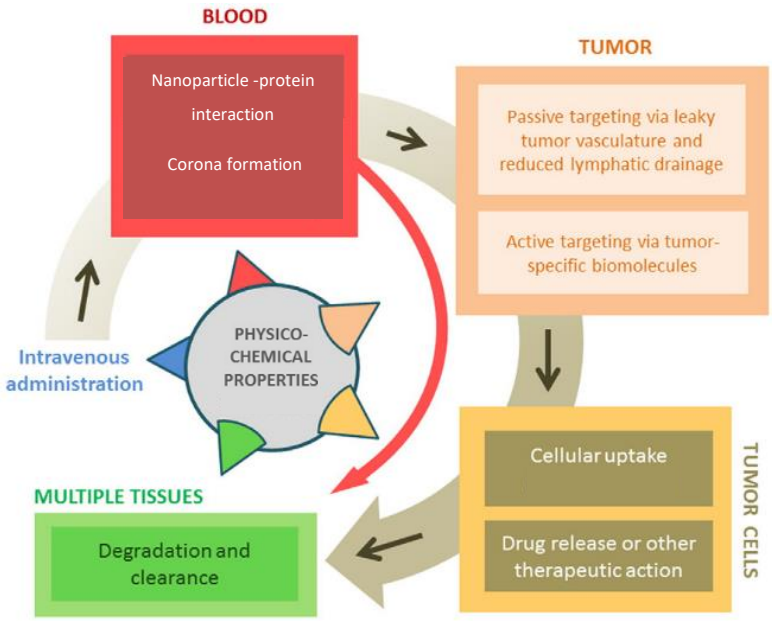

Figure 2- Schematic illustration of an intravenously administrated nanomedicine, highlighting the importance of its physicochemical properties and how they will influence the transport, distribution, efficacy, and toxicity of the nanomedicine in the patient, adapted from ref 16.

Size is one of the most important factors related with the development of this type of nanosystems since it will influence their distribution, accumulation in tumour cells, and clearance. Nanoparticles with sizes between 5 nm and 250 nm, are preferred since they can flow through the bloodstream more easily than larger nanoparticles. They also tend to accumulate more in cancer tissue since they can fluently pass through the leaky blood vessels. Nevertheless, the smaller sizes can also become a weakness since nanoparticles can outflow into healthy tissues and therefore affect and potentially cause unwanted side effects. On the other hand, nanoparticles with sizes above 250 nm do not extravasate as much as small nanoparticles into healthy cells but they also have difficulties in accumulating into the tumour tissue since they cannot pass through the leaky blood vessels [16,33,34]. After intravenous administration, nanoparticles with sizes bellow 10 nm can be rapidly cleared by the kidneys whereas larger nanoparticles tend to be cleared through the hepatobiliary route, and those that have sizes above or around 1000 nm tend to be retained in the lungs [16,35]. Optimization strategies that improve nanoparticles size are therefore crucial for the development of efficient nanosystems

The shape of the nanoparticles, as well as their size, will be decisive for the nanosystem to be successful in the delivery of the therapeutic cargo. There are several shapes of nanoparticles such as rod, sphere, cube, needle-like, and others. Amongst these, spherical nanoparticles are the most common ones, mainly because they have easier synthesis processes[35]. Additionally, the characterization methods employed to sphere-like nanoparticles are less challenging when compared to non-spherical nanoparticles. So far it is still unclear which shape has the best therapeutic outcomes since some of these improve circulation through the bloodstream, such as the cylindrical ones, while others tend to improve cellular uptake, such as the rod-like ones [20,33,35,36].

Alongside the size and the shape of the nanosystems, their surface charge will also affect their distribution and circulation through the bloodstream. Commonly, positively charged nanoparticles tend to be internalized more easily than negatively or neutrally charged nanoparticles[33]. This is due to the electrostatic interactions occurring between the cationic nanoparticles and the negatively charged surface of the cell membrane. However, nanoparticles that have a positive surface charge tend to be

rapidly cleared by macrophages while at the same time being toxic towards healthy cells [1,32,33,37].

The formation of a protein corona is a phenomenon that should be taken into account since it will influence the properties mentioned above and ultimately impact the therapeutic outcome of the nanocarrier[20]. This occurs when nanosystems, after systemic administration, come into contact with complex biological fluids, resulting in the interaction between the nanocarrier and multiple proteins present in these fluids. Therefore, the surface of the nanoparticle will become adsorbed with proteins forming the protein corona. This interaction will be dependent on several factors, some related with the nanocarrier, such as its size, shape and surface charge, and some related with the physiological fluid, such as its composition, exposure time and temperature [1,15,20]. For example, a recent study showed that there was a significant increase in protein adsorption on rod-like mesoporous nanoparticles when compared to sphere-like ones [38]. Additionally, Partikel *et al* tried to understand how nanoparticles sizes would affect protein absorption. It was demonstrated that in PLGA nanoparticles, with sizes between 100 nm and 200 nm, protein absorption wasn't dependent on their size, however they also demonstrated that PEGylation diminished the amount of bound proteins [39].

Although this phenomenon is not fully understood, all of the above properties are crucial and must be considered when designing nanotherapeutics that hope to achieve clinical applications.

2.3 Targeting mechanisms

In general, nanosystems, in order to have an effective therapeutic outcome, primarily need to reach the tumour tissue. For this reason, they need to have certain features that will allow them to have good circulation times while protecting the therapeutic agent, slow clearance times, and a way to reach and accumulate in the cancerous cells[15]. With this in mind, researchers need to take into account the tumour microenvironment characteristics and potentially use them to enhance unique physicochemical properties of the nanosystems to improve their efficacy. Overall, these

nanosystems have two types of targeting mechanisms, the passive targeting and active targeting (Figure 3) [1,15,19].

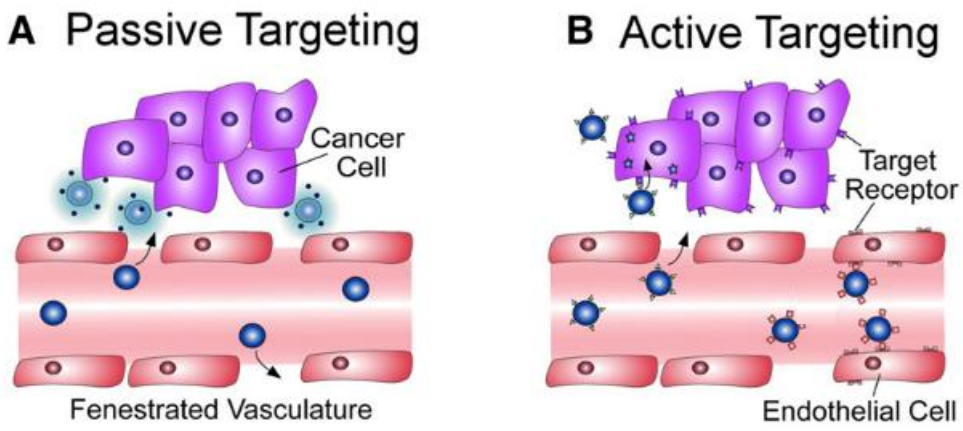

Figure 3- Mechanisms for nanosystems-mediated tumour targeting. (A) Passive targeting of the nanosystems occurs through the fenestrated vasculature surrounding the tumour tissue. (B) Active targeting of the nanosystems occurs trough the interaction of specific ligands, present in the surface of the nanoparticles, with overexpressed target receptors of the tumour cells, adapted from ref 21.

The passive targeting takes advantage of a phenomenon first discovered by Maeda and his colleagues, commonly known as the enhanced permeability and retention effect (EPR) [40–42]. This effect typifies what happens in solid tumours that are rapidly proliferating. The ever-growing tumour tissue requires essential nutrients and oxygen to supplement this abnormal growth. Therefore, a variety of growth factors such as vascular endothelial growth factor (VEGF) will be released by the tumour in order to stimulate the formation of new blood vessels, a process commonly known as angiogenesis[21,43]. However, these new supply vessels tend to be slightly different from the typical blood vessels, one of the main anatomical differences being the large fenestrae in between disorganized endothelium cells, creating leaky and irregularly shaped vessels. On the other hand, the lymphatic system in tumour tissues tends to be deficient, meaning that nanoparticles that enter through the leaky blood vessels remain retained and therefore accumulate more in cancerous tissues [1,16,21,43,44].

The active targeting approach is achieved with the surface functionalization of the nanoparticles with specific ligands that can recognize typically overexpressed receptors present in tumour cells[43]. This approach aims to minimize one of the major

limitations inherent to passive targeting, the inability to distinguish between tumoral and non-tumoral cells. For that reason, targeted nanomedicines tend to diminish unwanted side-effects by reducing non-specific interactions with healthy cells. A variety of ligands are being used to achieve this purpose, namely monoclonal antibodies, proteins, peptides, aptamers, and small molecules such as folic acid (FA). Selecting the appropriate ligand for a particular tumour could be a challenging process, nonetheless once selected it will make the overall properties of the nanosystem more suitable and consequently enhance its efficacy [16,21,43,45].

2.4 Stimulus-responsive nanosystems

The design and development of nanosystems that take advantage of active and passive targeting has allowed them to overcome existing barriers that therapeutic agents on their own would have difficulties to surpass. However, the relentless search for new approaches and strategies has led to the development of stimulus-responsive nanosystems. These differ from other nanotherapeutics in that they release the therapeutic agent in response to a certain stimulus. Generally, this trigger will lead to a conformational alteration of the nanocarrier in order to facilitate the release, for example, of a certain drug as close as possible to the tumour tissue while avoiding being released near healthy tissues and therefore reducing possible toxicity effects. These

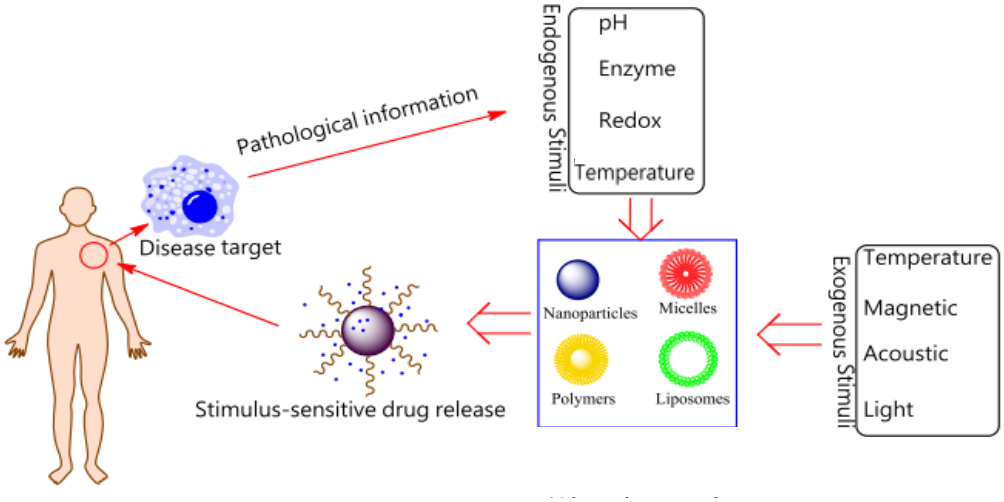

Figure 4- Schematic illustration of the endogenous and exogenous stimulus-responsive nanosystems, adapted from ref 49.

triggers or stimuli can be divided into two main types, the endogenous or exogenous stimuli (Figure 4) [15,18,46,47].

The endogenous or internal stimulus approach takes advantage of certain tumour characteristics that differ from healthy tissues, such as variations in pH value, overexpressed enzymes, redox activity, and temperature variations, to serve as release triggers for various nanocarriers [47–49]. Therefore, pathological information like those mentioned above will be crucial when designing these internal stimulus-mediated release nanosystems. Among the above stimuli, pH gradients are the most used when designing stimulus-responsive nanosystems[48]. It is well recognized that the extracellular pH in solid tumours is usually more acidic than that for normal tissues, normally bellow 7.0, due to the rapidly proliferating and hypoxic tumour microenvironment [50]. In addition to this difference, intracellular compartments such as lysosomes and endosomes also have lower pH values, around 5.0, when compared to the cytoplasm. Therefore, pH-sensitive nanocarriers under physiological values (pH=7.4) protect the therapeutic agent but they release it in acidic environments, assuring a much larger accumulation in the tumour tissue [1,21,48,49].

Typically, in pathological tissues, some molecules tend to have different expression patterns when compared to healthy tissues. Overexpressed enzymes found in tumour tissues, such as proteases and phospholipases, have been used as an internal stimulus. Using these enzymes as triggers offers some advantages, the most obvious of which is the location of the overexpressed enzyme in the diseased tissue. On the other hand, the catalytic reaction tends to be highly selective and specific due to the interaction between the enzyme and its substrate [1,51]. In the same way that previous nanosystems take advantage of differences between tumoral and healthy tissues, redox-responsive nanosystems take advantage of high levels of reductive agents found in cancerous cells. Glutathione (GSH) intracellular levels are significantly higher than in extracellular fluids, meaning that they can be used as a specific trigger for nanosystems that contain a redox -responsive moiety. The most common linker in redox-responsive nanosystems is the disulfide bond, where the linkage could be applied between a therapeutic agent and the nanoparticle. The cleavage of the linker will lead to the release of the therapeutic agent, that was previously inactive [48,52,53].

In the exogenous or external stimulus approach, the release of the therapeutic agent is triggered by the use of physical external factors such as light, ultrasound, magnetic fields, and induced temperature variations [1,52,54–56]. The use of external stimulus, in cases where there is limited knowledge about the tumour or when the internal triggers are reduced, has proven to be more reliable, mainly because the release trigger mechanism is ensured by specialized machines, which on the other hand, may be seen as a limitation or a constrain when designing these nanosystems due to their overall price [54]. Light-responsive nanosystems usually incorporate specific moieties that release the therapeutic cargo through different mechanisms such as photo-isomerization or photocleavage, amongst others[55]. Using light as an external trigger has proven to be beneficial for stimuli-release nanosystems since it is easy to manipulate, biocompatible, and offers great spatial and temporal control. However, the major limitation when using light as a trigger is the limited tissue penetration, inherited to this approach [52,54,55]. Using ultrasound waves as a trigger to release therapeutic agents has been highly investigated because of the proven benefits underlying clinical ultrasound for medical imaging, such as the use of non-ionizing radiation, safety, affordability, and significant tissue penetration[55]. Initially, microbubbles (MBs) loaded with a therapeutic agent were developed, but their overall efficacy was limited due to their relatively large size, restricting their ability to circulate through the bloodstream. Therefore nanometer-sized bubbles or nanobubbles (NBs) were developed to overcome these limitation [1,55,57].

Variations in temperature can be considered as an endogenous or exogenous stimulus. They are internal triggers because it has been documented that pathological tissues tend to have slightly higher temperature values when compared with healthy tissues. On the other hand, it could be considered as an external trigger when the tumour microenvironment is heated with the use of external factors, as it happens in photothermal therapy or magnetic hyperthermia. Overall, the goal of these thermosensitive nanosystems is to transport and contain the therapeutic cargo, under physiological temperature, and to be sensitive enough to release the therapeutic agent in the tumour tissue, where the temperature is increased [48,49,55,56].

2.5 Limitations and safety concerns regarding nanoparticles

Clinical translation of promising nanotherapeutics has proven to be one of the major hurdles that researchers and pharmaceuticals face. So far, very few successful nanocarriers have been approved for human clinical applications in comparison with the numerous research papers regarding nanomaterials[58]. One of the main drawbacks regarding the translation of these nanosystems is the economic/financial aspect that is crucial to address all of the regulatory fundamentals required while providing a safe, cost-efficient, and quality product[58]. On the other hand, precise physicochemical characterization of these nanosystems is essential to understand and predict possible toxicities while evaluating their consistency and reproducibility when they are being scaled-up for clinical applications. Often, and due to the batch-to-batch variations regarding the physicochemical properties, optimization or alternative strategies are employed to improve product quality and consistency while providing good manufacturing practices (GMP). Accumulation and toxicity of these nanosystems are some of the safety concerns that need to be assessed, before large scale-up synthesis processes, in order to understand the safety of the product. To do so, various *in vitro* and *in vivo* screening approaches need to be employed [17,21,22,58].

3. Gene Therapy

Gene therapy is an auspicious therapeutic modality involving the introduction of genetic material (DNA/RNA) into target cells in order to prevent or treat different types of diseases involving genetic factors such as cancer, monogenic diseases, viral infections, neurological and cardiovascular diseases [59,60]. The number of clinical trials using gene therapy has steadily increased in recent years demonstrating the interest of the scientific community for this therapeutic modality. Most of the gene therapy clinical trials have been directed to cancer (67%), which is justified by the high worldwide incidence of this pathology and by the lack of convenient therapeutic strategies that have the ability to eliminate this disease. Globally, the largest number of clinical trials

are taking place in the American continent, mainly in the USA with 65%, followed by Europe with 23.2% and Asia with 6,5% [59].

To date, and several years after the first gene therapy study was conducted in humans, significant progress has been made, with the approval of many gene therapy products. For example, the following gene therapies received approval either from the US Food and Drug Administration (FDA) or from European Medicines Agency (EMA). Glybera® was approved in 2012 by the EMA and is used to treat adults that have a rare condition of lipoprotein lipase deficiency. IMLYGIC® was approved by the FDA in 2015 and is used in patients with melanoma. Strimvelis® was approved by the EMA in 2016 and is used to treat patients with severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID). In 2017, three gene therapies were approved by the FDA, two of which, KYMRIAHR® and YESCARTAR®, are CD19-directed chimeric antigen receptor CAR T cell immunotherapies indicated to treat B-cell lymphoma, while the latter is also indicated for acute lymphoblastic leukaemia. Also, in 2017, LUXTURNAR® and in 2018, Onpattro® (patisiran), are the latest approved gene therapy products. The former is used for the treatment of patients with retinal dystrophy (biallelic RPE65 mutation) and the latter, a siRNA-based product, to treat polyneuropathy in individuals with hereditary transthyretin-mediated amyloidosis [59,61,62].

3.1 Gene Therapy approaches for cancer

Different gene therapy strategies have been used against cancer and can be subdivided, according to their overall mechanism of action, namely immunogenetherapy, oncolytic virotherapy, specific inhibition of gene expression, and gene transfer technology [59,63–66].

Immunogenetherapy aims to stimulate the immune system so that it can recognize and eliminate cancer cells, thereby making the body's natural defences more effective against cancer. There are several classes of immunogenetherapy such as cytokines, engineered T cells (CAR T cells), and DNA cancer vaccines [67–69]. Normally, cytokines, such as interferons (INFs) and interleukins (ILs), stimulate the immune system

in a more direct approach. Cancer vaccines, for example, are used to treat and not to prevent the disease because they stimulate the immune system to act upon cancerous cells. This can be accomplished, for instance, by using a vaccine containing tumour cells capable of expressing immunostimulatory molecules, such as antigens that will activate T-cells to attack the cancer cells [67,68].

Oncolytic virotherapy takes advantage of the ability to manipulate certain viruses so that they can recognize and infect cancerogenic cells. Once these are infected by the viruses, they will propagate and express certain proteins that will induce death mechanisms and result in cell lysis [70]. The introduction of certain immunostimulatory genes will lead to an enhanced immune response, meaning that their therapeutic efficacy relies on the combination of their selective cell killing and immunostimulatory ability. In 2015, one of the breakthroughs in oncolytic virotherapy was accomplished with the approval of IMLYGIC®, the first oncolytic virus therapy [70].

Specific inhibition of gene expression can be achieved using different molecules such as antisense oligonucleotides, ribozymes, and interference RNA (RNAi). Antisense oligonucleotides have different mechanisms of action and may inhibit transcription, with the formation of a triplex directly with the DNA, or translation, through a process of physical blockage or with the activation of RNase H. Ribozymes, on the other hand, act by binding to the target mRNA and cleaving it [71]. The most recent inhibition strategy is accomplished with the use of interference RNA. There are different types of RNAi, such as short interfering RNA (siRNA) and micro RNA (miRNA), and their mechanism of action will be dependent on the complementarity between the interference RNA and the target mRNA[71]. For example, miRNA, being small non-coding molecules, are key regulators in the development and progression of many cancers, with some miRNAs upregulated or downregulated. As it was stated before, the degree of complementarity between the miRNA and the target mRNA will be a key factor in their mechanism of action. They can either bind with a very high degree of complementarity to target mRNA sites, and therefore promote the cleavage of the transcribed mRNA, or inhibit the translation process when the degree of complementarity is lower [71–73].

Gene transfer technology has a diverse set of therapeutic options, from the inclusion of “suicide genes”, antiangiogenetic genes, or the inclusion of genes, that when

expressed, produce beneficial proteins. This technology is characterized by the introduction of a therapeutic gene into the cancer cell and/or other target cells. The use of "suicide genes" is also referred to as gene-directed enzyme prodrug therapy or GDEPT. This therapy is characterized by the expression of an enzyme that could metabolize an inactive prodrug into a cytotoxic derivative, inducing cellular death (Figure 5). The best-described suicide gene systems are the Herpes Simplex Virus Thymidine Kinase/Ganciclovir System, and Cytosine Deaminase /5-Fluorocytosine System (CD/5-FC). Herpes Simplex Virus-1 Thymidine Kinase (HSV-TK) has a strong affinity for the pro-drug ganciclovir (GCV), phosphorylating it to its monophosphate form. This form of the drug will be converted to its di- and triphosphate derivatives by cellular kinases. After this, DNA polymerase will use GCV triphosphate during DNA replication because it is a nucleoside analogue, and this will ultimately lead to the cell's death by polymerase inhibition and apoptosis induction. During the suicide gene therapy process some of the cancerous cells will not receive the desired gene and therefore are not able to convert the prodrug to its cytotoxic form. Nevertheless, this difficulty is overcome by the bystander effect, where the cytotoxic drug produced in a transfected cell is able to enter the neighbour/adjacent cells and enhance its overall cytotoxic effect [59,74,75].

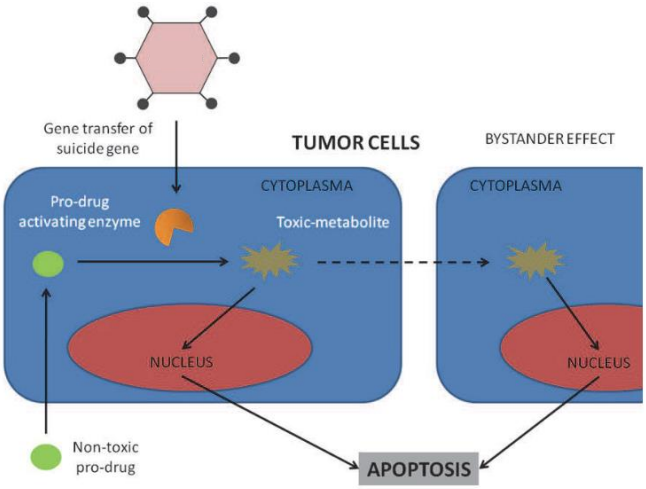

Figure 5- Schematic illustration of the mechanism of action of the gene therapy strategy, adapted from ref 65.

3.2 Delivery of the genetic material

A crucial aspect concerning gene transfer and its potential therapeutic application is the efficient delivery of the genetic material into the target cells. The introduction of "naked DNA" is possible but its transfection efficiency is hindered by its vulnerability to degradation by nucleases, the lack of cell specificity, and its difficulty to enter the cell due to the weak interaction with the cell membrane, attributed to the negative charge of the molecules. Hence the development of delivery systems, commonly known as vectors, is essential to enhance their therapeutic potential [60,76].

The two main delivery types of systems are the viral and non-viral vectors. Between these two, the viral vectors remain the most studied in clinical trials. Their high popularity in gene therapy is attributed to their high transduction efficiencies. Nonetheless, they have several drawbacks such as immunogenicity, limited DNA packaging capacity, challenging vector modification and/or production, and the possible activation of oncogenes[77,78]. Due to these side-effects, non-viral strategies have been increasingly developed, mainly because they have better safety profiles when compared to viral vectors. In addition to their safety, they are also easier to synthesize and are capable of loading more genetic material [59,75,77–80].

Non-viral vectors can be subdivided into physical and chemical methods. Physical methods exert a type of physical force that creates a more permeable cell membrane that will aid in the intracellular delivery of the genetic material. Direct needle injection, hydrodynamic delivery, gene gun and electroporation are some of the physical methods used [74,76,81].

The chemical methods, that are the ones selected in the scope of this work, use synthetic or natural compounds as vectors to deliver the desired genetic material into the target cells. They can be subdivided into lipid-, polymer- and inorganic-based vectors that usually exploit the physicochemical characteristics of the genetic material and of the vector that will carry it to the target cells to produce a non-viral delivery system. They generally take advantage of the negative charges of the genetic material to complex it with the positive charges of the vectors through electrostatic interactions [28,78]. The cationic polymers, cationic liposomes, and the combination of the two with the

negatively charged genetic material form the polyplexes, lipoplexes and lipopolyplexes, respectively. These are the most common chemical non-viral gene delivery systems [82,83]. The goal of these methods is to develop a vector with good biocompatibility, pharmacokinetics and biodistribution that is capable of recognizing the target cells and efficiently releasing the genetic material inside them. Correct translocation to the nucleus is necessary for gene expression to occur and condensation of the genetic material is essential to protect it against nuclease action. Due to their characteristics, chemical methods have become a viable alternative to viral methods, and enabled the development of a new class of nanosystems for theranostic applications, allowing the combination of gene therapy and diagnosis/imaging properties [76,81].

3.3 Biological barriers for gene therapy

The delivery of genetic material through non-viral vectors such as lipoplexes, polyplexes, lipopolyplexes or other non-viral chemical vectors, can be in some cases conditioned by the lack of cell specificity, interaction with serum proteins, cytotoxicity, and consequently its inefficient transfection[76,83]. In addition to these limitations, the entire process from their synthesis to the cell entry and further gene expression includes several barriers that the vectors have to overcome in order to be successful (Figure 6). As mentioned before, complexation or encapsulation of the genetic material and protection from nuclease degradation are essential steps to develop a successful delivery system. Nonetheless, blood circulation and selective accumulation at the tissue of interest, cellular internalization through the endocytic pathways, such as phagocytosis, macropinocytosis, clathrin-mediated, and caveolae endocytosis, and endosomal escape will also affect the overall success of the delivery system[83]. One of the most common modifications used to prolong circulation and reduce interactions with serum proteins is the conjugation with polyethylene glycol (PEG). On the other hand, conjugation with PEG could reduce the transfection efficiency because it shields the positive charges of the system and prevents the interaction with the cell membrane [76,82]. Considering the original non-specific nature of these nanosystems, the inclusion of certain targeting ligands, such as glycoproteins, proteins, peptides or antibodies, yields

them specificity to the target cells, improving their transfection efficiency and diminishing the undesired side effects [82]. This limitation is usually overcome through the use of targeting ligands. It was also shown that the noncovalent functionalization of lipoplexes with folic acid through electrostatic interactions resulted in a significant enhancement in DNA protection and biological activity [84]. In another study, hyaluronic acid (HA) modified lipoplexes loaded with siRNAs presented enhanced cellular uptake and were able to efficiently silence gene expression in an animal model [85]. Therefore, after the cellular internalization of the complexes, the endosomal escape becomes a crucial issue in order to avoid the degradation of the genetic material in the lysosomes [86,87]. So far, various nanosystems have been developed in order to overcome this barrier, either through the proton-sponge effect, the use of fusogenic compounds, or by using nanoparticles that swell in acidic environments[86,87].

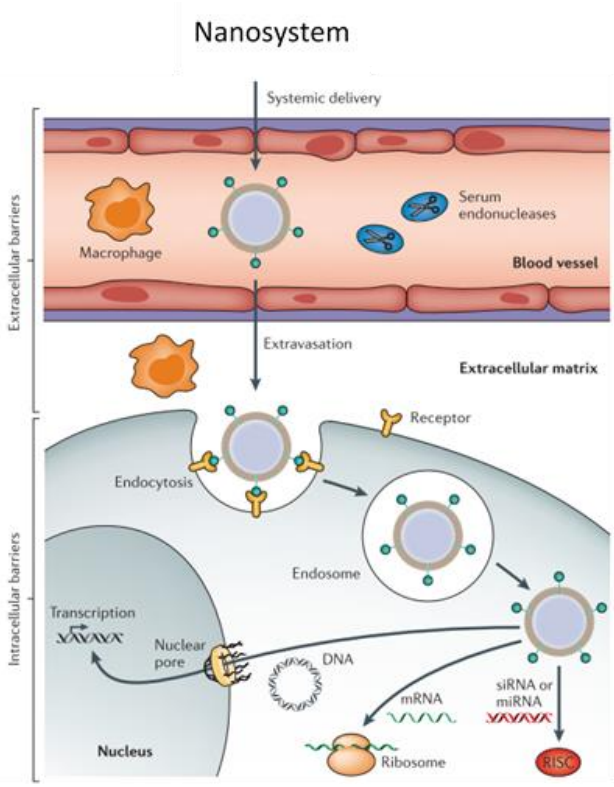

Figure 6- Biological obstacles for non-viral vectors-based gene delivery, adapted from ref 77.

When the genetic material that is being delivered to the cell is DNA-based and not RNA-based, there are extra barriers to overcome, the entry into the nucleus with the DNA release in order to be transcribed [76,77,83,88]. In this regard, transport through the

nuclear membrane can be accomplished by the nonspecific association of the DNA with proteins or peptides that contain the nuclear localization signal (NLS), thus promoting the entry of the genetic material. On the other hand, in actively dividing cells, the DNA benefits from the temporary rupture of the nuclear membrane to enter the nucleus [89]. The development of new formulations that will be able to overcome these drawbacks has drawn the attention of many researchers.

4. Nanoparticles as imaging and theranostic agents

The field of nanomedicine has contributed to new advances regarding the administration of therapeutic agents, with many formulations approved or in clinical trials, with one of the most well-known and emblematic approved nanotherapeutics being Doxil®, a PEGylated liposomal formulation encapsulating the chemotherapeutic drug doxorubicin (DOX) [90–92]. Although nanotechnology has contributed to enhance and diversify treatment options for cancer, one of the crucial and increasingly more important aspects in order to overcome this disease is its early diagnosis and monitorization. Currently, the use of diagnostic methods for cancer is considered a routine approach in order to perform a reliable diagnostic and for the careful management of the disease progression. For most cancer types early diagnosis and detection have improved patient's survival rates, allowing the treatment administration in an initial stage of the disease. In later stages, tumour metastasis lead to the establishment of secondary metastatic tumours where these options are used in a more palliative manner [93–95]. The disease can be evaluated in an indirect way with the use for example, of blood tests to determine abnormal physiological values that may indicate or imply that there is a disease, or with more direct visualization methods either *in vivo* with the use of imaging modalities or *ex vivo* using biopsies [96]. All of these approaches tend to complement each other to provide as much information as possible about the disease. Nonetheless, direct *in vivo* visualization through imaging modalities has proven to be beneficial as a complementary diagnostic tool [97].

4.1 Imaging modalities

The field of clinical diagnosis has been extremely important in the fight against cancer. Being essential in detecting and characterizing the disease as well as assessing whether the tumour is regressing, rapidly growing, and possibly metastasizing or recurring when it had been thought to be successfully treated. In an ideal scenario, these methods must be able to detect and distinguish molecular, physiological, or morphological variations between healthy and diseased tissues, therefore being extremely sensitive and selective in order to elucidate the physicians on the type and stage of the disease [93–95].

In this section, a brief overview of the various imaging modalities currently used in clinical practice and pre-clinical research will be covered. Included in this are magnetic resonance imaging (MRI), computer tomography (CT), the nuclear imaging modalities positron emission tomography (PET) and single photon emission CT (SPECT), as well as ultrasound (US), optical imaging (OI) and photoacoustic imaging (PAI). It is noteworthy to mention that due to the complex underlying physical principles regarding each imaging modality, an in-depth approach to each of them is beyond the scope of the thesis. However, a more general comparison between these imaging techniques will describe and distinguish variations between them, since they provide different information about the disease [94,97,98]. Table 1 summarizes the main advantages and limitations of these imaging techniques as well as some of the typical probes for each modality.

MRI was introduced into clinical practice in the early 80s and is based on the principles of nuclear magnetic resonance (NMR), where tomographic images are obtained when water and lipid protons present in various tissues and organs, placed in an external magnetic field, absorb the energy of radio frequency (RF) pulses. The corresponding macroscopic transverse spin magnetization is detected through the time-dependent voltage signal that it induces in a tuned RF coil, spatially localized using magnetic field gradients. The image contrast is obtained from differences in the proton relaxation processes, spin−lattice relaxation ($R_1 = 1/T_1$), and spin−spin relaxation ($R_2 = 1/T_2$). The outstanding diagnostic capabilities of MRI result mainly from the high degree

of spatial resolution of the images, produced non-invasively and without the use of ionizing radiation. MRI provides images with great soft tissue contrast, allowing the detailed visualization of organs and tissues, with rich anatomical and physiological information. However, the main constraints regarding MRI are its poor sensitivity, high costs, and its time-consuming nature[99–101].

The tissue contrast of MRI images can be enhanced, distinguishing smaller disease tissues from healthy ones, with the use of contrast agents. These agents can be subdivided in paramagnetic (T_1 or positive) agents, such as Gd^{3+} or Mn^{2+} complexes, and superparamagnetic (T_2 or negative) agents, such as small superparamagnetic iron oxide NPs (SPIONs) or ultrasmall superparamagnetic iron oxide NPs (USPIONs), since they will affect the intensity of the MRI signals of water protons located around them by shortening either their T_1 or T_2 relaxation times [31,90,100,102–104].

X-ray-computed tomography (CT) is one of the most traditional imaging techniques, that were introduced to clinical practice in 1972 [105]. Since then, it has been one of the most frequently used imaging techniques for the diagnosis of cancer, mainly due to its availability in hospitals and clinics. This availability and its relatively short scan times make this an attractive imaging modality while maintaining low costs. A CT image of a scanned area is obtained from the signals acquired from an X-rays beam source oriented at different angles, which are compiled and processed to reconstruct 3-D images. The CT image contrast results from different attenuation of the X-rays by different tissues, which increases with the linear attenuation coefficient, the density and the thickness of the materials present it the tissues, and also depends on the X-ray energy (E). The linear attenuation coefficient increases with the atomic number (Z) of the atomic nuclei present in the materials. The CT images provide great anatomical information, being more sensitive to denser tissues, or bones, which absorb more the X-rays, while soft tissues rely on contrast agents, made of high-Z materials, such as barium sulfate suspensions, iodinated compounds or gold nanoparticles. However, the main limitation of CT is the overexposure to radiation, being potentially harmful and carcinogenic towards the patients[93,96,105].

Nuclear medicine imaging, such as PET and SPECT, are highly sensitive imaging techniques that can visualize biological processes at the molecular and cellular levels.

Although their sensitivity is appreciated amongst clinicians, the anatomical information provided by these imaging techniques is relatively poor, mainly due to their low spatial resolution[46]. For both modalities' radiopharmaceuticals must be administrated and detected in order to produce an image. Radionuclides used in PET imaging decay by β^+ or positron emission. The emitted positron annihilates with an electron with the emission of two gamma rays that travel in opposite directions to one another. Detectors on opposite sides of the patient locate this and other coinciding events and backtrack and process them to produce an image that reflects the distribution of radioactivity within the tissues. For SPECT imaging, the radioisotopes used decay directly into single γ-rays, which are detected by a circular array of detectors to obtain the images. They are generally of lower energy while having longer half-lives ($t_{1/2}$) and therefore being more practical to use because they allow longer imaging times. Therefore, the main drawbacks of these nuclear medicine imaging techniques, besides the low spatial resolution, are the radiation exposure, the high costs, and the short imaging times due to the short half-lives of many radioisotopes [90,96,106].

Optical imaging (OI) is a non-invasive imaging technique that uses non-ionizing radiation, such as visible, ultraviolet, and infrared light, to obtain physiological and molecular information of a certain tissues or organs, therefore being different from other imaging modalities that use ionizing radiation and could be harmful to the patient. In addition to its safety and ease of use, it is a cost-effective and sensitive technique that can provide real-time imaging. This imaging modality includes various techniques that use light to obtain images from inside the body, tissues, or cells, such as endoscopy, optical coherence tomography (OCT), or fluorescent and bioluminescent imaging. The main limitation regarding this imaging modality is the poor tissue penetration hindering the clinical translation of some of these techniques. Nonetheless, fluorescent probes have been used for image-guided tumour surgery, while near-infrared (NIR) fluorescent fluorophores are being implemented to improve tissue penetration [90,93,102,107–109].

Ultrasound imaging is one of the most widely used imaging technologies for the diagnosis and staging of various pathologies in clinical practice. It is a relatively cheap, safe (no ionizing radiation) and unique imaging modality that can be used for real-time diagnostic, as well as therapeutic purposes (High Intensity Focused Ultrasound- HIFU).

This imaging technology uses pulses of ultrasound waves produced/emitted by ultrasonic transducers, which are transmitted through the body and are reflected according to the various boundaries between tissues, organs, or bones, and the echoes are received and recorded by the same transducer [110]. Theses echoes are processed digitally to reconstruct an image representing in a grey scale the two-dimensional cross-section of the body with a contrast that reflects the different echogenicities of the tissue structures originating the echoes. The key limitations of this imaging modality are the relatively low spatial resolution and limited tissue penetration[90,98,110,111].

Photoacoustic imaging (PAI), also known as optoacoustic imaging (OAI), is a diagnostics technique, based on the photoacoustic (PA) effect, which was first described in 1881 by Alexander G. Bell [110]. It consists of the generation of sound waves after light absorption within a media, and thus combines properties from two other imaging modalities, OI and US. In this imaging modality, short laser pulses are used to irradiate a target tissue, after which ultrasound transducers are used to capture photoacoustic waves produced due to the thermoelastic expansion and relaxation of the absorbing endogenous or exogenous chromophores[110,112–114]. Therefore, commonly used OI probes can be used for PAI applications, such as NIR dyes or gold nanoparticles.

The combination of various imaging modalities has proven to be a useful strategy to combine and overcome the advantages and limitations inherent to each modality, therefore providing more accurate diagnostics. Generally, modalities with high spatial resolution (MRI/CT) are combined with modalities with high sensitivity (OI/Nuclear imaging), complementing anatomical, and molecular/functional information[115,116].

Table 1- Overview and comparison of the advantages and limitation of the various imaging modalities, adapted from ref 96 and 110.

Imaging modality	Advantages	Limitations	Depth	Typical probes
MRI	High spatial resolution and non-invasive Excellent soft tissue contrast	Low sensitivity High costs and time consuming	Unlimited penetration depth	Paramagnetic, superparamagnetic nanoparticles
CT	High contrast resolution	Insufficient soft tissue contrast without	Unlimited penetration depth	Gold, silver, and iodine nanoparticles

	Affordable and time-efficient	injection of contrast agents Radiation exposure Low sensitivity to contrast agents		
PET	Very high sensitivity Quantitative	Low spatial resolution No anatomical information Radiation exposure High costs	Unlimited penetration depth	Radio-labelled nanoparticles (^{18}F, ^{68}Ga)
SPECT	Very high sensitivity Long-circulating radionuclides	Low spatial resolution No anatomical information Radioactive probes High costs	Unlimited penetration depth	Radio-labelled nanoparticles (^{99m}Tc, ^{131} I)
OI	High sensitivity for contrast agents Broad range of probes and cost-efficient	Penetration depth High background signal	Low penetration depth (<10 cm)	Quantum dots and fluorescent/dye-loaded nanoparticles
US	Good temporal resolution Rapidly operable Real-time imaging and cost-efficient	Limited spatial resolution Not appropriate for whole body imaging Limited to imaging soft tissues	Limited penetration depth	Microbubbles and nanobubbles
PAI	Broad range of probes and cost-efficient Real-time imaging	Limited to imaging soft tissues	Limited penetration depth	Gold nanoparticles, Fluorescent/dye-loaded nanoparticles

Acronyms: MRI, Magnetic resonance imaging; CT, Computed tomography; PET, Positron emission tomography; SPECT, Single photon emission computed tomography; US, Ultrasound; OI, Optical imaging; PAI, Photoacoustic imaging

4.2 Theranostic nanosystems

In the past decades, an increase in the complexity of nanocarriers has been observed from the earliest systems, that were limited to encapsulating and delivering the therapeutic agent, to nanosystems that mediated a targeted approach or were sensitive to certain stimuli to release their therapeutic cargo, and even to those that combined these previously mentioned approaches for cancer treatment. These new multifunctional nanosystems have become more complex to respond to the numerous mechanisms of cancer development and, to some extent, to minimize the variability between patients to deliver a more efficient therapy. On the other hand, and due to the

importance of early diagnosis, nanoparticles have also been applied to carry various contrast agents for different imaging modalities, where they are primarily intended to improve the diagnostic capabilities of the imaging modalities mentioned in the section above [94]. However, in 2002 Funkhouser introduced the scientific community to the term "theranostics" [117]. This term can be broadly defined as a material that combines diagnostic and therapeutic functions within a platform. In this regard, and due to the numerous achievements of using nanoscale materials for therapeutic or diagnostic purposes, the field of nanotechnology provides the opportunity for the development of nanotheranostic formulations for the simultaneous diagnostic and treatment of cancer[118,119]. Typically, these are organic and inorganic based materials that combine diagnostic and therapeutic agents in a single nanoplatform (Figure 7).

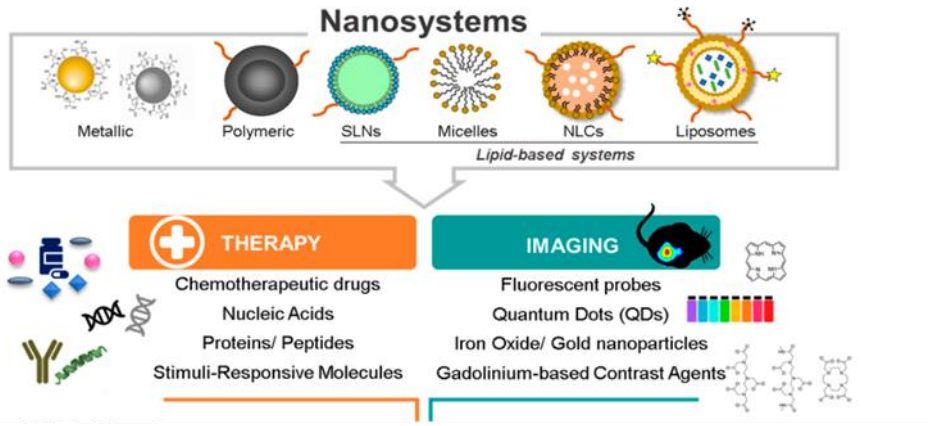

Figure 7- Organic and inorganic based nanosystems capable of combining diagnostic and treatment modalities, adapted from ref 119.

Using nanosystems for this purpose can result in a multitude of combinations due to the various possible functionalizations and properties that could be incorporated along with the broad range of therapeutic and diagnostic agents available. Therefore, different cancer treatment strategies such as chemotherapy, gene therapy, or photodynamic therapy (PDT) could be employed, depending on the therapeutic cargo included [118,119]. Regarding the diagnostic capability of these nanosystems, various imaging agents could be integrated into these platforms to promote different imaging modalities, such as magnetic resonance imaging, optical imaging, or nuclear imaging (PET/SPECT)[91,118–120].

The combination of these individual components in a single platform provides the ability to evaluate the efficiency of the treatment while monitoring and managing the progression of the disease (Figure 8). Furthermore, other aspects related to treatment can be accessed, such as the distribution and accumulation in the patient, specificity for a given target, through active or passive targeting, and the release of the therapeutic agent [46,91]. Therefore, and depending on the overall nanoformulation, physicians would be able to deliver a more personalized therapy that would take into account the variability between patients. On the one hand, they could adjust the dose administered according to the diagnostic information collected, and on the other, they could change or modify the therapeutic strategy with the early detection of possibly side effects [46,91,93,94,119,121].

As previously mentioned, there are several possible therapeutic strategies that could be mediated by theranostic nanosystems, on which several review articles can be consulted [91,119,121,122]. Here the main focus will be on theranostic nanosystems that involve gene therapy.

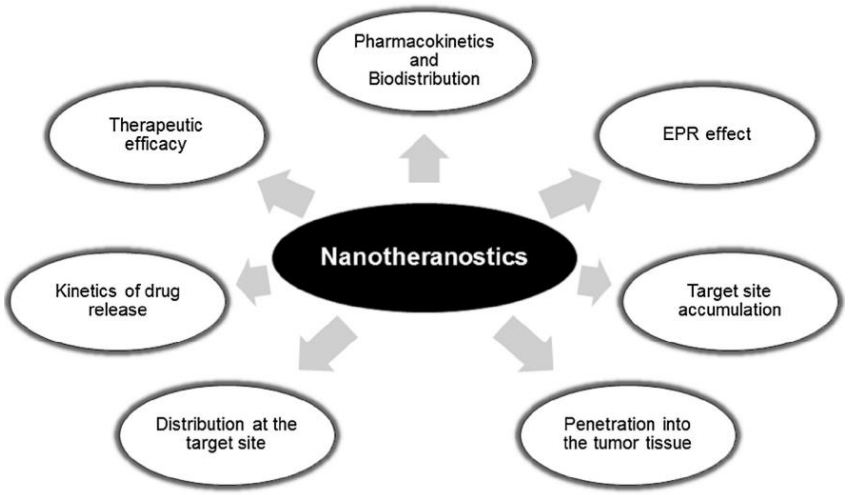

Figure 8-Important aspects evaluated by the combination of diagnostic and therapeutic properties within a single nanoplatform (nanotheranostics), adapted from ref 46.

5. Gene therapy based theranostic nanosystems

In the following sections various theranostic nanosystems, where the main therapeutic modality is gene therapy, are briefly described and divided according to the

imaging modality in which they are active, such as MRI, OI, US, and multimodal imaging. These were further subdivided if other therapeutic strategies, such as chemotherapy, PDT, or photothermal therapy (PTT), were used in combination with gene therapy. Table 2 lists some of the nanosystems mentioned here with *in vivo* applications.

Table 2-Gene Therapy based theranostic nanosystems with in vivo applications

Imaging modality	Nanosystem	Disease model	Targeting agent	Therapeutic cargo	Combined Strategy	Ref
MRI	^{PAE}MTN-transfected hMSCs	Glioblastoma	HA	pDNA TRAIL gene	N/A	126
	micelleplexes	Colorectal cancer	N/A	SN-38 and VEGF siRNA	Chemotherapy	127
	ALBTA	Glioblastoma	angiopep-2	TMZ and siTGF-β	Chemotherapy	128
	Gd-HM-Dox/34a	Breast cancer	N/A	DOX and miR-34a	Chemotherapy	129
	Fe_3O_4@PDA-siRNA@MSCs	Prostate cancer	N/A	Plk1 siRNA	PTT	132
	Sphere and rod-like M-MSNs	HCC	N/A	HSV-TK/GCV	Magnetic hyperthermia	135
OI	RVG-PNPs	Neuroblastoma	RVG peptide	Myc, Bcl-2, and VEGF siRNA	N/A	137
	Apt-QLs	Breast cancer	anti-EGFR aptamer	Bcl-2 and PKC-ι siRNA	N/A	139
	QD-HA-PEI	HCC	HA	anti-miR-27	N/A	140
	NIR polymeric NPs	Anaplastic thyroid cancer	N/A	siBRAF	N/A	141
US	siRNA micelles and MBs	Human cervical cancer	N/A	XIAP siRNA	N/A	147
	Dox-NBs/PPP/ P-gp shRNA	Breast cancer	N/A	Dox and P-gp shRNA	Chemotherapy	148
	PTX-NBs/siRNA	HCC	N/A	PTX and BCL-2 siRNA	Chemotherapy	149
MRI/CT	M-MSN(Dox/Ce6)/ PEM/P-gp shRNA	Breast cancer	N/A	Ce6, DOX and P-gp shRNA	PDT and Chemotherapy	153
PA/US	FCNPI/pDNA	Retinoblastoma	FA	HSV-TK/GCV	N/A	154
PA/IR	PSZ	Human cervical cancer	N/A	Blc-2 siRNA	PTT	155
	GNPs-hPD-L1 siRNA	Human lung cancer	N/A	PD-L1 siRNA	PTT	156

5.1 Combination of gene therapy and MRI

As previously mentioned, when designing nanosystems to be used as contrast agents for MRI, they are generally subdivided in T_1 or T_2 contrast agents. Typically, T_1 agents, based on paramagnetic materials such as Gd^{3+} or Mn^{2+}, are also referred to as positive contrast agents since they lead to brighter areas in T_1-weighted images. These metal ions, in order to be used for *in vivo* applications, need to be chelated with high thermodynamic and kinetic stability[31,100]. With this in mind, Gao *et al* developed a non-viral reducible nanocarrier for efficient gene delivery and MRI capability[123]. To do so, they first synthesised Gd-chelated reducible cationic poly(urethane amide) (GdCPUA) polymers, with the polymerized diethylenetriaminepentaacetic acid (DTPA) residues used to chelate Gd^{3+}. Then polyplexes where obtained using the cationic polymers mixed with the plasmid DNA (pDNA) at different nitrogen/phosphate (N/P) ratios. During the characterization process, the GdCPUA15 polyplexes, with 15 % DTPA residues, were able to bind to DNA at lower N/P ratios while at the same time releasing it when in the presence of dithiothreitol (DTT), used to mimic a reductive environment. High *in vitro* transfection efficiencies were observed with the above polyplexes at a 10/1 N/P ratio. After this, polyplexes prepared with a plasmid expressing a short hairpin RNA (shRNA) were used to silence the angiogenic factor VEGF. *In vitro* studies revealed that the above polyplexes were able to significantly silence VEGF expression in SKOV-3 cells (human ovary carcinoma), even when compared with commercially used complexes. Although *in vivo* studies were not accomplished, the GdCPUA15 polyplexes, at the above-mentioned ratio, produced brighter T_1-weighted images with higher relaxivity (r_1) values when compared with Magnevist, a small Gd^{3+}-based contrast agent[123].

T_2 or negative contrast agents, usually composed of superparamagnetic iron oxide nanoparticles (SPIONs), are referred in this way since they produce the opposite effect of the above-mentioned paramagnetic materials, thus producing darker areas in T_2 -weighted images[31,100]. In 2017, Luo *et al* designed and characterized a SPIONs based nanosystem for siRNA delivery against the programmed death ligand-1 (PD-L1)[124]. Here, SPIONs were encapsulated in nanosystems prepared with folic acid (FA)-(PEG)-conjugated polyethyleneimine (PEI) polymer. Targeted polyplexes, due to the FA

targeting ligand, were then formed at different N/P ratios. Although monoclonal antibodies have been used and are under clinical trials for checkpoint blockade of PD-L1 in various cancers types, here siRNAs were used to downregulate its expression. Polyplexes were able to complex siRNA when prepared with N/P ratios of 10 or higher, only exhibiting cytotoxicity with much higher ratios (50 or 60). Using the folate receptor-overexpressing gastric cancer cell line (SGC-7901 cells), the targeted polyplexes exhibited higher cellular internalization and transfection efficiency, as demonstrated by Prussian blue staining and confocal laser-scanning microscopy, than non-targeted ones. *In vitro* T_2-weighted images of transfected SGC-7901 cells with the polyplexes (N/P ratio of 10) revealed that the nanosystem could potentially be used as a negative contrast agent for MRI. PD-L1 silencing was accomplished using polyplexes prepared with four different PD-L1 siRNAs, named siRNA 1 to siRNA 4. The FA-PEG-SS-PEI-SPION/PD-L1 siRNA2 polyplexes were the most efficient downregulating nanosystems at the mRNA and protein levels [124].

In a similar study, Wu *et al* also designed and characterized a SPIONs based gene delivery and MRI nanosystem. However, instead of using a synthetic polymer as a coating material, they used amylose as a natural/biocompatible polymer[125]. Here FA-functionalized cationic amylose (CA) nanoparticles were loaded with SPIONs and complexed with siRNAs against the overexpressed apoptotic inhibitor survivin in hepatocellular carcinoma (HCC) cells. Nanocomplexes were formed with different *w/w* ratios of the cationic FA-CA-SPION and the siRNAs, those prepared with a *w/w* ratio of 12 displaying a slight positive surface charge and a mean diameter size of 150 nm, while also being able to condense the genetic material. Cellular uptake and transfection efficiency were higher when cells were incubated with FA-CA-SPION/siRNAs, at the above-mentioned ratio. Without the therapeutic siRNA, negligible toxicity was observed in HepG2 cells (human hepatocellular carcinoma). Survivin downregulation at the mRNA and protein levels was accomplished and revealed that FA-CA-SPION/siRNAs were the most efficient downregulating nanosystems when compared with the non-targeted nanosystems. Cells pre-treated with FA followed by FA-CA-SPION/siRNAs displayed similar results as the nontargeted nanosystem, therefore proving the importance of FA for the specific uptake, transfection efficiency, and downregulation of the targeted

gene. Darker T_2-weighted images were produced when HCC cells were incubated with FA-CA-SPION and therefore could potentially be used as MRI contrast agents[125].

Unlike previous works, Huang *et al* developed a magnetic gene delivery system referred to as magnetic ternary nanohybrid (MTN). The main goal of this nanohybrid system is to transfect human mesenchymal stem cells (hMSCs) that would in turn express the tumour necrosis factor-related apoptosis inducing ligand (TRAIL) and therefore be used to treat Glioblastoma (GBM) (Figure 9A). To do so, several non-viral MTN systems were prepared from hyaluronic acid (HA)-SPIONs mixed with various polyplexes and lipoplexes, with HA used to target the overexpressed CD44 receptors in hMSCs. Among the various systems, ^{PAE}MTN complexes, prepared from poly β-amino ester (PAE)/pDNA polyplex and HA-SPIONs, were the ones that exhibited higher transfection efficiencies and TRAIL expressions in hMSCs. These results were further enhanced when an external magnetic field (EMF) was applied, through a process known as magnetofection. The TRAIL expressing stem-cells (^{TRAIL}hMSCs), after magnetofection, were highly cytotoxic towards the human glioma cell line (U87MG) when cocultured. A migration transwell system and a three-dimensional U87MG spheroid were used to demonstrate that the transfected hMSCs, when compared with untransfected hMSCs, maintained their migration capability and were able to efficiently penetrate the tumour model. *In vitro* and *in vivo* images of HA-SPIO and of intracranial injected ^{PAE}MTN-transfected hMSCs, respectively, demonstrated the ability of the nanosystems to be used as MRI contrast agents (Figure 9B). Orthotopic glioma mice treated with ^{TRAIL}hMSCs showed a reduction of tumour growth and a significant survival increase, while *in vivo* T_2-weighted images further demonstrated their therapeutic effect when compared with PBS or untransfected hMSCs (Figure 9C)[126].

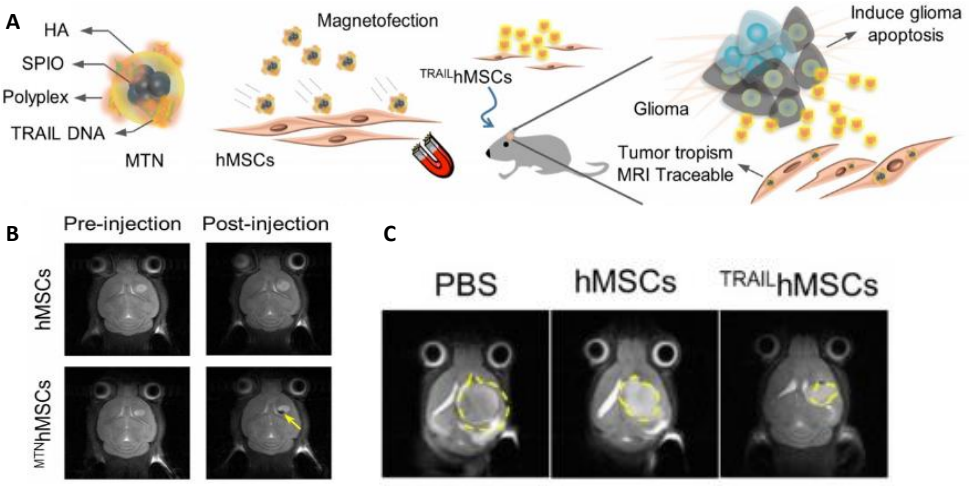

Figure 9- (A) Schematic illustration of ^{TRAIL}hMSCs preparation and application in Glioblastoma treatment. (B) In vivo T_2-weighted images after intracranial injection of hMSCs or ^{TRAIL}hMSCs. (C) In vivo T_2-weighted images of glioma-bearing treated mice 35 days after inoculation, adapted from ref 126.

5.1.1 Combination of gene therapy, MRI and chemotherapy

Regarding combined chemotherapeutic and gene therapy strategies using non-viral vectors, Lee *et al* developed a micelle based theranostic nanosystem[127]. Here amphiphilic PDMA-block-poly(ε-caprolactone) (PDMA-b-PCL) and mPEG-PCL micelles were used to encapsulate the chemotherapeutic drug, SN-38, and the USPION contrast agent. After this, micelleplexes were prepared via electrostatic interactions using the co-loaded micelles with a previously prepared VEGF siRNA-PEG conjugate. After drug loading optimization, the complexes prepared at polymer/siRNA *w/w* ratios of 8 or higher were able to efficiently complex the siRNA. *In vitro* studies with a high VEGF-expression human colon cancer cell line (LS174T) revealed that the nanosystem, prepared without the siRNAs, did not affect the cell viability when not loaded with SN-38 and that the therapeutic effect of the drug was not altered when loaded with USPIO. Micelleplexes were able to significantly silence VEGF expression even when compared with a commercially used transfection agent. *In vivo* biodistribution studies demonstrated that the micelleplexes could accumulate in the tumour region, even without a targeting agent, and therefore be used as an MRI contrast agent. Additionally, the highest therapeutic effect was observed when xenografic mice were treated with the micelleplexes [127]. It is worth mentioning that in the initially designed nanosystem without mPEG-PCL, the high surface charge observed influenced and compromised the

potential use of the nanosystem for *in vivo* experiments, further demonstrating the importance of nanoparticle properties for an efficient delivery.

In another combined strategy, Qiao *et al* developed a targeted theranostic nanosystem that combined a commonly used chemotherapeutic drug for glioblastoma treatment, temozolomide (TMZ), and a siRNA against the immunosuppressive cytokine, tumour growth factor β (siTGF-β), that hinders T-Cell and B-cell proliferation[128]. The nanosystem consisted of SPIONs loaded polymeric NPs prepared from positively charged ROS-responsive poly[(2-acryloyl)ethyl(p-boronic acid benzyl)diethylammonium bromide] (BAP) polymers. BAP/SPIONs@siTGF-β complexes were prepared in various N/P ratios and were further coated with lipid-based materials (LiB) during which TMZ was incorporated. Thereafter, the targeting peptide angiopep-2, which can target an overexpressed receptor in glioblastoma cells, was conjugated to obtain ALBTA (Ang-LiB(T+AN@siTGF-β) formulation. ALBTA displayed a mean diameter size of 120 nm, a slightly positive surface charge, and an r_2 value of 315.46 mM^{-1} s^{-1}, at an N/P ratio of 10. An *in vitro* blood brain barrier (BBB) monolayer established that the targeting peptide of ALBTA improved the transport efficiency and cellular uptake by GL261 cells in comparison with the non-targeted LBTA. In addition, *in vitro* antitumor results demonstrated that the combination of TMZ and siTGF-β in the ALBTA nanosystem resulted in the highest therapeutic effect while at the same time significantly down-regulating TGF-β levels. *In vivo* immune cells, obtained from the spleen of tumour bearing mice treated with ALBTA, revealed that cytotoxic T lymphocytes (CTL) and helper T cells were significantly increased while regulatory T cells where reduced. The mean survival time of treated animals was also improved when compared with the control groups. Furthermore, *in vivo* T_2-weighted images and Prussian-blue staining revealed that the nanosystem could be used as an MRI contrast agent and could accumulate in the tumour region [128].

In a different approach, a T_1 contrast agent (Gd-DTPA) was conjugated to polyethyleneglycol-polycaprolactone (Gd-PEG-PCL), which, in combination with polyethyleneimine-poly-caprolactone (PEI-PCL), was used to produce micelles to co-deliver Dox and the complexed microRNA-34a for breast cancer cells treatment. *In vitro*

and *in vivo* studies demonstrated the potential use of this theranostic nanosystem due to its promising imaging and therapeutic effects [129].

5.1.2 Combination of gene therapy, MRI, and PTT

Besides chemotherapeutic drugs, other therapeutic modalities could be combined with gene therapy to enhance the overall efficacy of the nanoplatform. One of these is photothermal therapy (PTT). This strategy has been extensively reviewed but is briefly characterized by the local increase in temperature due to the presence of a PTT agent that converts light into heat and consequently kills cancer cells [114,130,131]. Usually, near infrared (NIR) PTT agents, being either organic or inorganic based materials, are employed in order to lessen possible damages to healthy tissues [114]. Therefore, and inspired by natural biomimetic gene delivery platforms, Mu *et al* designed an imaging-guided photothermal and siRNA delivery nanosystem [132]. Here mesenchymal stem cell membrane-derived vesicles were used to wrap polydopamine (PDA)-coated hydrophobic iron oxide nanoparticles (IONPs) [132]. The therapeutic siRNA against an overexpressed Plk1 oncogene was adsorbed to the surface of the prepared Fe_3O_4@PDA NPs. After this, prepared vesicles, derived from stem-cell membranes, were mixed and co-extruded with the Fe_3O_4@PDA-siRNA NPs to produce the biomimetic nanosystem. The Fe_3O_4@PDA-siRNA@MSCs NPs exhibited a core-shell structure with a mean size of 109 nm and a surface charge (-30 mV) similar to that of stem cell vesicles (-33 mV). Characterization tests revealed that the nanosystem could be used for photothermal ablation when irradiated with NIR light, due to the photothermal conversion ability of the polydopamine shell, and used as a contrast agent for MRI with an r_2 value of 209.3 mM^{-1} s^{-1}. Furthermore, the nanosystems displayed good hemocompatibility, were not cytotoxic towards a healthy cell line (293t cells), and were capable of photothermal NIR induced ablation against a human prostate cancer cell line (DU145 cells). Significant Plk1 silencing was accomplished when the therapeutic siRNAs were delivered with the biomimetic nanosystem, enhancing cell apoptosis as confirmed by Annexin V-FITC and PI staining. *In vivo* tests in xenografic tumour mice revealed that the nanosystem plus laser irradiation could significantly halt tumour growth with no inflammation or lesions

observed in the major organs. Additionally, *in vivo* diagnostic tests revealed that the nanosystem prepared without the genetic material could also serve as a contrast agent for MRI [132].

5.1.3 Combination of gene therapy, MRI, and magnetic hyperthermia

As demonstrated in the previous section, iron oxide nanoparticles have been widely incorporated in various nanosystems to be used as contrast agents for MRI. However, due to their magnetic properties, when exposed to an alternating current magnetic field (ACMF) they can raise local temperature in order to be used in a therapeutic manner similar to that of PTT, through a process known as magnetic hyperthermia[133,134]. Recently Wang *et al* developed a theranostic nanosystem that could be used for both magnetic hyperthermia and gene therapy of HCC[135]. In this case, the gene therapy strategy employed was the suicide gene therapy. However, unlike the typically employed strategies where suicide gene and prodrug are separately delivered, this nanosystem incorporated an imaging agent for MRI along with the prodrug ganciclovir (GCV) and the suicide gene herpes simplex virus thymidine kinase (HSV-TK/GCV)[135]. To do so, sphere and rod-like magnetic mesoporous silica nanoparticles (M-MSNs), with a core composed of Fe_3O_4, were post-grafted with PEG-g-PLL to incorporate the prodrug and were latter complexed with the pDNA at various *w/w* ratios (Figure 10A). Both complexes were able to bind to the pDNA and protect it from DNase degradation. External magnetic fields (EMFs) enhanced the cellular uptake and transfection of both complexes with the rod-like ones displaying higher results in HepG2 cells. Furthermore, the EMFs enhanced the cytotoxic effect of the theranostic nanosystem, the most efficient therapeutic effects being observed when both EMF and ACMF were applied. *In vitro* relaxivity and *in vivo* MR images of tumour bearing mice demonstrated the potential of these nanosystems to be used as MRI contrast agents with EMF enhancing tumour accumulation. *In vivo* studies in xenografic tumour mice further demonstrated that both nanosystems + EMF + ACMF were able to significantly halt tumour growth, the rod-like ones displaying slightly better performances (Figure 10B). Additionally, T_2-weighted MR images were used to monitor the therapeutic effect

of the various formulations (Figure 10C), the safety tests further confirming the biocompatibility of the nanosystems[135].

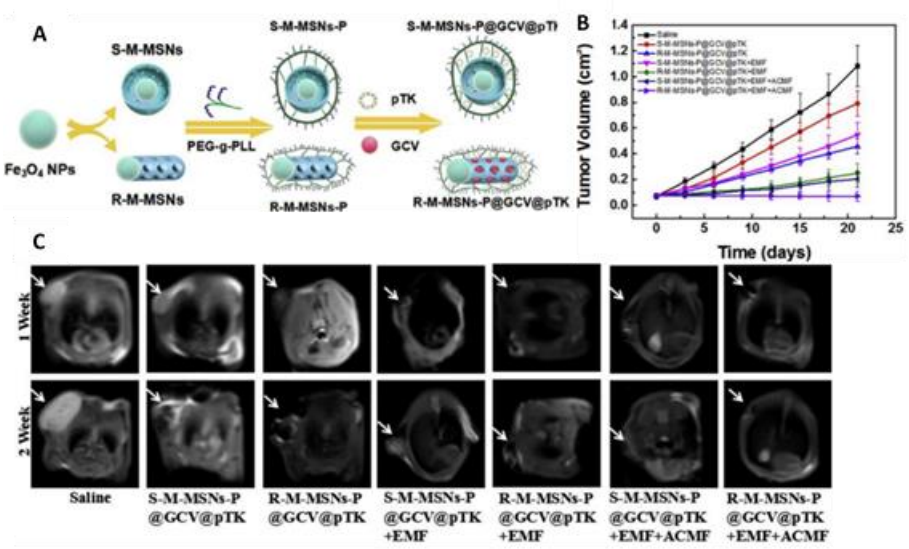

Figure 10-(A) Schematic illustration of the preparation of the differently shaped complexes. (B) Tumour inhibition curves and (C) representative MR images of the differently treated tumour bearing mice, adapted from ref 135.

5.2 Combination of gene therapy and OI

Optical imaging has been extensively used in cancer research in which various dyes, quantum dots, and metallic nanoparticles were used for this purpose[108,136]. Affordable small fluorescent chromophores are still one of the most used types of probes for OI, mainly because they are easily embedded or conjugated into nanosystems, allowing them to be tracked[108]. For example, polymeric nanoparticles were used to encapsulate a therapeutic cocktail of various siRNAs along with a fluorescent probe, 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate (Dil), for neuroblastoma therapy and imaging, respectively [137]. The genetic material cocktail containing siRNAs against Myc, Bcl-2, and VEGF, was chosen based on previous anticancer studies showing synergetic effects. These nanosystems, composed of poly(D,L-lactide-*co*-glycolide), were further functionalized with a rabies virus glycoprotein (RVG) used to target neural cells (RVG-PNPs). The targeted nanosystems, without the therapeutic siRNAs, displayed a mean size of around 200 nm and a negative surface charge, and did not affect the viability of a neuroblastoma cell line (N2a cells).

Binding and cellular uptake studies revealed that the RVG-PNP nanosystems were specific towards the above-mentioned cell line and that pre-treatment with RVG peptides significantly hindered their efficiencies. The nanosystems, after intravenous injection, were able to efficiently target and accumulate in the tumour region of xenografic mice, as demonstrated by the whole-body images. Furthermore, RVG-PNP nanosystems prepared with the siRNA cocktail were able to silence the target genes while hindering tumour growth in an animal model [137].

Similar to the fluorescent dyes, core-shell QDs have attracted interest from the scientific community mainly due to their small sizes, tunable fluorescent emission, and relatively high photostability[30]. Henceforth, nanoparticles incorporating these probes could potentially be used to monitor gene delivery systems. For instance, Kim *et al* developed and synthesised a theranostic lipid-based nanosystem containing fluorescent CdSe/ZnS Q-dots[138]. Liposomes, composed of a cationic lipid, cholesterol, and DSPE-mPEG2000 were prepared through a lipid film hydration method and used to incorporate QDs. After this, complexes with siRNA molecules were prepared at various N/P ratios. The targeted nanosystem Apt-QLs were obtained with a post-insertion method, using anti-EGFR aptamer conjugates and DSPE-mPEG2000, displaying a mean size of 165 nm and a slightly negative surface charge. Complexes prepared at a 4:1 N/P ratio were able to complex the siRNAs and protect them from RNase A degradation. Positive and negative EGFR expressing breast cancer cell lines transfected with Apt-QLs, prepared with labelled siRNA molecules, revealed a specific cellular binding to cell lines with the overexpressed receptor, that was significantly affected when cells were pre-treated with free anti-EGFR aptamers. *In vivo* imaging revealed that a higher fluorescent signal was obtained in xenografic mice administrated with the targeted nanosystems. Biodistribution of the above sacrificed mice further demonstrated the importance of the targeting ligand for the accumulation in the tumour region [138]. In a follow-up study, and due to the favourable results obtained, the Apt-QLs nanosystems were prepared with two therapeutic siRNAs, and the *in vitro* and *in vivo* therapeutic efficacy was assessed and compared with a non-targeted nanosystem and with an antibody-coupled nanosystem (immuno-QLs)[139]. In this study, anti Bcl-2 and PKC-ι siRNAs were chosen as the therapeutic molecules, mainly because the former is an apoptosis inhibiting protein

while the latter is associated with cancer metastasis. *In vitro* studies demonstrated that the Apt-QLs and the immuno-QLs were cytotoxic towards positive EGFR-expressing cells while being able to efficiently silence both gene expressions. Moreover, *in vivo* studies with both targeted nanosystems, co-delivering both siRNA, revealed that they were able to significantly supress tumour growth while also silencing gene expression. Although immuno-QLs displayed a slightly better therapeutic effect, no toxicity was observed when mice were treated with either of the formulations [139].

When using visible light, the application of nanosystems in human contexts is severely hindered mainly due to the limitations mentioned in section 4.1. Therefore, and to overcome these limitations, NIR probes, such as some QDs, (NIR, λ = 700-950 nm) have been developed. Probes that utilize the NIR region tend to minimize the light absorption by endogenous chromophores, thus improving *in vivo* imaging[108,109]. In a particular case, Zheng *et al* developed a targeted polymer-based theranostic nanoplatform for HCC therapy and NIR imaging[140]. For that purpose, a synthesized hyaluronic acid-polyethyleneimine (HA-PEI) conjugate was used to load QDs for NIR imaging. After this, the targeted nanosystems anti-miR-27a/QD-HA-PEI were assembled at various *w/w* (QD-HA-PEI to RNA) ratios. The nanosystems displayed similar emission peaks as the blank QDs along with an emission peak at 460 nm that was later confirmed to be due to the conjugation of HA-PEI. Efficient siRNA binding and protection from RNase A degradation were observed at a *w/w* ratio of 9:1 or above. The nanosystems were cytotoxic towards HepG2 cells while being able to significant silence mir-27a expression and consequently upregulating downstream targets of mir-27-a. *In vivo* imaging and biodistribution studies revealed that the nanosystems accumulated at the tumour site and that pre-treatment with HA confirmed the importance of the targeting ligand for tumour accumulation. Xenografic mice treated with the targeted nanosystems presented supressed tumour growth and displayed similar expression patterns as the *in vitro* results. Additionally, a biosafety evaluation demonstrated that mice treated with anti-miR-27a/QD-HA-PEI had no altered levels of several biomarkers and displayed normal tissue slices without lesions or inflammation[140].

In a slightly different approach, NIR nanosystems were prepared through a self-assembly nanoprecipitation method composed of a NIR polymer, a cationic lipid,

previously prepared by the same group, and DSPE-PEG3k to encapsulate siRNAs (NIR NPs) for anaplastic thyroid cancer (ATC) treatment (Figure 11A) [141]. The therapeutic RNAi was used to target an overexpressed V-Raf murine sarcoma viral oncogene homolog B (BRAF) due to a mutation in ATC. Nanoparticles displayed a spherical shape with a mean hydrodynamic size of 85 nm and a weak positive surface charge. *In vivo* NIR fluorescence images in tumour xenografic and orthotopic mice, prepared with $BRAF^{V600E}$-mutated 8505C cells, demonstrated that the nanosystems were able to accumulate in the tumour region (Figure 11B). *In vitro* studies, using the above-mentioned cell line, revealed that the NIR NPs could efficiently downregulate BRAF and significantly affect the cell proliferation while maintaining cell viability when they were prepared with control siRNAs. *In vivo* treatment of tumour xenografic and orthotopic mice with the NIR NPs could efficiently down-regulate BRAF and inhibit tumour growth. In addition, the nanosystems were also able to diminish the described metastatic capability of an orthotopic tumour model, prepared with GFP-expressing 8505C cells harbouring $BRAF^{V600E}$, as demonstrated by the green fluorescent protein (GFP) signals in the lungs (Figure 11C). *In vivo* studies with healthy mice demonstrated the good biocompatibility of the NIR NPs[141].

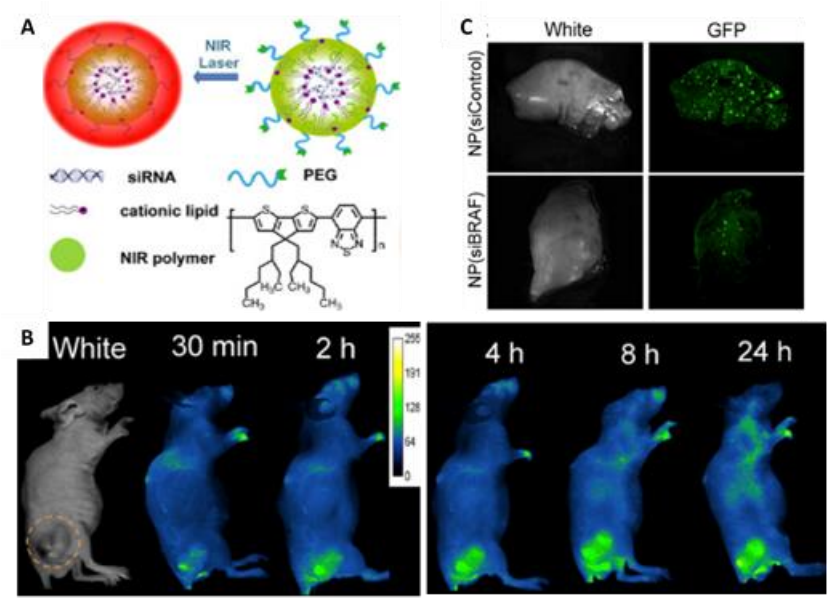

Figure 11-(A) Schematic illustration of the nanosystem. (B) In vivo NIR imaging of xenografic $BRAF^{V600E}$-mutated 8505C tumour-bearing mouse after a single-dose injection. (C) Lung images of the orthotopic tumour bearing mice treated with the experimental and control NPs, 1 month after injection with $BRAF^{V600E}$-mutated 8505C cells with GFP expression, adapted from ref 141.

5.2.1 Combination of gene therapy, OI, and chemotherapy

As it was previously mentioned, MDR is one of the main downsides of chemotherapy drugs for cancer treatment, affecting their overall therapeutic effect[142]. However, combined strategies of DOX and RNAi have proven to be a feasible option to enhance therapeutic efficiencies while minimizing the MDR effects[143]. In a recent study, a theranostic nanosystem was developed to combine DOX and siRNAs to silence a well-known MDR protein, P-glycoprotein (P-gp). To do so, fluorescent silica nanoparticles (SiNPs) were synthesized and loaded with DOX, after which SiNP-DOX/siRNA were prepared at various SiNPs/siRNA *w/w* ratios[144]. Compared with some of the previously mentioned nanosystems, this spherical-shaped one displayed a minimal hydrodynamic size of 7.2 nm. The nanosystems prepared with a *w/w* ratio above 150 were able to bind the siRNAs and protect them from RNase A degradation while being highly photostable. Interestingly, complexes prepared with siRNAs with a high G+C content had the highest silencing efficiency observed by the mRNA and protein levels. Cell viability with the same multidrug-resistance breast cancer cells (MCF-7/ADR cells) revealed that there was a significant increase in the cytotoxic effect of the co-loaded nanosystems when compared with free DOX and consequently there was a 37-fold decrease in the IC_{50} when compared with free DOX[144]. Further *in vivo* small animal studies are therefore required to determine if the nanosystems could be used for theranostic purposes and to evaluate its toxicity.

5.3 Combination of gene therapy and US

In a clinical context, US imaging is one of the modalities most used by clinicians mainly due to the practicality and ease of use. As with other imaging modalities, ultrasound contrast agents, such as gas-filled microbubbles (MBs) and nanobubbles, were developed to improve diagnostic outcomes[111]. Nonetheless, they can also be used to enhance gene transfection efficiencies when in combination with focused ultrasound (FUS), thereby opening the blood-brain barrier (BBB) and suppressing glioma growth due to the delivery of a short hairpin RNA by MB avidin-biotin conjugated lipoplexes[145].

In a similar study, to treat the same disease, Chang *et al* developed cationic MBs that were used to complex plasmid DNA. Here the suicide gene pHSV-TK was used in combination with the prodrug (GCV) instead of using the RNAi approach for therapeutic purposes[146].

Besides enhancing gene transfection, the modification of these agents may also serve as dual imaging-gene delivery systems. For example, Wang *et al* developed a microbubble-based system used for contrast enhanced US (CEUS) imaging and therapy of human cervical cancer[147]. In this study, commonly developed lipid shell MBs were first prepared and were further complexed with previously developed and characterized cationic polymeric micelles encapsulating siRNAs (N/P = 5). The siRNA was chosen to target the overexpressed anti-apoptosis protein, X-linked inhibitor of apoptosis protein (XIAP). The plain MBs displayed a negative surface charge whereas the complexes that were prepared at various P/P_1 values (which were the ratio of phosphate groups in siRNAs to that in DPPA of the MBs) had an increased zeta potential without altering the mean size of the formulation. The contrast enhanced ability of the gas-filled spherical shaped siRNA/MBs was similar to that of plain MBs. Human cervical cancer xenografic mice treated with intratumorally injected XIAP (siRNAs)/MBs and with US exposure (+) were able to significantly halt tumour growth and consequently had the highest survival rates (Figure 12A). *In vivo* CEUS images further demonstrated the therapeutic effect of the nanosystem when compared with the control groups (Figure 12B). The application of a low-frequency US was therefore essential to obtain a significant anticancer effect. Expression studies of the above mice revealed that the highest silencing effect, at the protein and mRNA levels, was observed when they were treated with the XIAP/MBs US (+)[147].

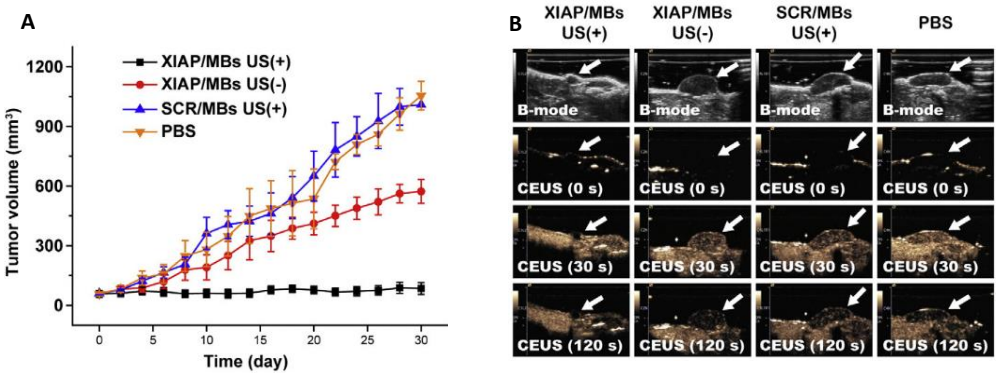

Figure 12- (A) Tumour inhibition curves and (B) B-mode and CEUS imaging of the differently treated tumour bearing mice, 30 days after the first treatment, adapted from ref 147.

5.3.1 Combination of gene therapy, US and chemotherapy

In another study to overcome MDR, a theranostic nanosystem was developed to co-deliver DOX and a plasmid expressing a short hairpin RNA (shRNA) against P-gp[148]. Here PLGA DOX nanobubbles were surface modified with PEI (DOX-NBs/PEI). The above NBs were further modified with a charge-reversal polyelectrolyte followed by a second PEI adsorption (DOX-NBs/PPP). After this, the pDNA was complexed with the DOX-NBs/PPP suspension at various *w/w* ratios (DOX-NBs/PPP/P-gp shRNA). The experimental nanobubbles were able to encapsulate DOX, successfully bind to the pDNA while also being able to release it under an acidic environment (pH=4.6) due to polyelectrolyte pH sensitivity. Uptake and silencing studies revealed that Dox-NBs/PPP/ P-gp shRNA could accumulate in both non-resistant (MCF-7) and resistant cell lines (MCF-7/ADR) while also being able to downregulate P-gp expression. The blank nanosystems were biocompatible in 3 cancer cell lines and in a healthy cell line while being highly cytotoxic to the resistant and non-resistant cell lines when co-delivering both therapeutics. The administration of nanosystems in MCF-7/MDR tumour-bearing mice significantly diminished tumour growth. The biosafety tests, which included a serum biochemistry assay, a complete blood count, and a hematoxylin and eosin (H&E) staining, revealed that the NB treated mice displayed similar levels as the PBS control group without histological damage to the major organs. Moreover, *in vivo* ultrasonic imaging revealed that the intratumorally injected DOX-NBs/PPP/P-gp shRNA could potentially be used as a contrast agent for US imaging[148].

In a similar study, paclitaxel (PTX) loaded NBs were assembled with Bcl-2 siRNA-complexed polymeric micelles to form a multifunctional US-sensitive nanocarrier for HCC treatment[149]. Promising *in vitro* and *in vivo* studies demonstrated the anti-cancer effect of the nanosystem when co-delivering both therapeutic cargos, inhibiting tumour growth, and downregulating the anti-apoptotic protein[149].

5.4 Combination of gene therapy and multimodal imaging

Although theranostic nanosystems hold the potential to be used for single imaging modalities, the incorporation of more than one probe may enable multimodal imaging, with all the advantages associated with it. Thus, in this section, some examples of multimodal and multifunctional theranostic nanosystems will be briefly described. It is worth mentioning that, due to their complexity, they will not be subdivided if other therapeutic strategies were introduced.

In addition to chemotherapy and PTT, gene therapy can also be combined with photodynamic therapy (PDT) for cancer treatment. Although being extensively reviewed, PDT can be briefly described as a treatment strategy that requires a light source, with the appropriate wavelength, to activate small molecules called photosensitizers (PSs), that will lead to the formation of reactive oxygen species (ROS) and consequently kill cancer cells[150–152]. In a recent study, a pH-responsive multimodal nanosystem was developed to co-deliver a photosensitizer chlorin e6 (Ce6), DOX, and a shRNA against P-gp for cancer treatment [153]. Here the core of the nanosystem, composed of Fe_3O_4–Au nanocrystals, allowed for MR and CT imaging, respectively. A mesoporous silica shell was used not only to wrap these crystals but also to encapsulate DOX and Ce6. This shell was further modified with a pH-sensitive polyelectrolyte, composed of biodegradable chitosan (CHI) and alginate (ALG), for pH-responsiveness and complexation of the shRNA (M-MSN(DOX/Ce6)/PEM/P-gp shRNA). The binding capabilities of the nanosystems improved when prepared with higher *w/w* ratios (M-MSN(DOX/Ce6)/PEM/P-gp shRNA), while the pH-responsiveness enabled the increased release of DOX and Ce6 when in acidic environments (pH 4.0). After laser irradiation, the M-MSN(DOX/Ce6)/PEM was able to generate ROS, in this case, the singlet oxygen (SO, $^1O_2$) and therefore be used for PDT. No hemolysis and cytotoxicity was observed when cells were incubated with the blank M-MSN/PEM. Uptake studies with MCF-7 cells confirmed the internalization of DOX and Ce6 when incubated with M-MSN(Dox/Ce6)/PEM nanoparticles. The highest antitumor effect, in three tumour cell lines (MCF-7, EMT-6, MCF-7/ADR), was observed when they were treated with M-MSN(Dox/Ce6)/PEM/P-gp shRNA + laser irradiation. Furthermore, *in vivo* EMT-6

tumour-bearing mice treated with the nanosystems + laser had the highest tumour growth suppression, which therefore confirmed the combined therapeutic effect. Major organ histology without inflammation or lesions demonstrated the biocompatibility of the nanosystems while T_2-weighted and CT images of MCF-7 cells incubated with the M-MSN(DOX/Ce6)/PEM nanoparticles demonstrated the potential dual-mode imaging of the formulation. In addition, xenografic mice intratumorally injected with M-MSN(DOX/Ce6)/PEM/P-gp shRNA displayed a negative/dark contrast in the tumour region[153].

Other imaging modalities, besides MRI and CT, could be combined for multimodal imaging and therapy. For example, Wu *et al* developed a targeted nanosystem for retinoblastoma (RB) therapy and dual-mode PAI/US imaging ability[154]. Here, a cationic lipid shell with conjugated folate was used to encapsulate liquid perfluorocarbon (PFC) and a NIR fluorophore, indocyanine green (ICG), for PA imaging. After this, the therapeutic plasmid DNA encoding HSV-TK was complexed to form the theranostic nanosystem (FCNPI/pDNA). Laser irradiation was used to trigger the liquid to gas phase transition of the PFC for US imaging, through a process known as optical droplet vaporization (Figure 13A). Dual-mode images *in vitro* demonstrated the potential use of the nanosystem as a contrast agent with a concentration-dependant signal increase, after laser irradiation. The nanosystems successfully loaded the pDNA while being stable and biocompatible towards the human retinoblastoma cell line Y79 RB. *In vitro* studies showed that the nanosystems could efficiently target the overexpressed receptors in the above cell line, the highest transfection being observed when cells were treated with FCNPI/pDNA + laser. *In vivo* biodistribution studies with fluorescently labelled FCNPI revealed that the targeting ligand enhanced the accumulation of the nanosystems in the tumour xenograft. Dual-mode images of mice injected with FCNPI, before and after laser irradiation, further demonstrated the importance of the targeting ligand, and of the laser irradiation to enhance the contrast capabilities of the nanosystems (Figure 13B, C). The highest therapeutic effects, *in vitro* and *in vivo*, were observed when treated with the targeted nanosystem + laser irradiation, which also resulted in the highest gene expression levels of TK. Biosafety tests revealed that the nanosystems displayed similar levels as the PBS control group

without histological damage to the major organs. In this study the authors further confirmed that ICG was not used to induce thermal ablation[154].

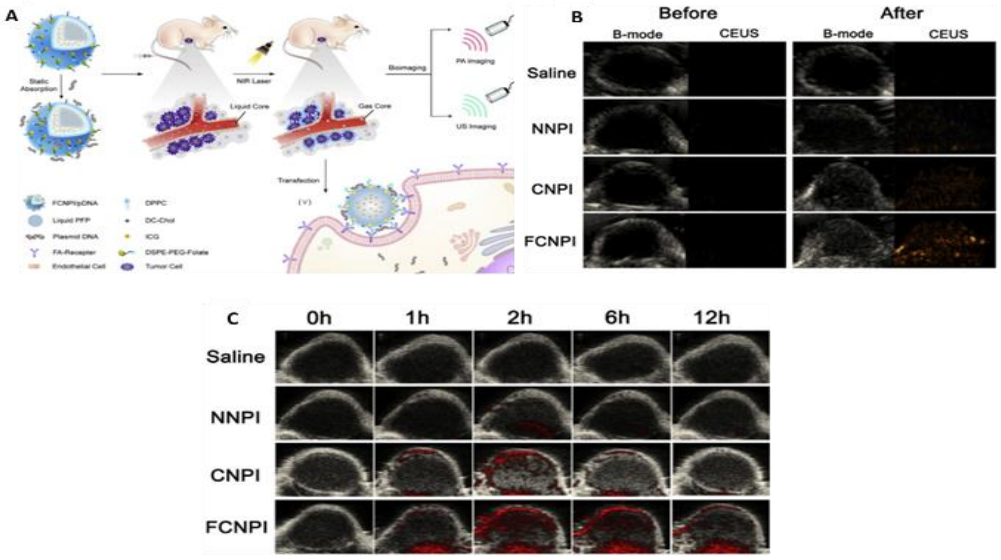

Figure 13-(A) Schematic illustration of the FCNPI theranostic system. (B) B-mode and CEUS imaging and (C) Photoacoustic imaging of the differently treated tumour-bearing mice, at various time points after laser irradiation, adapted from ref 154.

Recently Feng *et al* also developed a theranostic nanosystem formulation for cancer treatment that enabled PTT, siRNA delivery against Blc-2, and dual-mode PAI/ infrared (IR) imaging[155]. Synthesized polydopamine nanoparticles (PDAs) were used as a photothermal agent, as in a previously described MRI nanosystem[132], but also to allow dual-mode imaging. These were surface modified with a metal zeolitic imidazolate framework-8 (ZIF-8) to encapsulate the siRNA (PSZ). PSZ displayed a positive surface charge and a mean size of 176.8 nm. The pH-responsiveness of the nanosystem, due to the metal framework, enabled the increased release of the siRNA in acidic environments (pH 5.0). Recorded temperature variations and thermal images demonstrated that during laser irradiation PSZ displayed a similar increase in temperature as the PDAs and therefore could potentially be used for PTT. Uptake studies showed that fluorescently labelled NPs were able to accumulate in HeLa cells. The most efficient gene suppression, at the mRNA and protein levels, was observed when cells were treated with PSZ. Moreover, negligible *in vitro* cytotoxicity was observed when cells were treated with PSZ (without Blc-2 siRNA), while the highest therapeutic effect was observed when treated with PSZ and laser irradiation. *In vivo* PAI and IR thermal images demonstrated that PSZ

could accumulate in the tumour region and potentially be used as a dual contrast agent. *In vivo* tumour-bearing mice treated with PSZ + PTT significantly halted tumour growth, further demonstrating the efficient combined therapeutic effect of gene therapy and PTT. In addition, no hemolysis was observed, with blood biomarkers and histology sections of mice treated with PSZ being similar to that of mice treated with PBS[155].

In a similar manner, Liu *et a*l also combined gene therapy, PTT, and dual-mode PAI/IR imaging in a single nanoplatform [156]. However, synthesized gold nanoprisms (GNPs) were used instead of the above-mentioned melanin analogues (PDAs) to allow PTT and PAI/IR imaging. These were sequentially surface-decorated with negatively and positively charged polymers to enable the complexation of anti-human PD-L1 siRNA and to form the final nanoplatform, GNPs-hPD-L1 siRNA. Thermal images were used to demonstrate the photothermal ability of the nanosystem with a significant increase in temperature, after laser irradiation. Uptake studies, using a human lung cancer cell line (HCC827 cells) revealed that the nanosystems displayed similar internalization efficiencies as a commercially used agent. Meanwhile, *in vitro* and *in vivo* studies demonstrated that the nanosystems were able to efficiently silence hPD-L1 expression, demonstrated by the mRNA and protein levels, and that the highest therapeutic effect was observed when combined with the induced PTT. During *in vivo* treatment, thermal images were used to monitor temperature variations after laser irradiation, with GNPs-hPD-L1 siRNA and blank GNPs increasing tumour temperature to almost 42 ° C. Histology sections of the major organs were morphologically similar to that of mice treated with PBS. Additionally, before the *in vivo* therapeutic studies, photoacoustic images revealed that the nanosystems, after intravenous injection, could accumulate intratumorally [156].

6. Concluding remarks and future perspectives

To this date, there are no theranostic nanosystems in clinical trials. However, as demonstrated here, various gene therapy-based platforms are being developed and investigated for numerous therapeutic and imaging strategies. The nanomaterials chosen to design the final nanosystems may enhance the delivery of the therapeutic genetic material and/or other therapeutic molecules, provide targeting capabilities towards the tumour tissue, and enable monitorization through different imaging modalities, due to their intrinsic properties or by using encapsulated external probes. As such, these multifunctional and sometimes multimodal nanotheranostics hold great potential to be used in future clinical contexts, for example, to monitor the treatment application and therapeutic efficiency, and to evaluate the biodistribution. Nonetheless, to achieve clinical translation, some limitations regarding the toxicity and accumulation in the body need to be overcome with the design and combination of other materials or with improvements to existing nanosystems with promising results. To do so, these nanosystems need to be carefully characterized regarding their intrinsic properties, such as size, composition, structure, and surface charge.

In conclusion, and in order to push forward to clinical trials, knowledge regarding pharmacodynamics, pharmacokinetics and cellular interactions will be essential to design new and promising theranostic nanoplatforms, capable of both gene delivery and imaging in a clinical context.

7. References

1. Hossen, S. *et al.* Smart nanocarrier-based drug delivery systems for cancer therapy and toxicity studies: A review. *J. Adv. Res.* **15**, 1–18 (2019).
2. Senapati, S., Mahanta, A. K., Kumar, S. & Maiti, P. Controlled drug delivery vehicles for cancer treatment and their performance. *Signal Transduct. Target. Ther.* **3**, 1–19 (2018).
3. Li, W., Ng, J. M. K., Wong, C. C., Ng, E. K. W. & Yu, J. Molecular alterations of cancer cell and tumour microenvironment in metastatic gastric cancer. *Oncogene* **37**, 4903–4920 (2018).
4. Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA. Cancer J. Clin.* **68**, 394–424 (2018).
5. Fidler, M. M., Bray, F. & Soerjomataram, I. The global cancer burden and human development: A review. *Scand. J. Public Health* **46**, 27–36 (2018).
6. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2019. *CA. Cancer J. Clin.* **69**, 7–34 (2019).
7. Zhang, Q. Y., Wang, F. X., Jia, K. K. & Kong, L. D. Natural product interventions for chemotherapy and radiotherapy-induced side effects. *Front. Pharmacol.* **9**, (2018).
8. Wyld, L., Audisio, R. A. & Poston, G. J. The evolution of cancer surgery and future perspectives. *Nat. Rev. Clin. Oncol.* **12**, 115–124 (2015).
9. Tohme, S., Simmons, R. L. & Tsung, A. Surgery for cancer: A trigger for metastases. *Cancer Res.* **77**, 1548–1552 (2017).
10. Baskar, R., Lee, K. A., Yeo, R. & Yeoh, K. W. Cancer and radiation therapy: Current advances and future directions. *Int. J. Med. Sci.* **9**, 193–199 (2012).
11. El-Readi, M. Z. & Althubiti, M. A. Cancer Nanomedicine: A New Era of Successful Targeted Therapy. *J. Nanomater.* **2019**, (2019).
12. DeVita, V. T. & Chu, E. A history of cancer chemotherapy. *Cancer Res.* **68**, 8643–8653 (2008).
13. Yuan, R. *et al.* Natural products to prevent drug resistance in cancer chemotherapy: a review. *Ann. N. Y. Acad. Sci.* **1401**, 19–27 (2017).
14. Kreuter, J. Nanoparticles-a historical perspective. *Int. J. Pharm.* **331**, 1–10 (2007).
15. Li, H., Jin, H., Wan, W., Wu, C. & Wei, L. Cancer nanomedicine: Mechanisms, obstacles and strategies. *Nanomedicine* **13**, 1639–1656 (2018).
16. Bregoli, L. *et al.* Nanomedicine applied to translational oncology: A future perspective on cancer treatment. *Nanomedicine Nanotechnology, Biol. Med.* **12**, 81–103 (2016).
17. Shi, J., Kantoff, P. W., Wooster, R. & Farokhzad, O. C. Cancer nanomedicine:

Progress, challenges and opportunities. *Nat. Rev. Cancer* **17**, 20–37 (2017).

18. Wolfram, J. & Ferrari, M. Clinical cancer nanomedicine. *Nano Today* **25**, 85–98 (2019).

19. Tran, S., DeGiovanni, P.-J., Piel, B. & Rai, P. Cancer nanomedicine: a review of recent success in drug delivery. *Clin. Transl. Med.* **6**, (2017).

20. Salvioni, L. *et al.* Thirty years of cancer nanomedicine: Success, frustration, and hope. *Cancers (Basel).* **11**, (2019).

21. Wicki, A., Witzigmann, D., Balasubramanian, V. & Huwyler, J. Nanomedicine in cancer therapy: Challenges, opportunities, and clinical applications. *J. Control. Release* **200**, 138–157 (2015).

22. Anselmo, A. C. & Mitragotri, S. Nanoparticles in the clinic. *Bioeng. Transl. Med.* **1**, 10–29 (2016).

23. Mauricio, M. D. *et al.* Nanoparticles in medicine: A focus on vascular oxidative stress. *Oxid. Med. Cell. Longev.* **2018**, (2018).

24. El-Say, K. M. & El-Sawy, H. S. Polymeric nanoparticles: Promising platform for drug delivery. *Int. J. Pharm.* **528**, 675–691 (2017).

25. Yang, G., Phua, S. Z. F., Bindra, A. K. & Zhao, Y. Degradability and Clearance of Inorganic Nanoparticles for Biomedical Applications. *Adv. Mater.* **31**, 1–23 (2019).

26. Yingchoncharoen, P., Kalinowski, D. S. & Richardson, D. R. Lipid-Based Drug Delivery Systems in Cancer Therapy: What Is Available and What Is Yet to Come. *Pharmacol. Rev.* **68**, 701–787 (2016).

27. García-Pinel, B. *et al.* Lipid-based nanoparticles: Application and recent advances in cancer treatment. *Nanomaterials* **9**, 1–23 (2019).

28. Rai, R., Alwani, S. & Badea, I. Polymeric nanoparticles in gene therapy: New avenues of design and optimization for delivery applications. *Polymers (Basel).* **11**, (2019).

29. Singh, G., Faruk, A. & Bedi, P. M. S. Technology Overview and Current Biomedical Application of Polymeric Nanoparticles. *J. Drug Deliv. Ther.* **8**, 285–295 (2018).

30. H. R, C., Schiffman, J. D. & Balakrishna, R. G. Quantum dots as fluorescent probes: Synthesis, surface chemistry, energy transfer mechanisms, and applications. *Sensors Actuators, B Chem.* **258**, 1191–1214 (2018).

31. Xiao, Y. D. *et al.* MRI contrast agents: Classification and application (Review). *Int. J. Mol. Med.* **38**, 1319–1326 (2016).

32. Albanese, A., Tang, P. S. & Chan, W. C. W. The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems. *Annu. Rev. Biomed. Eng.* **14**, 1–16 (2012).

33. Zhao, J. & Stenzel, M. H. Entry of nanoparticles into cells: The importance of nanoparticle properties. *Polym. Chem.* **9**, 259–272 (2018).

34. Jo, D. H., Kim, J. H., Lee, T. G. & Kim, J. H. Size, surface charge, and shape determine therapeutic effects of nanoparticles on brain and retinal diseases.

Nanomedicine Nanotechnology, Biol. Med. **11**, 1603–1611 (2015).

35. Wei, Y., Quan, L., Zhou, C. & Zhan, Q. Factors relating to the biodistribution & clearance of nanoparticles & their effects on in vivo application. *Nanomedicine* **13**, 1495–1512 (2018).

36. Truong, N. P., Whittaker, M. R., Mak, C. W. & Davis, T. P. The importance of nanoparticle shape in cancer drug delivery. *Expert Opin. Drug Deliv.* **12**, 129–142 (2015).

37. Fröhlich, E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int. J. Nanomedicine* **7**, 5577–5591 (2012).

38. Madathiparambil Visalakshan, R. *et al.* The Influence of Nanoparticle Shape on Protein Corona Formation. *Small* **16**, (2020).

39. Partikel, K. *et al.* Effect of nanoparticle size and PEGylation on the protein corona of PLGA nanoparticles. *Eur. J. Pharm. Biopharm.* **141**, 70–80 (2019).

40. Thomas, O. S. & Weber, W. Overcoming Physiological Barriers to Nanoparticle Delivery—Are We There Yet? *Front. Bioeng. Biotechnol.* **7**, (2019).

41. Wakaskar, R. R. Passive and Active Targeting in Tumor Microenvironment. *Int. J. Drug Dev. Res.* **9**, 37–41 (2017).

42. Matsumura, Y. & Maeda, H. A New Concept for Macromolecular Therapeutics in Cancer Chemotherapy: Mechanism of Tumoritropic Accumulation of Proteins and the Antitumor Agent Smancs. *Cancer Res.* **46**, 6387–6392 (1986).

43. Attia, M. F., Anton, N., Wallyn, J., Omran, Z. & Vandamme, T. F. An overview of active and passive targeting strategies to improve the nanocarriers efficiency to tumour sites. *J. Pharm. Pharmacol.* **71**, 1185–1198 (2019).

44. Iyer, A. K., Khaled, G., Fang, J. & Maeda, H. Exploiting the enhanced permeability and retention effect for tumor targeting. *Drug Discov. Today* **11**, 812–818 (2006).

45. Muhamad, N., Plengsuriyakarn, T. & Na-Bangchang, K. Application of active targeting nanoparticle delivery system for chemotherapeutic drugs and traditional/herbal medicines in cancer therapy: A systematic review. *Int. J. Nanomedicine* **13**, 3921–3935 (2018).

46. Arranja, A. G., Pathak, V., Lammers, T. & Shi, Y. Tumor-targeted nanomedicines for cancer theranostics. *Pharmacol. Res.* **115**, 87–95 (2017).

47. Raza, F. *et al.* Cancer nanomedicine: focus on recent developments and self-assembled peptide nanocarriers. *J. Mater. Chem. B* **7**, 7639–7655 (2019).

48. Liu, M., Du, H., Zhang, W. & Zhai, G. Internal stimuli-responsive nanocarriers for drug delivery: Design strategies and applications. *Mater. Sci. Eng. C* **71**, 1267–1280 (2017).

49. Liu, D., Yang, F., Xiong, F. & Gu, N. The smart drug delivery system and its clinical potential. *Theranostics* **6**, 1306–1323 (2016).

50. Vander Heiden, M., Cantley, L. & Thompson, C. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science (80-.).* **324**, 1029–1033 (2009).

51. Hu, Q., Katti, P. S. & Gu, Z. Enzyme-responsive nanomaterials for controlled drug delivery. *Nanoscale* **6**, 12273–12286 (2014).

52. Yu, J., Chu, X. & Hou, Y. Stimuli-responsive cancer therapy based on nanoparticles. *Chem. Commun.* **50**, 11614–11630 (2014).

53. Han, L. *et al.* Redox-responsive theranostic nanoplatforms based on inorganic nanomaterials. *J. Control. Release* **259**, 40–52 (2017).

54. Wang, Y., Shim, M. S., Levinson, N. S., Sung, H. W. & Xia, Y. Stimuli-responsive materials for controlled release of theranostic agents. *Adv. Funct. Mater.* **24**, 4206–4220 (2014).

55. Kauscher, U., Holme, M. N., Björnmalm, M. & Stevens, M. M. Physical stimuli-responsive vesicles in drug delivery: Beyond liposomes and polymersomes. *Adv. Drug Deliv. Rev.* **138**, 259–275 (2019).

56. Yao, J., Feng, J. & Chen, J. External-stimuli responsive systems for cancer theranostic. *Asian J. Pharm. Sci.* **11**, 585–595 (2016).

57. Geers, B., Dewitte, H., De Smedt, S. C. & Lentacker, I. Crucial factors and emerging concepts in ultrasound-triggered drug delivery. *J. Control. Release* **164**, 248–255 (2012).

58. Satalkar, P., Elger, B. S., Hunziker, P. & Shaw, D. Challenges of clinical translation in nanomedicine: A qualitative study. *Nanomedicine Nanotechnology, Biol. Med.* **12**, 893–900 (2016).

59. Ginn, S. L., Amaya, A. K., Abedi, M. R., Alexander, I. E. & Edelstein, M. Gene therapy clinical trials worldwide to 2017 : An update. 1–16 (2018). doi:10.1002/jgm.3015

60. Jin, L., Zeng, X., Liu, M., Deng, Y. & He, N. Current progress in gene delivery technology based on chemical methods and nano-carriers. *Theranostics* **4**, 240–255 (2014).

61. Anguela, X. M. & High, K. A. Entering the Modern Era of Gene Therapy. *Annu. Rev. Med.* **70**, 273–288 (2019).

62. Adams, D. *et al.* Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N. Engl. J. Med.* **379**, 11–21 (2018).

63. Cross, D. & Burmester, J. K. Gene therapy for cancer treatment: Past, present and future. *Clin. Med. Res.* **4**, 218–227 (2006).

64. Caffery, B., Lee, J. S. & Alexander-Bryant, A. A. Vectors for glioblastoma gene therapy: Viral & non-viral delivery strategies. *Nanomaterials* **9**, (2019).

65. Wirth, T., Parker, N. & Ylä-Herttuala, S. History of gene therapy. *Gene* **525**, 162–169 (2013).

66. Steffin, D. H. M., Hsieh, E. M. & Rouce, R. H. Gene Therapy: Current Applications and Future Possibilities. *Adv. Pediatr.* **66**, 37–54 (2019).

67. Mu, W., Chu, Q., Liu, Y. & Zhang, N. A Review on Nano-Based Drug Delivery System for Cancer Chemoimmunotherapy. *Nano-Micro Lett.* **12**, (2020).

68. Riley, R. S., June, C. H., Langer, R. & Mitchell, M. J. Delivery technologies for cancer immunotherapy. *Nat. Rev. Drug Discov.* **18**, 175–196 (2019).

69. Ribas, A. & Wolchok, J. D. Cancer immunotherapy using checkpoint blockade. *Science (80-.).* **359**, 1350–1355 (2018).

70. Lawler, S. E., Speranza, M. C., Cho, C. F. & Chiocca, E. A. Oncolytic viruses in cancer treatment a review. *JAMA Oncol.* **3**, 841–849 (2017).

71. Rayburn, E. R. & Zhang, R. Antisense, RNAi, and gene silencing strategies for therapy: Mission possible or impossible? *Drug Discov. Today* **13**, 513–521 (2008).

72. Xu, X. *et al.* The role of MicroRNAs in hepatocellular carcinoma. *J. Cancer* **9**, 3557–3569 (2018).

73. Shah, M. Y., Ferrajoli, A., Sood, A. K., Lopez-Berestein, G. & Calin, G. A. microRNA Therapeutics in Cancer — An Emerging Concept. *EBioMedicine* **12**, 34–42 (2016).

74. Izmirli, M., Sonmez, D. & Gogebakan, B. The war against cancer: Suicide gene therapy. *Adv. Mod. Oncol. Res.* **2**, 139 (2016).

75. Reghupaty, S. C. & Sarkar, D. Current status of gene therapy in hepatocellular carcinoma. *Cancers (Basel).* **11**, (2019).

76. Al-Dosari, M. S. & Gao, X. Nonviral gene delivery: Principle, limitations, and recent Progress. *AAPS J.* **11**, 671–681 (2009).

77. Yin, H. *et al.* Non-viral vectors for gene-based therapy. *Nat. Rev. Genet.* **15**, 541–555 (2014).

78. Zheng, Q., Lin, D., Lei, L., Li, X. & Shi, S. Engineered Non-Viral Gene Vectors for Combination Cancer Therapy : A Review. **13**, 1565–1580 (2017).

79. Hardee, C. L., Arévalo-Soliz, L. M., Hornstein, B. D. & Zechiedrich, L. Advances in non-viral DNA vectors for gene therapy. *Genes* **8**, (2017).

80. Goker, F., Larsson, L., Del Fabbro, M., & Asa'ad, F. Gene Delivery Therapeutics in the Treatment of Periodontitis and Peri-Implantitis : A State of the Art Review. *Int. J. Mol. Sci.* **20**, 3551 (2019).

81. Ramamoorth, M. & Narvekar, A. Non viral vectors in gene therapy - An overview. *Journal of Clinical and Diagnostic Research* **9**, GE01–GE06 (2015).

82. Chen, W., Li, H., Liu, Z. & Yuan, W. Lipopolyplex for therapeutic gene delivery and its application for the treatment of Parkinson's disease. *Front. Aging Neurosci.* **8**, 1–10 (2016).

83. Rafael, D., Andrade, F., Arranja, A., Luís, S. & Videira, M. Lipoplexes and Polyplexes: Gene Therapy. *Encycl. Biomed. Polym. Polym. Biomater.* 4335–4347 (2015). doi:10.1081/e-ebpp-120050058

84. Duarte, S., Faneca, H. & Pedroso De Lima, M. C. Non-covalent association of folate to lipoplexes: A promising strategy to improve gene delivery in the presence of serum. *J. Control. Release* **149**, 264–272 (2011).

85. Leite Nascimento, T. *et al.* Hyaluronic acid-conjugated lipoplexes for targeted delivery of siRNA in a murine metastatic lung cancer model. *Int. J. Pharm.* **514**,

103–111 (2016).

86. Smith, S. A., Selby, L. I., Johnston, A. P. R. & Such, G. K. The Endosomal Escape of Nanoparticles: Toward More Efficient Cellular Delivery. *Bioconjug. Chem.* **30**, 263–272 (2019).

87. Bus, T., Traeger, A. & Schubert, U. S. The great escape: How cationic polyplexes overcome the endosomal barrier. *J. Mater. Chem. B* **6**, 6904–6918 (2018).

88. Zhang, X. X., McIntosh, T. J. & Grinstaff, M. W. Functional lipids and lipoplexes for improved gene delivery. *Biochimie* **94**, 42–58 (2012).

89. Lam, A. P. & Dean, D. A. Progress and prospects: Nuclear import of nonviral vectors. *Gene Ther.* **17**, 439–447 (2010).

90. Janib, S. M., Moses, A. S. & MacKay, J. A. Imaging and drug delivery using theranostic nanoparticles. *Adv. Drug Deliv. Rev.* **62**, 1052–1063 (2010).

91. Luk, B. T., Fang, R. H. & Zhang, L. Lipid- and polymer-based nanostructures for cancer theranostics. *Theranostics* **2**, 1117–1126 (2012).

92. Cagel, M., Grotz, E., Bernabeu, E., Moretton, M. A. & Chiappetta, D. A. Doxorubicin: nanotechnological overviews from bench to bedside. *Drug Discov. Today* **22**, 270–281 (2017).

93. Ehlerding, E. B., Grodzinski, P., Cai, W., & Liu, C. H. Big Potential from Small Agents: Nanoparticles for Imaging- Based Companion Diagnostics. *ACS Nano* **12**, 2106–2121 (2018).

94. Baetke, S., Lammers, T. & Kiessling, F. Applications of nanoparticles for diagnosis and therapy of cancer. *Br. J. Radiol.* **88**, (2015).

95. Mcphail, S., Johnson, S., Greenberg, D., Peake, M. & Rous, B. Stage at diagnosis and early mortality from cancer in England. *Br. J. Cancer* **112**, S108–S115 (2015).

96. Park, S. M., Aalipour, A., Vermesh, O., Yu, J. H., & Gambhir, S. S. Towards clinically translatable in vivo nanodiagnostics. *Nat. Rev. Mater.* **2**, 1–20 (2017).

97. Key, J. & Leary, J. F. Nanoparticles for multimodal in vivo imaging in nanomedicine. *Int. J. Nanomedicine* **9**, 711–726 (2014).

98. Shahbazi-Gahrouei D, Moradi Khaniabadi P, Moradi Khaniabadi B, S.-G. S. Medical imaging modalities using nanoprobes for cancer diagnosis: A literature review on recent findings. *J. Res. Med. Sci.* **24**, (2019).

99. Kamaly, N. & Miller, A. D. Paramagnetic liposome nanoparticles for cellular and tumour imaging. *Int. J. Mol. Sci.* **11**, 1759–1776 (2010).

100. Geraldes, C. F. G. C. & Laurent, S. Classification and basic properties of contrast agents for magnetic resonance imaging. *Contrast Media Mol. Imaging* **4**, 1–23 (2009).

101. Kasban, H., El-Bendary, M. A. M. & Salama, D. H. A comparative study medical imaging techniques. *Int. J. Latest Trends Eng. Technol.* **8**, (2017).

102. Li, X., Zhang, X., Li, X. & Chang, J. Multimodality imaging in nanomedicine and nanotheranostics. *Cancer Biol. Med.* **13**, 339 (2016).

103. Wahsner, J., Gale, E. M., Rodríguez-Rodríguez, A. & Caravan, P. Chemistry of MRI contrast agents: Current challenges and new frontiers. *Chem. Rev.* **119**, 957–1057 (2019).

104. Merbach, A., Helm, L. & Tóth, É. *The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging: Second Edition*. *John Wiley & Sons* (2013). doi:10.1002/9781118503652

105. Lusic, H. & Grinstaff, M. W. X-ray-computed tomography contrast agents. *Chem. Rev.* **113**, 1641–1666 (2013).

106. Pimlott, S. L. & Sutherland, A. Molecular tracers for the PET and SPECT imaging of disease. *Chem. Soc. Rev.* **40**, 149–162 (2011).

107. Weissleder, R. & Pittet, M. J. Imaging in the era of molecular oncology. *Nature* **452**, 580–589 (2008).

108. Haque, A., Faizi, M. S. H., Rather, J. A. & Khan, M. S. Next generation NIR fluorophores for tumor imaging and fluorescence-guided surgery: A review. *Bioorganic Med. Chem.* **25**, 2017–2034 (2017).

109. He, X., Gao, J., Gambhir, S. S. & Cheng, Z. Near-infrared fluorescent nanoprobes for cancer molecular imaging: Status and challenges. *Trends Mol. Med.* **16**, 574–583 (2010).

110. James, M. L. & Gambhir, S. S. A molecular imaging primer: Modalities, imaging agents, and applications. *Physiol. Rev.* **92**, 897–965 (2012).

111. Chong, W. K., Papadopoulou, V. & Dayton, P. A. Imaging with ultrasound contrast agents: current status and future. *Abdom. Radiol.* **43**, 762–772 (2018).

112. Kim, J., Lee, D., Jung, U. & Kim, C. Photoacoustic imaging platforms for multimodal imaging. *Ultrasonography* **34**, 88–97 (2015).

113. Xu, M. & Wang, L. V. Photoacoustic imaging in biomedicine. *Rev. Sci. Instrum.* **77**, (2006).

114. Liu, Y., Bhattarai, P., Dai, Z. & Chen, X. Photothermal therapy and photoacoustic imaging: Via nanotheranostics in fighting cancer. *Chem. Soc. Rev.* **48**, 2053–2108 (2019).

115. Lee, D. E. *et al.* Multifunctional nanoparticles for multimodal imaging and theragnosis. *Chem. Soc. Rev.* **41**, 2656–2672 (2012).

116. Lee, S. Y., Jeon, S. I., Jung, S., Chung, I. J. & Ahn, C. H. Targeted multimodal imaging modalities. *Adv. Drug Deliv. Rev.* **76**, 60–78 (2014).

117. Ahn, B. C. Personalized Medicine Based on Theranostic Radioiodine Molecular Imaging for Differentiated Thyroid Cancer. *Biomed Res. Int.* **2016**, (2016).

118. Kelkar, S. S. & Reineke, T. M. Theranostics: Combining imaging and therapy. *Bioconjug. Chem.* **22**, 1879–1903 (2011).

119. Silva, C. O. *et al.* Current Trends in Cancer Nanotheranostics: Metallic, Polymeric, and Lipid-Based Systems. *Pharmaceutics* **11**, 22 (2019).

120. Sharma, M. *et al.* Nanotheranostics, a future remedy of neurological disorders.

Expert Opin. Drug Deliv. **16**, 113–128 (2019).

121. Ryu, J. H. *et al.* Theranostic nanoparticles for future personalized medicine. *J. Control. Release* **190**, 477–484 (2014).

122. Sarcan, E. T., Silindir-Gunay, M. & Ozer, A. Y. Theranostic polymeric nanoparticles for NIR imaging and photodynamic therapy. *Int. J. Pharm.* **551**, 329–338 (2018).

123. Gao, X. *et al.* Biodegradable gadolinium-chelated cationic poly(urethane amide) copolymers for gene transfection and magnetic resonance imaging. *Mater. Sci. Eng. C* **65**, 181–187 (2016).

124. Luo, X. *et al.* Folic acid-functionalized polyethylenimine superparamagnetic iron oxide nanoparticles as theranostic agents for magnetic resonance imaging and PD-L1 siRNA delivery for gastric cancer. *Int. J. Nanomedicine* **12**, 5331–5343 (2017).

125. Wu, Z. *et al.* Magnetic cationic amylose nanoparticles used to deliver survivin-small interfering RNA for gene therapy of hepatocellular carcinoma in vitro. *Nanomaterials* **7**, 1–13 (2017).

126. Huang, R. Y. *et al.* Magnetic ternary nanohybrids for nonviral gene delivery of stem cells and applications on cancer therapy. *Theranostics* **9**, 2411–2423 (2019).

127. Lee, S. Y. *et al.* A theranostic micelleplex co-delivering SN-38 and VEGF siRNA for colorectal cancer therapy. *Biomaterials* **86**, 92–105 (2016).

128. Qiao, C. *et al.* Traceable Nanoparticles with Dual Targeting and ROS Response for RNAi-Based Immunochemotherapy of Intracranial Glioblastoma Treatment. *Adv. Mater.* **30**, 1–9 (2018).

129. Xie, X. *et al.* Polymeric Hybrid Nanomicelles for Cancer Theranostics: An Efficient and Precise Anticancer Strategy for the Codelivery of Doxorubicin/miR-34a and Magnetic Resonance Imaging. *ACS Appl. Mater. Interfaces* **11**, 43865–43878 (2019).

130. Jung, H. S. *et al.* Organic molecule-based photothermal agents: An expanding photothermal therapy universe. *Chem. Soc. Rev.* **47**, 2280–2297 (2018).

131. Zou, L. *et al.* Current approaches of photothermal therapy in treating cancer metastasis with nanotherapeutics. *Theranostics* **6**, 762–772 (2016).

132. Mu, X. *et al.* SiRNA Delivery with Stem Cell Membrane-Coated Magnetic Nanoparticles for Imaging-Guided Photothermal Therapy and Gene Therapy. *ACS Biomater. Sci. Eng.* **4**, 3895–3905 (2018).

133. Chang, D. *et al.* Biologically targeted magnetic hyperthermia: Potential and limitations. *Front. Pharmacol.* **9**, (2018).

134. Abenojar, E. C., Wickramasinghe, S., Bas-Concepcion, J. & Samia, A. C. S. Structural effects on the magnetic hyperthermia properties of iron oxide nanoparticles. *Prog. Nat. Sci. Mater. Int.* **26**, 440–448 (2016).

135. Wang, Z. *et al.* Shape-controlled magnetic mesoporous silica nanoparticles for magnetically-mediated suicide gene therapy of hepatocellular carcinoma. *Biomaterials* **154**, 147–157 (2018).

136. Etrych, T. *et al.* Fluorescence optical imaging in anticancer drug delivery. *J. Control. Release* **226**, 168–181 (2016).

137. Lee, J. *et al.* Optical Imaging and Gene Therapy with Neuroblastoma-Targeting Polymeric Nanoparticles for Potential Theranostic Applications. *Small* **12**, 1201–1211 (2016).

138. Kim, M. W. *et al.* Cancer-targeted Nucleic Acid Delivery and Quantum Dot Imaging Using EGF Receptor Aptamer-conjugated Lipid Nanoparticles. *Sci. Rep.* **7**, 1–11 (2017).

139. Kim, M. W. *et al.* Anti-EGF receptor aptamer-guided co-delivery of anti-cancer siRNAs and quantum dots for theranostics of triple-negative breast cancer. *Theranostics* **9**, 837–852 (2019).

140. Zheng, X. *et al.* Self-assembled dual fluorescence nanoparticles for CD44-targeted delivery of anti-miR-27a in liver cancer theranostics. *Theranostics* **8**, 3808–3823 (2018).

141. Liu, Y. *et al.* Theranostic near-infrared fluorescent nanoplatform for imaging and systemic siRNA delivery to metastatic anaplastic thyroid cancer. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 7750–7755 (2016).

142. Cui, Q. *et al.* Modulating ROS to overcome multidrug resistance in cancer. *Drug Resist. Updat.* **41**, 1–25 (2018).

143. Zhang, C. G. *et al.* Novel polymer micelle mediated co-delivery of doxorubicin and P-glycoprotein siRNA for reversal of multidrug resistance and synergistic tumor therapy. *Sci. Rep.* **6**, 1–12 (2016).

144. Guo, D. *et al.* Photostable and Biocompatible Fluorescent Silicon Nanoparticles for Imaging-Guided Co-Delivery of siRNA and Doxorubicin to Drug-Resistant Cancer Cells. *Nano-Micro Lett.* **11**, 1–13 (2019).

145. Zhao, G. *et al.* Targeted shRNA-loaded liposome complex combined with focused ultrasound for blood brain barrier disruption and suppressing glioma growth. *Cancer Lett.* **418**, 147–158 (2018).

146. Chang, E. L. *et al.* Angiogenesis-targeting microbubbles combined with ultrasound-mediated gene therapy in brain tumors. *J. Control. Release* **255**, 164–175 (2017).

147. Wang, P. *et al.* Ultrasound-responsive microbubbles for sonography-guided siRNA delivery. *Nanomedicine Nanotechnology, Biol. Med.* **12**, 1139–1149 (2016).

148. Yang, H. *et al.* Charge-reversal-functionalized PLGA nanobubbles as theranostic agents for ultrasonic-imaging-guided combination therapy. *Biomater. Sci.* **6**, 2426–2439 (2018).

149. Yin, T. *et al.* Tumor-penetrating codelivery of siRNA and paclitaxel with ultrasound-responsive nanobubbles hetero-assembled from polymeric micelles and liposomes. *Biomaterials* **35**, 5932–5943 (2014).

150. Mansoori, B. *et al.* Photodynamic therapy for cancer: Role of natural products. *Photodiagnosis Photodyn. Ther.* **26**, 395–404 (2019).

151. Xie, Z. *et al.* Emerging combination strategies with phototherapy in cancer nanomedicine. *Chem. Soc. Rev.* (2020). doi:10.1039/d0cs00215a

152. Kwiatkowski, S. *et al.* Photodynamic therapy – mechanisms, photosensitizers and combinations. *Biomed. Pharmacother.* **106**, 1098–1107 (2018).

153. Yang, H. *et al.* Chemo-photodynamic combined gene therapy and dual-modal cancer imaging achieved by pH-responsive alginate/chitosan multilayer-modified magnetic mesoporous silica nanocomposites. *Biomater. Sci.* **5**, 1001–1013 (2017).

154. Wu, M. *et al.* A laser-activated multifunctional targeted nanoagent for imaging and gene therapy in a mouse xenograft model with retinoblastoma Y79 cells. *Acta Biomater.* **70**, 211–226 (2018).

155. Feng, J. *et al.* Multifunctional siRNA-Laden Hybrid Nanoplatform for Noninvasive PA/IR Dual-Modal Imaging-Guided Enhanced Photogenetherapy. *ACS Appl. Mater. Interfaces* **12**, 22613–22623 (2020).

156. Liu, B. *et al.* Effects of gold nanoprism-assisted human PD-L1 siRNA on both gene down-regulation and photothermal therapy on lung cancer. *Acta Biomater.* **99**, 307–319 (2019).