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Preface

Cancer remains one of the leading causes of death worldwide, both in developed and in developing nations. It may affect people of all ages, even fetuses, but the risk for most cancer varieties increases with age. Current therapeutic approaches, which include surgery, chemotherapy, and radiotherapy, are associated with adverse side effects, arising from the lack of therapy specificity for tumor cells or tissues. Nanobiopharmaceuticals is the area that studies nanotechnology-based therapeutic agents and drug delivery systems. Drugs, nucleic acids, peptides, proteins, antibodies, and small molecules can be used in cancer therapeutic approaches, and their effect is potentiated when delivered in nanocarriers. The ability to differentiate malignant cells from nonmalignant cells and to selectively eradicate malignant cells is the central role of nanotechnology as it relates to cancer treatment. Moreover, abnormal epigenetic changes play a critical role in cancer development. In this way, nanotechnology can provide effective targets for cancer prognosis (contributing as models to understanding cancer development), diagnosis (as scaffolds to evaluate metastases), and therapy under different approaches.

Drug delivery is a widely explored strategy as a biomedical application in cancer therapy. Nanosystems and nanostructures based on a broad variety of materials (such as lipidic, polymeric, inorganic, among others) have been used to load/encapsulate drugs while promoting their targeted and tailored/controlled release. Pursuing this goal, scientists have focused their attention on the conception of stimuli-responsive, tunable, multitargeted, and codelivery systems as a powerful tool to deliver therapeutic molecules to their site of action. Several innovative strategies based on the delivery of monoclonal antibodies, proteins, and peptides resorting to nanotechnology have been developed for efficient cancer therapy. A relatively recent approach to cancer therapy involves the use of a natural process wherein coding or noncoding nucleic acids can regulate gene expression. Gene therapy has been explored to rearrange different genes and manipulate their functions providing the necessary information for gene correction, protein synthesis, and supplementation as well as for regulation of cell mechanisms. Mitochondrial gene therapy emerges as an exciting and promising technology to correct mitochondrial DNA mutations, by replacing mutated genes, therefore restoring the normal mitochondrial function.

Gene silencing using small RNA molecules shows potent ability in silencing oncogenic factors, has been considered as one of the most important breakthroughs and rapidly growing fields in science, and is used as a therapeutic approach against cancer and its progression to metastasis. Additionally, the combination of nanotechnology with chronobiology to adjust drug and/or gene delivery according to each patient's individual circadian rhythm emerges as a unique and original perspective in personalized cancer chronotherapy. Another approach is the human vaccination making use of nanosystems as immunopotentiators and delivery vehicles capable of eliciting humoral and cellular immunity against cancer. Nanovaccines have been characterized as methodologies of vaccination at the nanoscale, which could improve the stimulation of several immune system cells and immunomodulatory molecules to generate a potent antitumor immunity.

For an effective treatment, improved diagnostic and therapeutic techniques with minimal side-effects are required. An emerging trend in this direction is theranostics which represents a combinatorial diagnosis and therapeutic approach to cancer disease and aims to eliminate multistep procedures, reduce delays in treatment, and improves patient care. Nanoparticles, including fluorescent semiconductor nanocrystals (quantum dots) and magnetic nanoparticles, have proven their excellent properties for in vivo imaging techniques in a number of modalities such as magnetic resonance and fluorescence imaging, respectively. Nano-phototherapies (namely nano-photothermal, nano-photochemical, and nano-photoimmunological therapies) also bring strong potential for cancer treatment by inducing the local activation of nontoxic phototherapeutic agents upon light irradiation. Some innovative devices, such as microneedles, are in development to enhance cancer therapy applications.

Overall, this book presents an overview of research and innovation about the most potent cancer therapies, focusing on the role of nanotechnology as a powerful tool in diagnosis, imaging, and cancer treatment aiming to develop efficient, specific, and noninvasive approaches to restore the health and well-being of all cancer patients.

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Part I
Materials for Cancer Nanotechnology

Lipid Nanocarriers for Breast Cancer Treatment

Luciana B. Lopes, Alexsandra C. Apolinário, Giovanna C. Salata, Isabella D. Malagó, and Julia S. Passos

Abbreviations

A7RC	A7R-cysteine peptide
AUC	Area under the plasma concentration-time curve
DCIS	Ductal carcinoma in situ
DIM	3,3[1]-Diindolylmethane
DSPE	Distearoyl-sn-glycero-3-phosphorylethanolamine
EPR	Enhanced permeability and retention effect
ER	Estrogen receptor
FDA	Food and Drug Administration
HA	Hyaluronic acid
HER	Tyrosine-protein receptor kinase human epidermal growth factor
IV	Intravenous
LBL	Layer-by-layer coating
LCNP	Liquid crystalline nanoparticles
MDR	Multidrug resistance
ME	Microemulsions
NE	Nanoemulsions
NLC	Nanostructured lipid carriers
NRP-1	Neuropillin-1
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PG	Phosphatidylglycerol

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P-gp	P-glycoprotein
PI	Phosphatidylinositol
PLD	PEGylated liposomal doxorubicin
PR	Progesterone receptor
PS	Phosphatidylserine
SLN	Solid lipid nanoparticles
T_m	Phase transition temperature

1 Introduction

Breast cancer is the most prevalent type of cancer among women, except for non-melanoma skin cancer, and a frequent cause of cancer death among women (Winters et al., 2017). An estimate by the American Cancer Society and the Susan G. Komen Foundation shows that approximately 15% of women will be diagnosed with the disease in their lifetime. Data from GLOBOCAN (accessed in January 2022) demonstrate over 2 million cases diagnosed worldwide in 2020. Depending on their invasive characteristics, site of development, histology, and molecular profiling, several breast cancer classifications have been proposed. Based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), various targeted and efficient therapies were developed (Ensenyat-Mendez et al., 2021). The lack of expression of these receptors characterizes triple-negative breast cancer, which accounts for approximately 15% of the newly diagnosed breast cancer and is linked to the lack of effective treatments compared to other subtypes and poor prognosis (Łukasiewicz et al., 2021).

In addition to surgery, radiation therapy, oral estrogen receptor modulators, and a wide range of chemotherapies are available for adjuvant and neoadjuvant treatment, including taxanes and anthracyclines, especially in the case of invasive and metastatic cancer. HER2-positive and hormone receptor-positive tumors can be treated with targeted therapy consisting of monoclonal antibodies (such as trastuzumab), antibody-drug conjugates, and drugs that block cyclin-dependent kinases (such as palbociclib) (Jain et al., 2020). However, chemotherapy in general causes a large diversity of adverse effects that range in severity due to the lack of selectivity of most pharmacological agents. Another challenges relates to the acquisition of multidrug resistance (MDR), often related to the expression of efflux transporters, which reduces the effectiveness of single-dose treatments and chances of recurrence further complicate treatment (Muley et al., 2020).

To overcome these problems, several micro- and nanocarriers have been developed as drug delivery platforms. Since the approval of Doxil in 1995 by the US Food and Drug Administration (FDA), many other nanocarriers – which differ in composition and/or structure – were developed; several were approved since, and others are in clinical trials. In this chapter, we will focus on lipid nanocarriers for breast cancer treatment and theranostics and discuss the characteristics and properties that make them promising tools to improve therapy outcomes. This chapter will

be divided in systemic and local approaches, and for each of them, examples of lipid-based nanocarriers for treatment and diagnoses will be discussed. As lipid-based nanocarriers, we will consider systems that (i) contain lipids as structure-forming agent (such as liposomes and solid lipid nanoparticles) or (ii) contain lipids as one of the phases that form the nanocarriers (oil-in-water or water-in-oil dispersions, such as nanoemulsions).

2 Properties and Advantages of Lipid Nanocarriers for Breast Cancer Management

Being biocompatible and biodegradable, lipid-based nanocarriers have received increased attention due to their many advantages. These systems include liposomes, microemulsions, nanoemulsions, solid lipid nanoparticles, nanostructured lipid carriers, and liquid crystalline dispersions, among others. Nano- and microemulsions have also been classified as surfactant-based systems since surfactants are the structure-forming compounds, which may be of synthetic or natural origin (such as phospholipids) (Carvalho et al., 2019; Lopes, 2014). Several advantages of these systems are recognized. First, they can be obtained with a wide variety of biocompatible lipids, resulting in desirable safety profile (Apolinário et al., 2021a; Lopes, 2014). Second, their design may include lipids that confer specific properties, such as the ability to mimick cancer cell bilayers, improve uptake, and activate specific signaling cascades. For example, Abumanhal-Masarweh et al. demonstrated that the lipid composition of a nanoparticle affects its internalization into triple-negative breast cancer cells. The lipid headgroup was the main factor on cellular uptake; receptor-targeted headgroup and cationic amine headgroups were more efficient than zwitterionic (neutral) and negatively charged headgroups, while longer acyl chains facilitated liposomal uptake (Abumanhal-Masarweh et al., 2019).

Third, they can modify the pharmacokinetic profile of drugs, changing the distribution and the incidence of adverse effects. An improved passive targeting due to the enhanced permeability and retention (EPR) effect in tumors and other inflammation sites has been described, which is based on the leaky vasculature and lack of lymphatic drainage (Luan et al., 2021). In other words, the EPR effect represents a universal pathophysiological phenomenon that leads to the accumulation of macromolecules and nanocarriers in vascularized tumors. However, this concept has been challenged and is a matter of intense debate as the scientific community considered the role of tumor microenvironment and localized pressure, which would go against the relevance of the EPR effect for nanoparticle accumulation in tumors (Foulkes et al., 2020). Clinical studies based on theranostics have demonstrated that the EPR effect is present in human metastatic breast tumors, but largely variable (Lee et al., 2017). The strength of the EPR effect seems to depend on the type, blood flow, and localization of tumors; thus, several investigators advocate for the assessment of its contribution as part of a personalized approach for cancer treatment, identifying patients that would benefit from nanomedicine (Wu, 2021). Nevertheless,

nanoparticle accumulation has been described in several types of tumors and often leads to less drug amounts available in healthy tissues to produce off-target and adverse effects, improving the safety profile (Gabizon et al., 2014). This is especially important for antitumor drugs that generally present low selectivity.

Tumor targeting and selectivity can be further improved by functionalization of the nanocarrier surface: attaching ligands of highly expressed receptors of breast cancer cells (such as folic acid) or antibodies can improve targeting (Loureiro et al., 2015). In addition, surface modification with polyethylene glycol (PEG) increases the circulation time of nanocarriers, which has been described to aid tumor accumulation (Gabizon et al., 2014). It is important to mention that these potential benefits are not universal, but often dependent on the type of nanocarrier and tumor. For example, the clinical efficacy of Doxil® (PEGylated liposome containing doxorubicin) was superior than doxorubicin solution in AIDS-related Kaposi's sarcoma, but comparable in breast cancer (Harris et al., 2002; Northfelt et al., 1996). In addition, nanocarriers might have unique toxicity profiles as demonstrated by Doxil® ability to reduce cardiotoxicity but cause hand-foot syndrome (Luan et al., 2021).

Lipid nanocarriers have also been described to overcome biological barriers, as demonstrated in the skin, gastrointestinal mucosa, and blood-brain barrier (Apolinário et al., 2020a, 2021a; Carvalho et al., 2017b; Lin et al., 2016). This effect depends on the composition of the nanocarrier and its ability to improve the permeability of the biological barrier and to be transported by specialized mechanisms. For example, the presence of chemical penetration enhancers in nanoemulsions and liposomes has been described to improve drug delivery into the skin, which finds relevance for adjuvant treatment and prevention of recurrences using the breast skin as an administration route (Apolinário et al., 2020a; Mojeiko et al., 2019, 2021).

In addition, lipid-based nanocarriers are important tools to reverse MDR (Famta et al., 2021; Giacone et al., 2018; Han et al., 2018). MDR has been attributed to various mechanisms, including high expression of drug efflux transporter, modulation of cellular metabolism and anti-apoptotic proteins, alterations in DNA repair mechanisms, and chemical changes in target proteins/receptors, decreasing their affinity for the drug (Famta et al., 2021). Several efflux transporters have been identified, with breast cancer resistance protein (BCRP) and P-glycoprotein (P-gp) being two of the most common transporters in breast cancer cells. Nanocarriers have been demonstrated to reduce drug efflux depending on their composition and structure. For example, surfactants employed in nanodispersed lipid-based systems (like micro- and nanoemulsions) have been demonstrated to affect the membrane microviscosity and induce fluidization and, thus, conformational changes in P-gp and/or nonspecific steric hindrance of the drug-binding sites (Batrakova et al., 2004). Additionally, because they enable co-encapsulation of compounds with distinct physicochemical properties, it is possible to combine specific inhibitors of efflux transporters (such as elacridar) with antitumor agents, modify drug transport and distribution, and enhance efficacy in resistant cells (Giacone et al., 2018; Grabarnick Portnoy et al., 2021).

These properties will be further discussed in the following sessions in the context of specific types of nanocarriers.

3 Lipid-Based Nanocarriers for Breast Cancer: Approved and Under Development

The nanomedicine is a rapidly evolving field driven by innovations related to drug development and repurposing, delivery strategies, and treatment modalities. However, the approval process of nanotechnology-based products is lengthy with little regulatory guidance (Foulkes et al., 2020). Thus, before discussing new developments in lipid-based nanocarriers for breast cancer, it is important to consider approved nanomedicines and their use. A recent manuscript by Anselmo and Mitragotri accounts for over 30 nanomedicines approved by the FDA or European Medicines Agency since 1995 (Anselmo & Mitragotri, 2021).

Seven of those are lipid-based systems for cancer treatment, and one of them received FDA indication approval specifically for breast cancer: Myocet® is composed of 150 nm egg phosphatidylcholine/cholesterol (55:45 mole percent) liposomes containing doxorubicin. Nevertheless, other approved nanomedicines have been employed for breast cancer (in trials and off-label use) and demonstrated efficacy. The PEGylated liposomal doxorubicin Doxil® (United States) and Caelyx® (in Europe, Canada, and other countries) were approved for the treatment of AIDS-related Kaposi's sarcoma in 1995 and 1996, respectively (Jiang et al., 2017). Although the first indication of these liposome-based doxorubicin was sarcoma, their clinical application has been extended to metastatic breast cancer (Lee, 2019). Lipusu® (liposomal paclitaxel) was available for breast cancer treatment in China in 2003 (Apolinário et al., 2021a). Table 1 summarizes the main aspects of the liposomes approved to treat breast cancer.

There is a vast literature with new nanocarriers under development, in preclinical and clinical evaluation for the treatment of breast cancer. In the next sessions, new lipid-based nanocarriers aiming breast cancer treatment will be discussed based on

Table 1 Approved liposomes for breast cancer therapy

Marketed product/ regulatory agency	Formulation
Doxil/Caelyx® (FDA/EMA)	Formulation for intravenous infusion of doxorubicin hydrochloride encapsulated in PEGylated liposomes, composed of N-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3--phosphoethanolamine sodium salt, soybean phosphatidylcholine, and cholesterol
Myocet® (EMA)	Extemporaneous preparations for infusion of doxorubicin encapsulated in liposomes formed by egg phosphatidylcholine and cholesterol. The formulation consists of a three-vial system: Vial 1 – red lyophilized powder of doxorubicin HCl Vial 2 – white to off-white and homogeneous liposomal dispersion Vial 3 – buffer is a clear colorless solution/infusion
Lipusu® (Regulatory agency from China)	Formulation of paclitaxel encapsulated in liposomes composed of lecithin and cholesterol lyophilized powder for intravenous infusion.

their properties, advantages, and routes of administration. Three main routes will be included: intravenous, oral, and local in the breasts, which include subcutaneous (in and above the mammary tissue), topical (on the breast skin and nipples), and intraductal (in the ductal system).

4 Nanocarriers for Parenteral Administration

Intravenous (IV) administration provides rapid action of compounds and preserves stability, since it avoids oral digestion and first-pass metabolism and allows a personalized control of therapy (Parker & Davey, 1992). However, challenges in cancer IV chemotherapy include severe side effects and cancer recurrence (Chenthamara et al., 2019b). Many types of nanocarriers defined by 20–1000 nm structures have been shown to not only target the delivery and site of action of a drug but also decrease systemic acute toxicity, creating more tolerable therapy. Besides, nanocarriers can be designed to avoid opsonization or macrophage uptake in the intravenous route, prolonging its circulation and action (Chenthamara et al., 2019b). In the following subsections, the main types of conventional and targeted lipid-based nanocarriers studied for IV treatment of breast cancer will be introduced and be discussed.

4.1 Liposomes

Liposomes are lipid vesicles that consist of one or more concentric bilayers entrapping an aqueous core. Most often, they are classified according to the number of bilayers, but other criteria may be used, such as composition of the bilayer (Apolinário et al., 2021a). They represent the majority of nanomedicines approved for clinical use (Crommelin et al., 2020), including for breast cancer therapy with or without metastasis. Some of the most cited advantages of liposomes are biocompatibility (Mallick & Choi, 2014), possibility of co-delivery of hydrophilic drugs into the aqueous core and hydrophobic drugs into the bilayer (Joshi et al., 2016), well-known manufacturing process (Shah et al., 2020), and relevant regulatory discussions with a draft guidance from the FDA (Schlich et al., 2021).

From the simplest viewpoint, liposomes are vesicular structures (Fig. 1a). They can be formed from one lipid bilayer surrounding an aqueous core, which are classified as unilamellar and lead to minimum clearance especially for vesicles of less than 200 nm (Paliwal et al., 2011) (Fig. 1b), or be structured as a concentric sequence of multiple bilayers, being categorized as multilamellar vesicles (FDA, 2018). They may also be organized as a set of tightly packed non-concentric vesicles (Deng et al., 2016), which display much larger sizes (~3–30 μm) and are suitable to form depots for sustained-release drug delivery and utilized by non-vascular routes (Mantripragada, 2002).

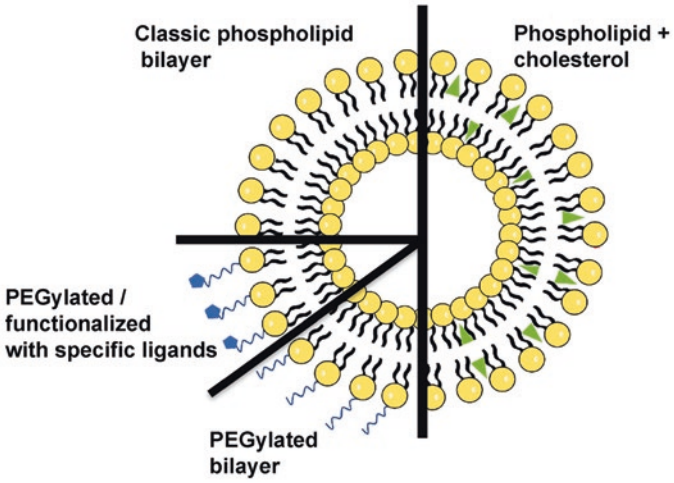

Fig. 1 Schematic representation of lipid vesicles. The figure depicts a vesicle formed by a classic bilayer (composed of classical phospholipids), bilayer formed by phospholipids and cholesterol, PEGylated and functionalized lipid bilayer. (Figure drawn using the image bank: Servier Medical Art by Servier, licensed with CC BY 3.0)

Cholesterol is frequently added to bilayers to improve the stability of phospholipids employed as structure-forming agents (Apolinario et al., 2017), helping to control fluidity, permeability, elasticity, strength, retention of encapsulated compounds, and stability (Nakhaei et al., 2021). The chemical versatility of the lipids enables the formation of complex liposomes with sophisticated physicochemical and biological properties that may optimize the outcomes for breast cancer therapy (Singh et al., 2021b). Drug-loaded liposomes can overcome the heterogeneity of the vasculature and high interstitial pressures in necrotic zones of tissue in solid tumors and deliver drugs to these sites (Park, 2002). Likewise, liposomes enable immunotherapy of breast cancer since they are useful for the encapsulation and protection of RNA and proteins (Apolinário et al., 2020b).

The main lipids employed to self-assemble liposomes are phospholipids classified as glycerophospholipids and sphingomyelins (Fig. 1c). Glycerophospholipids are composed of a glycerol backbone esterified with fatty acids that may differ in length and degree of saturation and may present distinct polar headgroups like choline for phosphatidylcholine (PC), ethanolamine for phosphatidylethanolamine (PE), glycerol for phosphatidylglycerol (PG), serine for phosphatidylserine (PS), and inositol for phosphatidylinositol (PI) as well as comprise a lipophilic tail (Li et al., 2015).

Charge and phase transition temperature (T_m) are key phospholipid properties for the design of liposomes and to ensure that they reach efficacy and safety for breast cancer treatment. Phospholipids can be zwitterionic and display a neutral net charge at physiological pH and be positively or negatively charged depending on the type of polar headgroup and pH of the medium (Drescher & Van Hoogevest, 2020).

Liposomes can also be formed from lipids other than the classical phospholipids such as fatty acids and other lipids synthetically obtained or found in natural sources like bacteria (e.g., archaeal lipids, found in Archaebacteria) (Apolinário et al., 2021a). Likewise, lipids can be modified with PEG or antibodies coupled to phospholipid headgroups for longer circulation time and active targeting (Sapra & Allen, 2003) or contain specific groups for stimuli-triggered drug delivery (Antoniou et al., 2021). These approaches will be better discussed in the next subsections.

Several studies have demonstrated the therapeutic advantages of modifying liposomes taking into consideration the tumor microenvironment of breast cancer (Dumont et al., 2019). The complex tumor microenvironment is a key aspect of tumor progression and affects the response to treatment, suggesting that the antitumor effects of several agents seem to be mediated through microenvironmental properties (Place et al., 2011). These properties encompass physiopathological phenomena such as hypoxia, acidosis, and abnormal expression of extracellular matrix-related proteins and enzymes (Fig. 2).

4.1.1 Co-encapsulation of Drugs

The use of liposomes to co-deliver drug combinations to cancer has led to pronounced advantages compared with monotherapy, enabling potentiation of therapeutic efficacy and reversal of MDR (Mo et al., 2014). For example, a co-encapsulation

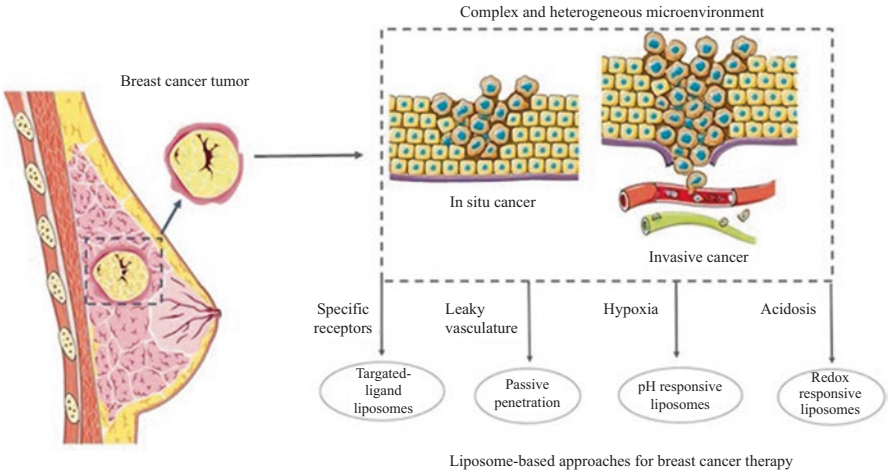

Fig. 2 Importance of the complex and heterogeneous microenvironment of breast cancer on the choice of liposome-based treatment strategy. The overexpression of the receptors on tumor cell surface can be targeted using functionalized liposomes. Passive transport through the leaky vasculature and tumor accumulation have been known as a central dogma for nanomedicine, although some studies have undermined the relevance of this mechanism. Physiopathological changes at the tumor microenvironment, such as hypoxia and acidosis, can trigger drug release from responsive liposomes. (Figure drawn using the image bank: Servier Medical Art by Servier, licensed with CC BY 3.0)

of vincristine and quercetin (at 2:1 molar ratio) in liposomes enhanced the antitumor activity in trastuzumab-resistant breast cancer and prolonged plasma circulation of the two drugs, further inhibiting tumor growth compared to the free drugs (Wong & Chiu, 2011).

Co-encapsulation of P-glycoprotein (P-gp) inhibitors in liposomes has been investigated. P-gp is encoded by MDR genes and reduces the intracellular accumulation of drugs, such as doxorubicin, performing a substantial role in resistance to chemotherapy (Nielsen et al., 1996). Co-encapsulation of doxorubicin and the P-gp inhibitor fluoxetine in stealth liposomes was performed using an ammonium sulfate gradient, resulting in an improved cell cytotoxicity compared to doxorubicin only in liposomes in vitro (Ong et al., 2011). In addition, pharmacokinetics data evidenced the potential of co-encapsulation to extend the half-life of the compounds and reduce systemic toxicity (Ong et al., 2011).

4.1.2 Long-Circulating Liposomes

It is crucial to reduce liposome uptake by the reticuloendothelial system, and, thus, PEG-coated liposomes have a crucial role to escape from the immune system and promote the accumulation of drugs in tumors (Symon et al., 1999). They are also called PEGylated, long-circulating, sterically stabilized, or "stealth liposomes"; this last term has been used in allusion to stealth bombers that can evade radar (Cattel et al., 2003). Both hydrophobic and electrostatic interactions at the liposome surface with a variety of blood components are sterically inhibited by the hydrated groups PEG (Gabizon, 2001b). The modulated biodistribution and pharmacokinetics markedly mitigate many of the toxicity of bolus drug administration (Park, 2002).

Stealth liposomes are formed by PEGylated lipids. For example, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol and soybean phospholipids (with 75% phosphatidylcholine) were employed in liposomes to encapsulate cisplatin. Addition of 5% PEG to these liposomes led to smaller diameter and higher drug encapsulation. The formulation increased the therapeutic index, reduced the required drug dose, and improved the cytotoxic effect compared to the free drug (Ghafari et al., 2020). Although PEG is the most frequent polymer employed for coating, other polymers have also been shown to protect liposomes from opsonization and prolong their circulation time (Cattel et al., 2003).

4.1.3 Stimulus-Responsive Liposomes

As it was previously reported, liposomes can be designed using lipids that respond to (i) stimulus from the tumor microenvironment, such as pH, redox potential, or presence of enzymes, and (ii) external stimulus, like increases in temperature. For example, lysolipids facilitate transient pore formation in lipid bilayers around T_m, promoting a faster drug release.

To promote thermoresponsive therapy to breast cancer, hyperthermia, i.e., tumor heating to a temperature ~ 40–43 °C, triggers increased uptake of liposomes under a suitable amount of time (Paliwal et al., 2011). To explore this possibility, liposomes of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and monopalmitoyl-2-hydroxy-sn-glycero-3-phosphocholine were obtained for the co-delivery of tamoxifen and imatinib. Release of over 80% of the drug in 30 min was observed above the transition temperature (39.4 °C), and the system reduced the viability of MCF-7 and MDA-MB-231 breast cancer cells (Jose et al., 2019). The liposomal doxorubicin ThermoDox® was developed for this goal. ThermoDox might be intravenously administered in combination with hyperthermia (caused by microwave or radiofrequency ablation), which promotes drug release at 39.5–42 °C. ThermoDox® is undergoing various phases of clinical trials: phase I for locally recurrent breast cancer and metastatic breast cancer and phase II for recurrent regional breast cancer and loco-regional relapse breast cancer (Jiang et al., 2022).

In addition to temperature variations, responsiveness to pH has been explored. The tumor microenvironment presents lower pH when compared to healthy tissue due to the production of lactic acid and hydrolysis of ATP, and a low pH is also found in the endosomal and lysosomal compartments within tumor cells (Gerweck & Seetharaman, 1996). Since lipids may self-assemble into different nanostructures according to the microenvironment pH, which influence net balance of intermolecular forces established by van der Waals, electrostatics, and hydrogen bonding, liposomes can be obtained using lipids that are stable at neutral environment but become soft and leaky upon pH reduction (Karve et al., 2009). Promising results have been published. For instance, the hydrazone bond presents high pH sensitivity and was used to produce pH-sensitive liposomes containing paclitaxel. These liposomes also included the R8 peptide, which is exposed after hydrolysis of hydrazone moiety under the low extracellular pH of tumors, leading to internalization mediated by R8 peptide (Zhang et al., 2015). The findings demonstrated high accumulation and internalization in breast cancer cells (Zhang et al., 2015).

Redox environment in the cytoplasm is another strategy that can trigger redox activation of liposomes formed by lipids with disulfide linkages, which can be disrupted by powerful thiolytic reducing agents (Lee & Thompson, 2017). Liposomes obtained with a redox-sensitive cationic oligopeptide lipid, natural soybean phosphatidylcholine, and cholesterol were employed for the co-delivery of paclitaxel and anti-survivin siRNA for the synergistic treatment of breast cancer and metastasis (Fouladi et al., 2017). This strategy resulted in rapid redox-responsive release of both molecules and a synergistic inhibitory effect on tumor growth.

Enzymes like phospholipase A2, matrix metalloproteinases, urokinase plasminogen activator, elastase, and cathepsin B are overexpressed in several cancer types, including breast cancer, and can be explored to trigger drug release and cytotoxic effects of liposomes (Fouladi et al., 2017).

4.1.4 Ligand-Targeted Liposome

The use of ligand-targeted liposomes is another strategy to improve tumor targeting and intracellular uptake. Antibodies, peptides, proteins, carbohydrates, and various other small molecules have been employed as ligands (Noble et al., 2014). Tumor vasculatures, stroma, cells, and the immune system can be sites for active targeting (Jiang et al., 2022).

For breast cancer, there are many possible options for ligands. One example is transferrin (Shindelman et al., 1981), which promotes iron uptake and is essential to the growth of breast cancer cells (Rychtarcikova et al., 2017). Another example is a folate, since folic acid receptors are preferentially expressed in cancers of epithelial origin (Hartmann et al., 2007). One can still cite ligands for CD44, which is a cell surface transmembrane protein and the main receptor for hyaluronic acid (McFarlane et al., 2015); it is overexpressed in breast cancer cells and associated with enhanced invasive properties and distant metastasis (Sheridan et al., 2006). To take advantage of these features, liposomes were conjugated with transferrin and promoted sirolimus active targeting of breast cancer cells. The intravenous injection of these liposomes resulted in superior antitumor effect with significant suppression of its growth compared to the free drug and non-conjugated stealth liposomes (Nandi et al., 2021). Higher tumor uptake and antitumor activity were observed by designing folate-coated, long-circulating, and pH-sensitive liposomes loading doxorubicin (de Oliveira Silva et al., 2019). Cationic liposomes loading curcumin and celecoxib for breast cancer treatment were coated on the surface with hyaluronic acid (HA) as CD44 targeting moiety and, after systematical administration in 4T1 breast tumor-bearing mice, exerted the most prominent effects on anti-inflammation, inhibition of macrophage recruitment, and antitumor effects compared to other liposomes (Sun et al., 2019).

Somatostatin receptor is also overexpressed in breast cancer cells. Pharmacokinetic studies performed with diacerein-loaded liposomes decorated with a synthetic analog of somatostatin demonstrated longer circulation time and improved the antiproliferative and anti-angiogenic effects of the drug (Bharti et al., 2017). Modified paclitaxel-loaded liposomes containing A7R-cysteine peptide (A7RC) on the surface were designed since the A7R peptide is a ligand of the NRP-1 receptor, which regulates the intracellular signal transduction related to tumor vascularization and tumor growth (Cao et al., 2015a). These liposomes enhanced cell uptake and showed improved cytotoxicity in vitro and higher accumulation in vivo (Cao et al., 2015a).

Targeting organelles is an additional approach explored in the liposomes field. Liposomes loading sunitinib and vinorelbine were produced with D-α-tocopheryl PEG 1000 succinate-triphenylphosphine conjugate, which presents a mitochondrial targeting effect. These vesicles presented prolonged blood circulation, and their uptake resulted in accumulation in the mitochondria of breast cancer cells (Shi et al., 2015).

4.1.5 Liposome-Based Gene Therapy

The development and high heterogeneity observed in breast cancers can also be related to gene mutations; thus, gene therapy has been proposed for the treatment of subtypes of breast cancer, especially triple negative. However, gene therapy is challenging mainly due to the low stability of the macromolecules, low expression after transfection, and high immunogenicity (Kaneda, 2001). Liposomes have been employed for the encapsulation of plasmids, oligonucleotides, and RNA based on the formation of a complex between vesicles (containing cationic lipids) and anionic macromolecules, which have also been referred to as lipoplex (Paliwal et al., 2011).

Many examples for breast cancer are reported in the literature. For example, liposomes complexed to plasmids to encode angiostatin or endostatin inhibited angiogenesis and the growth of MDA-MB-435 tumors. Injections of these liposomes reduced nearly 40% tumor growth in nude mice (Chen et al., 1999). Stealth liposomes produced with 1,2-dipalmitoyl-Sn-glycero-phosphatidylethanolamine--linked PEG moiety and conjugated at the distal end with 17-ß-estradiol (which acts as a targeting ligand for estrogen receptors) were loaded with pCMV-p53 DNA and induced cell death in breast cancer cells (Reddy & Banerjee, 2005).

4.1.6 Liposomes for Theranostics

Another strategy that has been receiving considerable attention is the application of nanocarriers to theranostics, which combines treatment and diagnosis. A variety of nanocarriers have been developed as they can deliver chemotherapy and contrast agents (such as radionuclides), enabling the monitoring of tumor growth/metastasis and treatment, contributing to personalized medicine. Liposomes have been employed for diagnostic and theranostic purposes, mainly to overcome drawbacks of imaging contrasting agents and nanoparticles, such as low tumor targeting ability due to short circulation time.

Jeon et al. (2021) reported a multifunctional dual-layered nanomaterial for theranostic and photothermal therapy, obtained by adding liposomal layer to liposomes coated with gold nanoparticles and modifying this external layer with ^{64}Cu to assess tumor accumulation. Shen et al. developed a liposome-based theranostic system for ruthenium polypyridine complex. A fluorescent signal is emitted when the complex is incorporated in lipid bilayers or DNA helix, which is useful for tumor imaging (Shen et al., 2017). Their use for the treatment of MDA-MB-231 breast cancer cells promoted double-strand DNA breaks and apoptosis and reduced tumor growth in a triple-negative breast cancer model, with over 20% of the dose accumulating in the tumor. Hybrid liposomes composed of L-α-dimyristoylphosphatidylcholine and polyoxyethylene dodecyl ether were used to incorporate indocyanine green and demonstrated enhanced tumor localization in orthotopic breast cancer model in mice (MDA-MB-453) (Ichihara et al., 2018). Melanin-based liposomes were developed to simultaneously enable photoacoustic and T1-weighted magnetic resonance imaging-guided photothermal ablation of tumors (Zhang et al., 2018). IRDye800CW

and ^{64}Cu were linked to liposomes loading doxorubicin and modified with PD-1 antibody (Du et al., 2017). The system enabled the visualization of 4-T1 tumors using NIRF/PET imaging and inhibited tumor growth.

To assess the EPR effect in the tumor accumulation of nanocarriers and identify patients who could benefit from the use of nanomedicine, HER-2-targeted and ^{64}Cu-labeled PEGylated liposomes encapsulating doxorubicin were administered in 19 patients with HER2-positive breast cancer (Lee et al., 2017). They were subjected to 2–3 PET/CT scans to assess distribution. Uptake of the liposomes was observed in the liver and spleen. In tumors, accumulation at 24–48 h varied 35-fold. Consistent with the existence of the EPR effect of nanoparticles, accumulation of the liposomes in non-tumor tissues was not observed; however, the results suggested the variable nature of the process. The authors suggested the feasibility of using tumor deposition of nanoparticles to identify patients that may benefit from nanomedicine. Identification of new and improved diagnostic imaging agents to enable accurate assessment of tumor delivery is necessary.

4.2 Solid Lipid Nanoparticles and Nanostructured Lipid Carriers

Solid lipid nanoparticles (SLN) are composed of a solid lipid core dispersed in aqueous phase by surfactants, giving the matrix a protective characteristic against chemical and UV degradation, as well as the property to modify drug release profiles (Apolinário et al., 2020b). Some authors relate SLN core to an alluring structure for co-delivering microRNA (miRNA), siRNA, plasmid DNA (pDNA), and antineoplastic drugs, such as paclitaxel, a potent microtubule stabilizing taxane employed as first-line therapy for breast cancer since the 1990s. The Yu group studied the application of the SLN as a co-delivery agent of paclitaxel and a model plasmid DNA (pDNA) expressing only EGFP – a green fluorescent compound – aiming at a new IV platform for breast cancer treatment (Yu et al., 2016). Thereunto, SLN was prepared by dispersion-sonication, and functionalized with hyaluronic acid using 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE) through anhydride linkage. The authors reported that the gene was delivered efficiently in the MCF-7 breast cancer cell line monolayer; and the uptake of HA-paclitaxel/pDNA SLN was 30% and 40% higher ($p < 0.05$) than paclitaxel/pDNA SLN and pDNA reagent solution, respectively. Likewise, cellular viability decreased approximately 29% after a 48 h exposure to 20 nM- HA-paclitaxel/pDNA SLN compared to a paclitaxel solution. In an in vivo xenograft model, MCF-7-inoculated mice were submitted to an i.v. injection of the formulations containing paclitaxel at 0.1 mg/kg every 3 days from day 6 to day 18. The inhibition of the tumor weight in HA-paclitaxel/pDNA SLN group was 52% higher than the paclitaxel solution group and 9% higher than paclitaxel/pDNA SLN group, demonstrating the potential of this delivery system for drug and gene co-delivery therapy.

In a similar manner, Wang and collaborators developed a HA-paclitaxel-SLN by hot homogenization and observed that, in MCF-7 tumor-bearing mice model, i.v. treatment with the loaded SLN reduced tumor growth rate in 2.7-fold compared to paclitaxel solution, maintaining it stable up to 14 days (Wang et al., 2017). The HA-paclitaxel-SLN-treated group also presented the higher drug concentration in plasma and on tumor site and exhibited maximum area under the plasma concentration-time curve and mean residence time, indicating that the system could serve as a potential treatment for breast cancer.

Nanostructured lipid carriers (NLC) are a modification of SLN, with an adjustment on the lipid core: both solid and liquid lipids are present, increasing the number of imperfections in the internal matrix and, thus, facilitating the incorporation of a greater amount of drug and preserving physical stability of the nanocarrier (Apolinário et al., 2020b). With this reasoning, Pedro et al. optimized NLCs intended for breast cancer IV administration that could incorporate paclitaxel in higher amounts (Pedro et al., 2019). The group reported that in a mixture of glyceryl behenate, medium-chain triglyceride, and polysorbate 80 composition, the nanocarrier containing the drug presented a more monodisperse size distribution as the solid lipid concentration increased. Also, the lipid matrix was shown to saturate, since 1 mg/mL of paclitaxel could reach about 93% of encapsulation efficiency, but increasing the drug concentration would not increase the amount of encapsulation. Besides, the NLC-paclitaxel demonstrated lower systemic toxicity, higher antineoplastic activity, and tumor accumulation in Ehrlich tumor-bearing Swiss mice.

Li and collaborators also took advantage of NLC features and developed a nanocarrier containing lapachone (a therapeutic agent reported to downregulate P-gp expression) and doxorubicin (an anthracycline anticancer drug utilized in the management of several types of cancer) as a new platform to overcome MDR associated with breast cancer treatment (Li et al., 2018). The authors prepared the nanocarrier by ultrasonic dispersion and modified the glyceryl behenate, soybean phosphatidylcholine, glycerin monostearate-containing NLC surface with PEG, to increase the circulation time and augment tumor retention. The release of both drugs encapsulated in the NLC reached 80% in 48 h, demonstrating a more prolonged release than the solution. In terms of cellular uptake, the formulation containing both drugs could successfully deliver doxorubicin to the nuclei, while only-doxorubicin NLC was kept in the cytoplasm of MCF-7 ADR cells (multidrug resistant cell line). In MCF-7 ADR tumor-bearing nude mice model at a regimen of IV administration every 2 days for 14 days and a doxorubicin dose of 5 mg/kg and lapachone of 25 mg/kg, the NLC containing both drugs presented a more pronounced tumor inhibition activity than the NLC containing only one of the drugs; the tumor volume remained constant in less than threefold in the group containing both drugs. Another approach for the breast cancer NLC therapy was taken by Liang's group, which designed a multi-stage complex for breast cancers that overexpresses HER2 (Liang et al., 2018). The nanocarrier, functionalized with a HER2 aptamer, contained a natural anticancer drug (epigallocatechin gallate) and an ATP aptamer, which would, upon HER2 receptor binding and endosomal internalization, trigger drug release and induce cellular apoptosis. In this study, the NLC played a protective

role, since it would prevent the extravasation of the content while being biocompatible. The NLC was shown to inhibit tumor growth by 11.5% in the unfunctionalized NLC, and a greater inhibition was noted (19.5%) in the functionalized NLC group.

SLN and NLC have also been employed for theranostics. A study carried out by Urandur et al. described the development of a monoolein-based NLC containing formononetin, a phytoestrogenic isoflavone (capable of inducing apoptosis in breast cancer cells), and tetraphenylethene (a molecule that has aggregation-induced emission effect and can be used to image the tumor) (Urandur et al., 2018). To detect the tumor with higher specificity and provide a detailed image, the group also conjugated anisamide (AA), a ligand of sigma receptors overexpressed on breast cancer cells, to Pluronic F-127. The NLC was capable of controlling the release of formononetin and tetraphenylethene and did not hinder the aggregation-induced emission effect. The uptake of the formulation containing AA after 12 h of exposure of MDA-MB-231 triple-negative breast cancer cells was almost twofold greater than the untargeted one, evidencing its crucial role in the theranostic nanoformulation. After a single IV injection on females BALB/c mice xenograft T41 model, the NLC with AA presented a 79% tumor inhibition rate, and tumor weight was about 3.3-fold lower than the free formononetin group and was able to prolong the survival of the animals. Besides, a higher fluorescence intensity in the tumor was noted in the AA NLC-treated group, and its specificity to the tumor site was reinforced by the lower NLC fluorescent signal distribution in other organs compared to untargeted NLC, demonstrating the targeting ability of the formulation.

4.3 Nanoemulsions and Microemulsions

Nanoemulsion (NE) consists of a colloidal dispersion of emulsified oil, water, and surfactants. It is an isotropic system thermodynamically unstable but can be kinetically stable (Carvalho et al., 2017b; Giacone et al., 2018). Besides, NEs can be appealing for the loading of lipophilic/hydrophobic drugs since it can encapsulate high quantities in a low oil concentration (Giacone et al., 2020). For example, in order to enhance the antitumor effect of the antineoplastic drug lapachol and reduce its severe adverse effects, Miranda et al. designed a nanoemulsion composed of soybean oil, polysorbate 80, and glycerol and produced by a hot homogenization method (Miranda et al., 2021). Cytotoxicity was evaluated at a mice breast cancer cell line (4T1), and the results pointed at a 24.7% enhanced toxicity in the NE-lapachol group compared to the free drug solution. To evaluate the antitumor effects of the formulation and compare it to the free drug, the group employed a 4T1 tumor BALB/c mice model with an intravenous lapachol dose concentration of 5 mg/kg. The nanoemulsion was capable not only to double the β-half-time of the drug but also to inhibit in about 53.7% and 23.1% the tumor growth compared to the untreated and treated with the drug solution groups, respectively, possibly due to the enhancement of the bioavailability and, consequently, uptake in tumor.

Other authors also took advantage of the higher encapsulation feature provided by nanoemulsions to co-deliver drugs aiming at overcoming MDR in breast cancer treatment (Meng et al., 2016). In that sense, Cao and collaborators developed (by high-pressure homogenization) a lipid nanoemulsion containing doxorubicin and a P-gp inhibitor (bromotetrandrine) (Cao et al., 2015b). Interestingly, the 1:1 ratio (doxorubicin/bromotetrandrine, w/w) presented the higher encapsulation efficiency: approximately 84.2% and 93.4% of doxorubicin and bromotetrandrine, respectively. Results in MCF-7/ADR tumor-bearing mice demonstrated that the co-loaded NE presented a better antitumor activity than the solution of both drugs and doxorubicin-NE, also reinforcing that the encapsulation in the nanocarrier did not preclude the effects of the drugs.

In contrast to the nanoemulsions, microemulsions (ME) are thermodynamically stable systems composed of two immiscible liquids (an oil and a polar phase that can be aqueous or non-aqueous) and stabilized by surfactants and co-surfactants (Carvalho et al., 2017a, b; Lopes, 2014; Nornoo et al., 2009). They are easily produced and can be sterilized by filtration. For their composition, microemulsions can be designed with biocompatible components and reduce toxic effects of some solvents, such as Cremophor®, present in Taxol®, the conventional dosage form for i.v. administration of paclitaxel. As an attempt to create a safer and less toxic form of administering the drug to breast cancer patients, Nornoo et al. produced two microemulsions containing either lecithin, butanol, Myvacet, and water or Capmul, Myvacet, and water (Nornoo et al., 2008). Compared to Taxol®, these MEs demonstrated to double the incorporation of paclitaxel (12 mg/g compared to 6 mg/g) and in MDA-MB-231 cell line, and both formulations seemed to slightly increase the drug's IC_{50} compared to Taxol®. This is possibly attributed to a slower release of the ME and is compensated for the better pharmacokinetic parameters in an intravenous injection in male Sprague-Dawley rats such as the two- to fivefold prolonged half-lives, and the formulations were three- to eightfold more distributed than the solution (Nornoo & Chow, 2008). The MEs have also proven to be safer than the commercial solution, since its hemolytic potential was lower than Taxol.

The group of Oh and collaborators demonstrated the feasibility of the application of a nanoemulsion for theranostics. The NE contained paclitaxel, a Bcl-2 siRNA, and Lipiodol, a poppy seed oil iodinated derivative used as contrast agent (Oh et al., 2013). In MCF-7 cells, the system was able to silence Bcl-2 expression in 52.1%, and cytotoxicity of the formulation containing the siRNA and paclitaxel at 140 nM was greater (19.6%) than the NE containing only paclitaxel, indicating the crucial role of Bcl-2 to the susceptibility of the cells. In an MCF-7 xenograft model of nude mice, the NE containing siRNA was capable to target the tumor site, unlike PBS- and unloaded NE treatment. The next step of this study is tumor targeting and treatment with the formulation containing both siRNA and paclitaxel.

4.4 Liquid Crystalline Phases

Liquid crystals are systems that combine properties of solid and liquid systems: they present the mobility of liquids but the structural organization of crystalline solids (Boyd et al., 2006; Ferreira et al., 2006). One of the compounds most frequently employed to form liquid crystalline phases is the polar lipid monoolein, which swells in water giving rise to several mesophases depending on its concentration, temperature, and ionic strength and presence of other compounds such as drugs and other additives (Caboi et al., 2001; Ferreira et al., 2006). In addition to monoolein, several surfactants can be employed to obtain the mesophases (Hosmer et al., 2011, 2013; Farkas et al., 2000; Borgheti-Cardoso et al., 2015). Since this chapter is focused on lipid-based nanocarriers, only systems formed by monoolein will be discussed.

Although more frequently employed in their bulk form, especially for topical delivery, which can be macroscopically visualized as gels, their dispersion in aqueous environment forms nanoparticles (liquid crystalline nanoparticles or LCNP) that have been increasingly investigated (Lopes et al., 2006; Hosmer et al., 2013; Santos et al., 2020). Some of the properties of the LCNP that have attracted interest include:

(i) Versatility, since it reunites both particulate and fluidic characteristics.
(ii) They can incorporate macromolecules and hydrophilic, hydrophobic, and amphiphilic drugs often at higher amounts than liposomes.
(iii) They enhance transport across biological barriers, especially when composed of the polar lipid monoolein as structure-forming compound (which serves as a penetration enhancer), or contain other penetration enhancers in their structure.
(iv) Suitable stability profile.
(v) Ability to sustain the release of incorporated compounds (Depieri et al., 2016; Lopes et al., 2007; Barauskas et al., 2005).

Aiming at testing whether layer-by-layer (LbL) coating with hyaluronic acid and chitosan would improve LCNP-rapamycin's antitumor effects on breast cancer, Freag and collaborators developed a monoolein-based LCNP by hydrotropic method (Freag et al., 2016). The chitosan-hyaluronic acid LbL formulation was capable of reducing the drug hemolytic potential in almost 80% compared to the formulation coated with chitosan only, although its toxicity in vitro in MDA-MB-231 cells demonstrated to be higher in the LbL group, possibly due to hyaluronic acid's targeting CD44+ cancer cells, which might contribute to the selectivity of the formulation. In a mice Ehrlich ascites tumor model, survival on the LbL-rapamycin-LCNP group was higher (83.3%) than animals treated with rapamycin-LCN (66.7%) and the solution of the drug (16.7%), and the tumor development was lower in the LbL-rapamycin-LCNP group.

Another interesting strategy for the treatment of breast cancer is to target cancer stem-like cells, since they are described to be the ones to proliferate and develop a

tumor, metastasis, or recurrence. In a study conducted by Singh et al., a monoolein-based LCNP containing paclitaxel and forskolin, a phytochemical diterpene reported to induce cancer stem-like cell differentiation and sensitize cells to chemotherapy, was developed (Singh et al., 2021a). Compared to a fluorochrome solution, the LCNP almost doubled the uptake, measured by cytometry, and spheroid penetration and distribution, reinforcing the LCNP advantageous properties. Also, the authors have shown that paclitaxel, when alone, can enrich the cancer stem-like cells in mammospheres and that the incorporation of forskolin can reverse the effect and aid tumor regression. The advantages of co-encapsulating both drugs can be further supported by the tumor growth follow-up, in which the solution of paclitaxel and paclitaxel + forskolin presented a similar rate after 82 days at equivalent single-dose treatment of 10 mg/kg of paclitaxel, while LCNP co-encapsulating both drugs presented tumor regression at 30 days after treatment. After the 82 days, the group treated with LCNP did not present tumor relapse.

5 Nanocarriers for Oral Administration

The oral route is the most used route of drug administration; it does not require training or a health professional to be applied, which makes it convenient and accessible to patients. However, it leads to systemic exposure to drugs, and the high systemic toxicity caused by antineoplastic drugs is often dose-limiting and results in low tolerability in patients. Besides, orally administered compounds must surpass the gastrointestinal physical, chemical, and immunological barriers and are often inactivated by first-pass liver metabolism, which limits therapeutic efficacy (Chenthamara et al., 2019a). Lipid-based nanocarriers have emerged as a strategy capable not only of reducing systemic adverse effects and enhancing oral bioavailability but also of promoting controlled release, protecting the encapsulated compounds from degradation, allowing the solubilization of lipophilic drugs, promoting tumor tissue targeting, and increasing compound concentration in the target site (Sharma et al., 2006).

5.1 Liposomes

Ağardan et al. (2020) developed 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and chitosan liposomes containing dimethyl-β-cyclodextrin (DM-β-CD) and (as absorption enhancer) for oral delivery of tamoxifen or raloxifene. These drugs are selective estrogen receptor modulators, approved by the FDA for clinical use, that act by interacting competitively with ER. Raloxifene has been employed in postmenopausal women to prevent osteoporosis. Both belong to Biopharmaceutical Classification System (BCS) class II, meaning they have poor solubility in water; thus, incorporation in liposomes can contribute to absorption. In vitro cell viability

studies, performed using MCF-7 and MDA-MB-231 breast cancer cell lines, demonstrated more pronounced viability reductions in MCF-7 (which are ER-positive), than for MDA-MB-231 cells. The pharmacokinetic study in mice showed that tamoxifen and raloxifene were still present in the blood 24 h after the administration of liposomes. Upon oral administration of 1200 μg/mL tamoxifen and raloxifene liposomes orally once a week for 8 weeks in mice subjected to tumor development by N-nitroso-N-methylurea, tumor reduction of 92.5% in the group treated with tamoxifen liposomes and 65% in raloxifene liposome-treated animals were observed.

5.2 Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers (NLC)

SLN and NLC are solid nanocarriers that appear promising for improving oral drug delivery, but there have been reports of inefficient oral delivery by SLNs due to their typical burst release profile in the stomach acidic environment, which leads to poor absorption. To overcome this, Baek and Cho (2017) developed a surface-modified SLN (NCC-SLN) for the oral delivery of curcumin, a natural lipophilic compound with known therapeutic and chemopreventive activity against breast cancer. The SLN was coated with the polysaccharide N-carboxymethyl chitosan, which is stable in stomach pH and dissolves in pH > 5. The in vitro release study showed that coating the SLNs inhibited the burst release in a simulated gastric fluid, while the release profile was not significantly altered in a simulated intestinal fluid. The pharmacokinetic profile in rats was also altered: the maximum plasmatic concentration (C_{max}) and area under the plasma concentration-time curve (AUC) values were higher for the coated SLN than for the non-coated SLN and free curcumin. Besides inhibition of the burst effect in the stomach and increased concentration of curcumin in the intestine, the improved oral bioavailability was also attributed to lymphatic uptake, which was significantly augmented in animals that received the coated SLNs.

Godugu et al. (2016) developed NLC formulations for the 3,3[1]-diindolylmethane (DIM) analogs DIM-10 and DIM-14, two anticancer compounds poorly soluble and therefore not well absorbed orally, as a novel oral treatment for triple-negative breast cancer. The NLCs were developed by the hot melt homogenization technique using water, Compritol 888, Miglyol 812, and vitamin E TPGS, all of which are FDA approved for clinical use. Both DIM-10 NLC and DIM-14 NLC presented a sustained-release profile in vitro and increased the maximum percentage of drug diffused through nitrocellulose membrane by 2.94- and 3.08-fold, respectively, compared to the free drug solutions. The formulations also increased both drugs' permeability by 2.59- and 2.56-fold for DIM-10 and DIM-14, respectively, in Caco-2 cells. In vivo pharmacokinetic studies showed that the NLCs were able to significantly increase oral absorption of both compounds and improve pharmacokinetic parameters such as C_{max}, half-life time ($t_{1/2}$), and AUC. In fact, C_{max}, $t_{1/2}$, and AUC values were higher for a 30 mg/kg dose of DIM-10 NLC than for a 100 mg/kg

dose of free DIM-10, suggesting that the dose necessary for comparable effect in the NLC formulation could be reduced to almost 1/3 of the free drug dose, which shows yet another advantage in the use of NLC formulations, especially when it comes to expensive drugs. Accordingly, the NLCs also had superior antitumor efficacy compared to the free drugs in an orthotopic TNBC model.

NLCs have also been developed to improve oral delivery of docetaxel, a taxane widely used for breast cancer treatment, by Fang et al. (2015). Oral bioavailability of docetaxel is low due to its poor water solubility, high efflux rates by the P-glycoprotein (P-gp), and metabolization by cytochrome P450. As the SLNs developed by Baek and Cho (2017), these NLCs were modified for functionalization. To increase the residence time of the NLC in the gastrointestinal tract, an amphiphilic bioadhesive thiomer was created and added to the particle's surface. Thiomers are thiolated polymers capable of making nanoparticles mucoadhesive, because they have sulfhydryl groups that form disulfide bonds with cysteine present in mucus. Besides the bioadhesive property, thiomers were included to increase drug permeation through the epithelium and suppress P-gp activity. For these reasons, the authors of this study conjugated cysteine with polyethylene glycol monostearate (PEG-MSA), an amphiphilic polymer, to synthesize thiolated PEG-MSA (Cy-PEG-MSA) which was used to modify NLC surface. In an in vitro bioadhesion model, mucin adsorption was 81.6% on Cy-PEG-MSA-modified NLCs (cNLCs) and only 51.9% on unmodified NLCs (uNLCs). Permeability of docetaxel in the intestine was also improved by the formulations, as shown in an in situ model of intestinal perfusion. The cNLC absorption rate was three- to fivefold the rate of free docetaxel solution and was also higher than the rate of uNLC. Pharmacokinetic parameters in vivo also indicated significantly improved oral bioavailability of cNLCs in comparison to uNLCs and especially to free docetaxel.

5.3 Micro- and Nanoemulsions

Omega-3 polyunsaturated fatty acid (PUFA) metabolites have been identified as potential anticancer agents. However, their administration is compromised by the low water solubility of these compounds. The synthetic omega-3 PUFA derivative 16-(4′-chloro-3′-trifluorophenyl)carbamoylamino] hexadecanoic acid (ClFPh-CHA) was selected for encapsulation in a nanoemulsion intended for oral delivery (Garrastazu Pereira et al., 2018). The ClFPh-CHA-loaded NE was administered orally to tumor-bearing animals once a day, 6 days a week. After 32 days, the 2.5 and 10 mg/kg doses reduced breast tumor's medium volume to approximately 60% of controls, while the 40 mg/kg dose promoted a reduction to 42%. ClFPh-CHA solution could cause significant reduction (approximately 40%) only at 40 mg/mL.

Choudhury et al. (2014) developed NEs with sustained-release profile in vivo to improve paclitaxel oral bioavailability. Based on paclitaxel solubility and thermodynamic stability of formulations, Capryol® 90 (propylene glycol monocaprylate) was selected as the lipid phase of the nanoemulsion. Polysorbate 20 and ethanol were

selected as surfactant and co-surfactant based on further physicochemical characterization. The in vitro release study showed that the NE was capable of enhancing paclitaxel diffusion through the dialysis membrane to 91.20 ± 4.20% after 8 h, compared to the i.v. commercial formulation of paclitaxel (Taxol®) (58.35 ± 2.91%) and to a paclitaxel suspension (7.50 ± 1.35%). Compared to solution, the NE increased paclitaxel permeability through Caco-2 monolayer in the apical-basolateral direction and reduced transport in the basolateral-apical direction, meaning it decreased drug efflux. In accordance with this result, the NE presented higher cytotoxicity than the solution in both MCF-7 and MDA-MB-231 breast cancer cells. In mice, the orally administered NE achieved 55.9% bioavailability of paclitaxel. Compared to the IV solution, $t_{1/2}$ and the time point when plasmatic paclitaxel was no longer quantifiable increased, suggesting the NE ability to promote sustained release in vivo.

Another NE for docetaxel was developed by Pandey et al. (2017). In this formulation, the oily phase was composed of soya oil and frankincense oil, which contains boswellic acids and keto-boswellic acids, both with anticancer and P-gp inhibitory properties. To study P-gp inhibition, cells were exposed to the P-gp fluorescent substrate rhodamine-123. In cells exposed to rhodamine only, the fluorescence was hardly observed, which can be attributed to its efflux. In contrast, the fluorescence was higher in cells exposed to rhodamine and frankincense oil or verapamil (a known P-gp inhibitor) suggesting the inhibitory activity of the oil over P-gp. In an in vitro cytotoxicity study with MDA-MB-231 cells, the nanoemulsion containing the oil and docetaxel was more cytotoxic than that containing only docetaxel, and both were more cytotoxic than Taxotere®: in cells treated with Taxotere® and the NE, 8.53% and 13.51% of cells were arrested in the G2/M phase after 24 h of treatment, respectively, and 28% and 72.45% after 48 h, suggesting that co-encapsulated docetaxel and frankincense oil are more efficient in arresting cell cycle than Taxotere®, in a time-dependent manner. In comparison to Taxotere®, the nanoemulsion enhanced apoptosis and necrosis in MDA-MB-231 cells. In mice, time to maximum plasma concentration (t_{max}), C_{max}, and AUC were higher for the nanoemulsion than for Taxotere®. Nanoemulsion efficacy, assessed in an in vivo model of tumor-bearing mice in which mammary carcinoma cells 4T1 were subcutaneously inoculated in the mammary gland, was higher, displaying a tumor inhibition ratio of 93%.

Taking advantage of the fact that some oils and surfactants are capable of self-emulsifying in contact with water under specific conditions, oral self-emulsifying drug delivery systems have been proposed for breast cancer treatment. As these formulations do not contain water, they can be inserted in capsules for commercialization and clinical use (Constantinides et al., 1994). Self-nanoemulsifying drug delivery systems have been developed for improved oral delivery or co-delivery of several drugs that are active against breast cancer, such as quercetin, resveratrol, and genistein (Tripathi et al., 2016); tamoxifen and naringenin (Sandhu et al., 2017); and docetaxel (Seo et al., 2013), as well as a self-microemulsifying system for exemestane (Singh et al., 2009). All have shown promising results in terms of enhanced oral bioavailability of compounds.

5.4 Liquid Crystalline Nanoparticles (LCNPs)

Swarnakar et al. (Swarnakar et al., 2014) developed bicontinuous cubic liquid crystalline nanoparticles (LCNPs) to improve the safety profile and efficacy of doxorubicin in cancer treatment. Doxorubicin is frequently employed in the chemotherapy of breast cancer. Despite its efficacy, it causes severe systemic toxicity, remarkably cardiotoxicity. Its oral bioavailability is also very low (less than 5%) due to low permeability, efflux by P-gp, and hepatic first-pass metabolism, and thus, it is clinically administered through the intravenous route. Phytantriol, a non-digestible lipid, was selected for the oil phase. Various surfactants were incorporated into the formulation for optimization, and, based on particle size, size distribution, zeta potential, and encapsulation efficiency, Pluronic® F-127 was selected. LCNPs were shown to be stable in simulated gastrointestinal fluids, maintaining their ability to retain high drug content even after 8 h. In the in vitro release study, LCNPs demonstrated a sustained-release profile, having been able to prolong drug release for up to 120 h (96.7 ± 2.1% doxorubicin released). In the Caco-2 cell uptake assay, while free doxorubicin and free doxorubicin with cyclosporine A (a known P-gp inhibitor) achieved intracellular concentrations of 42.81 ± 3.14 and 64.63 ± 5.79 ng/mL after 6 h, the concentration in cells treated with loaded LCNPs was 731.04 ± 38.51 ng/mL after only 2 h, which is equivalent to ~17.08- and 11.31-fold increases, respectively. Furthermore, cell viability was greater than 90% for the 1.0 μg/mL concentration tested, indicating low cytotoxicity in Caco-2 cells. In MCF-7 cells, the LCNPs promoted a ~ 4.57-fold increase in doxorubicin intracellular concentration in half the time compared to free doxorubicin. The LCNPs also increased doxorubicin cytotoxicity in MCF-7 cells drastically – for 24 and 48 h treatments, the IC_{50} values, which indicate the drug concentration needed for 50% reduction in cell viability, were 3.12- and 16.23-fold lower for loaded LCNPs than for free doxorubicin. This difference could be explained by the more efficient internalization of LCNPs due to higher permeability in comparison to free doxorubicin. The pharmacokinetic profile of LCNPs was assessed in female rats, at a 10 mg/kg dose of doxorubicin. In animals that received loaded LCNPs, C_{max} and AUC values were pronouncedly higher than in animals that received free doxorubicin, indicating the oral bioavailability improvement promoted by the incorporation of doxorubicin in the developed system. To verify the efficacy of LCNP's treatment, breast tumors were induced with 7,12-dimethylbenz [α] anthracene (DMBA) in rats, which were then treated with equivalent doses of 5 mg/kg doxorubicin. The animals received oral saline as control, oral loaded LCNPs, or intravenous clinically approved formulations Adriamycin® or Lipodox®. As expected, all treatments reduced tumor burden in comparison to control. LCNPs reduced tumor volumes more efficiently than Adriamycin® (~42% and ~31% reduction, respectively, after 30 days), but less efficiently than Lipodox® (~85%). On the other hand, cardiac biochemical and histological parameters were analyzed and revealed that LCNPs were significantly less cardiotoxic than Adriamycin® and Lipodox®, suggesting an improved safety profile of the developed formulation.

Phytantriol was also used by Jain et al. (2018), who developed LCNPs to improve the oral bioavailability and safety profile of tamoxifen. Either phytantriol (PLCNPs) or glyceryl monooleate (GLCNPs) were used in the oil phase, and oleic acid was added to enhance tamoxifen loading. The LCNPs were shown to be stable in gastrointestinal simulated fluids. In an in vitro drug release study, both LCNPs exhibited sustained-release profiles, prolonging tamoxifen release for up to 96 h, in contrast to 2 h for the free drug control solution. The fluorescent dye coumarin-6 (C-6) was used to evaluate LCNP's internalization in Caco-2 cells, and uptake for both formulations was clearly higher than for free C-6. MCF-7 cells were used to assess in vitro cytotoxicity of the tamoxifen-loaded LCNPs in comparison to free tamoxifen. PLCNPs and GLCNPs had 7.3- and 10.0-fold lower IC_{50} values relative to free tamoxifen after 24 h treatment, indicating their superior cytotoxicity. The permeability and cytotoxicity studies together suggest possible membrane-altering activity and some bioadhesive property of LCNPs. In vivo pharmacokinetic study in rats showed that the LCNPs had higher C_{max} values than free tamoxifen and bioavailability was 7.45- and 5.38-fold for PLCNPs and GLCNPs, respectively, relative to the free drug. Tumors were induced in rats by the administration of DMBA, and the animals were treated with oral doses equivalent to 3 mg/kg tamoxifen once every 3 days, for 30 days. At the end of this period, PLCNPs and GLCNPs caused greater reduction in tumor burden (67.96% and 48.87% reduction, respectively) than free tamoxifen (32.74% reduction) while reducing the drug hepatotoxicity. Between the two formulations, PLCNPs' reduction in hepatotoxicity was significantly higher than GLCNPs, reinforcing the safer profile of phytantriol in relation to glyceryl monooleate as previously suggested (Jain et al., 2015). Also, it is believed that LCNPs enhance absorption through the lymphatic system, reducing drug first-pass hepatic metabolism and hence leading to improved oral bioavailability and, at the same time, lessening oxidative stress in the liver, which could explain the minimized hepatotoxicity (Jain et al., 2018).

6 Nanocarriers for Localized Treatment

The standard of treatment for breast cancer has changed over the past decades, in search of less invasive methods and better outcomes (Kuang et al., 2020). As we saw in the previous sections, lipid nanoparticles are quite versatile and can be used for this purpose. However, most studies that investigate these nanocarriers for breast cancer therapy do not employ local routes of administration despite the clear advantages of these routes over systemic administration, such as fewer adverse effects and greater adherence to treatment (Kuang et al., 2020; Mojeiko et al., 2021; Murata et al., 2006; Zhang et al., 2014). The research developed over the last decades for drug localization in breast tissue was based on intraductal, topical-transdermal, and subcutaneous administration of lipid and polymeric nanocarriers. In the following subsections, we will focus only on lipid-based nanocarriers employed to localize drug delivery in breast cancer. A summary of the strategies that will be discussed in presented in Table 2.

Table 2 Lipid-based nanocarriers investigated for the intraductal, transdermal, and subcutaneous treatment of breast cancer

Administration route	Nanocarrier type	Advantages	References
Intraductal	Liposome	Increased drug retention in ducts Lower plasma drug levels Greater safety profile	Murata et al. (2006), Stearns et al. (2011)
	Nanoemulsion	Surface modification and viscosity modulation for localization in mammary ducts	Migotto et al. (2018), Carvalho et al. (2019)
Transdermal	Liposome	Deformability and permeation enhancers can increase transdermal drug delivery Drug depot formation	Bathara et al. (2020), Altamimi et al. (2021a, b), Apolinário et al. (2021a, b)
	Micro- and nanoemulsion	Surface modification promotes interaction with epidermal cells Penetration enhancers increase permeation across the stratum corneum	Dave et al. (2017), Mojeiko et al. (2019), Altamimi et al. (2021a, b), Saraiva et al. (2021)
	Lipid nanoparticles	Lipophilic matrix facilitates transdermal transport and depot formation in breast adipose tissue	Gadag et al. (2021)
	Liquid crystalline nanoparticles	Lipophilic matrix facilitates transdermal transport and depot formation in breast adipose tissue	Musa et al. (2017)
Subcutaneous	Microemulsion	Depot formation	Salata et al. (2021)
	Lipid nanoparticles	Subcutaneous peritumoral location of the drug	Guo et al. (2012)

6.1 Intraductal Therapy

The intraductal route emerged as a viable strategy capable of reaching the entire ductolobular system in an effective and minimally invasive way. In fact, considering that more than 95% of breast cancers originate in the epithelial cells that surround the breast ducts, the intraductal administration of compounds allows greater local exposure to the neoplastic lesions and fewer adverse effects, due to reduced systemic exposure of healthy tissues (Murata et al., 2006; Zhang et al., 2014; Kuang et al., 2020).

The feasibility of using the intraductal route has been previously demonstrated in several models of breast cancer in rodents for the delivery of different cytotoxic drugs, such as paclitaxel, 4-hydroxytamoxifen, doxorubicin, curcumin, methotrexate, 5-fluorouracil, and carboplatin (Gao et al., 2021a; Chun et al., 2012a; Okugawa

et al., 2005). Compared to control groups, intraductal administration of drugs reduced already established tumors and prevented the development of new tumors with comparable efficacy than the intravenous route and without evidence of systemic toxicity. In addition, clinical studies were carried out to prove the success and safety of the intraductal route in humans (Love et al., 2013). Mahoney et al. administered liposomal doxorubicin in 13 women with ductal carcinoma in situ (DCIS) (Mahoney et al., 2013). The safety of the intraductal route was demonstrated in breast cancer patients waiting mastectomy by Zhang and colleagues (Zhang et al., 2014). In this study, liposomal doxorubicin and carboplatin were administered in the mammary ducts after the application of a local anesthetic, resulting in high tolerability with only transient and medium discomfort (on a scale of 0–10, patients reported levels of pain between 0.2 and 1.3).

Despite great advances in intraductal therapy, the high ductal permeability causes small drugs to rapidly diffuse into the systemic circulation, and it is still a challenge to design formulations that take into account the specific characteristics of this route (Singh et al., 2012). Next, lipid nanocarriers employed in intraductal administration will be discussed.

6.1.1 Liposomes

Most studies that evaluate the use of liposomes in the context of intraductal administration use PEGylated liposomal doxorubicin (PLD), since its advantage over free doxorubicin is well established and it is approved by the Food and Drug Administration for clinical use (Doxil®). PLD is a doxorubicin formulation in PEG-coated liposomes that presents a unique toxicity profile and greater stability of the drug in the circulation, in addition to reducing drug distribution and promoting an increase in its location in the tumor tissues (Chun et al., 2012a, b; Gabizon, 2001a; Zhang et al., 2014).

Murata and colleagues first studied the intraductal administration of PEGylated liposomal doxorubicin in the prevention and treatment of breast cancer in rodents in N-methyl-N′-nitrosourea and spontaneous transgenic HER-2/neu models (Murata et al., 2006). Intraductal administration was able to reduce systemic exposure to the drug when compared to intravenous administration, in addition to being more effective in promoting the regression of induced tumors and reducing the appearance of new tumors in both models. Another study compared the efficacy of PEGylated liposomal doxorubicin with several other anticancer drugs (5-fluouroracil, carboplatin, methotrexate, and nanoparticle albumin-bound paclitaxel) (Stearns et al., 2011). The drugs were able to prevent tumor growth, and PLD remained for long periods in the mammary gland. Despite the advantages of intraductal administration of liposomal doxorubicin, subsequent studies of the group observed that after long periods of time, this treatment was capable of inducing malignant breast tumors, which indicated the need for more studies on drug release and metabolism in the breast (Chun et al., 2012b).

As mentioned at the beginning of this subsection, there are clinical studies describing the intraductal administration of drugs. Love et al. (2013) demonstrated the intraductal administration of carboplatin and liposomal doxorubicin in 30 women waiting for mastectomy; carboplatin was selected for its efficacy in several types of breast cancer, including metastatic one, and PLD was selected for being as effective as free doxorubicin in breast cancer treatment with an increased safety profile. Encapsulation of doxorubicin in liposomes seemed to promote drug retention in the ductal system, as no drug metabolites were detected in the bloodstream and there was no systemic toxicity. Subsequent studies have confirmed the effects of intraductal administration of PLD in the treatment of patients with DCIS and the ability of the formulation to aid drug retention in the ductal system (Mahoney et al., 2013).

The search on ClincalTrials.gov resulted in two studies that aimed to administer liposomal doxorubicin to women awaiting mastectomy with invasive and localized breast cancer (ductal carcinoma in situ), which were complete and recruiting, respectively. The first study used different doses of liposomal PEGylated doxorubicin and evaluated the maximum tolerated dose after intraductal administration and compared doxorubicin concentrations observed in blood and tissue with those obtained after intravenous administration of the drug (Stearns et al., 2011). Intraductal administration was able to reduce doxorubicin plasma concentration and increase drug concentration in the breast compared to those who received the agent intravenously. In addition, no severe adverse events were observed in the intraductal arm of the study.

6.1.2 Nanoemulsions

Nanoemulsions are colloidal dispersions formed by droplets of oil in water (o/w) or water in oil (w/o) capable of encapsulating and delivering drugs (Mattheolabakis et al., 2012). By modulating the nanoemulsion components and modifying its surface, it is possible to obtain systems with an adequate size distribution for the intraductal route and capable of increasing the residence time in the mammary tissue while reducing systemic exposure. Migotto et al. studied the possibility of increasing the permanence of C6 ceramide in the breast ducts by encapsulation in a bioadhesive cationic nanoemulsion as a local strategy for ductal carcinoma in situ therapy (Migotto et al., 2018). The nanoemulsion surface was modified with chitosan, which confers a positive charge and allows interaction with mucin and other negatively charged proteins expressed in the mammary ducts. Also, chitosan increased the viscosity of the system, which may contribute to greater retention time. In fact, intraductal administration of the nanoemulsion loaded with fluorescently labeled C6 ceramide promoted mammary tissue targeting and prolonged retention in the mammary region for more than 120 h compared to a drug solution and systemic administration.

In another study, drug mammary retention after intraductal administration of nanoemulsions modified with chitosan (cationic) or hyaluronic acid (anionic) was

compared (Carvalho et al., 2019). Despite the opposite charge of the polymers, both nanoemulsions presented bioadhesive properties and prolonged retention time in the mammary tissue, probably by complexation with mucus components or hydrogen bond formation.

6.2 Topical-Transdermal Therapy

The skin is another potential alternative route for local drug delivery to breast tissue and has been explored for adjuvant treatment and prevention of development and recurrence of breast cancer. The transdermal route has a series of advantages, such as reduced plasma concentration, possibility of self-application, improved patient compliance, and avoidance of first-pass liver metabolism (Manish et al., 2020; Mojeiko et al., 2019, 2021; Lee et al., 2015; Lee & Khan, 2016). Despite these advantages, the skin has an important barrier function, which hinders the permeation of drugs (Mojeiko et al., 2021). Lipid-based nanocarriers can be used for drug encapsulation to improve skin delivery.

6.2.1 Vesicles

There are several studies that propose the use of liposomes in the transdermal transport of cytotoxic drugs for the treatment of breast cancer. In a recent study, Altamimi and colleagues developed elastic liposomes for luteolin, a drug with an important antineoplastic action that has low water solubility and is unstable in the gastric lumen and, consequently, has low oral bioavailability (Altamimi et al., 2021a). The choice of system applied is justified by its high deformability, which facilitates permeation through the skin and transdermal drug delivery for the treatment of breast cancer. Liposomes were optimized using the design of experiments, and the final formulation was composed of phosphatidylcholine and Span 80, presenting high encapsulation efficiency (up to 92%), ability to sustain drug release for more than 12 h, and increased drug efficacy in MCF-7 cells. The optimized formulation was able to promote ex vivo permeation of 3270 μg/cm^2 of drug in rat skin, which represents a 6.2-fold increase compared to a drug solution. Furthermore, it was able to form a drug depot on the skin up to 4.5 times larger than the control solution.

In addition to deformability, other properties can be explored in the development of systems for transdermal administration. One strategy is the use of vesicles containing synergistic combination of permeation enhancers (SCOPE) to improve the skin permeation of drugs. Bathara et al. developed liposomes for docetaxel delivery and breast localization; sodium oleate, sodium lauryl ether sulfate, and propylene glycol were used as permeation enhancers (Bathara et al., 2020). These high permeation vesicles were able to sustain drug release for more than 48 h and to increase drug permeation in the skin by 2.4- and 1.7-fold compared to a drug dispersion in SCOPE and SCOPE-free liposomes. In vivo studies demonstrated that SCOPE

liposomes were able to reduce the size of tumors in an equivalent proportion to conventional intravenous treatment with docetaxel (Taxotere®), with the advantage of substantially lower systemic toxicity. These results demonstrate the applicability of the lipid vesicles in the transdermal treatment of breast cancer.

Ethosomes are vesicles that contain ethanol (10–50%) in its composition and can be used in the delivery of drugs to the skin as they promote drug penetration (Apolinário et al., 2021a). Apolinário and colleagues developed and systematically optimized liposomes for fenretinide local-transdermal administration (Apolinário et al., 2021b). The objective of the work was the chemoprevention of breast cancer, and this system was chosen to promote drug penetration into the skin, increase the drug localization in the breast tissue, and enhance the tolerability of the treatment. The ethosomes were able to increase fenretinide delivery into the skin by up to fivefold compared to a control solution; this effect was attributed to the nanometric size of the vesicles and the components of the formulation, such as ethanol and propylene glycol. Ethosomes were well tolerated, did not reduce the viability of non-tumor MCF-10A cells and presented similar IC_{50} to a drug solution in tumor cells (MCF-7) suggesting that nanoencapsulation does not hinder cytotoxicity.

6.2.2 Micro- and Nanoemulsions

Micro- and nanoemulsions have been frequently studied for topical-transdermal administration as they improve drug solubility and allow the incorporation of permeation enhancers, such as lipids and surfactants, promoting greater permeation across the *stratum corneum* (Nastiti et al., 2017; Hosmer et al., 2009; Giacone et al., 2020; Pepe et al., 2012).

The study of Altamimi and colleagues exemplifies the use of cationic nanoemulsions for the transdermal delivery of luteolin; the positive charge was obtained by cationic charge inducers (oleylamine and stearylamine) with the intention of promoting electrostatic interaction with epidermal cells and increasing permeation through the skin, in addition to promoting greater colloidal stability (Altamimi et al., 2021b). The cationic nanoemulsion was able to increase the cumulative amount of drug that penetrates into the skin of rats by 8.2- and 1.6-fold compared to a solution and an anionic nanoemulsion of the drug, respectively. The transdermal approach can also be used in the adjuvant therapy of triple-negative breast cancer, which is highly aggressive and metastatic and requires more effective and safer therapeutic strategies. Saraiva et al. developed edelfosine-loaded nanoemulsions and evaluated their efficacy against MDA-MB-231 triple-negative breast cancer cells in vitro and in vivo (zebrafish model) (Saraiva et al., 2021). The nanoemulsion was developed with edelfosine, Miglyol 812, and phosphatidylcholine, based on GRAS (generally recognized as safe by the US FDA), showing a good safety profile. In vitro tests revealed an increased cellular uptake of nanoemulsions and a twofold reduction in the IC_{50} in tumor cells compared to an edelfosine solution. The animal model demonstrated the nanoemulsions' ability to cross biological barriers

after topical application in zebrafish embryos and significantly reduced the proliferation of tumor cells compared to the untreated control.

To date, various microemulsions have been developed to deliver cytotoxic drugs to the skin for the treatment and/or chemoprevention of breast cancer (Mojeiko et al., 2019; Dave et al., 2017; Yehia et al., 2017). Recently, Mojeiko et al. combined the skin microneedling technique with microemulsions to increase the cutaneous delivery of celecoxib (Mojeiko et al., 2019). The formulation enhanced dermal and transdermal transport of celecoxib compared to a control solution by up to three- and fourfold, respectively. Furthermore, pretreatment of the skin with microneedles was beneficial for deeper penetration, as it results in a strong barrier disruption due to pore formation. Encapsulation of celecoxib in microemulsions reduced the IC_{50} in MCF-7 cells by 3.3-fold, suggesting that the formulation components may aid drug cytotoxicity. In another study, Dave et al. compared the transdermal administration of α-santalol-loaded microemulsions applied to the skin of the breast and the nipple region, in the chemoprevention of breast cancer (Dave et al., 2017). Microemulsions produced with PL-ME10 phospholipid applied into the nipple were able to increase in vitro drug permeation and tissue retention in porcine skin by up to ten- and eightfold, respectively, compared to control formulations (solution and cream). Next, they investigated the difference in in vivo transport of microemulsions applied to the skin, nipple, or whole breast of Sprague-Dawley rats and found that application to the whole breast promoted the greatest accumulation of α-santalol in the breast tissue; all administration routes were able to achieve therapeutic concentrations in breast tissue and reduce plasma concentrations to undetectable levels. Finally, the in vivo chemoprevention study demonstrated the ability of the formulation to reduce the incidence and multiplicity of tumors by 1.5 times, compared to the control.

6.2.3 Lipid Nanoparticles

In a recent study, Gadag and colleagues developed and optimized resveratrol-loaded nanostructured lipid carriers and evaluated its effectiveness in combination with microneedles as a strategy for the local delivery of drugs for breast cancer therapy (Gadag et al., 2021). Microneedling was used to overcome the skin barrier function and facilitate drug delivery into the skin, and nanostructured lipid carriers were the selected system because its lipophilic matrix is capable of creating a slow-release drug depot in breast adipose tissue. The nanoparticles were able to sustain drug release and, when combined with the microneedle technique, promoted a greater transdermal permeation of resveratrol compared to the free drug. Furthermore, the nanocarriers presented greater potency than free resveratrol, represented by the lower IC_{50} value obtained in the MDA-MB-231 breast cancer cell cytotoxicity assay. Finally, the pharmacokinetic study revealed increased values of C_{max} and AUC and a greater localization in the breast tissue compared to treatment with oral resveratrol, suggesting the applicability of the nanoparticle in local-transdermal drug delivery.

6.2.4 Liquid Crystalline Nanoparticles

Liquid crystalline nanoparticles can also be used in the transdermal therapy of breast cancer because, like micro- and nanoemulsions, they have the ability to incorporate penetration enhancers and potentially improve drug delivery through the skin, in addition to presenting suitable viscosity for topical application (El-Gendy et al., 2020; Hosmer et al., 2011). Furthermore, they are easy to obtain, allow the encapsulation of drugs with distinct physicochemical properties, and promote modulated drug release (El-Gendy et al., 2020; Musa et al., 2017). Musa et al. developed a liquid crystalline gel formulation for exemestane, an aromatase inhibitor currently used in breast cancer therapy in high-risk post-menopausal women (Musa et al., 2017). The size of the nanoparticles ranged between 120 and 466 nm, and they presented negative zeta potential (−17–25 mV) and high encapsulation efficiency (85–92%). The system successfully allowed the cutaneous delivery of exemestane in rats, which was attributed to the ability of surfactants to modify the structure and fluidity of the stratum corneum, overcoming its barrier function. In addition, the nanocarrier showed cytotoxicity in MDA-MB-231 cells at lower concentrations (12.5 and 25 μg/mL) than those used in clinical practice, suggesting that it is an efficient and safe delivery system.

6.3 Subcutaneous Delivery

In addition to intraductal and transdermal therapy, subcutaneous administration of cytotoxic drugs had also been explored for localized drug delivery in the breast. Subcutaneous therapy combines the advantages of localizing the drug in the breast tissue with greater adherence to treatment and reduced treatment time (Jackisch et al., 2016). Several clinical trials, such as HannaH, SafeHer, and PrefHer, had demonstrated the advantage of subcutaneous administration of trastuzumab in adjuvant and neoadjuvant therapy in patients with HER2-positive breast cancer; the long-term safety and efficacy of subcutaneous treatment were non-inferior to the conventional one, with greater patient convenience and preference (Jackisch et al., 2016; Heo & Syed, 2019; Jung et al., 2018). In 2020, subcutaneous therapy with trastuzumab, pertuzumab, and hyaluronidase-zzxf (Phesgo) was approved by the FDA for the treatment of patients with HER2-positive early-stage and metastatic breast cancer (Gao et al., 2021b). Despite the aforementioned advantages, few preclinical and clinical studies designed specific formulations for the subcutaneous administration of drugs. Here, we will discuss lipid nanoformulations applied for the subcutaneous administration of drugs, but efforts are still needed to understand how the formulation composition and physicochemical properties can influence the treatment outcomes.

6.3.1 Microemulsions

Microemulsion-based systems can be used in the subcutaneous delivery of drugs aimed at localized breast cancer therapy. Fenretinide is a potential candidate for breast cancer therapy and chemoprevention, since it accumulates in the breast tissue due to its high fat solubility (Sabichi et al., 2003). However, its low oral bioavailability, the need for high doses, and occurrence of severe adverse effects limit its clinical use. Salata et al. have recently developed bioresponsive microemulsions for subcutaneous administration of fenretinide and drug localization in breast tissue (Salata et al., 2021). The system was developed with phosphatidylcholine and propylene glycol aiming at the formation of a liquid crystalline system in vivo upon uptake of local fluids. The microemulsion was able to absorb water and form a gel after subcutaneous administration, forming a depot and prolonging the release of compounds for up to 30 days. In addition, it increased fenretinide in vitro cytotoxicity compared to a drug solution in MCF-7 and T47D breast cancer cell lines. In the in vivo model, the system was able to reduce tumor incidence by 4.7-fold.

6.3.2 Solid Lipid Nanoparticles

Solid lipid nanoparticles have been described to undergo rapid clearance from the circulation via the mononuclear phagocytic system, so that their subcutaneous administration represents a potential strategy for localization in tumors (Lu et al., 2006; Guo et al., 2012). Guo and collaborators investigated the subcutaneous administration of a thermosensitive hydrogel containing 2-methoxyestradiol-loaded solid lipid nanoparticles for the treatment of subcutaneous tumors, such as breast cancer (Guo et al., 2012). The system was able to increase cytotoxicity against T-41 cells by 6.4-fold and to promote localization around the injection site for more than 46 days. In addition, the subcutaneous peritumoral administration of the SLN-loaded system showed greater efficacy than just the hydrogel containing the drug, with no observed toxicity.

7 Conclusions

Since the approval of Doxil in 1995 by the US Food and Drug Administration, many lipid-based nanocarriers were developed, underwent clinical trials, and/or received approval for breast cancer treatment. Several strategies were employed to improve nanocarrier-based delivery and efficacy and to reduce the adverse effects of conventional chemotherapy. The advantage of using PEGylated, functionalized, pH- and temperature-sensitive nanocarriers has been demonstrated at least in preclinical trials. Other strategies include co-encapsulation of drugs and inclusion of efflux transporter inhibitors. Although some of these strategies have found their way to the clinic, there are many hurdles that still need to be addressed for a further

development of the nanomedicine field for breast cancer. For example, understanding the real influence of the EPR effect and the surface modification on the interactions of lipid-based nanocarriers with the immune system and plasma proteins, as well as further knowledge of the tumor microenvironment are essential to aid formulation development and enable advancements in the field.

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Polymeric Nanocarriers in Cancer Theranostics

Vanessa Carla Furtado Mosqueira, Marina Guimaraes Carvalho Machado, and Maria Alice de Oliveira

Abbreviations

AuNPs	Gold nanoparticles
AuNR	Gold nanorod
AuNS	Gold nanospheres
CNT	Carbon nanotubes
Doxo	Doxorubicin
EPR	Enhanced permeability and retention effect
ICG	Indocyanine green
MION	Magnetic iron oxide nanocrystals
MRI	Magnetic resonance imaging
NIR	Near-infrared
NIRF	Near-infrared fluorescence
NP	Nanoparticle
PAI	Photoacoustic imaging
PDT	Photodynamic therapy
PEG	Polyethylene glycol
PET	Positron emission tomography
PLA	Poly(*D,L*-lactide)
PLA-PEG	Poly(*D,L*-lactide)-*block*-polyethylene glycol
PLGA	Poly(*D,L*-lactide-*co*-glycolide)
PLGA-PEG	Poly(*D,L*-lactide-*co*-glycolide)-*block*-polyethylene glycol (PLGA-PEG)
PNP	Polymeric nanoparticle
PTT	Photothermal therapy

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QDs	Quantum dots
ROS	Reactive oxygen species
SPECT	Single photon emission computed tomography
SPION	Superparamagnetic iron oxide nanoparticles
US	Ultrasound

1 Introduction

One of the major problems of conventional cancer treatment is the lack of specificity of chemotherapeutic agents. When the disease can be at the same time monitored and combined with a more selective chemotherapy, the approach can be called "all-in-one" theranostic platforms. Theranostics are based on sophisticated and recent technologies. The monitoring is generally made by in vivo imaging and contributes to improving the efficacy and safety of new developed personalized treatments (Walia & Acharya, 2016). To overcome the main issues of conventional chemotherapy, the search of suitable delivery systems for small molecules, biomolecules, targeting moieties, and probes with high efficacy for the diagnosis and therapy got inspiration from Paul Ehrlich "magic bullet" theory more than a hundred years ago (Strebhardt & Ullrich, 2008). It is an efficient strategy that can lead to early disease detection, targeted treatment, and real-time monitoring therapeutic effect with potential to reduce toxicity in cancer therapy. The theranostic approach includes personalized medicine in clinics, such as bioproducts, molecular imaging, and very frequently a targeting strategy. In general, the best balance between risk and benefit for a wide range of patients is reached (Sharpe et al., 2020). The main applications of theranostics in medicine are the use of non-invasive molecular imaging method to evaluate disease heterogeneity, better monitoring of drug biodistribution and accumulation at a target site, more precise understanding of in situ drug release kinetics (at the target site), and prediction of drug response and associated adverse effects in special cases (Leeds, 1990). The main challenges of the field are to find emission probes, new molecules that can be used as photosensitizers, and imaging modalities associated with targeted medicines to obtain a successful patient outcome.

However, a better molecular understanding of the molecular targets and a better way to optimize the biodistribution and selectivity of the nanoconstructs for the success of theranostics technology are required. To achieve this goal, nanotechnology applied to the biomedical area has developed significantly in recent decades, especially due to its potential to revolutionize the treatment of serious diseases such as cancer and cardiovascular diseases. Nanocarriers decorated with ligands able to target specific cells have been investigated with the advantages of improving the therapeutic efficacy of drugs and drastically reducing, in some cases, toxicity and adverse effects (Nicolas et al., 2013; Banik et al., 2016). Molecular imaging techniques have evolved a lot in recent years. Examples include near-infrared fluorescence (NIRF), positron emission tomography (PET), and magnetic resonance imaging (MRI) as diagnostic tools (Zhou et al., 2016). The imaging modality based on the NIR fluorescence is one of the most promising, and it is nowadays intensively investigated,

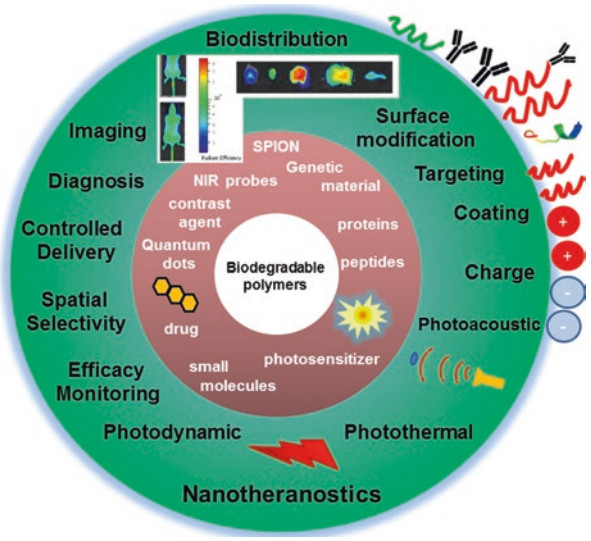

Fig. 1 Schematic representation of the diversity of properties of polymeric nanoparticles used as nanotheranostics in cancer therapy and diagnosis

particularly using the small molecules and nanodevices to increase spatial resolution and targeting properties (Fig. 1). Herein, it will be presented promising preclinical results that have been reported more recently and discussed in the context of their biological applications (Tuguntaev et al., 2022).

2 Nanostructured Carriers and Cancer Treatment

The use of nanotechnology has been extensively studied to obtain improved accumulation of drug concentrations at the tumor site, destroying cancer cells and dramatically decreasing the effect on healthy cells. This has shown potential to revolutionize cancer treatment, tumor detection, and diagnosis (Misra et al., 2010). The physiologic base for the nanostructure accumulation in tumor sites is related to the process of neovascularization in tumors, leading to the rapid and irregular development of new vessels. The newly formed vessels present much larger fenestrations (100–780 nm) when compared to normal vascular endothelium, which contains fenestrations of size between 5 and 10 nm (Hobbs et al., 1998; Maeda et al., 2000). Therefore, nanoparticles with an average size of 200 nm can permeate the larger fenestrations of tumor neo-vessels more easily than the narrow fenestrations of the endothelium of normal tissues (Fig. 2). Furthermore, lymphatic drainage is compromised in these sites, by the lack of lymphatic vessel development near tumors. This mechanism is known as enhanced permeability and retention (EPR) effect (Torchilin, 2011). In some cases, this phenomenon can increase the concentration of drug encapsulated in nanoparticles in tumors by up to 70 times (Liechty & Peppas, 2012).

The EPR phenomenon alters the distribution of nanocarriers to the tumor without necessarily increasing its ability to reach the specific pharmacological target, an

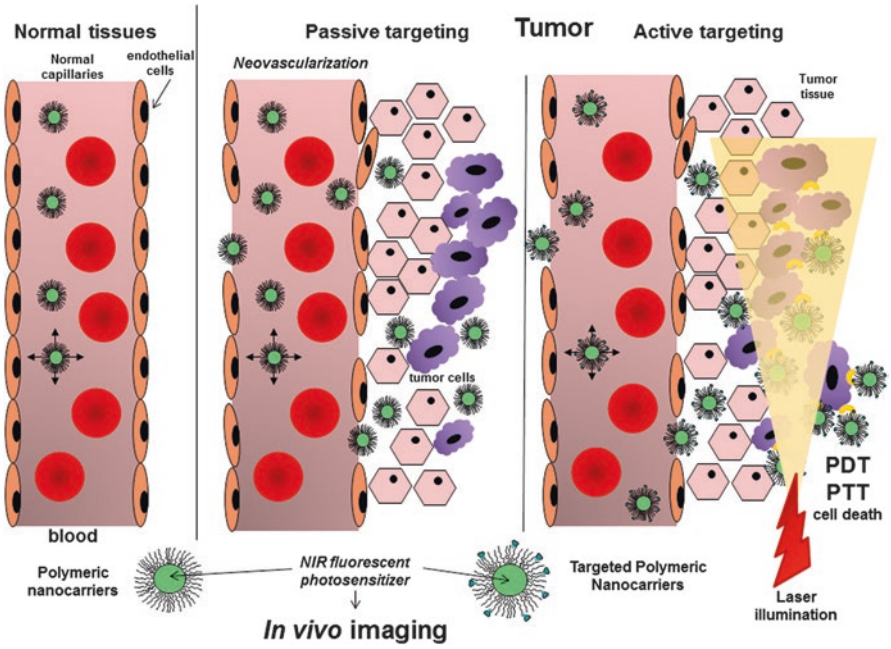

Fig. 2 Schematic representation of physiologic differences in the endothelium of normal and tumor tissues and the strategies of targeting, passive and active, applied in cancer treatment with polymeric nanoparticles used as theranostics

approach called passive targeting (Fig. 2). In contrast, nanoparticles have the potential to accumulate at the tumor site compared to normal tissues via surface modification with ligands in "active targeting" approach and via particle size and other physical properties' optimizations. Therefore, to increase the affinity of nanoparticles for tumor cells and, consequently, increase their internalization, active targeting has been used frequently. In this, the nanoparticles are modified through the insertion of ligands with affinity for specific receptors highly expressed in the target tissue, which are thus able to interact with the target cells through receptor-mediated endocytosis (Fig. 2) (Bertrand et al., 2014). The complexity of cancer requires research to expand our understanding of the interactions of nanomaterials with the tumor microenvironment (Bertrand et al., 2014).

Imaging/diagnosis modalities associated with the use of nanosystems can be useful tools to contribute to the advancement of research in antitumor chemotherapy (Xue et al., 2018). In this complex field of oncology, theranostic strategy has the potential to make the treatment of tumors faster, more efficient, and safer, since the same nanocarrier can detect and kill tumor cells, allowing a non-invasive diagnosis and more accurate therapy. Nanoformulations can be tracked using different imaging modalities so that their targeted accumulation and delivery to the tumor site can be monitored (Fig. 1). Therefore, one of the challenges in this area is the development of nanoparticles with multiple functions (Misra et al., 2010). Imaging after the

administration of the nanotheranostic is important to monitor and verify biodistribution and the amount of drug reaching the target site from a pharmacokinetic point of view and also to determine an optimal time for its therapeutic activation (Choi, 2020). Activation of the therapeutic molecule in the specific target tissue improves the therapeutic efficacy of drugs and drastically reduces toxicity and adverse effects on healthy tissue. To activate the therapeutic module of an image-guided nanostructure, different strategies can be applied, such as photothermal and photodynamic activation.

3 Photodynamic (PDT) and Photothermal (PTT) Cancer Therapies

Recently, cancer treatment with photodynamic (PDT) and photothermal (PTT) therapies and photoacoustic imaging (PAI) in diagnosis has emerged (Ai et al., 2016; Walia & Acharya, 2016). Photodynamic therapy consists of the incidence of laser light at the tumor site, activating the molecule of the photosensitizer that transfers energy to the oxygen present in the tumor environment, leading to the formation of singlet oxygen ($^1O_2$), which is cytotoxic by generating highly reactive radical species that lead to the death of underlying cells (Aires-Fernandes et al., 2022). Photothermal therapy, on the other hand, occurs through the production of heat from the irradiation of a molecule by laser light (Dolmans et al., 2003). Photoacoustic imaging (PAI) offers rich optical contrast and a high depth-to-resolution ratio, revealing anatomical information (Jablonowski et al., 2017; Upputuri & Pramanik, 2020). In all these specific techniques using light-based technologies, the fluorescent probes act also as photosensitizers and can have their photophysical properties modified by association with polymeric nanoparticles. Most of them have been investigated in the biomedical field for cancer treatment.

Phototherapies such as photodynamic (PDT) and photothermal (PTT) associated with delivery systems such as polymeric nanoparticles have shown great potential in the treatment of tumors, since non-toxic molecules are photoactivated by laser illumination at a specific wavelength, making the treatment more site selective. Thus, seeking to increasingly refine research in this area, new strategies based on nanotechnology improved the efficiency of PDT and PTT (Gao et al., 2020). For this, it is necessary to use fluorescent molecules in imaging techniques and with photosensitizing capacity to act in PDT and PTT, with multiple functions.

3.1 Photodynamic Therapy (PDT)

PDT has been considered a minimally invasive modality of cancer treatment, and it is the result of the combination of a chemical substance capable of being photoactivated (called a photosensitizer) with light and oxygen molecules (Fig. 3). The Fig. 3

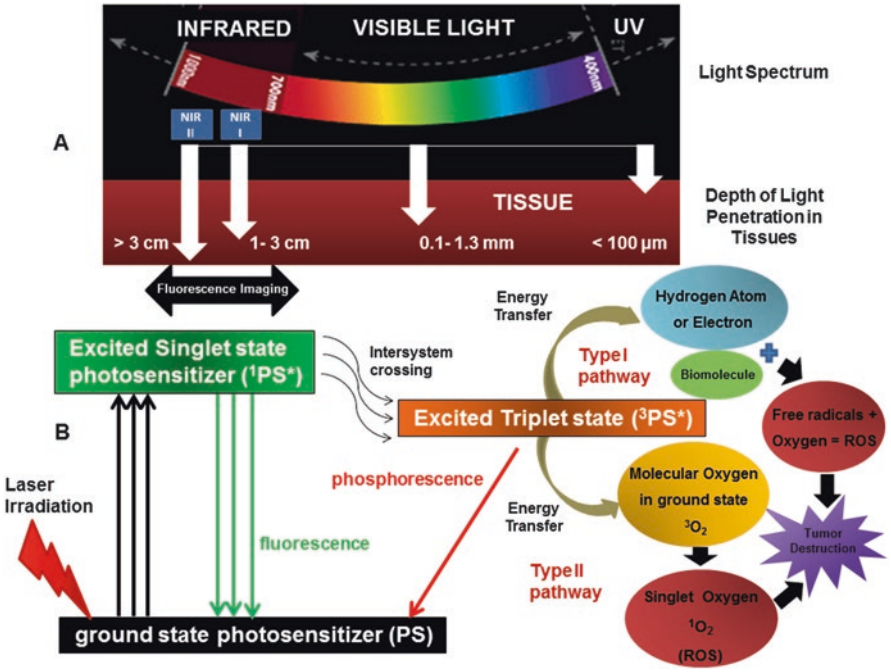

Fig. 3 In (**a**) the wavelength range used for imaging of nanotheranostics and depth of light penetration in tissues. In (**b**) the schematic representation of the two pathways of tumor destruction in photodynamic therapy of cancer using a photosensitizer

summarizes the general mechanisms of PDT. The incidence of a laser, at the appropriate wavelength, at the site of interest after intravenous or topical administration of formulations containing the photosensitizer (PS) causes the transfer of energy from the molecule to the oxygen present in the environment, leading to the formation of singlet oxygen ($^1O_2$). Reactive oxygen species (ROS) are produced leading to cell death (Gao et al., 2020). ROS can be produced by two different pathways, but that occur at the same time. In the type I reaction, from the excited state, the photosensitizer can also transfer energy to the biomolecules around it, such as lipids and proteins, leading to the formation of free radicals and anionic radicals from the photosensitizer and the substrate. Electrons interact with oxygen molecules, which leads to the production of ROS (Kwiatkowski et al., 2018). In the type II reaction, the photosensitizer in its excited triplet state ($^3PS^*$) reacts with molecular oxygen in triplet ground state ($^3O_2$), in a process called triplet-triplet annihilation. PDT also induces hypoxia in the tumoral region, contributing to cell death. This therapy is considered much less invasive for tumor treatment than that performed by surgery and much more selective than radiotherapy and chemotherapy due to the illumination of the selected tumoral regions (He et al., 2018a).

Photodynamic activation is an important strategy in the development of nanotheranostics since it led to ROS-mediated cytotoxicity and nanoparticulate systems allow the controlled release of photosensitizer and has been associated with an

optical imaging modality (Fig. 3a). The in vivo imaging monitoring of biodistribution is due to the fluorescence emission of the photosensitizer or metal-based photoactive nanomaterials, being frequently applied in image-guided delivery (Zhang et al., 2018).

The generation of ROS in the target tissue (tumor) causes damage to tumor cells and blood vessels, triggering cell death by necrosis, if there is strong damage to the cell membrane (Fig. 3). On the other hand, apoptosis preferentially occurs if damage is more related to cell organelles. In addition, activation of innate and adaptive immunity can also be induced, and necrosis can stimulate non-specific immune reactions, thus contributing to an antitumor immune response (Gao et al., 2020).

3.2 Photothermal Therapy (PTT)

PTT is a more recent and promising treatment, based on the use of a photothermal agent that, after absorbing light of a specific wavelength, is excited and dissipates the absorbed energy in the form of heat, inducing an increase in the local temperature. It causes irreversible cell damage by hyperthermia or thermal ablation (Gao et al., 2020). PTT has a simpler action mechanism and does not depend on oxygen to have a cytotoxic effect on the cells (Alsaab et al., 2020). Thus, even if the molecule has a low fluorescence quantum yield and low efficiency in the production of ROS, after laser irradiation, it may still be capable of generating hyperthermia (Gao et al., 2020). Not only fluorescent molecules can be used for photothermal activation; other classes of nanomaterials like gold nanoparticles (AuNPs), gold nanorod (AuNR), hollow gold nanosphere (AuNS), black phosphorus, CuS, and MoS_2 nanosheets are able to produce localized heat through plasmonic photoactivation (Choi, 2020). Another possibility is the photothermal drug release that occurs through pore opening or nanoparticles disassembly.

The general requirements for fluorescent probe use in molecular imaging and in PDT, PTT, and PAI are to present (I) a remarkable contrast effect, i.e., a high signal-to-noise ratio under real physiological conditions; (II) pronounced in vivo stability under the effect of enzymes in serum or targeted tissue; (III) fast clearance from healthy organs; and (IV) low cost of production under eco-friendly conditions. Several research groups are exploring the association of fluorescence probe imaging agents with nanoparticles as better contrast agents to overcome current drawbacks that impair the full development of the different bioimaging techniques.

4 Imaging Agents and Techniques in Cancer Theranostics

The combination of diagnosis and treatment has been a trend for clinical application (Tuguntaev et al., 2022). Coupled with advances in the study of tumor treatment, it was also necessary to develop superior imaging modalities. Different types of

imaging techniques have been widely used for tumor detection, such as magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), X-ray, ultrasound (US), and NIR in vivo imaging (Yuan et al., 2013). Although these techniques are widely used and sensitive in some cases, in all of them, there are limitations. Thus, new diagnostic and therapeutic tools are always the subject of intense investigation.

The use of gold nanoparticles (AuNPs), iron oxide nanoparticles (IONP), carbon nanotubes (CNTs), quantum dots (QDs), or mesoporous silica has been intensively investigated. However, they have a limitation of short drug release time (<6 h) and in some approaches require drug covalent attachment to the metal nuclei of the particles (Bagalkot et al., 2007). On the other hand, the acquisition of images using NIR fluorescence through the excitation of fluorophores had extraordinary growth in recent years. This is because many of the photosensitizers used in PDT have fluorescent properties in NIR that allow in vivo imaging (Gao et al., 2020).

Optical imaging is a high sensitivity technique, non-invasive, nonionizing, and based on the detection of low levels of light emitted by specific molecules from the body. Molecules of interest emit light such as bioluminescence and fluorescence that are acquired by special equipment providing optical imaging (Reisch & Klymchenko, 2016). Bioluminescence is based on the identification of specific reactions in the body by light-emitting proteins. Fluorescence uses an external light to activate specific molecules that emit light in another wavelength. Both types have some limitations for their application in vivo, such as small penetration depths, interference of tissue autofluorescence, and light scattering (Fig. 3). Thus, new and more modern probes for optical imaging agents, which present outstanding photophysical properties, have been investigated in order to overcome these limitations (Table 1), though few fluorophores acting as photosensitizers in PDT are approved for human use in photodynamic therapy.

5 Fluorophores as Photosensitizers in Theranostics

The presence of a fluorophore tracer on the theranostic nanoparticles allows monitoring tumor location, disease evolution, and treatment efficiency. The indocyanine green (ICG) molecule was approved for diagnostic application by the US regulatory agency Food and Drug Administration (FDA) more than 50 years ago (Yi et al., 2014). However, some problems which are related to the use of its free form have been reported, such as inadequate optical properties, aggregation problems, and strong adsorption to plasma proteins, which can affect its biodistribution. Furthermore, several attempts were made to carry this molecule into different types of nanoparticles. However, these studies resulted in low encapsulation efficiency or poor stability over time or after injection (Jacquart et al., 2013).

Organic fluorescent dyes have enormous potential for nanotheranostic applications and can be used in fluorescence and photoacoustic imaging techniques and in photodynamic and photothermal therapies. However, most of these dyes have low

Table 1 Examples of fluorescent probes used for cancer imaging diagnosis and therapy

Class	Probes (λex/λem) (nm)	Advantages	Limitations	Applications
Cyanines	Indocyanine green (ICG) (789/814 nm) Cy3 (554/565 nm) Cy5 (651/670 nm) Cy7 (756/779 nm)	Narrow absorption bands High molar extinction coefficient	Usually have low photostability Low fluorescence quantum yield in aqueous medium Aggregation in aqueous media	Widely used as active ingredients in semiconductor materials, laser materials, dyes, and biomarkers for nucleic acids and proteins
Modified cyanines	IR783 (633/780 nm) MHI-148 (782/802 nm) IR780 (780/808 nm) IR808	High molar extinction coefficient	High hydrophobicity Low stability in aqueous media	Potential for in vitro and in vivo imaging and rapid detection of kidney tumors Ability to target tumor cells Detects small lesions and metastases
BODIPY and derivatives (boron-dipyrromethene)	Classic BODIPY (505/516 nm) Aza-BODIPY Iodo-substituted aza-BODIPY derivative (AZB-I) (680/720 nm)	High molar extinction coefficient and fluorescence quantum yield Thermal and photochemical stability	Excitation and emission wavelengths outside the NIR region Low tissue penetration Low water solubility Low molar extinction coefficient Small stokes' shifts	Photodynamic therapy Molecular photonic wires
Phthalocyanines and porphyrin derivatives	Zinc phthalocyanine (ZnPc) (674/678 nm) Chloroaluminium phthalocyanine (AlClPc) (610/679 nm) Porfimer sodium (Photofrin®) (500, 540 and 560/615 and 680 nm) Chlorin e6 (400 /665 nm)	High thermal and chemical stability Excellent photophysical properties	High hydrophobicity and low bioavailability	Photosensitizers for photodynamic therapy

aqueous solubility. For this reason, these molecules are commonly associated with nanocarriers, such as liposomes and polymeric nanoparticles, which can improve the dispersity of these molecules in water, alter their distribution profile, and protect against degradation. In addition, other molecules can be associated with these carriers, such as drugs and targeting molecules.

Recently, photosensitizers with emission at near-infrared (NIR) region have been exploited in the non-invasive imaging technique and have been shown to be a good option for tumor detection due to its high sensitivity. There is a "biological window," a range of wavelengths from 650 to 1700 nm for tissue imaging (Fig. 3). In the near-infrared (NIR) region (700–900 nm), there are less interference from tissue autofluorescence and deeper penetration of light into the tissue. Therefore, the fluorescence imaging technique allows real-time monitoring of biological samples in vitro and in vivo (Yi et al., 2014).

Penetration depth of NIR light in tissue depends on the NIR wavelength, as shown in Fig. 3. To obtain images by NIRF, it is necessary to use fluorescent probes that absorb in the NIR region with adequate chemical and photophysical properties. The markers usually used for this purpose are divided into two groups, organic and inorganic molecules. However, due to the cytotoxic potential and accumulation of inorganic molecules, organic molecules are more attractive (Luo et al., 2011). These molecules can absorb NIR light at a specific wavelength to reach an excited state. Then part of the energy of the excited state dissipates as light with a longer wavelength, called fluorescence. Therefore, NIR fluorescent molecules can be applied in vivo to monitor tumors using imaging techniques (Yuan et al., 2013). Obviously, theranostic polymeric nanoparticles possess physical limitations with respect to penetration deep of light due to scattering. Other limiting factors are the power of the excitation wavelength source, its distance to the NPs, rapid photobleaching of some fluorophores, self-quenching, the biodistribution of NPs within the body, and the sensitivity of the luminescent detection because of low depth of light penetration in tissue (Frangioni, 2003; He et al., 2018b). In particular, NIRF-based imaging has shown to be very promising, becoming an important technique for the study and monitoring of tumor evolution, therapeutic response, and diagnosis, being applied non-invasively in vivo to preclinical studies in small animals (Kim et al., 2016). Fluorescence imaging in the NIR windows (first NIR-I 700–900 nm and second NIR-II, 1000–1700 nm) offers unique advantages in terms of reduced photon scattering, deep tissue penetration, high signal-to-background ratio, high sensitivity, low scattering light, and low cost of small organic molecules (Frangioni, 2003; Adams et al., 2007; Qian et al., 2012; Yuan et al., 2012; Guo et al., 2014; Diao et al., 2015; Ding et al., 2018; He et al., 2018b; Jiang et al., 2020).

The fluorescent probes have some disadvantages such as low aqueous solubility and rapid elimination from the organism, which can be circumvented by association with polymeric nanocarriers to increase dispersion in biological media, obtaining better photophysical stability (de Paula et al., 2013) and controlled release properties (Alves et al., 2018). Currently, indocyanine green (ICG), methylene blue (MB), and 5-aminolevulinic acid-induced protoporphyrin IX are Food and Drug Administration (FDA)-approved dyes for clinical applications.

NIR fluorescence imaging has been used in preclinical and fundamental research studies based on the availability of fluorophores, such as cyanines (Cy5, Cy7, indocyanine green) and methylene blue. The cyanine-based dyes are tunable absorption and emission spectra obtained by varying the chain length of conjugated polymethine that lead to a bathochromic shift of about 100 nm (Dahal et al., 2021).

Table 1 summarizes the PDT photosensitizers with excellent photothermal conversion efficiencies that produce in vivo efficient inhibition of tumor growth with biocompatibility and prominent integrated photoacoustic imaging performance and with a high contrast in living mice at a low systemic injection mass. Encapsulation and covalent attachment of photosensitizers to polymers is a strategy to obtain highly efficient polymeric NIR photothermal agents for high desirable cancer phototheranostic nanoplatforms.

Tetrapyrrole-based compounds are fluorescence probes that have been widely used as agents for photodynamic therapy, such as porphyrins, chlorins, benzochlorins, and phthalocyanines (Zhang et al., 2012). Another class is the borondipyrromethene, known as BODIPYs. They are probes that exhibit very high fluorescence quantum yield, relatively sharp absorption and emission, and high photo- and chemical stability under physiological conditions (Ulrich et al., 2008). However, their low water solubility, relatively low molar extinction coefficient, small Stokes' shifts, and emission limitations in visible to deep red regions restrict their wide range use for NIR imaging applications.

Among the available fluorescent probes with good photophysical properties for PDT and PTT, IR780 iodide, an indocyanine heptamethine with a maximum absorption peak at 780 nm, stands out for its ability to preferentially accumulate in the mitochondria of tumor cells (Zhang et al., 2010; Alves et al., 2018). In an attempt to better elucidate the mechanism by which this occurs, Zhang et al. (2014) demonstrated in a comparative study that the cellular uptake of IR780 was significantly higher in tumor cells than in non-tumor cells, probably mediated by OATP1B, an organic anion transporter peptide (OATP) subtype overexpressed in tumor cells. Furthermore, there was a high degree of co-localization of IR780 and Mito-tracker Red, confirming their targeting to the mitochondria of tumor cells. In photodynamic therapy with IR780, there is an incidence of a laser, usually at 808 nm, at the tumor site after intravenous administration of formulations containing the photosensitizer. This irradiation causes the photosensitizer to transfer energy to the oxygen present in the tumor environment, leading to the formation of singlet oxygen ($^1O_2$), which is cytotoxic and leads to the death of cells at the site and surroundings, through the oxidation of proteins, lipids, and DNA (Fig. 3, type II reaction) (Dolmans et al., 2003). IR780 has been used successfully in photodynamic (PDT) and photothermal (PTT) therapies in preclinical investigations for cancer therapy, since after lighting it has the ability to generate reactive oxygen species (ROS) and increase the local temperature (Pais-Silva et al., 2017; Alves et al., 2018). IR780, for example, can be also properly detected by imaging techniques through NIRF. It also allows for ex vivo and in vivo tumor imaging in small animals, investigation of the pharmacokinetics of antitumor drugs, and monitoring of tumor response to treatment over time (Luo et al., 2011).

6 Polymeric Nanoparticles as Nanotheranostics

Despite having promised and distinct optical and physicochemical properties, the use in the biological field of inorganic nanomaterials is limited due to their low biodegradability and the possibility of accumulation of these materials in the body. Thus, organic and biodegradable nanomaterials are considered more appropriate as carriers for the different molecules used in chemotherapy of different diseases and in diagnosis.

Polymeric nanoparticles (PNPs) are particles obtained from polymers that can be obtained from natural sources or produced by synthesis or semi-synthesis. Polymeric nanosystems used in therapy of cancer are generally organized and self-assembled with a nanometric size (10–300 nm) (Banik et al., 2016). PNPs possess high diversity of physicochemical properties and attract great attention as multifunctional nanocarriers in drug delivery systems (El-Say & El-Sawy, 2017).

Depending on the preparation method, drugs can be entrapped, encapsulated, or bound to polymeric NPs. Polymeric NPs are organized as matrix systems, called nanospheres, or vesicular systems, called nanocapsules (Fig. 4). There are also self-aggregated polymers that form micellar systems crosslinked that are closely related to nanoparticles. Nanospheres (NS) are colloidal particles that entrap the drug inside their matrix by physical dispersion, by dissolution of molecules in polymeric matrix, or by adsorption of the actives on the nanoparticle surface. Nanocapsules (NC) are systems consisting of an oily core cavity with an encapsulated drug and polymeric shell surrounding it.

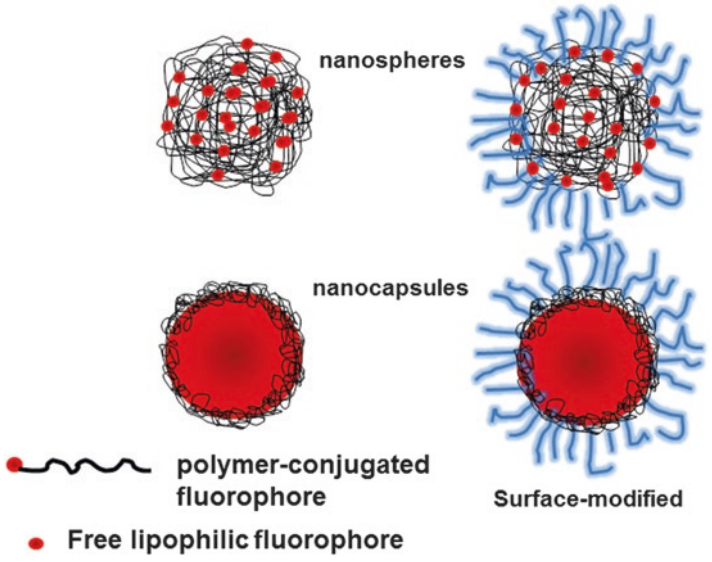

Fig. 4 Schematic representation of polymeric nanoparticles, the nanospheres (matrix systems) and nanocapsules (vesicular systems), and the dual mode of probe association to the nanoparticles

Polyesters such as poly(*D,L*-lactide) (PLA) and poly(*D,L*-lactide-*co*-glycolide) (PLGA) and their amphiphilic copolymers poly(*D,L*-lactide)-*block*-polyethylene glycol (PLA-PEG) and poly(*D,L*-lactide-*co*-glycolide)-*block*-polyethylene glycol (PLGA-PEG) are biocompatible polymers that allow flexibility in the dosage of the associated drug, being easily degraded in the human body to lactic acid and glycolic acid, which is metabolized to pyruvate and enters the Krebs cycle, where it is catabolized to CO_2 and water (Bazile et al., 1995). Nanoparticles (NPs) obtained from these biodegradable polymers have shown to be promising nanocarriers due to their ability to promote a controlled release, dependent on the degradation of its polymer matrix. In addition, PEG confers good physicochemical stability during storage and in biological fluids increasing the applications as drug delivery systems (Fig. 4). PEG also delays the adsorption profile of serum proteins on the surface of polymeric nanoparticles (Mosqueira et al., 2001; Sharma et al., 2015). Recently, an interesting review reported the use of polyesters for medical imaging and theranostic applications (Nottelet et al., 2015). Despite the potential of polymeric nanoparticles containing encapsulated fluorophores for application in imaging techniques using NIRF, few are suitable for in vivo imaging applications.

Table 2 summarizes selected polymeric nanoparticles designed as theranostics evaluated in vivo. The nanocarriers present several advantages such as outstanding properties for guided therapy, imaging monitoring using suitable probes encapsulated or covalently linked, and triggered release of encapsulated cargos for antitumor therapy. Most nanocarriers used in theranostics are built with strategies that comprise a physical interaction of the polymeric network with the NIR probes (Pound-Lana et al., 2019) or the covalent attachment of a probe to the polymeric matrix (de Oliveira et al., 2019; Machado et al., 2020).

7 Inorganic-Based Probes in Polymeric Nanoparticles for Imaging

Nanostructured inorganic-based fluorescent probes such as quantum dots (QDs) (Ag_2S QDs, PbS QDs), rare earth-doped nanoparticles (RENPs), and single-walled carbon nanotubes (SWCNTs) have been developed as alternatives to NIR small-molecule fluorophores for imaging in the NIR-II window. However, the biomedical application of these nanoparticle-based fluorophores, including SWCNTs, is limited due to their dose-dependent toxicity and metabolism (Ema et al., 2016; Jin, 2019). Gold nanoparticles (AuNPs) were used experimentally for non-invasive in vivo Raman monitoring because they show a strong absorbance in the phototherapeutic window (650–850 nm). Iron oxide-based nanoparticles ensure detection by magnetic resonance imaging (MRI) with lower toxic effects.

Superparamagnetic iron oxide nanoparticles (SPION) have been applied to design theranostic polymeric micelles for targeted cancer therapy and diagnosis using magnetic resonance imaging technique. The effects of SPIO clustering on the

Table 2 Polymeric nanoparticles investigated as theranostics

Nanosystem	Polymer	Imaging probe	Drug	Targeting agent	Physicochemistry	Tumor cell model	Mainly results in vitro and in vivo	References
Nanoparticle	PEG-PLA	SPION	Doxorubicin (Doxo)	–	Clustered SPIO affects MR imaging properties	–	Enhanced Doxo plasma AUC and half-life	Jiang et al. (2022)
Nanoparticle	PLGA	SPION	Doxorubicin	–	–	Glioma cells	Accumulation of the NP within the tumoral tissue Reduction of tumor growth rate in orthotopic U87 nude mice	Luque-Michel et al. (2021)
Nanoparticle	PEG-PCL	Iodo-substituted aza-BODIPY (AZB-I)	–	–	660 nm red LED lamp irradiation	4T1 breast cancer cell	49.8% tumor growth inhibition in 4T1 tumor-bearing mice	Treekoon et al. (2021)
Polymeric micelles	Poly(2-oxazoline)-based polymeric micelles	–	Paclitaxel	–	Super-low-frequency alternating current magnetic field	MCF-7	Improved tumor and lymph node accumulation and signal reduction in vivo (2.7% in tumor; 8.5% in lymph node)	Seo et al. (2022)
Nanoparticle	PEG-PLGA	Sulfo-cyanine 5.5	Paclitaxel	Cyclic (cRGDfK)	–	4T1	Clear NIRF images for biodistribution Improved tumor drug accumulation Stronger anticancer activity Targeting ability and synergistic effect of the drugs	Kim et al. (2021)

Nanosystem	Polymer	Imaging probe	Drug	Targeting agent	Physicochemistry	Tumor cell model	Mainly results in vitro and in vivo	References
Nanoparticle	PLGA	Chlorin-e6	L-arginine	–	–	MDA-MB-231	Alteration in mitochondrial energy metabolism, rendering tumor cells more sensitive to phototherapy by blocking ATP synthesis Enhance photoacoustic (PA) image contrast in vivo Alleviate hypoxia and enhance sensitization of tumor cells	Xiang et al. (2021)
Polymeric micelles	Chlorin-e6-modified glycol chitosan (GC)	Manganese dioxide (MnO_2)	Catalase (CAT)	–	–	HeLa	Enhance PDT efficacy in subcutaneous HeLa tumors Enhance PDT efficacy toward HeLa cells	Zhu et al. (2020)
Nanoparticles	PLGA	Cy7 cyanine	Doxorubicin	CRGDK homing peptide	–	MDA-MB-231	NP accumulation in the tumor area Reduction of tumor volume in orthotopic xenotransplant mouse model using TNBC cells	Cano-Cortes et al. (2020)

physicochemical and biological properties of polymeric micelles were evaluated using PEG-PLA micelles loaded with SPIO and doxorubicin (DOXO-loaded SPIO-micelles). The SPIO clusters in the micelles hydrophobic core significantly affected the physicochemical, biological and theranostic properties of the DOXO-loaded SPIO-micelles. The presence of SPIO in the micelle core resulted in (1) an increase in micelle size proportionally to the weight rate of SPIO added to the formulation; (2) a reduction of critical micelle concentration (CMC); (3) a reduction of the in vitro dissociation constant of micelles; (4) an increased DOXO loading efficiency; and in (5) a modification of the DOXO release profile, showing a more sustained release profile. In vitro biological analyzes showed that cellular uptake by H1299 non-small cell lung carcinoma cells was reduced with increasing SPIO weight ratio in micelles. However, increasing SPIO concentration in micelles improved the MR sensitivity of the formulation after its uptake by cells. Pharmacokinetic studies showed that polymeric micelles enhanced the area under the curve (AUC) and prolonged Doxo half-life in mice plasma (Jiang et al., 2022). PLGA polymeric nanoparticles loaded with SPION and Doxo (SPION-DOXO PNP) were developed for the theranostics for glioblastoma. Glioblastoma multiforme (GBM) is the most common and aggressive form of glioma, a type of brain tumor, and has a very poor prognosis with an expected survival of around 15 months. Targeting using an external magnetic field resulted in a significant increase in SPION-DOXO PNP accumulation in brain tumor tissue after IV administration in mice, as evidenced by MRI. This increase in the particles concentration in the tumor region resulted in a significant reduction in the rate of tumor growth (Luque-Michel et al., 2021).

The tri-functionalized NPs carrying doxorubicin, near-infrared cyanine tracer (Cy7), and a homing peptide (CRGDK) were designed, which can actively recognize the neuropilin-1 receptor, overexpressed in triple-negative breast cancer. They were evaluated in vitro in human breast cancer model and in vivo using an orthotopic breast cancer xenotransplant mouse model. These NPs accumulated in the tumor area and induced a reduction of tumor volume (Cano-Cortes et al., 2020).

Poly(2-oxazoline)-based polymeric particles loading paclitaxel (>40% w/w) were developed as a novel theranostic system designed to capitalize on the magnetic nanoparticle properties as imaging agents, called NanoFerrogels (Seo et al., 2022). NanoFerrogels improved tumor and lymph node accumulation and signal reduction in vivo (2.7% in tumor; 8.5% in lymph node) compared to clinically approved imaging agent ferumoxytol (FERAHEME®) 24 h after administration, demonstrating that this system has potential as remotely actuated theranostic platform for cancer diagnosis and treatment.

The fluorescent conjugated polymer nanoparticles (CPNs), poly(9,9-dioctylfluorene-alt-benzothiadiazole) loaded with octaethylporphyrin, have emerged as advanced polymeric nanoplatforms in biomedical applications by virtue of extraordinary properties including high fluorescence brightness, large absorption coefficients of one and two photons, excellent photostability, and colloidal stability

in water and physiological medium. CPNs were synthesized by incorporating a metal oxide magnetic core (5 nm Fe_3O_4-$NiFe_2O_4$ NP) into their matrix by nanoprecipitation to allow in vivo monitoring of CPNs in glioblastoma-bearing mice using MR imaging and intravital fluorescence, techniques widely used for intracranial tumor evaluation (Arias-Ramos et al., 2021). In the orthotopic glioblastoma model, the nanoparticles' size hinders an effect on intratumorally T2-weighted image signals, without affecting PDT. Other types of polymeric magnetic iron oxide nanocrystals (MIONs) were designed as potent theranostic nanoplatforms due to their photophysical properties with simple and cost-effective nanoengineering through self-assembly, biocompatibility, and attractive near-infrared photothermal properties (Kolokithas-Ntoukas et al., 2021). In vitro studies highlighted a synergy-amplified photothermal effect that significantly reduces the viability of A549 cancer cells upon 808 nm laser irradiation.

Different strategies of O_2-replenishing were used to improve PDT in solid tumors, because sub-physiological hypoxia in tumors is caused by dysregulated tumor vasculature. Furthermore, the intracellular antioxidant glutathione (GSH) can reduce the generated $^1O_2$. Nanoparticles composed of catalase (CAT) and manganese dioxide (MnO_2) integrated within chlorin-e6-modified glycol chitosan (GC) were developed as an intelligent multifunctional synergistic nanoplatform (CMGCC) for T_1-weighted magnetic resonance (MR) imaging-guided with enhanced PDT activity (Zhu et al., 2020). This system could improve the chlorin-e6 accumulation within the tumor region, catalase effectively re-oxygenated the hypoxic tumor via catalyzing endogenous hydrogen peroxide to O_2, and MnO_2 consumed the intracellular GSH while simultaneously producing Mn^{2+} as a contrast agent for T_1-weighted MR imaging. Both in vitro and in vivo experiments demonstrate that this polymeric NP exhibits significantly enhanced PDT efficacy toward HeLa cells and subcutaneous HeLa tumors as a promising synergistic theranostic nanoplatform for cancer therapy. Using the same strategy, PLGA biodegradable polymeric nanoparticles (PLGA NP) in which the outer polymeric layer embeds chlorin-e6 (Ce6) and incorporates L-arginine (L-Arg) and nitric oxide (NO) were developed as a nanotheranostic nanoplatform (Xiang et al., 2021). These NPs accumulated in solid tumors by the enhanced permeability and retention (EPR) effect, locally released L-Arg that is oxidized by the abundant H_2O_2 to produce NO. Furthermore, they reduced hyperactive O_2 metabolism of tumor cells by NO-mediated mitochondrial respiration inhibition raising endogenous O_2 tension to counteract hypoxia. In addition, NO hindered oxidative phosphorylation causing intracellular adenosine triphosphate (ATP) depletion, which inhibited tumor cell proliferation and turning cells more sensitive to PDT under NIR light irradiation. NO-based nanoplatform alleviated hypoxia and sensitized tumor cells to amplify the efficacy of phototherapy guided by photoacoustic imaging (PAI).

8 Polymeric Nanoparticles with Organic Fluorescent Tracers and Photosensitizers

Acid-responsive polymeric metal organic framework nanoparticle has been constructed for co-delivering doxorubicin (Doxo) and phototherapy agent, indocyanine green (ICG), for breast carcinoma theranostics (Zhang et al., 2021). Specifically, the polymeric nanoparticles respond to the acidic microenvironment in cells, delivering drugs into target tumor sites. They effectively accumulated in tumor sites and induced powerful immunogenic cell death effect by combined photochemotherapy using multimodal imaging monitor. These NPs demonstrated to be promising in image-guided synergistic tumor therapy.

An iodo-substituted aza-BODIPY derivative (AZB-I) was encapsulated in poly(ethylene glycol)-*block*-poly(caprolactone) (PEG-b-PCL) nanoparticles (AZB-I@PEG-b-PCL) aiming at the development of a formulation for the theranostic of breast cancer. In addition to being monitored by imaging through near-infrared (NIR) light, in vitro studies showed that the formulation was able to significantly reduce the viability of 4T1 breast tumor cells after irradiation with a 660 nm red LED lamp of time- and dose-dependent manner. The production of ROS by cells after exposure to light was also evidenced. The in vivo PDT efficacy was evaluated in a murine model of orthotopic 4T1 breast cancer. Treatment with AZB-I@PEG-b-PCL associated with irradiation with 660 nm light resulted in an inhibition of tumor growth by 49.8% and a suppression of tumor growth, 3 and 14 days after PDT, respectively (Treekoon et al., 2021).

Wang et al. (2016) studied the effect of IR780 in simultaneous PDT/PTT therapy, as they have a synergistic effect, because the incidence of the laser also leads to the photothermal effect (PTT). In the other study, the generation of ROS by IR780 in albumin nanoparticles was demonstrated by Lian et al. (2017) after laser irradiation at 808 nm in human prostate tumor cells. According to Zhang et al. (2010), IR780 can also be used to visualize breast, cervical, lung, and osteosarcoma tumors in mice, since the fluorescence signals observed in the tumor after intravenous administration were much higher when compared to normal tissues. It was also found that cellular uptake of IR780 was significantly higher in tumor cells than in non-tumor cells, probably mediated by OATP1B3, a subtype of organic anion transporter peptides (OATP) (Zhang et al., 2014). Thus, due to this ability to target tumor cells and the high intensity of fluorescence, when compared to ICG, this fluorescent dye stands out in relation to other clinically approved dyes (Alves et al., 2018). IR780 present poor solubility in biological media, rapid elimination from the organism, low stability in aqueous media, and acute toxicity at high doses (1 mg/kg in mice) as limiting factors for its direct use in the treatment of tumors or in the diagnosis (Jiang et al., 2015).

To solve these physicochemical limitations, several types of nanostructures were proposed in the literature to encapsulate IR780 and improve its properties (examples in Table 3). Alves et al. (2018) published a review about IR780 and its applications when encapsulated in different nanomaterials, such as micelles (Pais-Silva

Table 3 Nanostructures containing the IR780 fluorescent probe/photosensitizer

Nanostructure	Particle design	Tumor cell model	Main effects in vitro and in vivo	References
Solid lipid nanoparticles	cRGD ligand targeting integrin receptor $\alpha_v\beta_3$	U87MG	Free or encapsulated IR780 showed no toxicity without laser irradiation Reduction of cell viability to 52% after laser irradiation (IR780 at 2.5 mg/L) Greater accumulation of nanoparticles with ligand at the tumor site Fluorescence signal drop after 24 h	Kuang et al. (2017)
Transferrin nanoparticles	Binds to highly expressed transferrin receptor on tumor cells such as breast and prostate	CT26	Increased cellular internalization and tumor targeting Tumor suppression Increased tumor accumulation 48 h after injection	Wang et al. (2016)
Polymeric micelles	PEG-SH linked to IR780 and C13 chains	CT26	It only showed cytotoxicity after laser irradiation Increased tumor accumulation 24 h after injection Total tumor disappearance after 3 days of PDT treatment	Yuan et al. (2015)
Multifunctional nanostructure	IR780 and cRGC linked and loaded the anti-CRC chemotherapeutic agent, 7-ethyl-10-hydroxy-camptothecin (SN38)	DLD-1 $CaCO_2$ HCT116	Dose-dependent reduction in cell viability IC_{50} reduction for HCT116 cells Significant tumor growth inhibition	Tsai et al. (2017)
Thermo- and pH-responsive polymeric micelles	Poly(ethylene glycol)-b-poly(acrylamide-co-acrylonitrile-co-vinylimidazole) micelles with IR780 and doxorubicin	4T1	Reduction of cell viability after laser irradiation Strong capacity for inhibiting cell migration after laser irradiation Complete tumor elimination and suppress lung metastasis without any obvious adverse effects	Yang et al. (2018)

(continued)

Table 3 (continued)

Nanostructure	Particle design	Tumor cell model	Main effects in vitro and in vivo	References
Polymeric micelles	CRGDK ligand targeting and containing perfluorooctyl bromide (PFOB)	MDA-MB-231 MCF-7	Increased cellular internalization Reduction of cell viability only after laser irradiation Increased ROS production after laser irradiation	Zhao et al. (2018)
Polymeric nanoparticles	IR780-conjugated PLA polymer in PEG-PLA nanocapsules	MCF-7 MDA-MB-231	Increased cell death by apoptosis Reduction of cell migration Increased ROS production after laser irradiation	Machado et al. (2020, 2022)
Polymersomes	NO-containing *cis*-aconitine functionalized copolymer (mPEG-PNTC-CA) polymersomes co-loaded with IR780 and DOX·HCl	MCF-7/R	NIR irradiation accelerated NO release by increasing the microenvironmental temperature Together with NIR-induced PTT/PDT effects on antitumor activity, the formulation presented significantly synergistical roles in cancer therapy on drug-resistant cancer in vivo	Liu et al. (2021)
Polymeric micelles	Amphiphilic FHSV copolymer co-loaded with paclitaxel (PTX) and IR780	S180	The temperature of IR780/PTX/FHSV micelles (50 μg/mL of IR780) under NIR laser irradiation was increased by nearly 25 °C demonstrating to be effective for PTT and PDT with a large amount of ROS produced The system was efficiently endocytosed in tumor cells via specific receptor-mediated endocytosis Good tumor targeting ability, and effectively ablate tumors, with a synergistic antitumor activity	Yang et al. (2021b)
Nanobubbles (NBs)	IR780 iodide and docetaxel (DTX) co-loaded into the lipid shells of nanobubbles	Mia-Paca2	Cell viability decreased in 71.2% with the increase in the concentration of IR780-NBs-DTX 15 days after treatment, the tumor size gradually decreased for IR780-NBs-DTX and NBs-IR780 IR780-NBs-DTX targeted the tumor site at 1 h after injection via caudal vein	Yang et al. (2021a)

et al., 2017), polymeric nanoparticles (Bazylińska et al., 2014), and nanostructured lipid carriers (Li et al., 2016), and even in hydrophobic regions of some proteins such as albumin (Jiang et al., 2015) and transferrin (Wang et al., 2016). Multifunctional nanocarriers have also been developed, where, in addition to IR780, ligands are inserted to increase selectivity for tumor cells, such as the cRGD peptide that directs to integrins present in tumor vessel cells (Kuang et al., 2017) and/or drugs to increase cytotoxicity and improve efficacy in the treatment of tumors such as docetaxel (Lin et al., 2017) and doxorubicin (Yan et al., 2016).

However, in most of the published studies, IR780 is merely physically encapsulated in nanostructures used as theranostic. In the first attempts to covalently bond IR780 to polymers, Yuan et al. (2015) inserted a PEG_{2k} chain through reaction with the chlorine atom of the IR780 molecule, and this structure was able to self-organize in the form of micelles. There was an improvement in the apparent solubility of IR780 and its photothermal properties, and the toxicity was significantly reduced after intravenous administration, in addition to making it more stable against repeated laser irradiation.

In another different approach, to ensure that the fluorescent label IR780 was covalently linked to the nanoparticle, IR780 was conjugated with polylactide (PLA), which was called IR-PLA (de Oliveira et al., 2019). For this, IR780 was derivatized and conjugated (covalently linked) to PLA by means of the one-pot azide-alkyne cycloaddition reaction promoted by cycle stress (SPAAC). Such chemical synthesis was proposed to ensure that when nanoparticles prepared with this modified polymer were applied to in vivo imaging (biodistribution) studies, the nanoparticle and the fluorescent probe will be co-located, ensuring a real tracking of the particles. This covalent attachment prevents IR780 leak from the nanocarrier and provides stable monitoring of the nanostructures in vitro and in vivo. Recently, comparative studies conducted with IR780 physical versus chemical association to nanocarriers in cell culture have shown dramatic differences in distribution profiles and protein association (Machado et al., 2020, 2022). The use of the cyanine heptamethine IR780 as a theranostic agent for breast cancer has been successfully demonstrated by Yue et al. (2013). IR780 was encapsulated in heparin-folic acid conjugate self-assembly nanoparticles (HF-IR780 NP). The presence of folate on the particle surface promoted the targeting to tumor cells that have an overexpressed folate receptor, allowing the accumulation of particles at the tumor site, which was determined by in vivo fluorescence imaging and biodistribution analysis. The increase in temperature at the tumor site, after irradiation with laser light, promoted thermal ablation of the tumor.

9 Concluding Remarks

Nanotheranostics has been widely studied nowadays as a tool for the treatment and diagnosis of different tumor types, such as skin and breast cancer. Polymeric nanocarriers are very flexible and can be designed to target several types of tumors due

to their small size, stability in biological media, and surface versatility. Furthermore, polymers can be conjugated with ligands to provide active recognition of tumor cells or to release drugs upon *stimuli*. Fluorophore conjugation with polymeric nanoparticles plays a major role for the reliable tracking of the nanosystems in biodistribution studies in the field of nanotheranostics. Different alternatives and probes are available. In this sense, several preclinical studies have been conducted with the objective of developing alternative, safe, and economical approaches for the simultaneous therapy and imaging of tumors. The theranostics of cancer in the early stages of the disease is important for the treatment to be more effective, in addition to contributing to a positive prognosis, increasing the patient survival rates (Bhushan et al., 2021).

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Functionalization of Nanosystems in Cancer Treatment

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Abbreviations

^{1}H NMR	Proton nuclear magnetic resonance
ACUPA	2-[3-((*S*)-5-Amino-1-carboxypropyl)ureido)pentanedioic acid
ASGP	Asialoglycoprotein
ASGP	Asialoglycoprotein receptors
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
Bio-MOFs	Metal-organic frameworks
CuAAC	Copper-catalyzed azide-alkyne cycloaddition
DCC	Dicyclohexylcarbodiimide
DSPE-PEG200-MAL	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]
DSPE-PEG-COOH	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000]
DUPA	2-[3-(1,3-Dicarboxypropyl)ureido]pentanedioic acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGFR	Epidermal growth factor receptor
EGFR	Epidermal growth factor receptor
EPR	Enhanced permeability and retention

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FR	Folate receptor
FTIR	Fourier transform infrared
GA	Glycyrrhetinic acid
GLUT	Glucose transporter
HER1	Human epidermal growth factor receptor 1
HER2	Human epidermal growth factor receptor 2
IC50	Half-maximal inhibitory concentration
LHRH	Luteinizing hormone-releasing hormone
LRP-1	Low-density lipoprotein receptor-related protein 1
mAb	Monoclonal antibody
MMP	Matrix metallopeptidase
mPEG-*b*-PMAGP	Methoxy-poly(ethyleneglycol)-bock-poly(6-O-methacryloyl-D-galactopyranose
mPEG-MAL	Methoxypolyethylene glycol maleimide
NGR	Asparagine-glycine-arginine
NHS	N-Hydroxysuccinimide
NLC	Nanostructured lipid carriers
PABA	p-Aminobenzoic acid
PAMAM	Poly(amidoamine)
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLGA	Poly(glycolide-co-lactic acid)
PmAb	Panitumumab
PSMA	Prostate-specific membrane antigen
PXRD	Powder X-ray diffraction
Qdots	Quantum dots
SATA	N-Succinimidyl S-acetylthioacetate
SGLT	Human sodium-glucose transporters
SMCC	Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
SMVT	Sodium-dependent multivitamin transporters
SPAAC	Strain-promoted azide-alkyne cycloaddition
SPDP	N-Succinimidyl 3-(2-pyridyldithio)-propionate
TfR	Transferrin receptor
TKRs	Typical tyrosine kinase receptors
TmAb	Trastuzumab
TOSD	D-α-Tocopheryl succinate dichloromethane
TPGS	D-α-Tocopherol polyethylene glycol 1000 succinate
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 Introduction

In the last decades, researches have been conducted to understand cancer physiology and improve its treatment. However, cancer remains a major public health problem worldwide, with a high rate of incidence and lethality (Yan et al., 2019; Siegel et al., 2021). Despite the great antitumoral potential of chemotherapeutics (e.g., paclitaxel, docetaxel, doxorubicin, epirubicin), they lack specificity to tumoral cells, which results in several systemic side effects during cancer therapy. Moreover, the emergence of multidrug resistance has made treatment increasingly challenging (Nguyen et al., 2021).

To overcome these limitations, nanosystems have been extensively investigated for drug delivery to the tumor microenvironment. In this context, numerous nanostructure systems have been developed (e.g., polymeric nanoparticles, magnetic nanoparticles, liposomes, micelles, solid lipid nanoparticles, gold nanoparticles). They have demonstrated remarkable benefits compared to conventional therapy, including improved solubility and delivery of hydrophobic drugs, facilitated permeation across biological membranes, protection of drugs against degradation, and reduced toxicity of drugs to healthy cells (Yan et al., 2019; Nguyen et al., 2021; Rasool et al., 2022).

Targeting of nanostructures to tumor cells can be achieved by two different mechanisms: passive and active targeting. The passive targeting is based on the enhanced permeability and retention (EPR) effect, in which the fenestrated vessels formed during the process of angiogenesis allow the passive penetration of nanostructures into the tumor microenvironment. In addition, the absence of efficient lymphatic drainage allows nanostructures to remain at the tumor site for longer. Despite the greater accumulation of them at the tumor site, the nanostructures accumulated in the tumor by the EPR effect alone are not able to specifically distinguish between normal and tumor cells (Nguyen et al., 2021). To enhance their selection for tumor cells, the surface of the nanostructures has been modified with the addition of targeting ligands that can be specifically recognized and internalized by receptors overexpressed in the tumor cell, a phenomenon called active targeting (Alshaer et al., 2017).

For this purpose, a wide range of targeting ligands has been extensively studied, such as antibodies, antibodies' fragments, peptides, vitamins, carbohydrates, nucleic acids, and glycoproteins (Kumari et al., 2016). This chapter addresses the main strategies employed to functionalize nanostructures with different targeting ligands. Furthermore, it is illustrated their potential application in the treatment of several cancers.

2 Strategies for Functionalization of Nanostructures

As aforementioned, the surface of nanocarriers can be modified with specific ligands to increase their selectivity and affinity for cancer cells. This functionalization has demonstrated the potential to improve the antitumoral activity of several

chemotherapeutic agents, increasing their accumulation in the tumor microenvironment and their uptake in tumoral cells. For this, antibodies, proteins, peptides, and small molecules have shown great potential to target nanocarriers, and, therefore, they are currently the most used targeting ligands (Table 1) (Kumari et al., 2016; Nguyen et al., 2021).

Table 1 List of studies that used ligands for active targeting tumoral cells and the effect of functionalization in cancer treatment

Nanocarrier	Surface modification	Therapeutic molecule	Effect of functionalization	References
Liposomes	Cetuximab	Magnetic nanoparticles and doxorubicin	Increased cellular uptake and cytotoxicity in breast cancer cells overexpressing EGFR (SKBR-3)	Dorjsuren et al. (2020)
Liposomes	Cetuximab	Docetaxel	Greater cellular uptake in cells overexpressing EGFR (DU-145) than in cells with weak expression of EGFR (PC-3)	Eloy et al. (2020)
Polymeric nanoparticles	Panitumumab	Temozolomide	Greater internalization capacity in U-87 MG cells than unmodified formulation	Banstola et al. (2020)
Nanostructured lipid carriers	Trastuzumab	Docetaxel	Reduced cell viability of HER2-positive cells (BT-474). Higher cellular uptake in BT-474 cells than in MDA-MB-4686 (HER2-negative) cells	Varshosaz et al. (2018)
Polymeric nanoparticles	Trastuzumab	Cisplatin	Selective delivery to HER2-positive cells (SKOV-3). Greater cytotoxicity in SKOV-3 cells than unmodified formulation	Domínguez-Ríos et al. (2019)
Silica nanoparticles	Bevacizumab	Doxorubicin	Increased cellular uptake and bioaccumulation in neuroblastoma tissue	Zhu et al. (2017)
Iron oxide nanoparticles	Anti-VEGF	Doxorubicin	Greater cellular internalization in 4T1 cells than unmodified formulation. Targeted nanoparticles showed tumor regression and increased median survival time	Semkina et al. (2018)

(continued)

Table 1 (continued)

Nanocarrier	Surface modification	Therapeutic molecule	Effect of functionalization	References
Liposomes	Anti-VEGF	Docetaxel	Functionalization increased cellular uptake, cytotoxicity, and apoptotic index when compared to free docetaxel	Jain et al. (2021)
Carbon nanotubes	Anti-PSMA	Paclitaxel	Improved cytotoxicity and cellular uptake in PSMA-positive cells (LNCaP)	Comparetti et al. (2020)
Iron oxide nanoparticles	Anti-PSMA	Docetaxel	Enhanced the cellular uptake by about 25% more than unmodified nanoparticles	Rivero-Buceta et al. (2019)
Copper sulfide nanoparticles	Transferrin	Disulfiram	Targeted nanoparticles suppressed the tumor growth in vivo (inhibition rate of ~85%) and prolonged the survival rate	Lan et al. (2021)
Porous silicon nanoparticles	Transferrin	Doxorubicin	Greater internalization of targeted formulation in TfR-positive cells (U-87 MG and hCMEC/D3 cells) than in cells with weak expression of TfR (HaCaT)	Luo et al. (2019)
Polymeric nanoparticles	Transferrin	Amodiaquine	Greater cytotoxicity in 2D and 3D culture models than unmodified formulation, with reduction of spheroid volumes	Parvathaneni et al. (2021)
Lipid-polymer nanoparticles	RGD peptide	Paclitaxel and cisplatin	Increased cellular uptake in A549 cells and greater inhibition of tumor growth in vivo with low systemic toxicity	Wang et al. (2018)
Liposomes	cRGDfk peptide	Gemcitabine	Tumor targeting in SKOV3 cells in vitro and tumor growth reduction in vivo	Tang et al. (2019)
Lipid-polymer nanoparticles	cRGDfK peptide	Tangeretin and atorvastatin	Greater HT-29 cells tumor accumulation and growth inhibition in vivo, with low systemic toxicity	Bao et al. (2020)

(continued)

Table 1 (continued)

Nanocarrier	Surface modification	Therapeutic molecule	Effect of functionalization	References
Polymeric nanoparticles	cRGDfk peptide	Raloxifene	Apoptosis induction and angiogenesis inhibition in vitro and breast cancer tumor growth reduction in vivo	Yadav et al. (2020)
Selenium nanoparticles	RGD peptide	Doxorubicin	Suppression of the VEGF-VEGFR2-ERK/AKT signaling pathway and inhibition of MCF-7 xenograft tumor growth	Fu et al. (2016)
Polymeric nanocapsules	LHRH peptide	Docetaxel and quercetin	Enhanced in vitro cytotoxicity and inhibition of prostate cancer tumor growth (PC-3 cell) in vivo	Shitole et al. (2020)
Polymeric nanoparticles	GE11 peptide	Docetaxel and curcumin	Potentiated in vitro cytotoxicity and in vivo tumor reduction in xenographic tumor model with LCNaP cells	Yan et al. (2017)
Polymeric nanoparticles	Angiopep-2	Doxorubicin	Greater accumulation in brain tissue and increased survival in mice bearing intracranial SU-DHL-2-LUC lymphoma	Shi et al. (2020)
Polymeric nanoparticles	Angiopep-2	Ginsenoside-Rg3	Crossed the blood-brain barrier, potentiated the cytotoxic effect of Rg3 and cell uptake in C6 cells	Su et al. (2020)
Hybrid silica nanoparticles	Folic acid	Paclitaxel and ^{sh}RNA	Greater biocompatibility and increased cell uptake, cytotoxicity, apoptosis, and silencing gene in cells FRα positive	Jia et al. (2021)
Transfersomes	Folic acid	Docetaxel	Increased cellular uptake, target, and cytotoxicity in 2D and 3D culture models using U-87 MG cells	Luiz et al. (2021)
Metal-organic frameworks (bio-MOFs)	Folic acid	Curcumin	Greater cellular internalization in 4T1 cells and receptor-specific targeting	Alves et al. (2021)
"Multi-seed" polymeric liposomes	Biotin-mPEG2000-polypeptide	Asulacrine	Increased targeting in vitro and in vivo (4T1 cells), tumor reduction in xenograft tumor, increased nanosystem clearance out of tumor tissue	Jin et al. (2020)

(continued)

Table 1 (continued)

Nanocarrier	Surface modification	Therapeutic molecule	Effect of functionalization	References
Polymeric nanoparticles	Biotin and folic acid	Doxorubicin and $siRNA^{IGF1R}$	Increased cellular uptake, apoptosis rate, and antitumor activity in vitro decreased cell migration, and reduction in IGF1R protein level in vitro	Li et al. (2019)
PAMAM dendrimers	Glucose	Methotrexate	Increased cytotoxicity and cellular uptake in vitro cells, decreased cytotoxicity in normal cells in vitro	Torres-Pérez et al. (2020)
Liposomes	Glucose and biotin	Paclitaxel	Increased cellular uptake in brain cells in vitro and in vivo model of C6 xenograft cells. Increased crossing of the blood-brain barrier	Liu et al. (2021)
Polymeric nanoparticles	Galactose	Doxorubicin	pH-responsive degradation increased higher internalization and intracellular distribution and higher cytotoxicity in ASGP receptor-positive cells	Sun et al. (2017)
Polymeric nanoparticles	Galactose	Apigenin	Increased time release, increased cellular uptake, apoptosis rate, and cytotoxicity in HepG2 cells. Increased targeting in xenograft mice model, reduced tissue damage and nodules	Ganguly et al. (2021)
Lipid-polymer nanoparticles	Urea-derivative ACUPA acid	Wogonin	Uptake cellular increase, cytotoxicity, and apoptosis rates in PSMA-positive PCa cells	Zhang et al. (2016)
Polymeric nanoparticles	Hyaluronic acid	Cisplatin and doxorubicin	Increased cellular uptake and cytotoxicity in 2D and 3D culture models of CD44 receptor-positive tumor cells (4T1). Increased targeting to tumor tissue in 4T1-xenografted mice with increased cell apoptosis and decreased tumor volume	Yu et al. (2020)

Legend: *ASGP* asialoglycoprotein receptor, *EGFR* epidermal growth factor receptor, *HER2* human epidermal growth factor receptor 2, *PSMA* prostate-specific membrane antigen, *TfR* transferrin receptor, *VEGF* vascular endothelial growth factor receptor

It is important to optimize the process of development and functionalization of nanocarriers to ensure the potential of targeting ligands, given that changes in these steps can interfere with their physicochemical features, pharmacokinetic properties, and pharmacological activities. Therefore, it is decisive the selection of an appropriate conjugation method to guarantee that the function and orientation of the targeting moiety are preserved and allow its recognition by the target receptor. In addition, research has demonstrated that ligand density on the surface of nanocarriers can also interfere in their recognition by a receptor on tumor cells (Yao et al., 2016).

Wonder et al. (2018) developed RGD peptide-modified cationic liposomes for delivering DNA for cancer gene therapy. The authors investigated the influence of peptide density (1, 2.5, 5, and 10 mol% peptides) on the total biding and cellular internalization of nanocarriers in prostate cancer cells (PC3). Results indicated an increase in total biding and internalization from 1 to 2.5 mol% peptides. However, both parameters decreased when liposomes with 5 and 10 mol% peptides were analyzed. The authors suggested that high peptide density results in a thicker hydrophilic corona, which made it difficult for the RGD peptide to bind to the $\alpha_v\beta_3$ integrin receptors (Wonder et al., 2018).

Yong et al. (2020) evaluated the influence of orientation and density of anti-epidermal growth factor receptor (anti-EGFR) antibodies on quantum dots (Qdots) targeting efficiency. In vitro results indicated that changes in antibody orientation enhanced around eightfold the cell targeting and 10–30% antibody density on Qdots surface gives the optimal cell biding. As observed by the previous study, high antibody density reduced its ability to bind to the specific receptor due to the thicker hydrophilic corona (Yong et al., 2020). Similar results were also observed by Gong et al. (2019) when optimizing the folate density on the surface of polymeric nanoparticles (Gong et al., 2019), demonstrating that there is an optimal molar content of targeting ligands on the surface of nanocarriers to achieve great targeting efficiency to cancer cells.

The functionalization of nanocarriers can occur by several methods, including those that are performed before and after the synthesis of nanoparticles. In presynthesis methods, the targeting ligand previously conjugated to a nanocarrier component (e.g., surfactant, polymer, lipid) is used in the nanocarrier production step. These methods have the advantage that it is not necessary to perform the functionalization synthesis whenever the nanocarrier is produced and the targeting ligand conjugated to the formulation component can be stored and used in various preparations. However, it is important to ensure the correct orientation of the ligand to the outside of the nanocarrier (Kanth et al., 2020). Luiz et al. (2021) produced folate-modified docetaxel-loaded transfersomes using a presynthesis method. The author conjugated folic acid with TPGS (a PEGylated vitamin E) and used it during transfersomes' preparation. As folic acid was linked to the hydrophilic PEG moiety of TPGS, it was preferentially exposed to the outer aqueous side of the transfersomes, which enables a higher cellular internalization and spheroids' permeation than unmodified transfersomes (Luiz et al., 2021). By contrast, postsynthesis methods are those in which the functionalization is carried out after the production of the nanocarrier. Thus, these methods give more control over the surface

functionalization of nanocarriers, which makes them the most used (Kanth et al., 2020). In general, these modifications can be achieved via covalent and non-covalent functionalization. In this section, we address the main functionalization techniques that have been used for the production of functionalized nanocarriers.

2.1 Non-covalent Functionalization

Non-covalent functionalization involves the adsorption of the targeting ligand on the surface of the nanocarrier by reversible interactions, including electrostatic interaction, hydrophobic effect, and hydrogen bonds (Fig. 1). The main limitation of these methods is the risk of premature ligand release due to the weak nature of this interaction. However, the surface modification of nanocarriers using non-covalent interactions has the advantage of being a rapid and simple procedure that can be performed in one step without the need to carry out a purification step (Nieto et al., 2020; Osman et al., 2021; Chatterjee & Chanda, 2022).

Zhang et al. (2019) developed docetaxel-loaded polymeric nanoparticles functionalized with trastuzumab for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer. The authors conjugated the antibody via electrostatic interaction with the positive surface charge of the nanoparticles generated by polyethylenimine. In vitro assays indicated that the functionalized formulation increased cytotoxicity in HER2-positive cells, but not in HER2-negative cells. In addition, the cellular uptake of this formulation was dependent on HER2 expression, demonstrating that this functionalization strategy was efficient in actively targeting the nanocarrier to HER2-positive breast cancer cells (Zhang et al., 2019).

Avidin-biotin and streptavidin-biotin complexes have been one of the strongest non-covalent interactions used in the functionalization of nanocarriers. The high specificity and affinity between them enable a robust and stable interaction (Yao et al., 2016). Yong et al. (2020) developed anti-EGFR antibody-modified Qdots via streptavidin-biotin complex. For this purpose, the biotinylated anti-EGFR antibody was incubated overnight with Qdots 655 streptavidin conjugate. Functionalization was confirmed by gel electrophoresis, and its efficiency was demonstrated in in vitro antitumoral assays (Yong et al., 2020).

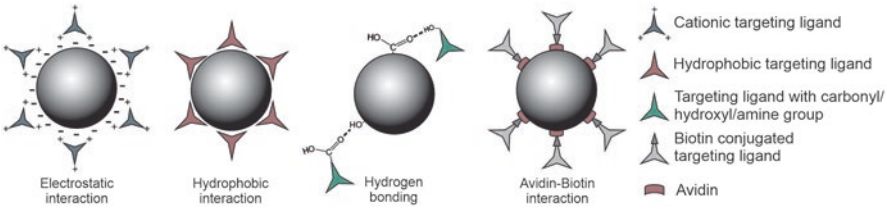

Fig. 1 Non-covalent interactions of targeting ligands and nanosystems

2.2 *Covalent Functionalization*

Covalent conjugation has been the most popular method for functionalizing nanocarriers with targeting ligands due to its high stability and fabrication of various modified nanocarriers. Thus, unlike non-covalent interaction, hardly a ligand covalently bound to the surface of nanocarriers will have a premature release (Nieto et al., 2020). The main chemical groups used to bind ligands in nanocarriers include amide, ester, thiol, carbamate, bisulfite, and polycyclic. If these functional groups are not present in nanomaterial composition, they can be introduced prior to the conjugation step. For the functionalization of nanocarriers, several coupling reactions can be used (Molavipordanjani & Hosseinimehr, 2019; Osman et al., 2021). This section addresses the most common covalent strategies used to functionalize nanocarriers, including carbodiimide chemistry, maleimide chemistry, and click chemistry.

2.2.1 Carbodiimide Chemistry

Carbodiimide chemistry is a widely used reaction to bind targeting ligands on the surface of nanocarriers due to its simplicity and high binding stability. Generally, this process is based on the reaction of the activated carboxyl group (-COOH) of nanocarriers with the primary amine group of the targeting ligand, forming an amide bond (Fig. 2). As biomolecules (e.g., peptides and proteins) have several amine groups, it is difficult to predict which one will be linked with the carboxyl group of the nanocarriers. It represents the main drawback of this functionalization process, given that changes in the ligand orientation can interfere with its binding to the tumor cell receptor (Yao et al., 2016; Nieto et al., 2020; Yong et al., 2020).

To carry out this reaction, the carboxyl group must be activated by adding the crosslinking agent (e.g., dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)) to produce O-acylisourea esters, which is a highly reactive group. However, the instability of O-acylisourea esters makes the incorporation of N-hydroxysuccinimide (NHS) necessary to make the reaction semi-stable, avoiding intra- and inter-molecular bonds between the functional group of the targeting ligands. After the activation process, the targeting ligand containing the amine group can be added to finish the functionalization process (Molavipordanjani & Hosseinimehr, 2019; Nieto et al., 2020).

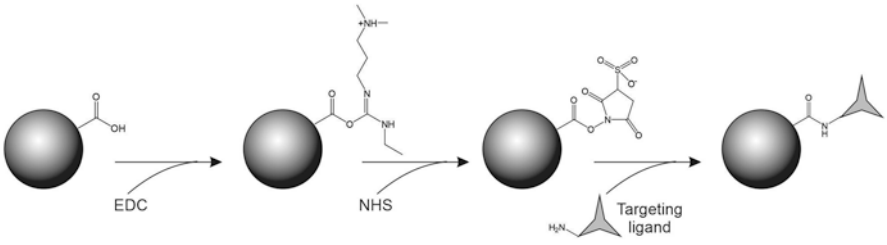

Fig. 2 Schematic representation of nanoparticles' functionalization via carbodiimide chemistry

2.2.2 Maleimide Chemistry

Maleimide chemistry is based on the reaction of the sulfhydryl functional group of targeting ligands with the maleimide group of nanocarriers through a stable thioether linkage (Fig. 3). Since this functional group is not as abundant in targeting ligands as amine groups, fewer variations in the ligand orientation in the nanocarrier are observed. Sulfhydryl group is susceptible to oxidation; thus, they form a disulfide bond in proteins that couple pairs of cysteines, which are responsible for the formation of their ternary and quaternary structure. Thus, the reduction of disulfide bond is required to expose the reactive group before the conjugation process with nanocarriers. For this purpose, the sulfhydryl-addition reagents 2-imonothiolane (Traut's reagent) and N-succinimidyl S-acetylthioacetate (SATA) are commonly used. After sulfhydryl reduction, the targeting ligand is incubated with the nanocarriers containing maleimide crosslinking reagent in their composition, such as NHS-PEG-maleimide, methoxy PEG maleimide (mPEG-MAL), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG_{2000}-MAL), succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (Molavipordanjani & Hosseinimehr, 2019; Chatterjee & Chanda, 2022). The main disadvantages of this chemical reaction are the loss of the covalent bond between cysteines, which can destabilize the structure and function of proteins, and the reaction of maleimide with serum proteins, resulting in unstable bioconjugates in vivo (Nieto et al., 2020).

2.2.3 Click Chemistry

The "click chemistry" term was introduced by Kolb et al. (2001) and refers to a group of reactions that are irreversible, simple to perform, with high yields, and with no release of byproducts. In addition, these reactions can be carried out in aqueous conditions and require no or minimal purification steps. Thus, the mild conditions of click chemistry reactions have the advantage of retaining the structural and functional integrity of targeting ligands (Kolb et al., 2001; Alves et al., 2021; Chatterjee & Chanda, 2022).

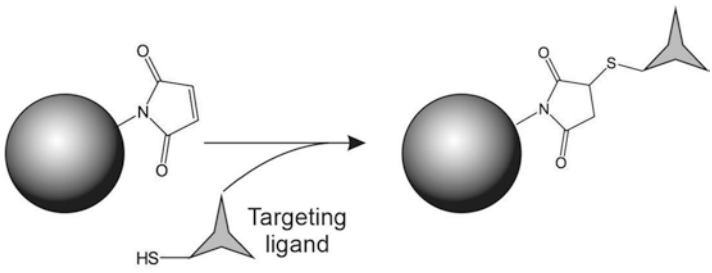

Fig. 3 Schematic representation of nanoparticles' functionalization via maleimide chemistry

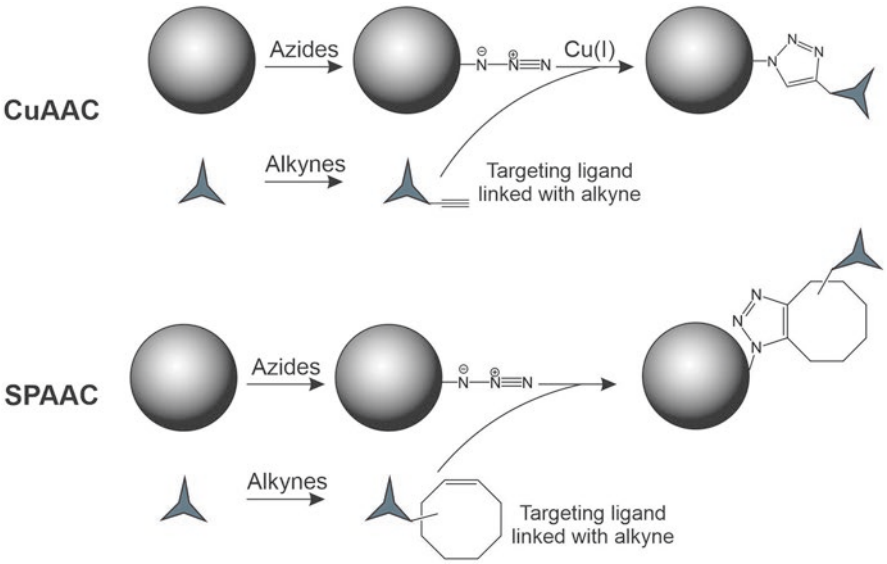

Fig. 4 Schematic representation of nanoparticles' functionalization via click chemistry. Above, CuAAC reaction, and below, SPAAC reaction

The first click chemistry reaction described in the literature was the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction (Fig. 4). In this functionalization, the cooper catalyzes the reaction between the azide and alkyne groups, which is previously conjugated with nanocarrier and targeting ligands. The interaction between the azide and alkyne functional groups is more precise than those observed in the abovementioned amine and sulfhydryl group bonds, which eliminates the risk of nonspecific conjugation (Chatterjee & Chanda, 2022). However, the employment of copper as a catalyzer reagent is associated with in vivo toxicity. Thus, to overcome this limitation, copper-free click chemistry was developed by the strain-promoted azide-alkyne cycloaddition (SPAAC) reaction (Fig. 4), in which a cycloalkyne can react with the azide group without adding copper (Molavipordanjani & Hosseinimehr, 2019).

3 Active Targeting Strategies

3.1 Proteins

The use of functionalizing agents of protein nature is one of the first and most extensively studied. Nowadays, proteins have been used to target nanosystems to tumor cells through the specific recognition of overexpressed receptors, including the transferrin receptor, the human epidermal growth factor (EGF) receptor family, and

prostate-specific membrane antigen (PSMA) (Biffi et al., 2019). Among the proteins used targeting ligand, monoclonal antibodies (mAbs) stand out due to their high antigen-specific interaction, being an attractive approach for designing cancer-targeted nanocarriers. Besides whole mAbs, antibody fragments such as Fab and scFv can be used for constructing targeted nanocarriers. One of the biggest advantages when using monoclonal antibodies is the improvement in drug delivery due to the increased cellular internalization of nanocarriers (Allen, 2002). Thus, the key factors that must be considered when choosing antibodies as functionalizing agents are receptor expression level on tumors, receptor binding affinity, and cellular internalization capacity. One tool that greatly boosted the creation of new binders for active targeting was the mastery of molecular techniques of cloning and purification of proteins, allowing the targeting of different receptors in cancer therapy (Yu et al., 2010; Shargh et al., 2016; Li et al., 2018; Marques et al., 2020; Zhao et al., 2020). However, the use of Abs as a targeting ligand has some imitations, including immunogenicity, high cost, and large ligand size (Allen, 2002; Yu et al., 2010).

The EGF receptor (EGFR/HER1) is a member of the human epidermal receptor family and binds to closely related ligands, including EGF or TGF-a (Fay & Scott, 2011). Activation of HER1/EGFR promotes proliferation, migration, and adhesion of tumor cells. In addition, the activation can enhance angiogenesis and decrease cell apoptosis (Arteaga, 2003). As these mAbs have high affinity and specificity to HER1/EGFR, they have been extensively used to target nanosystems to tumor cells. The main mAbs used for blocking EGFR activation are cetuximab (Erbitux®), panitumumab (Vectibix™), necitumumab (TheraCIM®), and matuzumab. The most commonly used mAb is cetuximab, which has been conjugated to nanocarriers to promote active targeting to EGFR (Yu et al., 2017).

Dorjsuren et al. (2020) developed cetuximab-modified thermo-sensitive liposomes co-loaded with magnetic nanoparticles and doxorubicin for breast cancer therapy, combining photo-thermal therapy and chemotherapy. The authors covalently conjugated the mAb with the carboxylic group of the lipid DSPE-mPEG-COOH via carbodiimide-mediated reaction. The conjugation efficiency of mAbs in liposomes (50%) was confirmed by the BCA protein assay. The authors performed cytotoxicity and cellular uptake assay using the breast cancer cell lines SKBR-3 (overexpression of EGFR) and MCF-7 (low expression of EGFR). Targeted liposomes improved the cellular uptake in SKBR-3 cells when compared to unmodified liposomes. In addition, the internalization of targeted liposomes was greater in SKBR-3 cells than in MCF-7, indicating the potential of functionalization with cetuximab. The cytotoxicity study has indicated that cytotoxicity of targeted liposomes was higher in SKBR-3 cells than MCF-7 cells. The active targeting of cetuximab-modified liposomes was also confirmed in in vivo tumor-bearing mice model (Dorjsuren et al., 2020).

Eloy et al. (2020) developed docetaxel-loaded immunoliposomes functionalized with cetuximab for the treatment of prostate cancer. The mAbs were covalently conjugated to the lipid 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG_{2000}-MAL) via maleimide-mediated reaction. The efficiency of cetuximab conjugation (~55.3%) was confirmed

using the BCA assay. In vitro studies indicated greater cellular uptake of immunoliposomes when compared to unmodified liposomes in EGFR-overexpressing DU-145 cells. Furthermore, lower internalization of immunoliposomes was observed in PC-3 cells (low expression of EGFR), indicating the important role of functionalization in cellular uptake. Moreover, targeted and non-targeted liposomes had similar cytotoxicity activity in PC-3 cells. However, immunoliposomes were higher cytotoxic in DU-145 cells due to the overexpression of EGFR (Eloy et al., 2020).

Another study conjugated panitumumab (PmAb) in polymeric nanoparticles loaded with temozolomide for glioblastoma multiforme treatment (Banstola et al., 2020). PmAb was conjugated to the surface of PLGA nanoparticles using carbodiimide-mediated reaction, with a conjugation efficiency of 65.5% in the BCA assay. In vitro studies were performed in U-87 MG and LN229 glioma cells. U-87 MG cells exhibited 4.2-fold higher expression of EGFR than LN299 in Western blot assay. PmAb-modified nanoparticles showed greater internalization capacity than unmodified formulation. Furthermore, all formulations showed similar cellular uptake in LN299, demonstrating that functionalization with PmAb can improve internalization due to its recognition by EGFR (Banstola et al., 2020).

Human epidermal growth factor receptor 2 (HER2/neu) is a member of the EGF receptor family with tyrosine kinase activity. The activation of HER2 by cognate growth factors regulates cell growth, proliferation, and differentiation (Arteaga, 2003; Iqbal & Iqbal, 2014). The most commonly used anti-HER2 mAbs are trastuzumab and pertuzumab, which have been used as functionalizing agents in nanosystems due to their high specificity to HER2 overexpressed in tumor cells (Yu et al., 2017).

Varshosaz et al. (2018) functionalized nanostructured lipid carriers (NLC) with trastuzumab (TmAb) to selectively deliver docetaxel to HER2-positive breast cancer. The authors used non-covalent (physical adsorption) and covalent (maleimide-mediated reaction) methods for functionalizing NLC. The conjugation efficiency was assessed by the Bradford method. The physical method showed significantly higher coupling efficiency than the covalent method. TmAb physically adsorbed in NLC showed lower viability in B-474 cells (HER2-positive cells) than unmodified formulation and free docetaxel. However, TmAb chemically bound to NLC showed the greatest reduction in cell viability. Furthermore, cellular uptake indicated that functionalized formulation had more internalization in BT-474 cells than in MDA-MB-468 cells, which demonstrated the selectivity of the developed formulation for HER2-positive tumor cells (Varshosaz et al., 2018).

Domínguez-Ríos et al. (2019) have also functionalized chitosan-coated PLGA polymeric nanoparticles with trastuzumab for cisplatin delivery for ovarian cancer therapy. Nanoparticles were functionalized using trastuzumab via carbodiimide chemistry conjugation. Fluorescence spectrophotometry was used to determine the conjugation efficiency of TmAb in nanoparticles' surface, and the results demonstrated that, on average, each nanoparticle was coated with 6.5 TmAb. Functionalized nanoparticles strongly reduced cell viability in SKOV-3 cells (HER2 positive) than unmodified formulations. Furthermore, no significant difference was observed for

targeted and non-targeted nanoparticles in HCC70 cells (HER2 negative). In addition, TmAb-modified nanoparticles showed greater internalization in SKOV-3 cells than in HCC70 cells, indicating the selectivity of targeted nanoparticles (Domínguez-Ríos et al., 2019).

Vascular endothelial growth factor receptors (VEGFRs) are typical tyrosine kinase receptors (TKRs) (Shibuya, 2011). The specific bind of vascular endothelial growth factor (VEGF) to VEGFRs stimulates the process of angiogenesis by promoting the proliferation of endothelial cells, enhancing the permeability of vessels, and recruiting vascular precursor cells (Yang et al., 2018). Two FDA-approved anti-VEGF antibodies are commonly used as targeting agents in nanocarriers, bevacizumab (Avastin®) and ranibizumab (Lucentis®). Another anti-VEGFR mAb currently available is the ramucirumab (Gao & Yang, 2020).

Zhu et al. (2017) proposed the use of VEGF-targeted inorganic composites based on SiO_2 (SiO_2@LDH) to deliver doxorubicin for anti-neuroblastoma treatment. Bevacizumab was covalently conjugated to SiO_2@LDH via carbodiimide-mediated reaction. According to the BCA assay, 4.59 μg of antibody produced 1 mg of targeted SiO_2@LDH. In vitro and in vivo experiments demonstrate that functionalization using bevacizumab was mandatory to achieve higher cellular uptake rates and bioaccumulation in neuroblastoma tissue due to active VEGF targeting. The formulation also exhibited an anti-angiogenic effect both in vitro and in vivo. Another advantage related to active targeting and accumulation of neuroblastoma tissue is the decrease in hepatic toxicity of doxorubicin (Zhu et al., 2017).

Semkina et al. (2018) conjugated anti-VEGF antibodies with bovine serum albumin-coated PEGylated iron oxide nanoparticles for promoting active targeting of nanoparticles to breast cancer theragnostic. Anti-VEGF was conjugated with nanoparticles via carbodiimide-mediated reaction. After the synthesis process, the unreacted antibodies were separated by gel permeation chromatography, and the effective binding of the antibodies to the nanoparticles was confirmed by ELISA assay. The flow cytometry data showed that targeted nanoparticles had the highest accumulation in 4 T1 cells (15.5%) than unmodified nanoparticles (5.2%), facilitating doxorubicin delivery to tumor cells. In vivo mice model showed tumor regression and 50% increased median survival time compared to non-targeted groups (Semkina et al., 2018).

Jain et al. (2021) also tested an alternative for breast cancer treatment, based on anti-VEGF-modified pH-responsive liposomes for docetaxel delivery. The VEGF mAb was linked onto liposomes' surface using carbodiimide chemistry and the linker 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy (polyethylene glycol)-2000] (DSPE-PEG-COOH). The functionalization increased cellular uptake (~3.17-fold), cytotoxicity (~5.78-fold), and apoptotic index (1.70-fold) when compared to free docetaxel. In addition, targeted nanoparticles showed lower IC_{50} (IC_{50} = 1.25 μg/ml) and greater cellular uptake than unmodified formulation (IC_{50} = 3.84 μg/ml). The anti-angiogenic potential was confirmed by the inhibition of human vascular endothelial cell proliferation in vitro. In vivo rat tumor model was used to assess the anti-tumor efficacy of immunoliposomes; data have shown a significant 35% reduction in tumor extension compared to Taxotere® (Jain et al., 2021).

Prostate-specific membrane antigen (PSMA) is a type II membrane protein with folate hydrolase activity produced by the prostatic epithelium (Niaz et al., 2020). PSMA possesses as substrates poly-γ-glutamated folates, which are essential nutrients, and *N*-acetylaspartylglutamate (Bühler et al., 2009). In prostate cancer cells, PSMA is upregulated and migrates to the cell plasm membrane during the transition to androgen independence, usually associated with high-grade and metastatic progression (Kiess et al., 2015). PSMA antibodies have been used as functionalized agents in drug delivery systems (Sun et al., 2021), with antibody-drug conjugates being a very promising strategy for clinical investigation of anticancer medicines. The prostate-specific membrane antigen (PSMA, also known as folate hydrolase-1) has been widely studied as a functionalization agent (Niaz et al., 2020).

Comparetti et al. (2020) developed multiple-walled carbon nanotubes functionalized with anti-PSMA mAb for the delivery of paclitaxel against prostate cancer. Anti-PSMA was bound to the surface of carbon nanotubes through a carbodiimide-mediated reaction. In vitro cytotoxicity assay indicated that both prostate cells (PSMA positive) and colorectal cancer cells (PSMA negative) were more susceptible to targeted and non-targeted carbon nanotubes than free paclitaxel. However, the functionalization of nanotubes with anti-PSMA improved the toxicity in PSMA-positive cells (LNCaP), indicating the selectivity of the developed formulation by PSMA. Also, the use of anti-PSMA antibodies improved carbon nanotubes' uptake and interaction on the cell surface (Comparetti et al., 2020).

Rivero-Buceta et al. (2019) developed docetaxel-loaded superparamagnetic iron oxide nanoparticles conjugated to anti-PSMA using carbodiimide chemistry. The amount of conjugated anti-PSA in nanoparticles (11 μg per mg of nanoparticles) was quantified by the Bradford protein assay. The targeted formulation presented greater cellular uptake in PSMA-positive cells (95%) compared to PSMA-negative prostate cancer cells (70%). Furthermore, the conjugation of anti-PSMA to nanoparticles improved the cytotoxicity effect in LNCaP cells (PSMA positive) 2- and 16-fold more than unmodified formulation and free docetaxel (Rivero-Buceta et al., 2019).

Liposomes co-loaded with genistein and plumbagin were functionalized with anti-PSMA by maleimide chemistry for prostate cancer therapy. The authors investigated the influence of anti-PSMA functionalization through the in vitro cellular uptake assay using PSMA-positive cells (LNCaP) and PSMA-negative cells (PC3). Results indicated that targeted liposomes enhanced the internalization in LNCaP when compared with the unmodified formulation. In addition, its uptake in PC3 cells was less expressive than LNCaP cells, suggesting that targeted liposomes were internalized preferentially through the receptor-mediated process (Tian et al., 2021). A similar improvement in uptake in PSMA-positive cells was found in functionalized hybrid nanoparticles carrying enzalutamide (Thangavel et al., 2018).

The transferrin receptor (TfR, CD71) is a type II transmembrane glycoprotein composed of two disulfide-linked subunits. It is involved in the transport of iron, the essential micronutrient used in the activation of oxygen transport, energy production, cell growth, and DNA synthesis (Daniels et al., 2012). It is overexpressed in cancer cells with higher metastatic potential and aggressive development (Li et al., 2019). The overexpression of TfR in cancer cells has been demonstrated to be an

important tool to target nanosystems to cancer cells (Daniels et al., 2012; Tortorella & Karagiannis, 2014).

Lan et al. (2021) functionalized disulfiram-loaded copper sulfide nanoparticles with transferrin for glioma treatment, given that TfR is overexpressed in the blood-brain barrier and tumor cells. For this purpose, transferrin was non-covalently functionalized to nanoparticles through electrostatic interactions. The cellular uptake behavior of targeted and non-targeted nanoparticles was explored using C6 (TfR-positive) and HUVEC (weak TfR expression) cells. Targeted nanoparticles showed high accumulation in C6 cells in comparison with unmodified formulations. Furthermore, less internalization of targeted nanoparticles was observed in HUVEC cells. These findings suggested that targeted nanoparticles were preferably internalized by the TfR-mediated process. In vivo studies indicated that targeted nanoparticles suppressed the tumor growth (inhibition rate of ~85%) and prolonged the survival rate (Lan et al., 2021).

Another study has also used transferrin to functionalize porous silicon nanoparticles loaded with doxorubicin for glioblastoma multiforme treatment. The transferrin was covalently bonded to nanoparticles via carbodiimide chemistry. The functionalization was confirmed by infrared spectroscopy by the appearance of peaks at 1657 and 1455 cm^{-1}. The selectivity of cell targeting by transferrin-modified nanoparticles was assessed through the fluorescence intensity in the microplate reader and laser scanning confocal microscopy. All analyses indicated greater internalization of targeted formulation in TfR-positive cells (U-87 MG and hCMEC/D3 cells) than in cells with weak expression of TfR (HaCaT). Furthermore, in vitro BBB models using hCMEC/D3 cells in Transwell inserts indicated that targeted nanoparticles were able to cross the BBB and efficiently deliver doxorubicin to U-87 MG cells (Luo et al., 2019).

Parvathaneni et al. produced transferrin-modified polymeric nanoparticles loaded with amodiaquine for non-small cell lung cancer treatment. Transferrin was bonded to PLGA via carbodiimide chemistry. The conjugation efficiency was assessed by the fluorescence intensity of transferrin, which indicated 90–95% transferrin conjugation efficiency. The transferrin-modified formulation showed greater targeting capability in A549 and H1299 cells than unmodified formulations after 3 and 24 h of incubation. In vitro cytotoxicity assay indicated higher toxicity of targeted nanoparticles in two-dimensional and three-dimensional culture models when compared with free drug and unmodified nanoparticles, with a significant reduction of spheroid volumes (1.9, 11.9, and 13.6 mm^3, respectively). These findings demonstrated the potential of transferrin functionalization for the active targeting of nanoparticles to tumor cells (Parvathaneni et al., 2021; Luo et al., 2019).

3.2 Peptides

The surface modification of organic and inorganic molecules with peptides is an excellent strategy for promoting the active targeting of drugs to different types of cancers. These ligands can be easily synthesized and show good stability, specific

recognition of target receptors, non-immunogenicity, and low toxicity. However, its use has some limitations, such as the susceptibility to be cleaved by proteases in vivo, rapid clearance, and high manufacturing cost (Marqus et al., 2017; Sun et al., 2018). Among the different peptides explored in targeting drug delivery nanosystems, this chapter will exemplify the RGD, angiopep-2, GE11, and LHRH peptides, which are the most used (Yan et al., 2017; Wang et al., 2018; Zhang et al., 2018; Tang et al., 2019; Bao et al., 2020; Shitole et al., 2020; Yadav et al., 2020).

RGD is a tripeptide containing arginine (Arg), glycine (Gly), and aspartic acid (Asp). RGD peptide can present linear and cyclic structures (e.g., cycle Arg-Gly-Asp-D-Phe-Lys) (cRGDfK), cycle(Arg-Gly-Asp-D-Phe-Cys) (cRGDfC), and cycle(Arg-Gly-Asp-D-Tyr-Lys (cRGDyK)), in which the cyclic structures show greater conformational stability than the linear (Vhora et al., 2015). These peptides of RGD are widely exploited due to their specificity and selectivity for binding to integrin receptors (αvβ3) overexpressed in the tumor cells and vasculature (Yadav et al., 2020).

Chitosan nanoparticles loaded with raloxifene were functionalized with cRGDfK peptide to selectively target breast cancer cells overexpressing integrin receptors. The peptide was conjugated with the chitosan through the thiolation reaction using the heterobifunctional crosslinker N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) before the development of nanoparticles. The covalent bond of RGD with the amine group of chitosan was confirmed by the FTIR spectrum, demonstrating peak reduction in the NH region of chitosan (from 1539 to 1532 cm^{-1}). Increased cellular uptake was observed in cancer cell lines (4T1 and MDA-MB-231 cells) that express αvβ3 receptor, followed by a reduction in cellular viability (<20%) and induction of apoptosis. The functionalized formulation inhibited cell migration, cell proliferation, and angiogenesis by the dysregulation of p-Akt and reduced expression of c-Fos, MMP, and VEGF. In addition, in vivo studies with a 4T1 xenograft model indicated a significant reduction in the tumor volume (Yadav et al., 2020).

Hybrid nanosystems composed of lipid and polymer were co-loaded with tangeretin and atorvastatin and functionalized with cRGDfK peptide to target colon cancer cells. To functionalize the nanosystems, PEG was conjugated with D-α-tocopheryl succinate dichloromethane (TOSD) and used as a linker to obtain the cRGDfK-PEG-TOSD; the reactions were obtained via carbodiimide chemistry. The synthesis was confirmed by ^{1}H NMR spectroscopy (signal at δ 8.02 ppm corresponding to NH of the amine linkage) and the reaction product used during the production of the nanosystems. The functionalized nanosystem showed pronounced concentration-dependent cytotoxicity in HT-29 cells. In the in vivo model of colon cancer xenograft, the developed formulation demonstrated greater targeting ability to tumor tissue and inhibition of tumor growth (76.8%) when compared to non-functionalized nanosystems (57.3%) and free drugs (22.2%), demonstrating the potential of functionalization with cRGDfK peptide (Bao et al., 2020).

Tang et al. (2019) produced liposomes functionalized with cRGDfK for delivering gemcitabine to ovarian cancer cells. The authors used cRGDfK previously synthesized with the lipid DSPE-PEG_{3500}-maleimide through a maleimide reaction. The efficiency of the reaction was confirmed by the molecular mass of 5146 Da observed

by time-of-flight electrospray mass spectrometry (TOF/MS/ES+). Then, the cRGDfK-PEG_{3500}-DSPE was used during the development of gemcitabine-loaded liposomes. Functionalized liposomes showed increased cellular uptake and cytotoxicity, with a significant upregulation of pro-apoptotic proteins (BAX) and downregulation of anti-apoptotic protein (BCL-2) in ovarian cancer cells (SKOV3 cells). Furthermore, in vivo studies indicated an increased accumulation of functionalized liposomes in the SKOV3 xenograft tumor, evidencing their targeting ability to cancer cells. Moreover, this formulation reduced the tumor volume by about 3.5-fold more when compared to the unmodified liposomes (Tang et al., 2019).

Wang et al. (2018) performed two carbodiimide-mediated reactions to produce paclitaxel-PEG-RGD molecule (RGD-ss-PTX) for functionalizing lipid-polymer nanoparticles to specific targets specifically the lung cancer cells. The synthesis of the RGD-ss-PTX complex was confirmed by ^{1}H NMR spectroscopy (signal near to 8.0 ppm of the NH of the amine linkage). The RGD-ss-PTX complex was combined with cisplatin during the development of lipid-polymer nanoparticles (RGD-ss-PTX/CDDP LPN) by emulsification and sonication technique. RGD-ss-PTX/CDDP LPN had a greater cytotoxicity effect (IC_{50} of 26.7 μg/ml) on A549 cells when compared to free drugs (IC_{50} of 75.3 μg/ml). In addition, in vivo studies indicated high accumulation of functionalized nanoparticles in the tumor than in normal tissues and a reduction of tumor volume from 1486 to 263 mm^3. Therefore, the results indicated the potential of the formulation to target tumor cells (Wang et al., 2018).

Selenium nanoparticles loaded with doxorubicin were also functionalized with RGD peptide (RGD-NP) to target specifically the tumor vasculature. The peptide was conjugated with chitosan using a 3-maleimide propionic acid-N-hydroxysuccinimide ester. The covalent binding of RGD-NP was characterized by UV-VIS spectroscopy, with the absorbance peak of RGD at 562 nm, and Fourier transform infrared (FTIR) spectroscopy, with signals of the amine band from RGD at 1540.8 and 1465.5 cm^{-1}. In vitro and in vivo studies indicated the ability of RGD-NPs to inhibit cell migration and angiogenesis by suppressing the VEGF-VEGFR2-ERK/AKT signaling pathway. In addition, in an in vivo xenograft model of MCF-7 cells, RGD-NPs inhibited the tumor growth and angiogenesis in a dose-dependent manner when the treatments with 2.5, 5, and 7.5 mg/kg were investigated (Fu et al., 2016).

The asparagine-glycine-arginine (NGR) is a peptide that can specifically bind to CD13 receptors, which is highly expressed on the endothelial cells of tumor angiogenic vessels. Gu et al. (2017) functionalized docetaxel-loaded pH-sensitive liposomes with NGR to promote specific targeting to solid tumors. For this purpose, the NGR peptide was covalently conjugated with CHEMS-mPEG_{2000}-COOH by a carbodiimide-mediated reaction and characterized using ^{1}H NMR. In vitro assays showed that NGR-modified liposomes have greater cytotoxicity and cellular uptake effect on HT-1080 cells when compared to free docetaxel and unmodified liposomes. Furthermore, in vivo studies have indicated the antitumoral potential of NGR-modified liposomes, with a significantly greater reduction in tumor volume compared to free docetaxel and unmodified liposomes. The results suggest that the functionalization of liposomes with NGR peptide is an interesting strategy to improve the active targeting of docetaxel to solid tumors (Gu et al., 2017).

Lyp-1 is a cyclic peptide that can be specifically recognized by p32/gC1q-R/HABP1 receptor overexpressed on the tumor cells. In addition, this peptide can penetrate tumor tissue. Zhang et al. (2020) developed LYP-1-modified fibroin-based nanoparticles to target quercetin to lung metastasis of breast cancer. The authors covalently conjugated the LyP-1 to nanoparticles using a carbodiimide-mediated reaction, with a conjugation amount of 36.2 μg of Lyp-1 per mg of nanoparticles. Lyp-1-modified nanoparticles showed 1.8-fold higher internalization in 4T1 cells than unmodified nanoparticles after 1 h of incubation. Furthermore, the functionalized formulation significantly reduced the IC_{50} values 2.2 and 3.0 times lower than those obtained by unmodified formulation and free quercetin. The great potential of the functionalization of nanoparticles with Lyp-1 was confirmed in in vivo studies, with a reduction in tumor growth and lung metastasis (Zhang et al., 2020).

Angiopep-2 (TFFYGGSRGKRNNFKTEEYC) is a peptide that binds to low-density lipoprotein receptor-related protein 1 (LRP-1) overexpressed in the blood-brain barrier (BBB) and brain tumors, being an excellent targeting ligand capable of crossing the BBB and reaching the tumor cells (Shi et al., 2020; Su et al., 2020). Su et al. (2020) covalently conjugated Angiopep-2 to PCL-PEG-maleimide via maleimide-thiol reaction for active targeting ginsenoside-Rg3 to glioma. The synthesis was performed after nanoparticle production and characterized using a BCA protein quantification kit. Angiopep-2-modified nanoparticles (Ang-Rg3-NP) showed 39.5% conjugation efficiency by the BCA quantification method. Ang-Rg3-NP improved the cellular uptake and cytotoxicity in C6 glioma, reducing the IC_{50} from 434 μg/ml (unmodified nanoparticles) to 348 μg/ml. Furthermore, Ang-Rg3-NP overcame the BBB, which was evidenced by the strong inhibition of C6 cells in an in vitro Transwell-based BBB model using BMEC cells (Su et al., 2020).

Another study has also used angiopep-2 as a targeting ligand for promoting active targeting of doxorubicin-loaded nanoparticles to primary central nervous system lymphoma. Angiopep-2 was synthesized with Mal-PCL-b-PEG by maleimide-thiol linkage (ANG-PEG-b-PCL); it was used in the preparation of nanoparticles. The conjugation efficiency was confirmed by the absence of the maleimide peak at 6.7 ppm in the ^{1}H NMR spectrum of ANG-PEG-b-PCL. The angiopep-2-loaded nanoparticles (APP@DOX) showed higher cellular uptake in brain endothelial cells (bEnd.3) than the unmodified formulation. In the orthotopic model of lymphoma, APP@DOX showed the smallest tumor area and an increase in animal survival (58%), while free doxorubicin and the unmodified formulation showed an increase in the survival of 9.7% and 16.4%, respectively (Shi et al., 2020).

Luteinizing hormone-releasing hormone (LHRH) receptors have low expression in healthy cells, while they are overexpressed in malignant tumors, being a potential target for the selective delivery of antitumoral drugs. Nanocapsules co-loaded with docetaxel and quercetin were functionalized with LHRH peptide (PPL-DQ NC) for the treatment of prostate cancer. The [D-lys^6]-LHRH-NH_2 was conjugated to PLGA-PEG by carbodiimide-mediated reaction with EDC/NHS. The synthesis was confirmed by the presence of an additional peak in 1556 cm^{-1} (corresponding to N-H) and the increase in peak intensity at 1640 cm^{-1} (corresponding to C=O of the amide bond) in the FTIR spectrum. PPL-DQ NC showed a greater cytotoxicity effect than

unmodified nanoparticles, with IC_{50} of 3.86 and 1.29 times lower in LNCaP and PC3 cells, respectively. Moreover, functionalized nanoparticles had a greater accumulation, in tumor tissue in PC-3 tumor-bearing mice, with an 85% tumor inhibition rate (Shitole et al., 2020).

The epithelial growth factor receptor (EGFR) is a target commonly used to deliver nanosystems for prostate cancer cells. Yan et al. (2017) developed polymeric nanoparticles co-loaded with docetaxel and curcumin and functionalized with GE11 peptide (GE11-DC NP). The peptide GE11 (YHWYGYTPQNVI) was conjugated to PLGA-PEG by the maleimide reaction. The synthesis was confirmed by the ^{1}H NMR spectrum through the presence of peaks corresponding to the amide and carboxylic groups. GE11-DC NP had the greatest cellular uptake (~80% after 12 h) in LNCaP cells, which contributed to potentiate its cytotoxicity (IC_{50} of 0.058 μM using docetaxel/curcumin ratio of 1:10 w/w). The xenographic tumor model of LNCaP cells showed a tumor inhibition of 85% when GE11-DC NP was used in the treatment, indicating the potential of the functionalization with GE11 in the prostate treatment (Yan et al., 2017).

3.3 Small Molecules

Small molecules are promising targeting ligands for nanosystems due to their small size, stability, ease of chemical synthesis, and lack of immunogenicity. The main challenge of these ligands is their small size and tissue diffusion that can induce non-targeted distribution, rapid clearance, and decreased tumor specificity due to problems in molecular interaction with the target. In addition, adjustment of the ligand density may be sufficient to restrict diffusion and maintain functionality (Muro, 2012). Furthermore, the variety of small molecules confers a range of promising targeting possibilities in cancer therapy; among them are small molecules based on vitamins (e.g., folic acid and biotin), monosaccharides (e.g., glucose, mannose, fructose, and galactose), urea derivatives (e.g., glutamate urea), and others (Huo et al., 2017; Singh et al., 2018; Jin et al., 2020; Ganguly et al., 2021; Liu et al., 2021; Luiz et al., 2021).

Folic acid is a member of the B vitamin family and is the most widely used small molecule targeting agent. Its structure has a pteridine ring, p-aminobenzoic acid (PABA), and glutamate moieties. In mammalian cells, it is an essential donor of carbon for the synthesis of nucleic acid bases, and intracellular transport is through folate receptors (FR). The isoform α of the FR (FRα) is overexpressed about 100–300 times higher in cancer cells than in normal tissues, being a specific strategy for targeted drug delivery into the tumor microenvironment (Zhao et al., 2009; Sun et al., 2015; Teixeira et al., 2019).

Hybrid nanoparticles co-loaded with paclitaxel (PTX) and P-shRNA (shRNA against P-glycoprotein) were functionalized with folic acid for overcoming the multidrug resistance of breast cancer. Folic acid was first activated using EDC cross-linking agent, NHS ester, and NH_2-PEG-NH_2 to obtain FA-PEG-NH_2. In sequence,

folic acid and polyethylenimine (PEI) were covalently bound in the modified (CS-COOH) and activated (EDC, NHS) chitosan. The successful synthesis of the copolymer was confirmed by the characteristic peak at δ 8.2–8.6 ppm of the newly formed -CONH- a bond of folic acid and PEI on CS in the ^{1}H NMR spectrum. Increased cell uptake was observed in multi-resistant cells (MCF-7/ADR) expressing FRα, fluorescence rate of 93% at 4 h. The shRNA plasmid carried in the copolymer caused MDR1 gene silencing, 78% decrease in MDR1 mRNA. Cytotoxicity was 1.99-fold higher in MCF-7/ADR cells, and apoptosis rate increased by 2.18-fold in the targeted nanoparticles (80 nM paclitaxel) (Jia et al., 2021).

Folate-modified TPGS transfersomes containing docetaxel demonstrated the efficiency of folic acid as a target ligand for glioblastoma multiforme treatment. Folic acid was linked to the hydrophilic PEG moiety of TPGS by esterification reaction and then used for the preparation of transfersomes. The esterification of FA and TPGS was confirmed by the presence of signals in the region between 6.5 and 9.0 ppm ^{1}H NMR spectrum and the presence of 1736 and 169 cm^{-1} peaks in FTIR spectrum, corresponding to carbonyl group of TPGS and carboxylic groups of FA, respectively. In vitro assays using 8–87 MG cells cultured in a two-dimensional model indicated higher cytotoxicity and cellular uptake effect of folic acid-modified transfersomes (IC_{50} of 5.064 nM) when compared to unmodified transfersomes (IC_{50} of 3.807 nM). Furthermore, cytotoxicity assay in multicellular tumor spheroids (U-87 MG) showed 6.26- and 1.33-fold higher cytotoxicity of folic acid-modified transfersomes when compared with free docetaxel and unmodified formulation. The permeation assay in U-87 MG spheroids demonstrated the potential of the functionalization to improve the permeation through the spheroids' layers (Luiz et al., 2021).

Alves et al. (2021) produced folate-modified metal-organic frameworks (bio-MOFs) loaded with curcumin for breast cancer treatment. The authors prepared the azide-modified bio-MOF and alkyne-functionalized folic acid for further conjugation via click chemistry. The functionalization was confirmed by ^{1}H NMR and powder X-ray diffraction (PXRD). Folate-modified bio-MOF showed greater cellular internalization when compared to unmodified bio-MOF and free CCM using 4T1 cells (triple-negative breast cancer). Thus, these results demonstrated that targeted bio-MOF exhibited excellent receptor-specific targeting (Alves et al., 2021).

Biotin (vitamin H or coenzyme R) is a cofactor associated with metabolic processes that promote cell growth. Similar to the FRα receptor, biotin receptors – sodium-dependent multivitamin transporters (SMVT) – are overexpressed in cancer cells. The upregulation of SMVT in cancer cells and the characteristics of biotin (e.g., low cost, non-toxic, non-immunogenic, and easy to modify) have made it an interesting targeting ligand for nanosystem delivery (Zempleni et al., 2009; Vadlapudi et al., 2012).

"Multi-seeded" polymeric liposomes were developed with asulacrine-loaded micelles as seeds in their aqueous cavities. The liposomes were modified with a biotin-linked polypeptide polymer cleavable by matrix metallopeptidase 9 (MMP-9). Biotin was anchored to Fmoc-NH2-mPEG2000-polypeptide activated by the cross-linking agent. Pre-insertion and post-insertion methods were used to anchor the

ligand in liposomes. In both methods, the success of the synthesis was confirmed by detecting the molecular weight (M_w 3424) of biotin-mPEG2000-polypeptide using time-of-flight mass spectrometry and by the hydrophobic interactions of polypeptides and liposomes from the increase in β-sheet by circular dichroism. There was increased cellular uptake in 4T1 spheroids over 8 h (~2.2-fold increase in fluorescence) that resulted in increased cytotoxicity. There were increased tumor tissue uptake and increased cell apoptosis with an 80% reduction in tumor size in the xenograft mice model. In addition, there was an increased clearance of the liposomes outside the tumor tissue in the presence of avidin, demonstrating the efficiency of MMP-9 cleavage in clearing the nanosystems distributed outside the tumor tissue (Jin et al., 2020).

Self-assembled folate-biotin-quaternized starch nanoparticles of doxorubicin and $siRNA^{IGF1R}$ were developed for target in human lung adenocarcinoma cells (A549 cells). The nanoparticles were prepared by a one-pot synthesis procedure using a crosslinking agent to anchor biotin and folate. The ^1H NMR spectra indicated the success of the reaction by the characteristic peaks of ring folate (6.60 and 7.60 ppm), methylene group of biotin (1.55–1.85 ppm), and methyl protons in the quaternized groups (3.12 ppm). Targeted nanoparticles showed higher antitumor activity in vitro when compared to doxorubicin (IC_{50} of 7.85 and 5.21 mg/l, respectively). The increase in cellular uptake was mainly through the caveolae-mediated endocytosis pathway. There were an increased apoptosis rate and a decreased cell migration observed by the wound-healing rate of only 0.03%. There was a 2.72-fold dose-dependent reduction in IGF1R protein level in A549 cells (Li et al., 2017, 2019).

Cancer cells adapt their metabolism to use glycolysis as the main source of energy for cell maintenance and survival. The high demand for glucose causes overexpression of receptors involved with tumor metabolisms such as glucose transporter (GLUT) and human sodium-glucose transporters (SGLT). Thus, the conjugation of monosaccharides to nanocarriers is an interesting strategy for their active targeting of solid tumors. In addition, these molecules are non-toxic and non-immunogenic, and their chemical structure allows stable conjugation with the amino-terminal groups of the nanocarriers (Szablewski, 2013; Pliszka & Szablewski, 2021).

Methotrexate-loaded PAMAM dendrimers linked to d-glucose via glycosylation reaction with PAMAM previously activated by the crosslinking agent EDC were prepared by targeting triple-negative cancer cells (MDA-MB-231). The binding of glucose to the amine group of PAMAM was confirmed by the FTIR spectrum, revealing a pronounced peak in the secondary NH region (from 3435 to 1638 cm^{-1}). Targeted dendrimers reduced cell viability up to 20% in MDA-MB-231 cells, being significantly inert in HaCaT cells (human keratinocytes). The functionalized formulation had a twofold higher cellular uptake in cancer cells than in normal cells, with predominant localization in the cell cytoplasm. The great antitumoral potential of functionalized nanoparticles is related to the overexpression of GLUT transporters present in cancer cells (Torres-Pérez et al., 2020).

Multi-targeted liposomes modified with biotin and glucose loaded with paclitaxel were evaluated for their ability to cross BBB to treat gliomas. Biotin and

glucose molecules were bound to cholesterol in the presence of EDCI and DMAP to obtain Bio-Chol or Glu-Chol ligands that were subsequently used to prepare liposomes. The binding between the ligands and cholesterol was confirmed by ^{1}H NMR spectra (Qu et al., 2012, 2014). The targeted liposome increased cellular uptake 4.04- and 3.49-fold in brain endothelial cells (bEnd.3) and glial cells (C6), respectively. The uptake was mediated by GLUT1 and the SMVT transporter. There was an increased targeting to glioma in vivo C6 xenograft cell model demonstrated by stronger fluorescence intensity of the dual-targeted liposome (Liu et al., 2021).

Galactose has also been used as a targeting ligand due to its ability to target asialoglycoprotein receptors (ASGP), primarily in mammalian liver cells. These receptors can endocytose saccharides or glycoproteins containing D-galactose or N-acetyl-D-galactosamine to hepatocytes (Cavallaro et al., 2017; Sun et al., 2017).

Galactose-based polymeric nanoparticles loaded with doxorubicin were synthesized by covalent conjugating of methoxy-poly(ethylene glycol)-bock-poly(6-O-methacryloyl-D-galactopyranose (mPEG-*b*-PMAGP) with doxorubicin using a 4-nitrophenyl chloroform crosslinker. The binding between galactose and doxorubicin was confirmed by the absorption peak at 3368 cm^{-1} (hydroxyl group) of galactose and a peak at 1639 cm^{-1} (-CO-NH-) of doxorubicin in the FTIR spectrum. The targeted polymeric nanoparticles have higher internalization (~3-fold) in HepG2 cells (ASGP positive) than MCF-7 and A549 cells (ASGP negative) with distribution in both nuclei and cytoplasm. Furthermore, functionalized nanoparticles showed higher in vitro antitumor efficacy in HepG2 cells than in MCF-7 cells (IC_{50} of 1.22 and 2.97 μg/ml, respectively), demonstrating the potential of this functionalization to promote specific targeting of the nanoparticles to tumor cells expressing ASGP receptor (Sun et al., 2017).

Apigenin-loaded galactosylated-PLGA nanoparticles were synthesized through esterification reaction of the galactose hydroxyl group with the carboxyl group of PLGA. The ^{1}H NMR spectra showed an additional peak at δ 4.32 ppm due to the galactose unit. Galactosylated nanoparticles had 1.5 times less initial burst release. Galactosylated nanoparticles increased the cellular uptake in HepG2 cells, which was demonstrated by higher fluorescence intensity. There was greater cytotoxicity in HepG2 cells with targeted nanoparticles than non-targeted nanoparticles (IC50 = 37.1 ± 3.4 μM and 77.2 ± 6.4 μM, respectively), with a ~ 1.2-fold higher apoptosis rate for targeted nanoparticles than non-targeted nanoparticles. The presence of nodules on the liver surface in the HepG2 mice xenograft model was reduced by ~3.43-fold compared with the control group (Ganguly et al., 2021).

Small urea-based molecules are promising ligands for the detection of prostate-specific membrane antigen (PSMA). PSMA is overexpressed in prostate cancer cells, making it an interesting strategy to selectively deliver drugs to prostate cancer cells while protecting normal cells that under-express PSMA. PSMA has a high affinity for 2-[3-(1,3-dicarboxypropyl) ureido]pentanedioic acid (DUPA) (K_i = 8 nM) and some chemical mimetic derivatives of DUPA such as

2-[3-((*S*)-5-amino-1-carboxypropyl)ureido)pentanedioic acid (ACUPA) (Roy et al., 2015; Lim et al., 2019).

Lipid polymer nanoparticles loaded with wogonin and modified with ACUPA were synthesized using the EDC crosslinking reagent for conjugation of ACUPA and Chol-PEG and subsequent formation of the nanoparticles by self-assembly assemblies with wogonin. The ^{1}H NMR spectrum showed a large peak at about δ 3.5 ppm of PEG fragments, multiple peaks from δ 0.6 to 1.2 ppm of cholesterol fragments, and peaks around δ 2.0 and δ 1.40–1.38 ppm of ACUPA fragments. ACUPA-modified nanoparticles increased the cellular uptake in PSMA-positive prostate cancer cells (PCa cells) (4.3-fold higher fluorescence intensity than unmodified nanoparticles). Targeted nanoparticles were more cytotoxic than non-targeted nanoparticles (IC_{50} = 15.83 and 45.65 μg/ml, respectively) with an apoptosis rate of 89.92% in PCa cells from the labeled nanoparticles. There was an increase in Bax and cytochrome c protein levels, suggesting that the induced apoptosis depends on the intrinsic mitochondria-related pathway (Zhang et al., 2016).

Hyaluronic acid (HA) is an unsulfated glycosaminoglycan. HA is a component of the extracellular matrix with overexpressed HA-biding receptors in several cancer cells, including the CD44, receptor for HA-mediated mot (RHAMM), and the HA receptor for endocytosis (HARE). Therefore, it is also an interesting strategy to target drug delivery in cancer cells (Kim et al., 2018).

HA-modified nanoparticles crosslinked with cisplatin and loaded with doxorubicin were synthesized as CD44-targeting anti-breast cancer cells (4T1). Hyaluronic acid was conjugated with doxorubicin using cisplatin as a crosslinker agent before the preparation of self-assembly nanoparticles. FTIR spectrum confirmed the success of synthesis by absorption peaks in 1300–1000 cm^{-1} due to the -CO- bond stretching vibration in doxorubicin and 3300–3200 cm^{-1} due to the amines of cisplatin. There was increased uptake of HA-targeted nanoparticles in 4T1 cells positive for CD44 than 3T3-negative cells. Targeted nanoparticles were more cytotoxic in 4T1 CDD-positive cells (IC_{50} = 1.10 μg/ml) than 3T3 CD44-negative cells (IC_{50} = 3.07 μg/ml). The 4T1 spheroid volumes were significantly lower than in the 3T3 groups, demonstrating the efficacy of the ligand, in addition to the improved targeting in 4T1-xenografted Balb/c mice (2.1-fold fluorescence) with 66% tumor inhibition and a 2.23-fold higher apoptosis rate at 12 h compared to control (Yu et al., 2020).

Several other small molecules have been studied for the specific targeting of nanosystems to tumor cells or the tumor microenvironment. Glycyrrhetinic acid (GA) derivatives are being used for hepatocarcinoma targeting because of the presence of GA receptors on hepatocytes. Another small molecule target has been the expression of carbonic anhydrase IX in solid tumors using sulfonamide derivatives as ligands. In addition, small molecule benzamides such as anisamide have been used as ligands targeting $Sigma^{-1}$ receptors (Krall et al., 2014; Huo et al., 2017; Singh et al., 2018).

4 Conclusion and Future Perspectives

In recent decades, the functionalization of nanosystems with targeting ligands has been a promising strategy to improve the selective delivery of drugs to tumor cells due to their specific recognition by receptor overexpressed in these cells. For this purpose, different functionalization procedures can be explored, including hydrophobic interactions, electrostatic interactions, biotin-avidin interaction, maleimide chemistry, carbodiimide chemistry, and click chemistry. Among them, carbodiimide and maleimide chemistry has been the most used due to the simplicity, high bind stability, and abundance of functional groups in targeting ligands. In addition to the choice of the functionalization method, other parameters are decisive to guarantee the desired biological activity, such as the orientation of the targeting moiety and the density of ligands on nanosystems. The correct orientation of the targeting ligand on the surface of nanosystems is crucial to ensure that their recognition and binding to receptors occur for later internalization of the nanosystems. Furthermore, the density of ligands on nanosystems should be optimized, given that a small number of molecules may be insufficient to promote specific targeting, whereas high ligand density can result in a thicker hydrophilic corona. Taking these aspects into account, the functionalization of nanosystems with targeting ligands (e.g., antibodies, peptides, and small molecules) has demonstrated the great potential to promote the specific delivery drug to tumors. Several in vitro assays demonstrated an improvement of cellular uptake and cytotoxicity effect when functionalized nanosystems were applied in tumor cells with overexpression of the target receptor. Furthermore, in vivo studies have confirmed the great potential of the active targeting, indicating their higher accumulation in tumor tissue and reduction of tumor volume. Therefore, the development of this technology has been an important strategy for improving cancer treatment and reducing side effects.

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3D Bioprinting for Cancer Models

Virginia Brancato

1 Introduction

Bioprinting technology is a branch derived by the need of regenerative medicine and tissue engineering to fabricate functional tissue substitutes (Datta et al., 2020; Kim et al., 2021). The global market for bioprinted medical products has been foreseen to reach about 1.3 billion dollars by 2022 (Ricles et al., 2018). Tissue engineering paved the way to the development of scaffold-based methodology to grow cells in vitro (Benam et al., 2015). In the last decades, tissue engineering tools become more and more sophisticated and allowed to shorten the distance between the native tissues and bioengineered ones. Scaffold or hydrogel-based approaches do not allow the combination of different types of cells with a spatial distribution and precise compartmentalization that mimic the tridimensional architecture of living tissues (Singh et al., 2018). Modular building block's assembly supports the construction of complex tissue starting from controlled microscopic units that could be obtained by means of different approaches (scaffold or hydrogels, cell aggregation, or cell sheet formation) (Monteiro et al., 2022). Bioprinting plays a leading role among these methodologies due to the versatility of fabricating bioengineered constructs with accurate spatial organization. Many bioprinting techniques have been developed for different purposes (Lobo et al., 2021). Bioengineering advances several materials have been improved starting from natural materials (gelatin, collagen, alginate, hyaluronic acid) or synthetic polymers (poly(e-caprolactone), poly(lactic acid), poly(glycolic acid), and poly(lactic-co-glycolic acid)), offering a wide range of mechanical properties and tunable biochemical cues. Starting from these materials, several bioinks have been developed that allow the cell proliferation and differentiation by means of biological and physical stimuli (Skardal & Atala, 2015; Boularaoui

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et al., 2020; Zhang et al., 2021). Therefore, an overview of 3D bioprinting technology applied to the most aggressive and complex cancer is proposed in this chapter as a tool to fabricate viable constructs by depositing living cells with tunable biomaterials.

2 Tumor Microenvironment Multicellularity

The definition of cancer given by the National Cancer Institute is not appropriate because it focuses only on cancer cell, considering them the only responsible for the abnormal cellular division and the invasion to the surrounding tissue. Hence, a wider definition of the cancer disease is necessary, taking into account the genetic and epigenetic mutational landscape that underpins cancer progression (Ungefroren et al., 2011; Lee & Chaudhuri, 2017). An extensive overview of the main features of cancer disease has been summarized in hallmarks of cancer. When, in 2011, reprogrammed metabolism and immune escape became the emerging hallmarks, it was not possible to consider cancer plasticity and non-mutational epigenetic reprogramming, due to the lack of genomic and epigenomic data on cancer evolution. Moreover, it is now evident that cancer disease is also strictly connected to the microbe community dwelling in the human body (Hanahan & Weinberg, 2000, 2011; Hanahan, 2022). Intratumor heterogeneity is the reading key for the investigation of cancer in order to understand which are the triggers that drive the tumor cells from early lesions to later stage disease. Cancer cells are dynamic entities able to modify themselves and the surrounding microenvironment. Cancer cells are also able to disseminate in other organs far from the primary tissue; hence, they exploit the vascular network and in some cases are able to escape the immune system defense (Mehlen & Puisieux, 2006). When the cancer cells reach another site, metastasis occurs, increasing the poor outcome of the disease (Klein-Goldberg et al., 2014). It is clear that human tumor main feature is the dynamic and complex organization based on the interactions among different cells and the extracellular matrix. Understanding this complexity will give novel insight in the puzzle of the biological and mechanical features of cancer. Recently, the tumor microenvironment gained interest and is investigated as a target for innovative anti-cancer drugs (Quail & Joyce, 2013).

3 Bioprinting Living and Non-living Entities for Functional Organotypic Models

The progress in the knowledge of the tumor microenvironment pushed the researchers to find new methodologies to investigate cancer, since cell culture on plastic dish is not sufficient to recapitulate the cancer complexity (Klein-Goldberg et al., 2014). During the latest decades, biomaterials have been widely studied and developed.

Moreover, biomaterials are good allies in facilitating the growth of cancer cells in 3D architecture, mimicking the tumor microenvironment. Nowadays, there is not a uniformity in the 3D tumor models due to large arrays of biomaterials, in the form of hydrogels or scaffold, so a unique standard is not available as it happens for the 2D cell culture methodology (Hickman et al., 2014; Rodenhizer et al., 2018). However, this is a positive aspect because a one-size-fits-all approach is no more compatible with the representation of the complexity of the tumor microenvironment. Among all the 3D tumor models available, this chapter will focus on the bioprinted models. The advancement of 3D bioprinting technologies enlarged the possibility of applications for mimicking cancer microenvironment in vitro. Hence, the combination of manufacturing novel bioinks and ECM-mimetic biomaterials enhances the performance of biofabricated constructs, improving the biochemical and biophysical properties that leads to more realistic read-outs for drug screening (Fig. 1). Moreover, bioprinting offers a solution to obtain spatially controlled organization of different types of cells, along with the possibility to replicate cell gradients at high resolution incorporating epithelial, endothelial, and stromal cells. Bioprinting is defined as an additive manufacturing technology that exploits the stacking of different layers of cells and materials. These constructs could rapidly maturate into tissue and organs with complex and spatially precise structure. Different methodologies have been developed to fabricate bioprinted constructs, and they can be summarized as follows: extrusion-based printing, inkjet-based printing, stereolithography/digital light processing bioprinting, and laser-assisted printing (Germain et al., 2022).

The most widespread technique for bioprinting is the extrusion-based one, where the bioink is extruded out of the nozzle by means of pneumatic pressure exerted by a piston or a screw. The bioink is deposited on a cell culture plate with a precise computer-aided design. The bioprinted constructs need to be then crosslinked to maintain the mechanical properties by means of photo/thermal or chemical crosslink.

Other printing techniques have been developed to improve the manufacturing of medical devices and are not properly defined as bioprinting where bioink and cells are mixed together (Davoodi et al., 2020). At this purpose, we mentioned the inkjet bioprinting technique that is considered a non-contact printing technique where the bioink is dispensed as droplets in a predesigned pattern by means of thermal or piezoelectric processes. Stereolithography/digital light processing-based bioprinting polymerize the materials by means of projection light, offering a better resolution efficiency and working condition. This technique is particularly used for the reproduction of medical models, and the artificial tissues or organs have high biocompatibility and allow high cell viability. This type of 3D printing is also exploited to fabricate medical device customized for precise drug delivery in a less invasive and more controlled manner. An innovative 3D printing technology is the laser-induced forward transfer approach, a digital printing method where a pulsed laser beam triggers the printing of materials from a thin donor layer to a receiving one. This approach is compatible with a wide range of materials and is becoming widely popular in applications as electronic devices and sensors (Zhang et al., 2017; Monteiro et al., 2022).

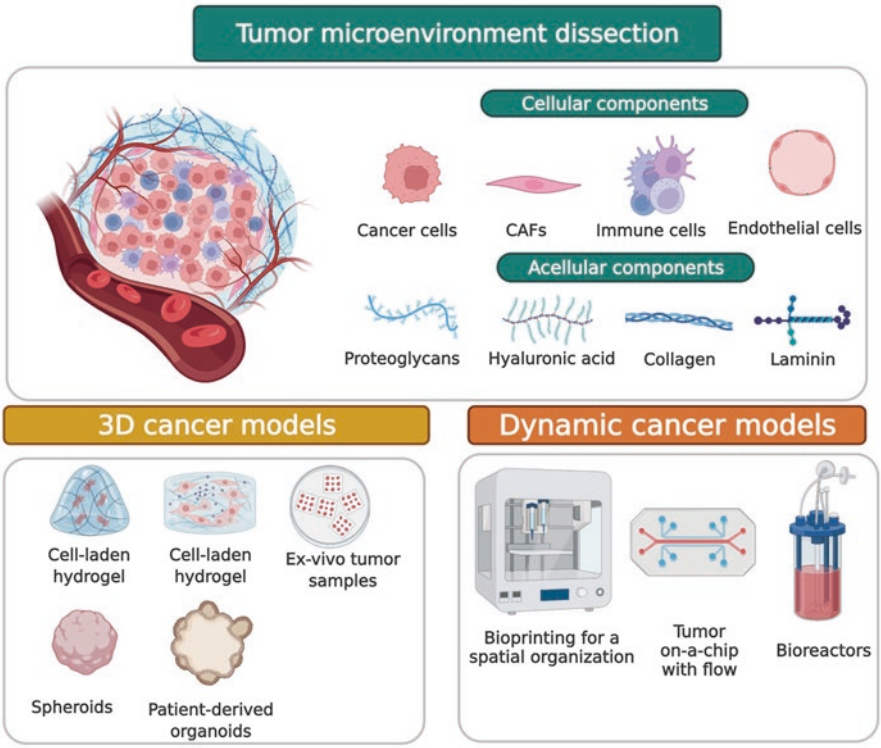

Fig. 1 The reductionist view of tumor tissue as an aggregate of cancer cells is nowadays outdated. Tumor microenvironment is composed of cellular and acellular components that are schematized in the upper part of the figure. In solid tumors, cancer cells are surrounded by a dense extracellular matrix that is mainly produced by cancer-associated fibroblasts. The extracellular matrix is composed of acellular molecules such as collagen, laminin, hyaluronic acid, and proteoglycans. The tumor growth is supported by vasculature formed by endothelial cells. Immune cells such as lymphocytes and macrophages could inhibit the tumor growth or support it according to the tumor stage. The advancement in culturing cancer cells is given by the combination of biomaterials (in the form of hydrogels or scaffold) in order to mimic the extracellular matrix. 3D architecture is relevant to obtain a more realistic and specific model for spheroids and patient-derived organoids that could be embedded in more complex models based on additive manufacturing technology such as bioprinting or microfluidic or being cultured in bioreactors

3.1 Breast Cancer

Breast cancer still remains the first cause of death for women due to metastasis of breast cancer cells to other organs. Breast cancer is a solid tumor characterized by a fibrotic and dense microenvironment that plays an essential role in cancer progression and affects the response to the drug. For this reason, it is necessary to fabricate 3D in vitro models that can mimic the heterogeneity of the tumor microenvironment, including both cancer and stromal cells such as fibroblasts and immune and endothelial cells (Sharifi et al., 2021). Many attempts have been made to fabricate

bioprinted breast cancer spheroids starting from cell lines in gelatin or Matrigel-based hydrogels. Bioprinting approach guaranteed the uniformity in size of the spheroids, an important parameter when these models are used to predict the drug response in vitro. As demonstrated for other 3D tumor models, also the bioprinted breast cancer spheroids showed higher resistance to drugs as paclitaxel that may be related to the enrichment of stem cells in the spheroids (Ling et al., 2015; Swaminathan et al., 2019). Bioprinting is a cutting-edge technology, and to obtain the most from this approach, researchers combined patient-derived cells and bioinks from extracellular decellularized matrix in order to enhance the native cancer microenvironment (Fig. 2a). The mammary-derived hydrogel sustains the growth of breast organoids and tumoroids, paving the way for the study of interaction between cancer cell and extracellular matrix (Fig. 2b) (Mollica et al., 2019). Beside the extracellular matrix, what makes breast cancer cells more aggressive is also the interaction with stromal cells. It has been demonstrated that the presence of osteoblasts or mesenchymal stem cells enhances the secretion of VEGF. This model recapitulates the breast to bone metastasis process and could shed light on the mechanism underpinning breast cancer cell invasion (Zhou et al., 2016). The role of adipose stem cells in triggering cancer invasion is under debate. Tumor stiffness could be recapitulated by bioprinted hydrogels; in particular, a 5% alginate hydrogel is able to reflect the breast cancer microenvironment and the co-printing of epithelial cells and adipocytes demonstrating the capability to grow in the bioprinted construct up to 10 days (Chaji et al., 2020).

3.2 Ovarian Cancer

Ovarian cancer genomic profile, unraveled by advanced sequencing technology, led to a deeper molecular classification of this cancer that is the seventh most lethal cancer in women. As solid tumor, also in ovarian cancer, the stromal component plays a relevant role in the cancer progression and invasion; hence, it is necessary to fabricate 3D in vitro predictive models that could recapitulate also the interaction between cancer cells and fibroblasts. A platform for high-throughput drug screening is obtained by means of a droplet-based bioprinting using fibroblasts and ovarian cancer cells mixed to Matrigel in order to copycat the multicellular microenvironment of ovarian cancer at micro-scale (Xu et al., 2011). The concept that cells grown on plastic dish have a different gene and protein expression profile is nowadays accepted. However, there is no universal accepted 3D in vitro model that can substitute the 2D cell culture methodology. Researchers have still a long way to go to recapitulate in vitro ovarian cancer even if bioprinting technology has been used for organ replacing in this field.

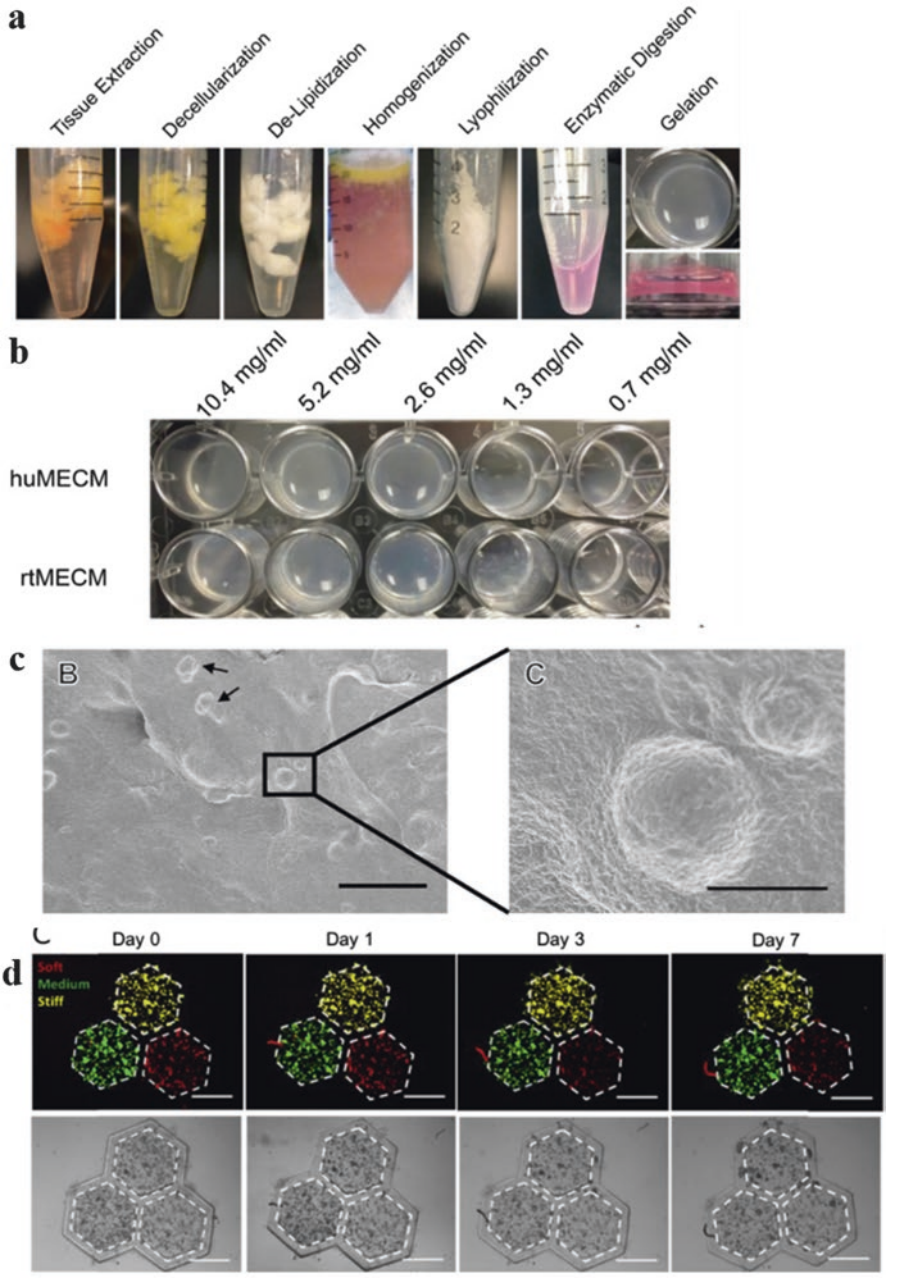

Fig. 2 The new generation of bioinks derived from decellularized extracellular matrix, such as murine or human ones. The mammary tissue is enzymatically digested through several steps that preserve the structural components. After the neutralization, the tissue extracts spontaneously polymerized (**a**) and can be used at different concentrations (**b**). (Reproduced and adapted with

3.3 Pancreatic Cancer

Pancreatic ductal adenocarcinoma is one of the most silent cancers, with poor prognosis at the stage of the diagnosis. The current therapies for pancreatic cancer are not efficient also due to the desmoplastic microenvironment of the pancreatic tumor that hinders the drug treatment to reach the inner tumor core. Many biomaterials have been used as bioinks for supporting the 3D growth of pancreatic cancer cells in vitro. Methacrylate gelatin allows the investigation of the early stage of pancreatic cancer, in particular the differentiation process that leads exocrine pancreatic acinar cells to remodel the pancreatic microenvironment and trigger in some cases early lesion occurring in cancer (Hakobyan et al., 2020). A more sophisticated approach to recapitulate pancreatic cancer by means of bioprinting is the fabrication of biomimetic materials based on cellulose nanofibrils and photo-crosslinkable galactoglucomannan methacrylates. These materials are highly tunable when combined together; hence, the stiffness of the tumor tissue can be achieved, so the pancreatic cancer cells can proliferate and be studied (Xu et al., 2019). The technical advances in bioprinter allow the fabrication of complex 3D culture models suitable for high-throughput screening of new drugs. An example is the technology that allows the drop of pancreatic cancer cells and 4-arm PEG-based polymers in 96-well plate in a short time (seconds), enhancing the biocompatibility of the printing process by means of bioprinter with eight independent addressable nozzles (Utama et al., 2021). Going toward fast and high-throughput approach is the key to push bioprinting as standard for the preclinical studies. An attempt to recapitulate the interaction of pancreatic cancer and stroma cell is provided by the fabrication of bioprinted organoids in the absence of extracellular matrix in low-attachment 384- or 1536-well plates. The organoids are obtained by bioprinting technology coupled with magnetic force and allow the testing of about 3300 approved drugs and proof of concept for the system (Hou et al., 2018).

3.4 Brain Cancer

Brain cancer, in particular glioblastoma, has a poor outcome due to its aggressiveness. Brain cancer develops resistance to the drugs caused by the intratumoral heterogeneity. Beside the presence of different populations of cells, the brain tumor microenvironment is also playing an important role in driving the progression of the

Fig. 2 (continued) permission from reference (Mollica et al., 2019)). Scanning electron microscopy images at day 3 of a 3D culture of bioprinted glioblastoma at two different magnifications (400× and 3500×) revealed the formation of spheroids in the bioprinted construct (**c**). (Reproduced and adapted with permission from reference (Lee et al., 2019)). Digital-light processing bioprinting allows the fabrication of a 3D model of hepatocarcinoma. Merged fluorescence and brightfield images show the HepG2 cells at day 7 of culture in soft (red), medium (green), and stiff condition (yellow) (**d**). (Reproduced and adapted with permission from reference (Ma et al., 2018))

disease. For this reason, 3D bioprinting offers an interesting platform to recapitulate this heterogeneity. A 3D glioblastoma model is obtained bioprinting glioblastoma cell together with a fibrin-based bioink by means of an Aspect Biosystems RX1 Bioprinter that minimized the shear stress allowing the growth of the cells for 12 days (Fig. 2c). Moreover, the cells formed spheroids, and the bioprinted constructs are challenged with novel treatment previously tested in 2D, demonstrating the higher resistance of the 3D glioblastoma model in comparison to the cells grown on plastic dish (Lee et al., 2019). Another attempt to model glioblastoma in vitro is achieved using a digital light processing bioprinter. This model is interesting because it combines patient-derived glioblastoma stem cells, macrophages, neuronal progenitor cells, and astrocytes. In this case, the bioprinting process ensures a spatial organization of the cells mimicking the brain tumor microenvironment and the tumor-immune cell interactions. This model highlights the role of macrophage in the chemoresistance and cancer invasion (Tang et al., 2021). A model based on RGD-modified alginate bioink mixed with stem and stromal cells, both derived from glioblastoma patient donors, reveals cisplatin resistance that has not been raised when the cells are cultured in 2D condition. The model obtained by means of extrusion-based bioprinting highlights the role of stromal cells in the response to the drug (Hermida et al., 2020). Recently published, another glioblastoma model combines two bioinks, fibrinogen and gelatin for the tumor part and the Pluronic F127, a thermos-reversible synthetic polymer, for the vascular part. The Pluronic F127 acts as a sacrificial material in order to provide the vascular network inside the tumor part. The bioprinted glioblastoma is exploited to test mechanical and biological properties, such as growth kinetics, invasion capabilities, response to therapies, and tumor genetic signature. The model is able to recapitulate better the glioblastoma progression and the response to therapy in comparison to 2D cells and can be a promising platform for preclinical research (Neufeld et al., 2021).

3.5 Hepatic Cancer

Liver cancer is the fifth most lethal cancer one, so there is an urgent need to generate in vitro models able to help in finding predictive markers for early diagnoses or understanding the mechanism of cancer progression and metastasis. Moreover, the liver is also the site of metastasis of many cancers. The HepG2 cell line is the most common choice to recapitulate liver cancer in vitro. The combination of these cell lines together with a gelatine/alginate based bioink has been used to develop a bioprinted model for hepatocarcinoma that has been characterized by immunofluorescence, gene expression, and transcriptome sequencing. This model demonstrates an overexpression of gene related to liver function and invasion. The researchers conclude that these differences in the molecular signature between 2D and 3D culture correlate with the difference in drug response in the models, making the 3D in vitro bioprinted model more eligible for realistic drug testing (Sun et al., 2020). If from one side cell lines are widely used for investigating cancer mechanisms, from the

other side, it is widely accepted that a model including patient-derived cells is more reliable in terms of drug response and the study of chemoresistance. At this aim, a bioprinted model using patient-derived cells of hepatocarcinoma has been maintained for long-term culture, and interestingly, it retained the same molecular features of the parental hepatocarcinoma from which the cells are derived. The model is proposed as a platform for the reliable study of drug screening (Sun et al., 2020). Liver extracellular matrix is essential for the environmental cues that trigger invasion in cancer. For that, researchers tried to print a liver decellularized extracellular matrix by means of rapid light-based bioprinting technology (Fig. 2d). The expression of invasion-associated markers in the bioprinted model resulted in higher than 2D culture cells (Ma et al., 2018). Hepatocarcinoma microenvironment is quite complex and not composed only of epithelial cells. A model embedding not only parenchymal cells is obtained bioprinting HiPSC-hepatocyte spheroids together with mesenchymal and endothelial cells. These bioprinted constructs showed better liver functionality when stromal cells support hepatocytes during the 18 days of the experiments. This promising approach pave the way for the bioprinting of heterocellular model for hepatocarcinoma modelling in vitro (Goulart et al., 2020).

4 Current Limitations in 3D Bioprinting

Bioprinting is a technology in progress since it is still far from the concept of a widely accepted and validated 3D tissue bioprinted model. Cell viability can be hindered by the mechanical pressure of the printing process, also because the cells are usually not immersed in a culture medium. Optimizing the printing time could affect less the cell viability and obtain functional constructs, or another strategy could keep the bioprinted constructs slightly hydrated during the process. One limitation for bioprinting living tissue is the resolution of the existing methods for bioprinting that cannot reproduce the nanoscale hierarchical organization of the human extracellular microenvironment. Due to the limits in the maximum resolution reachable in printing (40–50 μm), it is necessary to highlight that with the current bioprinting approaches, it is not always possible to print more than two to three cell types simultaneously. Some tissues, instead, are made up of more than three cell types; hence, it becomes clear that they cannot ensure the totality of the biological and structural interactions that may exist among all the cell types and the surrounding microenvironment. The native complexity is still not recapitulated, so it is needed an ultimate effort in improving the technology and the know-how behind the bioprinting technology. Moreover, when culturing two or more than one cell type, it is interesting to point out that different types of cells could need different types of culture media and growth factors that are not always compatible with all the other cells in the bioprinted construct. The easiest solution is to distribute the cell culture media in different ratios following the proportionality among the cells. However, the medium is mixed but not spatially distributed, so it represents a very specific challenge that needs to be addressed likely combining 3D bioprinting with

compartmentalized on-chip or bioreactor technologies. As a consequence of the medium choice and in the case of working on undifferentiated cells, it is important to delineate which stage of differentiation the cells should have. In fact, the bioprinted tissue could serve as a differentiation niche for stem cells, but also mature cells can be bioprinted in order to study the later stage of a process, such as cancer evolution.

5 Conclusion and Future Perspectives

Recent advances in tissue engineering lead to a large portfolio of bioprinted technologies that allow to recapitulate different cancer types at different stages. In this way, bioprinting improved the relevance of models for drug screening and testing along with the investigation of mechanisms underpinning cancer progression, drug resistance, and metastasis.

The bioprinted mimicking constructs support cell viability and maintain the functionality of the tissue they are mimicking. To recapitulate the complexity and cellular heterogeneity, more than one bioprinting technique can be needed coupled with different bioinks. At this purpose, bioinks can be mixed with tissue-specific decellularized ECM to improve the physiological relevance of the models.

Present challenges for a wider implementation of bioprinting technology are technically related to the development of new bioprinters with a higher spatial resolution and more physiologically performing bioinks. From this side, it is expected that bioengineering will continue supporting bioprinting progress and improvement with new materials with tunable properties that will allow a cell response to external stimuli for a dynamic temporal control on the cancer evolution in vitro.

Bioprinted living constructs, in particular the ones mimicking multicellular tumor niches, could be integrated in microfluidic chip or bioreactors that could provide by means of sensors interesting data regarding pH levels, glucose or lactate measurement, and gas exchange monitoring in real time both from the culture media and inside the bioprinted tissue.

Along this, it will be necessary to make software for the bioprinted design more and more friendly for users from different backgrounds, in order to move bioprinting from the bench to the clinical and industrial practice in the near future.

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Author Contribution VB: conceptualization, writing original draft, and funding acquisition.

Conflict of Interest The author declares that the chapter was written without any commercial or financial relationships that could represent a potential conflict of interest.

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Monoclonal Antibodies in Nanosystems as a Strategy for Cancer Treatment

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Abbreviations

2-MEA	β-Mercaptoethylamine
5-FU	5-Fluorouracil
A549	Adenocarcinomic human basal alveolar epithelial cells
Abs	Antibodies
Ab-SH	Antibody with sulfhydryl thiolation reaction
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
AMF	Magnetic fields
anti-GPC3	Anti-glypican-3 antibody
AuNPs	Inorganic gold nanoparticles
BME	β-Mercaptoethanol
BsAbs	Bispecific antibodies

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BT474	Human breast carcinoma with HER2 overexpression
BVZ	Bevacizumab
C	Constant
Calcein-AM	Calcein acetoxymethyl ester
CDC	Antibody-mediated complement-dependent cytotoxicity
CDR	Complementarity-determining regions
CET	Cetuximab
Chi	Chitosan
CMD	Carboxymethyl dextran
CNBr	Cyanogen bromide
CPT	Camptothecin
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CuAAC	[3+2] Azide-alkyne cycloaddition reaction catalyzed by copper(I)
DA	Diels-Alder reaction
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DSPE	1,2-Distearoyl-sn-glycero-3-phosphorylethanolamine
DSPE-PEG2000	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]
DSPS	1,2-Distearoyl-sn-glycero-3-phosphocholine
DTT	Dithiothreitol
DTX	Docetaxel
EDC	1-Ethyl-3-(-3-dimethylaminopropyl) carbodiimide
EGFR vIII	Epidermal growth factor receptor variant III
EGFR	Epidermal growth factor receptor
EPR	Enhanced permeability and retention
Fab	Antigen-binding fragments
Fc	Crystallizable fragment
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GPC3	Glypican-3
H	Heavy
HACA	Human anti-chimeric antibodies
HAMA	Human anti-mouse antibodies
HCC	Hepatocellular carcinoma
HCC827	Lung cancer cell line
HCT 116	Human colon cancer cell line
HepG2	Hepatocellular carcinoma cell line
HER	Herceptin
HER2	Human epidermal growth factor receptor-type 2

HGC-27	Human gastric carcinoma cell line derived from the metastatic lymph node of gastric cancer (undifferentiated carcinoma)
HKH-2	Human colon cancer cell line
ICP-MS	Inductively coupled plasma mass spectrometry
iEDDA	Inverse electron demand hetero-Diels-Alder reaction
L	Light
LN	Lipid nanoparticles
mAb	Monoclonal antibodies
MAC	Membrane attack complex
Mal	Maleimide
MCF-7	Human breast cancer cell line
MDA-MB-231	Breast cancer strain isolated from pleural effusion with low expression of HER2
MDA-MB-453	Human breast cancer cell line
MEA	2-Mercaptoethylamine
MES	2-(N-Morpholino)ethanesulfonic acid
MGC-803	Human gastric cancer cell line
MKN-45	Gastric adenocarcinoma cells
MMAE	Monomethyl auristatin E
MRI	Magnetic resonance imaging
MSNPs	Mesoporous silica nanoparticles
MTT	Bromide of (4,5-dimetilltiazol-2-il)-2,5-difeniltetrazolium
mV	Millivolts
NB	Nanobubble
NHS	N-Hydroxysuccinimide
NK	Natural killer cell
NLC	Nanostructured lipid carriers
nm	Nanometer
NP	Nanoparticle
P123	Pluronic
PANC-1	Human pancreatic cancer cell line isolated from pancreatic carcinoma of ductal cell origin
PCL	Poly(ε-caprolactone)
PD-1	Programmed cell death receptor 1
PD-L1	Programmed death ligand 1
PDLA	Poly(D-lactic acid)
PDLLA	Poly(D,L-lactic acid)
PEG	Polyethylene glycol
PGA	Poly(glycolic acid)
pH	Hydrogen potential
pI	Isoelectric point
PLA	Poly(lactic acid)
PLGA	Poly(lactide-co-glycolide)
PLLA	Poly(L-lactic acid)
PTT	Photothermal therapy

PTX	Paclitaxel
QD	Quantum dots
RAPA	Rapamycin
RES	Reticuloendothelial system
SAMSA	S-Acetylmercaptosuccinic anhydride
SATA	N-Succinimidyl S-acetylthioacetate
SATP	N-Succinimidyl S-acetylthiopropionate
scFv	Single-chain variable fragment
SFB	Sorafenib
SIA	N-Succinimidyl iodoacetate
SKBR-3	Human breast cancer cell lines that overexpress HER2
SLNs	Solid lipid nanoparticles
SMCC	Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
SMPT	4-Succinimidyloxycarbonyl-α-methyl-α-[2-pyridyldithio] toluene
SPAAC	Strain-promoted [3+2] azide-alkyne cycloaddition reaction
SPDP	N-Succinimidyl 3-(2-pyridyldithio)propionate
SPIONs	Superparamagnetic nanoparticles
Sulfo-LC-SMPT	Sulfosuccinimidyl 6-[α-methyl-α-(2-pyridyldithio)tolua-mido]hexanoate
Sulfo-LC-SPDP	Sulfosuccinimidyl 6-[3′-(2-pyridyldithio)propionamido] hexanoate
Sulfo-NHS	N-Hydroxysulfosuccinimide
Sulfo-SMCC	Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
TCEP	Tris-(2-carboxyethyl)phosphine
TCO	Trans-cyclooctene
THF	Tetrahydrofuran
THPP	Tris-(3-hydroxypropyl)phosphine
Tmab	Trastuzumab
TMZ	Temozolomide
TPGS	D-α-Tocopheryl polyethylene glycol succinate
TPGS-COOH	Carboxyl-terminated TPGS
Tz	Tetrazine diene
U87MG vIII	Glioblastoma cells expressing mutant epidermal growth factor VIII receptor
U87MG	Cell line with epithelial morphology isolated from malig-nant gliomas
US	Ultrasound
V	Variable
VEFGA	Vascular endothelial growth factor A
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

1 Introduction

Cancer is the term used to designate a group of diseases, whose origin occurs with an uncontrollable and disordered multiplication of cells (WHO, 2021). Thus, depending on the embryonic origin from which this cell aggregate originates, the cancer will have different nomenclatures, for example, when derived from the epithelial secretory glands, the tumors is called adenocarcinoma. According to the World Health Organization (WHO), in 2020, an average of 18 million new cases of cancer were observed in the world, with lung cancer in men (1,435,943) and breast cancer in women being more frequent (2,261,419). Following the average growth of the world population, for the year 2030, it is estimated that the world will register 24.6 million new cases of cancer, increasing to 30.2 million in 2040 (GCO-WHO, 2020; Kumar et al., 2013).

Depending on the type and stage of the tumor, location, and health status of the patient, among other factors, some cancer treatment options can be listed, such as surgery, chemotherapy, radiotherapy, hormone therapy, targeted therapy, immunotherapy, or a combination of more of a treatment modality (WHO, 2021). However, despite the wide range of possibilities, cancer therapy still presents a series of challenges, whether due to tumor resistance mechanisms (highly mutable) or high toxicity, with low preservation of other organs, leading to a series of side effects and consequent reduction in the patient's quality of life.

When a cancer cell appears, tumor antigens are expressed on its surface. In this way, the immune system, through innate (natural killer cells and macrophages) and adaptive (CD4 and CD8 lymphocytes and antibodies) immunity, recognizes and destroys this potential tumor cell. However, these tumor cells can undergo mutations or modifications, known as evasion mechanisms, allowing their survival in the face of the immune system attack. In addition, there may also be failure of the immune response due to tumor growth and proliferation overcoming the ability of the immune system to control. Thus, malignant tumor lethality is associated with dysregulated proliferative activity, resistance to death by apoptosis, and ability to invade tissues and metastasize to other sites (Abbas et al., 2019).

Antibodies can activate the complement system, mediate antibody-dependent cytotoxicity, or serve as drug carriers. Recombinant monoclonal antibody, such as trastuzumab, an anti-HER2 mAb, can act in monotherapy or drug combination, causing cancer cell death (Cameron et al., 2017). Radiolabeled monoclonal antibodies, in turn, are conjugated to radioisotopes and can be used both for early diagnosis and for cancer therapy, for example, ibritumomab tiuxetan, which has a radioactive isotope (yttrium-90 or indium-111), used in the treatment of non-Hodgkin lymphoma (Theuer et al., 2004). Acting similarly, antibody-drug conjugates (ADCs) have chemotherapeutic drugs attached, such as brentuximab vedotin, an antibody that targets the CD30 antigen, linked to the antimicrotubule agent monomethyl auristatin E (MMAE) (Eichenauer et al., 2018). The ADC technology has high affinity with a specific receptor present on the surface of tumor cells, releasing the drug in the region of interest and consequently protecting other organs and

tissues from cytotoxic effects; examples of this technology are brentuximab vedotin (Adcetris®) and trastuzumab emtansine (Kadcyla®) (Mitra et al., 2015).

In recent years, nanocarriers have attracted the attention of the scientific community. Nanocarriers are extremely small structures, from 0 to 1000 nm, developed with the aim of increasing the bioavailability of drugs by protecting the drug from physiological degradation, allowing control of the release, and, mainly, directing the therapy to have greater selectivity at the pathological site. In this sense, in cancer treatment, nanocarriers help the anticancer drug to accumulate in the tumor tissue, avoiding exposure to cytotoxicity to the rest of the body (Ernsting et al., 2013). Among the carriers, micelles, polymeric nanoparticles, liposomes, solid lipid nanoparticles, and several inorganic nanoparticles stand out, which will be addressed herein. Some formulations using nanocarriers have already been approved by the FDA for clinical use, such as Doxil®, a liposomal formulation of doxorubicin used to treat Kaposi's sarcoma, refractory ovarian cancer, and refractory breast cancer, and Abraxane®, in which the chemotherapy paclitaxel is efficiently associated with albumin nanoparticle for the treatment of breast cancer and pancreatic cancer (Northfelt et al., 1996; Saif, 2013).

Thus, in this chapter, nanoparticle targeting with mAbs will be discussed, which consists of functionalized nanostructures to deliver antineoplastic agents efficiently and specifically to tumor cells/tissues/organs. These can be chemically modified with several ligands for specific targeting the tumor and controlled release of the active substance, facilitating the uptake of tumor cells and increasing the retention and accumulation of nanoparticles in the tumor microenvironment. In this sense, suitable bioconjugation strategies to immobilize antibodies on the surface of nanoparticles will be addressed, demonstrating the fundamental principles of the most widely used functionalization methods. We discuss the methodologies of adsorption, covalent conjugation (carbodiimide chemistry, maleimide chemistry, and "click" chemistry), in addition to the biotin-avidin interaction. Furthermore, the chapter will cover the main physicochemical characteristics of functionalized nanoparticles, as well as their biological performance, both in vitro and in vivo.

2 Monoclonal Antibodies in Cancer Therapy (mAbs)

2.1 Antibody Structure

Only approximately three decades after the experiment by Tiselius and Kabat, which demonstrated that immunoglobulins belong to the gamma fraction of serum and that they had specificity against particular antigens, the structure of antibodies was elucidated (Singh et al., 2020). It appears in a Y shape, where the arms are represented by two antigen-binding fragments (Fab) and the base by a crystallizable fragment (Fc) (Fig. 5.1) (Parakh et al., 2020; Singh et al., 2020). An antibody is composed of two identical light (L) chains and two identical heavy (H) chains. Each

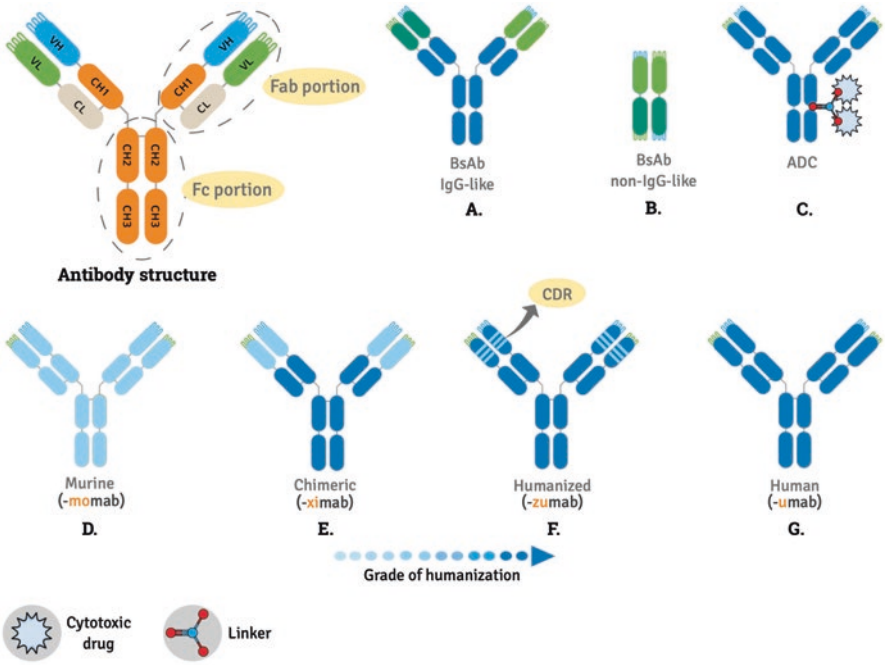

Fig. 5.1 General structure of antibodies and different mAbs types used in cancer therapy. Schematic representation of bispecific mAbs (BsAbs) IgG-like (**a**), non-IgG-like (**b**), and antibody-drug conjugates (ADC) (**c**). Development of monoclonal antibodies (**d**–**g**) represented in light blue (murine regions) and dark blue (human regions)

of the four polypeptide chains has constant regions (C) and variable regions (V), with respect to the nature and sequence of amino acids present between different antibodies (Eloy et al., 2017c). Together, the variable domains of the L and H chains constitute the antigen-binding site. The ability of antibodies to recognize and demonstrate binding specificity for a wide range of targets is related to the presence of complementarity-determining regions (CDRs) in variable domains. In all, a single Fab portion concentrates six CDRs which, in turn, form the antigen-binding loop (Parakh et al., 2020). The Fc portion is composed only of two pairs of paired heavy chain domains (Binyamin et al., 2006). It mediates interactions between antibodies and most cells of the immune system and is therefore essential for the maintenance of Immunoglobulin effector functions. It is also the genetic nature of the constant domains of the H chain of an antibody that determines its class, which will have individual biological structure and function. The IgA, IgD, IgE, IgG, and IgM classes have H chains called α (alpha), δ (deltha), ε (epsilon), γ (gamma), and μ (micro), respectively (Eloy et al., 2017c). In addition to being the most abundant isotype in the human body, IgG is also the most used in the production of therapeutic mAbs and drugs based on them, being subdivided into four subclasses, IgG1, IgG2, IgG3, and IgG4 (Kumar et al., 2017).

2.2 *Biotechnological Synthesis Techniques*

Hybridoma technology was a pioneer in the development and isolation of mAbs, and, even almost 50 years after its emergence, it is still widely used (Moraes et al., 2021). However, the success of this technique in the synthesis of mAbs has also brought to light a central problem in this field, the generation of human anti-mouse antibodies (HAMA) in response to their administration to humans (Liu, 2014; Stern & Herrmann, 2005). This is because the immunoglobulins from rodents, animals initially used, are foreign to the human organism and, therefore, like any unknown antigens, are seen as a threat. As a result, the search for technologies that allow the production of humanized or fully human mAbs for therapeutic use has intensified (Parray et al., 2020). Therefore, these technologies can be used both in upstream processing operations and in downstream processing operations (Carrara et al., 2021). In the first case, genetic engineering is used to obtain human antibody sequences without the need for further modifications. This translates into the use of synthesis techniques that allow the direct generation of fully human antibodies, such as the transgenic mouse technology and antibody phage display libraries (Carrara et al., 2021; Lonberg, 2008).

2.2.1 Hybridoma

The principle of the primary technique described by Kohler and Milstein in 1975 is based on three main steps. Initially, the animal of a particular species is exposed to the antigen of interest. From then on, the host's own immune system will be responsible for establishing an immune response, leading to the generation of B lymphocytes that produce antibodies against specific epitopes of the antigen. Once synthesized in the living organism, the short-lived, mature antibody-producing B lymphocytes are fused to immortalized myeloma cells (Liu, 2014; Parray et al., 2020). For the fusion of both cells, polyethylene glycol polymer (PEG), fusogenic viruses, or the application of an electric field can be used (Moraes et al., 2021). The resulting hybrid cell is called a hybridoma, which will be equipped with the ability to secrete highly specific monovalent antibodies and, at the same time, will possess "immortality." However, the population of hybridomas initially generated is not homogeneous, as it results from fusion to different clones of host B lymphocytes, each of which secretes individual antibodies specific for a particular epitope of the antigen (Liu, 2014). For this reason, the last central step of this technique consists of the selection of clones secreting the antibodies of interest and their subsequent large-scale cultivation (Moraes et al., 2021).

2.2.2 Transgenic Mouse

The transgenic mouse technology was described in the 1990s by two different research groups that generated animals genetically modified to be able to harbor a repertoire of human immunoglobulins (Parray et al., 2020). To make this possible,

endogenous murine heavy and light chain genes were silenced, while transgenic human genes encoding the same structures were introduced into the animal genome (Lonberg, 2008; Moraes et al., 2021). A method widely used for this purpose is pronuclear microinjection, where the human DNA fragment is inserted into the pronucleus of fertilized eggs of the mouse and, therefore, propagates through the cell germ line (Lonberg, 2008). As a result, the animal would preserve its ability to generate binding specificity from recombination of genes from the variable portion of the chains, immunoglobulin class switching, as well as their affinity maturation (Liu, 2014; Thomas, 2015). However, once immunized against an antigen of interest, the B cells of the transgenic animal would lead to the production not of murine antibodies, but of human antibodies against a variety of targets. The use of transgenic animals also allows access to the ease and reproducibility of mAb isolation using hybridoma technology, since human antibody-producing B cells can be collected and fused to myeloma cells (Moraes et al., 2021).

2.2.3 Phage Display

Another important methodology described in the 1990s applicable to the generation of fully human monoclonal antibodies is the phage display technology, originating from the homonymous concept initially proposed by George Smith in 1985 (Moraes et al., 2021; Smith, 1985). The basis for this technique emerged from the discovery that foreign gene fragments could be incorporated into the DNA of filamentous bacteriophage viruses and expressed on their surface along with their endogenous fusion proteins (Lonberg, 2008). Thus, once a repertoire of immunoglobulin variable region gene clones with diverse specificity is inserted into these viral particles, one has a vast library of antibody phage display. Therefore, with the antigen of interest in hand, this library can be accessed for affinity selection of target-specific fragments in a process called biopanning (Moraes et al., 2021). The bacteriophage, or phage, that expresses the compatible antibody portion is used as a vector for the insertion of the gene into prokaryotic organisms, such as *E. coli*, where it will be replicated and expressed, leading to the formation of new phage copies (Kumar et al., 2019). The cycle is repeated a few times, in order to obtain a population of fragments as homogeneous and specific as possible, as the lower affinity phages are removed (Lu et al., 2020). The antibody fragments most used in this methodology are the single-chain variable fragment (scFv) and the Fab, which can be directly applied in cancer therapy and diagnosis, but are preferentially converted into other formats, such as IgG or bispecific mAbs, which will be addressed later (Alibakhshi et al., 2017; Kumar et al., 2019).

2.3 Engineered Therapeutic mAbs

In addition to the synthesis strategies that allow the direct generation of human mAbs, such as the transgenic mouse and phage display techniques, the humanization of murine antibodies can be performed in the immunoglobulin downstream processing phase, which involves, among other steps, the modification and optimization of the final molecule (Carrara et al., 2021; Lonberg, 2008). This synthesis route is used on a large scale, since, despite being little applicable for therapeutic use in humans, mouse mAbs can be produced efficiently, quickly, and less expensively (Lu et al., 2020). Using molecular engineering techniques, it was possible to combine critical sequences from human antibodies to the structure of murine antibodies, preserving the essential characteristics for therapeutic activity and reducing the immunogenicity of the resulting hybrid molecule (Binyamin et al., 2006; Lu et al., 2020). Through the nomenclature assigned to each mAb used in the clinic, the species from which it originates can be identified. Murine, chimeric, humanized, and human antibodies are given the suffixes -momab, −ximab, −zumab, and -umab, respectively (Eloy et al., 2017c; Lu et al., 2020). Examples of monoclonal antibodies approved for cancer therapy are listed in Table 5.1.

2.3.1 Chimeric mAbs

Murine antibodies can be converted into chimeric mAbs through a process called chimerization, which consists of joining the variable domains of the mouse antibody to the constant portion of a human antibody (Fig. 5.1) (Carter, 2006). Although they still involve the risk of developing HAMA or, as they are called, human anti-chimeric antibodies (HACA) (Ternant & Paintaud, 2005) when administered to humans, chimeric mAbs are safer and more efficient when compared to mouse mAbs (Singh et al., 2020). This is due to the fact that chimeric immunoglobulins have approximately two-thirds of the human structural sequence, in addition to a half-life of close to 10 days, almost seven times longer than that shown by mouse mAbs (Kumar et al., 2019; Singh et al., 2020). The first mAb approved by the Food and Drug Administration (FDA) for the treatment of cancer, rituximab, is a chimeric IgG1 that, even two decades after its arrival on the market, remained among the ten mAbs that led the ranking of sales by pharmaceutical companies, moving almost seven billion dollars in 2018 (Lu et al., 2020).

2.3.2 Humanized mAbs

In humanized mAbs, only the complementarity-determining regions (CDR) of the variable portion of the molecule are of murine origin, resulting in an approximately 95% human molecule (Stern & Herrmann, 2005). Consequently, in addition to reduced immunogenicity compared to chimeric mAbs, humanized mAbs also

Table 5.1 FDA-approved mAbs for cancer treatment

mAb	Target	Type	Technology	Indication	FDA first approval	Reference
Rituximab	CD20	Chimeric IgG1	Hybridoma	Non-Hodgkin lymphoma, chronic lymphocytic leukemia	1997	Lu et al. (2020), Si et al. (2021)
Trastuzumab	HER2	Humanized IgG1	Hybridoma	HER2-positive breast cancer, stomach cancer	1998	Lu et al. (2020), Si et al. (2021)
Alemtuzumab	CD52	Humanized IgG1	Hybridoma	Chronic lymphocytic leukemia	2001	Lu et al. (2020), Si et al. (2021)
Ibritumomab tiuxetan	CD20	Murine IgG1	Hybridoma	Non-Hodgkin lymphoma	2002	Lu et al. (2020), Si et al. (2021)
Cetuximab	EGFR	Chimeric IgG1	Hybridoma	Metastatic colorectal, metastatic NSCL, and head and neck cancer	2004	Lu et al. (2020), Si et al. (2021)
Bevacizumab	VEGF-A	Humanized IgG1	Hybridoma	Colon, lung, and renal cancer, glioblastoma	2004	FDA (2022), Lu et al. (2020), Si et al. (2021)
Panitumumab	EGFR	Human IgG2	Transgenic mice	Colorectal cancer	2006	Lu et al. (2020), Si et al. (2021)
Ofatumumab	CD20	Human IgG1	Transgenic mice	Chronic lymphocytic leukemia	2009	Lu et al. (2020), Si et al. (2021)
Ipilimumab	CTLA-4	Human IgG1	Transgenic mice	Melanoma	2011	Lu et al. (2020), Si et al. (2021)
Pertuzumab	HER2	Humanized IgG1	Hybridoma	HER2-positive breast cancer	2012	Lu et al. (2020), Si et al. (2021)
Obinutuzumab	CD20	Humanized IgG1	Hybridoma	Chronic lymphocytic leukemia	2013	Lu et al. (2020), Si et al. (2021)

(continued)

Table 5.1 (continued)

mAb	Target	Type	Technology	Indication	FDA first approval	Reference
Ramucirumab	VEGFR-2	Human IgG1	Phage display	Gastric cancer	2014	Lu et al. (2020), Si et al. (2021)
Blinatumomab	CD19, CD3	Murine bispecific scFv	Hybridoma	Acute lymphoblastic leukemia	2014	Lu et al. (2020), Si et al. (2021)
Nivolumab	PD-1	Human IgG4	Transgenic mice	Melanoma, NSCL cancer, Hodgkin lymphoma, renal cell carcinoma	2014	Lu et al. (2020), Si et al. (2021)
Pembrolizumab	PD-1	Humanized IgG4	Hybridoma	Melanoma, NSCL, head and neck squamous cell, gastric, cervical, and hepatocellular cancers, Hodgkin lymphoma, urothelial carcinoma	2014	FDA (2022), Lu et al. (2020), Si et al. (2021)
Necitumumab	EGFR	Human IgG1	Phage display	NSCL cancer	2015	Lu et al. (2020), Si et al. (2021)
Dinutuximab	GD2	Chimeric IgG1	Hybridoma	Neuroblastoma	2015	Lu et al. (2020), Si et al. (2021)
Daratumumab	CD38	Human IgG1	Transgenic mice	Multiple myeloma	2015	Lu et al. (2020), Si et al. (2021)
Elotuzumab	SLAMF7	Humanized IgG1	Hybridoma	Multiple myeloma	2015	Lu et al. (2020), Si et al. (2021)
Atezolizumab	PD-L1	Humanized IgG1	Hybridoma	NSCL cancer	2016	FDA (2022), Lu et al. (2020), Si et al. (2021)
Avelumab	PD-L1	Human IgG1	Phage display	Merkel cell carcinoma	2017	Lu et al. (2020), Si et al. (2021)

(continued)

Table 5.1 (continued)

mAb	Target	Type	Technology	Indication	FDA first approval	Reference
Durvalumab	PD-L1	Human IgG1	Transgenic mice	Urothelial carcinoma, NSCL cancer	2017	FDA (2022), Si et al. (2021)
Cemiplimab	PD-1	Human mAb	Transgenic mice	Cutaneous squamous cell carcinoma, basal cell carcinoma, NSCL cancer	2018	FDA (2022), Si et al. (2021)

Abbreviation: *NSCL* non-small cell lung cancer

demonstrate a longer half-life of approximately 12–20 days (Kumar et al., 2019). For its production, one of the most used techniques is CDR grafting, in which the antigen-binding loops of mouse antibodies are allocated to a human antibody. Therefore, the same human immunoglobulin structural sequence can be used for the synthesis of mAbs against different targets, since its binding specificity will be determined by the grafted CDR sequence (Winter & Harris, 1993). However, in some cases, the recognition and binding affinity of the immunoglobulin antigen do not depend only on the CDRs but also on amino acids present specifically in the structural sequence of the murine antibody, but not necessarily in the human antibody. When this occurs, to reverse the impairment of target binding activity, the CDR grafting technique can be accompanied by "human back to mouse" mutations (Lu et al., 2020; Winter & Harris, 1993).

2.3.3 Human mAbs

Almost 10 years after the approval of rituximab, the first fully human mAb aimed at cancer therapy, IgG2 panitumumab, arrived on the market (Lu et al., 2020). Despite having a half-life similar to that of humanized mAbs, from 15 to 20 days, fully human mAbs are the most suitable for therapeutic purposes because they share the same structural framework as antibodies physiologically produced in the human body (Kumar et al., 2019). In the oncological therapeutic context, the two main techniques used for its production are the phage display and the use of transgenic mice, already discussed above (Lu et al., 2020; Si et al., 2021; Carter, 2006).

2.3.4 Bispecific mAbs

Another format of mAbs that emerged in the therapeutic horizon of cancer as a product of antibody engineering was the bispecifics (BsAbs). These are designed so that the variable region of each Fab portion of the molecule expresses specificity for

a particular antigen (Rodgers & Chou, 2016). Structurally, BsAbs are divided into two main groups, the IgG-like, which have the Fc portion in their molecule, and the non-IgG-like, which present themselves as fragments without the Fc portion, but the possibilities of conformational variation within each of them are many (Krishnamurthy & Jimeno, 2018). Figure 5.1 shows some examples of possible structural conformations. As a reflex, while IgG-like BsAbs are able to mediate Fc-dependent effector functions, described in the next topic, non-IgG-like BsAbs are more applicable to the simultaneous interruption of multiple cell signaling pathways by blocking receptors or of their respective ligands. Furthermore, because of their smaller size, BsAbs without the Fc portion exhibit better tissue penetration, but shorter half-life and stability when compared to IgG-like bispecific antibodies (Lim et al., 2021). The only bispecific mAb that currently has approval for the treatment of cancer is blinatumomab, a single-chain variable fragment BsAb (scFv), but which should soon compose a vast list as hundreds of clinical studies involving bispecific mAbs for cancer therapy are ongoing (Lim et al., 2021; Rodgers & Chou, 2016).

2.4 Therapeutic Applications of mAbs in Cancer Therapy

In the treatment of cancer, monoclonal antibodies can exert their therapeutic effect by direct mechanisms, commonly mediated by the variable portions of the molecule; by indirect mechanisms, mediated by its Fc portion; or even by both mechanisms concomitantly (Foltz et al., 2013). Direct mechanisms involve the binding of the mAb to the target, which can be a specific tumor marker, a receptor or protein expressed on the surface of the tumor cell, or even a growth factor, for example. In contrast, indirect mechanisms refer to the cascade of immunological events that are carried out by cells of the host immune system in response to the binding of the mAb to the target antigen and that similarly result in the death of cancer cells (Lee et al., 2018). For indirect mechanisms, particularly, the Fc portion of the molecule and the subtype determined by it are closely related to the cytotoxic ability demonstrated by the mAb, with IgG 1 and 3 being the most used for this proposal (Foltz et al., 2013; Hafeez et al., 2018; Rodgers & Chou, 2016). In this topic, the effector mechanisms of therapeutic mAbs will be discussed when in monotherapy, as well as in their conjugated form to other cytotoxic molecules, such as drugs, toxins, or radioisotopes (Binyamin et al., 2006).

2.4.1 Blocking Cell Signaling

Tumor development and progression are closely dependent on the occurrence of several pathophysiological events mediated by different cell signaling pathways. It is from the interaction between chemical mediators and their respective receptors that processes related to angiogenesis, proliferation, growth, and cell survival begin

(Rodgers & Chou, 2016). For this reason, blocking cell-to-cell signaling represents one of the mechanisms that make mAbs valuable therapeutic tools in the context of cancer. Within this proposal, mAbs can be designed to bind to soluble mediators in the tumor microenvironment, such as growth factors and cytokines, or to their respective receptors located on the surface of the cell membrane (Shuptrine et al., 2012). In both cases, signaling is disrupted and so are cellular responses mediated by receptor-ligand binding. For example, bevacizumab, a humanized IgG1, is a mAb against vascular endothelial growth factor A (VEFGA) that steals it from the microenvironment by preventing it from interacting with its receptor on the tumor cell (Hafeez et al., 2018). Cetuximab, a chimeric IgG1, is a widely studied epidermal growth factor receptor (EGFR) inhibitor that occupies the outer portion of the receptor, preventing its ligand from doing so.

In addition to cetuximab, other mAbs against EGFR, also known as erbB1 or HER1, and against HER2/erbB2, another member of the erbB family of receptors, have also been linked to increased internalization, downregulation, and degradation of target receptors, either individually or in association with other mAbs. However, the mechanism by which they exert this effect is still unclear and remains under investigation (Jaramillo et al., 2006; Jones et al., 2020).

2.4.2 Antibody-Dependent Cellular Cytotoxicity (ADCC)

In ADCC, the mAb acts as a mediator between the cancer cell and an effector cell of the immune system. With the former, it establishes an antigen-specific binding through its Fab domain, while, with the latter, it interacts through a link between its Fc domain and Fcγ receptors present on the surface of immune cells (Hafeez et al., 2018). Fcγ receptors are divided into activating and inhibitory, and the action of immune cells on the tumor cell depends on a favorable balance to the activation stimuli for it to occur. IgG1 and IgG3 MAbs, in particular, are able to bind to activation receptors present in the membrane of natural killer (NK) cells of the immune system, in greater proportion, and of monocytes and macrophages, to a lesser extent (Shuptrine et al., 2012). Upon recognizing the monoclonal antibody attached to the cancer cell membrane, the effector cells secrete mainly granzymes and perforins, substances that lead to cell lysis and death by different mechanisms (Rodgers & Chou, 2016; Shuptrine et al., 2012). Trastuzumab and pertuzumab, both humanized IgG1 anti-HER2, are examples of mAbs that concentrate the ability to exert direct mechanisms of action on the antigen, as well as to generate ADCC (Foltz et al., 2013; Hafeez et al., 2018).

Some studies carried out in the last 20 years were successful in correlating the antitumor activity of mAbs that act by this mechanism and the functionality of Fcγ receptors. In summary, the presence of functional activating Fcγ and the absence or poor expression of inhibitory Fcγ have been shown to be determinant factors of the therapeutic efficacy of rituximab and trastuzumab in vivo. Furthermore, the presence of polymorphic inhibitory Fcγ that potentiate the affinity of effector cells for

the Fc portion of antibodies also proved to be an important marker of response to ADCC-triggering mAbs (Shuptrine et al., 2012).

2.4.3 Antibody-Mediated Complement-Dependent Cytotoxicity (CDC)

Similar to ADCC, in the complement-dependent cytotoxicity mechanism, mAbs need to communicate with a component of the immune system to cause cell death, but in this case, they are not effector cells, but a group of plasma proteins. The complement system, as it is called, is composed of a series of enzymes that are inactive when soluble, but which, once activated, initiate or continue a proteolytic cascade that culminates in the lysis of the target cell (Shuptrine et al., 2012). Also as in ADCC, the most efficient IgG subtypes in the recruitment of complement system proteins are the IgG1 and IgG3 isotypes (Rodgers & Chou, 2016). In the presence of two or more mAbs coupled to the tumor cell membrane, the classical pathway of activation of the complement system begins with the binding of the C1 protein complex to the Fc domains of both immunoglobulins. From then on, in sequence, other proteins of the complement system are recruited and cleaved, one by one, into two fragments. While one of them is returned to the blood plasma to perform other biological functions, the other is covalently bound to the membrane of the target cell and conjugated to the proteolytic complex that is formed there. As the final event of this cascade, the formation of the membrane attack complex (MAC) occurs, which allows the opening of pores on the surface of the tumor cell and, consequently, its rupture and death (Shuptrine et al., 2012). Examples of therapeutic mAbs that can generate CDC are human IgG1 ofatumumab and humanized IgG1 alemtuzumab (Foltz et al., 2013; Lu et al., 2020).

2.4.4 Cancer Immunotherapy

Although its theoretical bases originated in discoveries dating from the nineteenth century, it was only from evidence obtained in the last three decades that immunotherapy was consolidated as an arm of oncotherapy (Hou et al., 2022). Among the evidences, there is the understanding of the immunoediting process, described for the first time in 2001, which establishes that the host's immune system acts not only in surveillance of tumor development in order to restrict and eradicate it but also shaping the progression of the neoplasm as it establishes mechanisms to circumvent it (Desai et al., 2022). For this reason, it is divided into three different dynamic and independent stages: elimination, equilibrium, and escape (Hou et al., 2022). Elimination is represented by the coordinated action of the innate and adaptive immunity of the host to identify and eradicate cancer cells at a faster rate than they multiply. Once this fails, the tumor enters the equilibrium phase, represented, metaphorically, by a "tug of war" between the cancer cell, which seeks to develop increasingly specialized survival strategies, and the individual's immune defense, which tries to continuously overlap these and contain tumor growth (Desai et al.,

2022). It is when the immune system loses the ability to recognize and fight the tumor that it reaches the escape phase, where there is uncontrolled and progressive growth of the neoplasm (Koebel et al., 2007). One of the mechanisms of tumor escape is the activation of the so-called immunological checkpoints, negative regulators of the immune response that act as brakes to prevent the attack on self-molecules, but which, in this case, are used by tumor cells as tools of disguise (Hou et al., 2022). In cancer immunotherapy, mAbs are used as checkpoint inhibitors, and the most widely studied targets to date are programmed cell death receptor 1 (PD-1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4).

Both CTLA-4 and PD-1 are part of the group of inhibitory receptors responsible for ensuring the tolerance of T lymphocytes, the main representatives of the adaptive immunity of the immune system, to self-antigens. In a very simplified way, after repeated contact with the antigen, T cells begin to express inhibitory checkpoints in order to prevent them from remaining activated indefinitely, causing damage to healthy tissues. After interacting with their respective ligands, present in other cells of the immune system or even in tumor cells, CTLA-4 and PD-1 become active and drive the T lymphocyte to its non-responsive state. What the mAbs used in cancer immunotherapy are intended to do is block this interaction, by binding either to the inhibitory checkpoint itself or to its respective ligand, thereby preventing the suppression of T lymphocyte activities (Desnoyer et al., 2020; Hou et al., 2022). Although many monoclonal antibodies are showing promising results in clinical trials, ipilimumab is, to date, the only FDA-approved anti-CTLA-4 mAb for cancer immunotherapy and was also the first immune checkpoint inhibitor approved in 2011 (Desai et al., 2022). Nivolumab, pembrolizumab, atezolizumab, and durvalumab are approved by the FDA for blocking the PD-1 signaled pathway, the first two targeting PD-1 and the last two anti-PD-L1, its ligand (Desnoyer et al., 2020; Lu et al., 2020). Interestingly, the clinical success of immunotherapy has been observed with the individual use of checkpoint inhibitors, but notably from the association between anti-PD-1 and anti-CTLA-4 mAbs (Dougan et al., 2021).

2.4.5 Antibody-Drug Conjugate (ADC)

In cancer therapy, mAbs can also be conjugated, via a linker, to other cytotoxic drugs to target them specifically to cancer cells. Therefore, this therapeutic modality minimizes the deleterious effects on healthy tissue and promotes the widening of the drug's therapeutic window, since the cytotoxic load is mainly concentrated at the tumor site (Desai et al., 2022; Hasan et al., 2022). ADC toxicity can be caused by any of its components (cargo, mAb, or linker) and can be manifested by target-dependent mechanisms, from the interaction between the mAb and its target, which is commonly represented by a membrane receptor, overexpressed, or target-independent, upon premature drug release, binding to Fc-portion receptors, or pinocytosis, for example. In target-dependent pathways, when targeted to a membrane receptor, binding between the mAb and this is followed by the internalization of the conjugate into endosomes, disruption of the linker, and release of the cytotoxic load

inside the cell (Hasan et al., 2022; Kumar et al., 2017). On the other hand, the adoption of antigens dispersed in the tumor microenvironment as a target is also used as a strategy, especially in solid tumors, since, once the linker is broken, the free charge can penetrate the tumor mass more easily than the ADC complex (Hasan et al., 2022). FDA-approved ADCs for cancer therapy are described in Table 5.2.

Table 5.2 ADCs approved for cancer therapy

ADC	Payload	Target	Type	Indication	FDA first approval	Reference
Brentuximab vedotin	MMAE	CD30	Chimeric IgG1	Hodgkin lymphoma and systemic anaplastic large cell lymphoma	2011	FDA (2022), Hasan et al. (2022)
Trastuzumab emtansine	DM1	HER2	Humanized IgG1	HER2-positive breast cancer	2012	FDA, (2022), Hasan et al. (2022)
Inotuzumab ozogamicin	Calicheamicin	CD22	Humanized IgG4	B-cell acute lymphoblastic leukemia (ALL)	2017	FDA (2022), Hasan et al. (2022)
Gemtuzumab ozogamicin	Calicheamicin	CD33	Humanized IgG4	CD33-positive acute myeloid leukemia (AML)	2017	FDA (2022), Hasan et al. (2022)
Moxetumomab pasudotox	Pseudotox	CD22	Murine IgG1	Hairy cell leukemia (HCL)	2018	FDA (2022), Hasan et al. (2022)
Polatuzumab vedotin	MMAE	CD79β	Humanized IgG1	Diffused large B-cell lymphoma	2019	FDA (2022), Hasan et al. (2022)
Enfortumab vedotin	MMAE	Nectin-4	Human IgG1	Locally advanced or metastatic urothelial cancer	2019	FDA (2022), Hasan et al. (2022)
Trastuzumab deruxtecan	Deruxtecan	HER2	Humanized IgG1	Unresectable or metastatic HER2-positive breast cancer	2019	FDA (2022), Hasan et al. (2022)

(continued)

Table 5.2 (continued)

ADC	Payload	Target	Type	Indication	FDA first approval	Reference
Sacituzumab govitecan	SN-38	TROP2	Humanized IgG1	Metastatic triple-negative breast cancer	2020	FDA (2022), Hasan et al. (2022)
Belantamab mafodotin	MMAF	BCMA	Humanized IgG1	Multiple myeloma	2020	FDA (2022), Hasan et al. (2022)
Loncastuximab tesirine-lpyl	PBD dimer	CD19	Humanized IgG1	Large B-cell lymphoma	2021	FDA, (2022), Hasan et al. (2022)

Abbreviations: *MMAE* monomethyl auristatin E, *DM1* maytansine derivative, *MMAF* monomethyl auristatin E, *SN-38* active metabolite of the topoisomerase I inhibitor irinotecan

3 Functionalization Strategies

The modification of the surface of nanoparticles through conjugation with biomolecules or functional groups is called functionalization, and, in the case of antibodies, this conjugation can occur by binding to the entire macromolecule or its fragments. The unique structural features of antibody molecules provide several options for modification and conjugation schemes. The chemistry used for the formation of the conjugate must be chosen in order to maintain, in the best possible way, the ability to bind the antigen. For this, the coupling method should preferably produce a stable, sterically unimpeded, and properly oriented binding, in which the antigen-binding fragment (Fab) is available for its interaction with the receptor (Manjappa et al., 2011; Sandeep et al., 2020; Thiruppathi et al., 2017). The functionalization of nanoparticles with immunoglobulins can be performed using different strategies, which is based on the nature of the interactions between these structures, and can be divided into two different groups: non-covalent and covalent methods (Petrilli et al., 2020).

Non-covalent methods are one of the simplest conjugation techniques available, as they do not require modification of the antibody structure or the nanoparticle. The simplest non-covalent immobilization method is adsorption, which is fundamentally based on physical adsorption or ionic bonding. In physical adsorption, antibodies are bound to the surface of nanoparticles through hydrogen bonds, van der Waals forces, or hydrophobic interactions. The nature of the forces involved in non-covalent attachment results in a condition that can affect the strength of the interaction (namely, pH, pI, and ionic strength). Adsorption fixation is a mild and

easy-to-perform process and commonly preserves the functionality of the biomolecule (Trilling et al., 2013; Zhang et al., 2020). The principle of bioaffinity between biomolecules is also explored within the context of functionalization methodologies. The most established procedure is the avidin/streptoavidin-biotin interaction, which configures one of the strongest non-covalent affinities known (Liébana & Drago, 2016).

Covalent methodologies tend to preserve the biological activity provided by the antibody for a longer time due to greater stability of the bonds; therefore, they are the most used strategies. The types of reactions involved in the formation of the antibody/nanoparticle bond depend on the reactivity of the nanoparticle components and the side chains of the amino acids that make up the antibody and can be mediated by catalysts, activators, or intermediate ligands (Manjappa et al., 2011). Depending on the coupling chemistry selected, bond formation with or without chemical modification of the antibody is possible. This additional step favors a targeted immobilization in which the antibody keeps its functional portion exposed (Marques et al., 2020).

The different covalent methodologies can be guided by the chemistry of the functional groups of the side chains of the amino acids present in the antibodies from (i) primary amino groups of glycine, ε-amino of lysine, and α-amino N-terminal of polypeptide chains; (ii) of aspartic or glutamic acid side chain carboxyl groups; or (iii) through mercapto groups (also known as sulfhydryl or thiol) of the cysteine side chain, which may come from the reduction of disulfide bonds present in the hinge region, as well as from the connection between the heavy and light chains of antibodies. Thiol groups can also be inserted into the antibody structure through reactions with primary amines (Eloy et al., 2017c; Guo et al., 2020; Manjappa et al., 2011; Werengowska-Ciećwierz et al., 2015).

In view of the limitations of reactivity of these functional groups, covalent bonding often requires prior activation of the nanoparticle or the reactive groups of the side chains of the amino acids that make up the antibodies. In this way, it is not as straightforward as the non-covalent methodology. Among the possible reactions related to the formation of covalent bonds, we can highlight carbodiimide chemistry, maleimide chemistry, and click chemistry (Juan et al., 2020; Parracino et al., 2019; Santos et al., 2021).

3.1 Non-covalent Methods

3.1.1 Adsorption

Adsorption or physisorption is a non-covalent coupling strategy between nanoparticles and antibody that includes physical adsorption and ionic binding. In physical adsorption, the antibody is immobilized on the surface of the nanoparticle (NP) through weak intermolecular forces, such as hydrogen bonding, hydrophobic interactions, van der Waals forces, and electrostatic interactions (Sivaram et al., 2018)

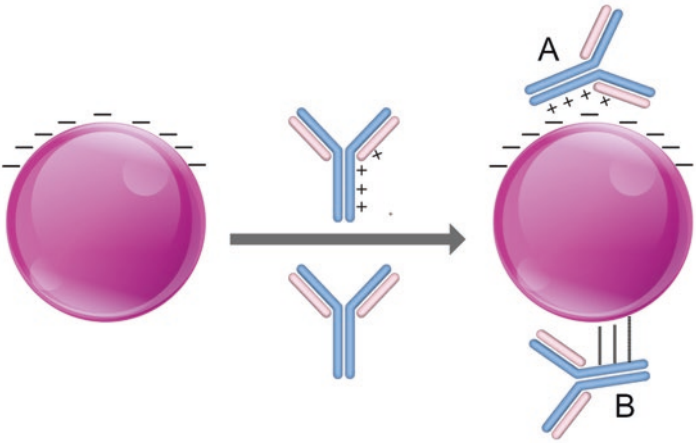

Fig. 5.2 Conjugation of antibodies on nanoparticles via (**a**) electrostatic adsorption and via (**b**) hydrophobic interaction

(Fig. 5.2). It is the simplest and most direct conjugation method, since the technique applied is simple and takes little time, as it does not involve any chemical modification of the antibody or nanoparticle, occurring by mixing the two components, leading to conjugation (Marques et al., 2020; Trilling et al., 2013).

For the preparation of antibody-NP bioconjugates by adsorption, some parameters must be considered, such as the isoelectric point (pI) – pH value where there is equivalence between the positive and negative charges – of the antibody, the pH, and the amount of antibody added (Zhang et al., 2020). In general, protein adsorption is enhanced when the pH is close to or relatively above its pI. The optimal pH value for coupling and for a given antibody must be determined by measuring its pI range. The vast majority of antibodies are better adsorbed at pH 8–9, in general. Charged groups are common in antibodies; positively charged amino acids (e.g., lysine and arginine residues) and N-terminal groups are present, favoring an electrostatic interaction between these groups and the charged surface of the nanoparticle (Jazayeri et al., 2016). The electrostatic attraction fundamentally depends on the number of charged groups on the surface of nanoparticles and antibodies. Antibody orientation depends on the positions of charged regions within the protein, so the antibody is expected to be immobilized across the surface region with the highest density of charged residues, where it is thus most likely to be adsorbed in the proper orientation. Therefore, this approach is heavily influenced by the pH of the solution and the isoelectric point of the antibody, and even a subtle variation in pH can result in the removal of antibodies from the nanoparticles. Therefore, it is important to work at a pH greater than or close to the isoelectric point of the antibody to promote proper ion adsorption.

Despite its ease, antibody adsorption to nanoparticles has some disadvantages: adsorption results in randomly oriented antibodies, which may lead to a partial loss of antigen-binding capacity due to the direct interaction between the nanoparticle

and the antigen-binding sites or by steric hindrance (Zhang et al., 2020). Antibody binding is relatively weak, and adsorption techniques require a high concentration of antibodies, making the method disadvantageous as the antibody is a high-cost biomolecule (Jazayeri et al., 2016). Furthermore, with regard to hydrophobic interactions, they can cause conformational changes in the three-dimensional structure of antibodies, which can lead to protein denaturation and consequent loss of biological activity (Marques et al., 2020).

Adsorption is a dynamic process between solution and bound antibody phases. Antibodies retained on the surface of the nanoparticle can be easily desorbed in the presence of another protein having a higher charge or a greater number of hydrophobic regions. This is attributed to weak and reversible interactions, so inefficient antigen binding can happen even if a greater amount of antibodies is adsorbed onto the nanoparticle. In addition, randomly adsorbed antibodies have a low affinity for their antigens (Shen et al., 2017). Therefore, to circumvent these problems, antibody immobilization strategies through covalent binding are preferred.

3.1.2 Avidin-Biotin System

One of the most popular methods involving adapter molecules is the non-covalent interaction between avidin and biotin molecules (Marques et al., 2020). This system is based on the bioaffinity of the interaction between biotin and a biotin-binding protein (e.g., avidin and its derivatives). Biotin is a B-complex vitamin, with a size of 244 Da, also known as vitamin H, soluble in water, which has a structure containing a tetrahydroimidizalone ring (ureido ring) with a tetrahydrothiophene ring. It plays a key role in cell signaling (Jain & Cheng, 2017). Avidin is a tetrameric protein obtained mainly from the egg white of oviparous vertebrates. It is highly stable (molecular weight of 66 kDa); its side chain is made up of portions of N-acetylglucosamine and mannose residues (Sakahara & Saga, 1999). Each of the four subunits binds biotin with high specificity and affinity, with a dissociation constant of about 10^{-15} (Sperling & Parak, 2010), being one of the strongest non-covalent interactions known. Avidin is an extremely basic protein, having an isoelectric point (pI) of approximately 10.5 (Jain et al., 2017). Thus, the strong positive charge on the protein, at physiological pH (pH ~ 7.4), causes ionic interactions with more negatively charged molecules. Furthermore, carbohydrate-binding proteins (for example, lectins, selectins, and galactins) in cells can interact with the polysaccharide moieties on the avidin molecule to bind them to chemical species other than (Jain & Cheng, 2017; Liébana & Drago, 2016). These non-specific interactions limit the application of avidin. Thus, non-glycosylated avidin analogues are most often chosen (e.g., streptavidin). Streptavidin is an avidin-like biotin-binding protein derived from *Streptomyces avidinii*. Similar to avidin, streptavidin contains four subunits, each with a unique biotin-binding site. Unlike avidin, streptavidin is not a glycoprotein, and its isoelectric point is much lower (pI 5–6) (Jain & Cheng, 2017), which implies a decrease in non-specific electrostatic interactions and the

elimination of non-specific bonds to molecules and carbohydrate-binding receptors (Jain et al., 2017).

In the context of the interaction between biotin and avidin/streptavidin, a fairly common technique is the modification of antibodies with biotin (biotinylation) (Prantner et al., 2013). In this approach, functional groups of antibodies, such as amines, hydroxyls, or sulfhydryls, can be modified with a range of biotinylation reagents, such as the sulfhydryl reagent reactivates biotin-BMCC or 1-biotinamido-4-[4′-(maleimidomethyl)cyclohexane-carboxamido]butane. Biotinylization in the Fc portion of the antibody guarantees oriented immobilization, mainly via free sulfhydryl groups or obtained by reducing disulfide bonds for reaction with maleimide-biotin reagents or by portions of polysaccharides, through the oxidation of hydroxyl groups to aldehydes for reaction with hydrazine-biotin (Trilling et al., 2013). Afterward, to form the biotin-avidin complex, the functionalized avidin/streptavidin nanoparticles are incubated with the biotinylated antibodies.

3.2 Covalent Bonding

3.2.1 Carbodiimide Chemistry

Carbodiimide compounds are used as crosslinking agents for carboxylic acids and promote the conjugation of the antibody with the nanoparticle by activating carboxyl groups. Its reaction with the carboxylic acid makes the carbonyl carbon more susceptible to nucleophilic attack by primary amines, leading to covalent bond formation by amide formation. Carbodiimides are considered zero-length crosslinkers, as no part of their chemical structure remains after amide formation in the final conjugated molecule (Elzahhar et al., 2019; Eroğlu & Ibrahim, 2020).

It is interesting to note that the carboxylic acid group may come from aspartic or glutamic acid residues of antibodies, which should react with the primary amino group present in the nanoparticle. However, the most usual strategy is the activation of carboxyl groups on the surface of nanoparticles, as primary amines are abundant on the surface of antibodies (Guo et al., 2020; Sandeep et al., 2020).

The most commonly used carbodiimide in these conjugation reactions is 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). It is a water-soluble compound that, upon reacting with a carboxylic acid group, forms the active intermediate O-acylisurea, and after being easily displaced by the nucleophilic attack of the primary amine to the carbonyl carbon, it is released in the form of a soluble derivative of urea. This reaction by-product can be removed easily by dialysis or gel filtration. The O-acylisurea intermediate is unstable in aqueous solutions and can undergo hydrolysis regenerating the carboxylic acid (Elzahhar et al., 2019; Werengowska-Ciećwierz et al., 2015) (Fig. 5.3 – Reaction A).

Another approach in carbodiimide chemistry is the use of N-hydroxysuccinimide (NHS), or N-hydroxysulfoxuccinimide (sulfo-NHS), with EDC reactions. These compounds are added to increase the efficiency of conjugation by forming an

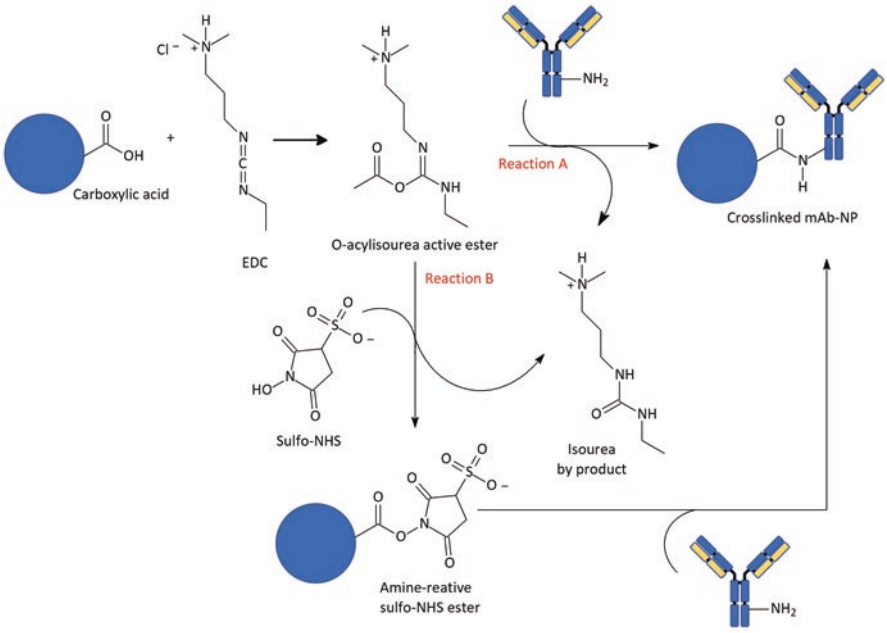

Fig. 5.3 mAb-nanoparticle conjugation via carbodiimide chemistry. Reaction A: reaction between EDC and the carboxylic group leading to the production of the O-acylisourea ester which then undergoes nucleophilic attack by a primary amine present in the antibody to form an amide bond. Reaction B: EDC couples sulfo-NHS to carbonyls, forming a sulfo-NHS ester, which has greater stability than the O-acylisourea intermediate, thus allowing a more efficient conjugation with the amine group of mAbs

amine-reactive intermediate, but more stable than O-acylisurea. And although not necessary for the one-step reaction, this modification reduces hydrolysis reactions and avoids intra- and intermolecular antibody reactions. Sulfo-NHS esters are water soluble, and their use with EDC increases the stability and solubility of the reactive intermediate. Although it also provides EDC activation, NHS decreases its solubility in water, and activation using EDC/sulfo-NHS is used in preference to the EDC/NHS protocol (Elzahhar et al., 2019; Hermanson, 2013a) (Fig. 5.3 – Reaction B).

Due to the presence of carboxyl and amine groups on the entire surface of the antibody, random and self-polymerization reactions that lead to loss of antigen-binding activity are possible. The presence of binding with antibodies oriented in the "head-on" and "side-on" positions makes binding with the antigen difficult and can lead to the loss of its biological activity. Thus, although chemical carbodiimide conjugation is a relatively simple and straightforward method, as there is no need for chemical modification of the antibody, other strategies are preferred due to the lack of control over the orientation of the antibody when bound to the nanoparticle (Guo et al., 2020; Marques et al., 2020).

3.2.2 Maleimide Chemistry

Another covalent conjugation strategy is through the reaction of maleimide with mercapto or sulfhydryl (-SH) groups which leads to the formation of thioether bonds in aqueous solution (Guo et al., 2020). Thiols (RSH) are sulfur analogues of alcohols (ROH) with good nucleophilic characteristics, and their deprotonated form (RS-) at physiological pH is an even better nucleophile (Eroğlu & Ibrahim, 2020; Juan et al., 2020). Mercapto groups are generated in antibodies via reaction with primary amines or by reduction of native antibody disulfide bonds (Juan et al., 2020) (Fig. 5.4). These groups are oriented to react with the amine ends of the conjugated molecules and in acylation, alkylation, or redox reactions. In general, it is known that most thiol reactions are fast and produce a high yield of the reaction (Eroğlu & Ibrahim, 2020; Gordon et al., 2015). The degree of antibody modification, i.e., the amount of sulfhydryl groups generated or inserted, can be quantified using Ellman's reagent (Gordon et al., 2015; Riener et al., 2002).

In antibodies, the frequency of free native sulfhydryl groups is low when compared to amine and carboxyl groups. In IgG1 antibodies, cysteine residues form four interchain disulfide bonds – two connecting the light and heavy chains and two connecting the two heavy chains in the hinge region, holding the two halves of the antibody together. In addition to these, there are also 12 intrachain disulfide bonds (Hoffmann et al., 2018; Juan et al., 2020). Interchain disulfide bonds, which are generally not critical for IgG1 stability, are more susceptible to reduction than intrachain disulfide bonds and are potential conjugation sites (Sandeep et al., 2020; Tsuchikama & An, 2018). Each disulfide bridge gives rise to two sulfhydryl groups, which, depending on the pH, can be free (deprotonated) or reduced (protonated) sulfhydryls (Manjappa et al., 2011).

The formation of sulfhydryl groups from the reduction of disulfide bonds can be carried out through enzymatic digestion, acidification or by the use of reducing agents such as dithiothreitol (DTT), 2-mercaptoethylamine (MEA), or tris(2-carboxyethyl)phosphine (TCEP). Methods using reducing agents and acidification can cause separation between light and heavy chains (Eloy et al., 2017c; Eroğlu & Ibrahim, 2020).

Enzymatic digestion is useful as it produces antibody fragments and maintains their ability to bind antigen (Manjappa et al., 2011). Pepsin and papain, and other enzymes such as ficin, bromelain, and trypsin, cleave the antibody molecule at the hinge region, and, depending on the cleavage site, portions of the antibody heavy chain may or may not remain attached to the antigen-binding fragments. Papain is activated in the presence of a reducing agent, and enzymatic digestion using this sulfhydryl protease produces two Fab fragments, each containing an antigen-binding site, and the Fc portion that corresponds to the bottom of the two heavy chains on the immunoglobulin molecule. In the case of enzymatic digestion using pepsin, immunoglobulin cleavage produces a single fragment with the two Fab portions linked, called F(ab')2, and small fragments from extensive degradation of the Fc portion (Eloy et al., 2017c; Hermanson, 2013b). The use of antibody fragments

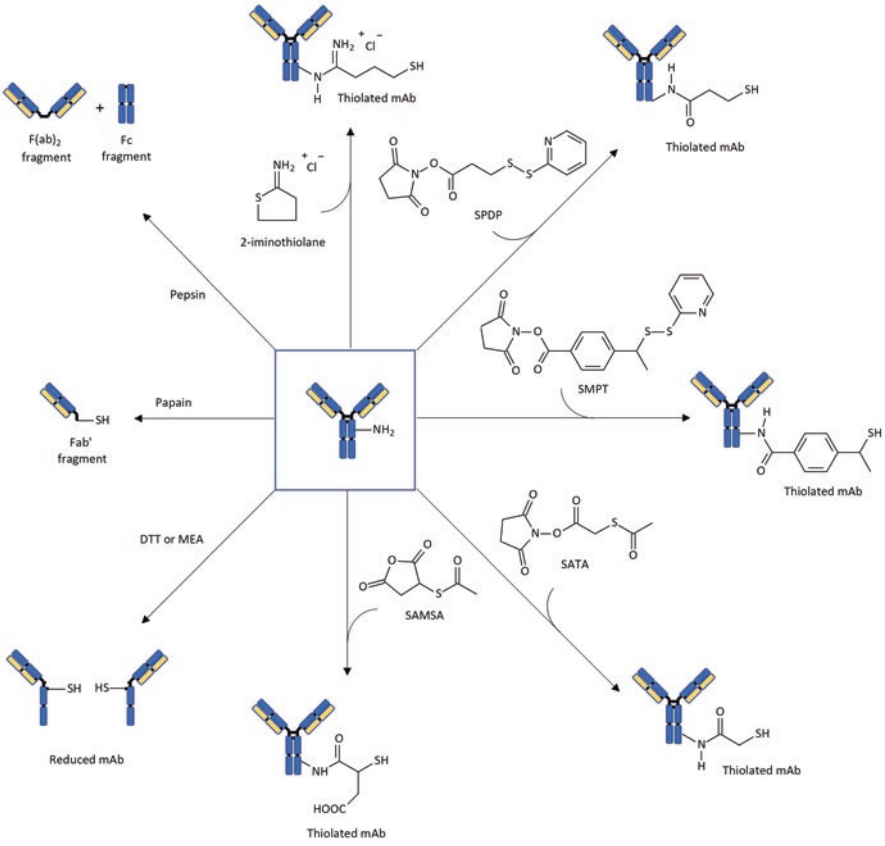

Fig. 5.4 Antibody thiolation methods. DTT dithiothreitol, MEA 2-mercaptoethylamine, SPDP N-succinimidyl 3-(2-pyridyldithio)propionate, SMPT succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene, SATA N-succinimidyl S-acetylthioacetate, SAMSA S-acetylmercaptosuccinic anhydride

has a lower immunogenic effect due to the absence of the Fc portion that is recognized by phagocytic cells (Manjappa et al., 2011).

Another method of generating sulfhydryl groups is by breaking the disulfide bonds of antibodies with mild reducing agents. The most popular traditional reducing reagents are dithiothreitol (DTT), β-mercaptoethanol (BME), and β-mercaptoethylamine (2-MEA) and soluble alkylphosphines such as tris-(2-carboxyethyl)phosphine (TCEP) and tris-(3-hydroxypropyl)phosphine (THPP) (Kantner & Watts, 2016). These reagents selectively cleave disulfide bonds present in the hinge region forming two halves of the antibody molecule, each of which has an antigen-binding site (Hermanson, 2013b). DTT and BME reagents generally require narrow neutral pH conditions (≥7) compared to TCEP ($1.5 < pH < 8.5$) and have poor stability in the presence of metal ions. In addition, excess unreacted DTT must be removed through an additional purification step as they readily react with

sulfhydryl-reactive agents (Kuan et al., 2016). Only 3.25 and 2.75 molar equivalents of DTT and TCEP per mole of antibody are required to achieve the reduction of two interchain disulfide bonds of a monoclonal IgG. This limited reduction strategy keeps the specificity of the antibody molecule intact while providing discrete sites for conjugation via thiols (Hermanson, 2013c).

In addition to generating sulfhydryl groups from the reduction of disulfide bonds, thiol groups can also be created or inserted using thiolating reagent systems that insert -SH groups into antibodies by modifying the amino residues of lysine and glycine. The reagents 2-Iminothiolane (Traut's reagent), N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), and N-succinimidyl S-acetylthioacetate (SATA) are the most common (Juan et al., 2020).

Thiolation of antibodies by Traut's reagent (2-iminothiolane) is the most popular approach (Eloy et al., 2017c). In this method, the cyclic imidothioester (2-iminothiolane) reacts with primary amines, opening the ring structure to introduce sulfhydryl groups (-SH) maintaining similar charge properties to the original amino group (Sandeep et al., 2020). Traut's reagent is completely soluble in water and reacts with amines in the pH range 7–10. It is stable to hydrolysis at acidic pH, and its half-life in solution decreases as the pH rises above neutrality. At pH values above 10.0, 2-iminothiolane also reacts with aliphatic and aromatic hydroxyl groups. However, the reaction rate with these groups is about 100 times slower than with primary amines (Hermanson, 2013c). The use of Traut's reagent can lead to the formation of secondary reactions in addition to oxidation to disulfides. After modification of the amine with 2-iminothiolane, terminal mercapto group can attack the amidine carbon forming new cyclic derivative, which can make the thiol group unavailable for conjugation. The loss of significant amounts of available sulfhydryl groups can occur within a few hours. For this reason, once forming the thiolation product as 2-iminothiolane, one should immediately proceed to the conjugation reaction in order to avoid substantial loss of activity (Manjappa et al., 2011).

N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) is a heterobifunctional crosslinking agent for introducing sulfhydryl groups in proteins and other molecules. The NHS ester end of SPDP reacts with amine groups to form an amide bond, and the other end containing the 2-pyridylthiol group can react with sulfhydryl residues to form a disulfide bond (Sandeep et al., 2020). After reaction with SPDP, the antibody molecule acquires the pyridyldisulfide group which can undergo reduction with DTT (or other disulfide reductants) forming free sulfhydryl groups and releasing pyridine-2-thione as a leaving group (Manjappa et al., 2011). The reagent 4-succinimidyloxycarbonyl-α-methyl-α-[2-pyridyldithio]toluene (SMPT) reacts similar to SPDP, except for the additional methyl group in its structure that makes the disulfide bond more sterically impeded, resulting in more stable conjugations (Gordon et al., 2015). These reagents are not soluble in aqueous solutions and must first be dissolved in an organic solvent (such as DMSO, DMF, or acetonitrile) and an aliquot of this stock solution transferred to the reaction solution (Manjappa et al., 2011). There are commercially available analogues of these reagents that are water soluble, such as sulfo-LC-SPDP and sulfo-LC-SMPT which contain an extended spacer chain. Sulfo-LC-SMPT is not as stable as SMPT. The sulfo-NHS ester is

more susceptible to hydrolysis in aqueous solution, and the pyridyl disulfide group is more easily reduced to free sulfhydryls. The main disadvantage of using these thiolating agents is the need for the additional step using reducing agents. These reducing agents can affect native disulfide bonds that are important for maintaining the structure and activity of these proteins (Gordon et al., 2015; Manjappa et al., 2011).

The disadvantage of these two molecules was solved by the development of the N-succinimidyl S-acetylthioacetate (SATA), N-succinimidyl S-acetylthiopropionate (SATP), and S-acetylmercaptosuccinic anhydride (SAMSA) reagents. The use of these reagents also requires an additional step, now for the deprotection of acetylated sulfhydryl, but the deprotection of the acetyl group is triggered by the addition of excess hydroxylamine, which does not confer structural instability to the mAb (Gordon et al., 2015).

N-Succinimidyl S-acetylthioacetate (SATA) is a versatile reagent for thiolation reactions. Similar to SPDP, SATA has an NHS ester active end that reacts with amino groups to form a stable amide. The other end of the molecule has a sulfhydryl group that remains protected by an acetyl group. Once modified by SATA, the protein (mAb) can be stored without degradation and deacetylated when necessary. The deprotection step requires excess hydroxylamine (Hermanson, 2013c). Most polyclonal antibody molecules can be modified to contain up to about six SATA molecules per immunoglobulin with minimal effect on antigen-binding activity. Some sensitive monoclonal antibodies, however, may be susceptible to modification and should be tested on a case-by-case basis (Manjappa et al., 2011). Although thiolation by modification of reactive amino groups, such as SATA, results in random attachment to the antibody surface, unlike other reduction strategies (which cleave the antibody molecule), the conjugates formed from the use of SATA maintain the two sites of antigen binding, making this approach advantageous (Hermanson, 2013b). Because many organic solvents are freely soluble, normally a stock solution is prepared in DMSO, DMF, or methylene chloride, and an aliquot of this solution is added to an aqueous reaction mixture containing the protein to be modified (Manjappa et al., 2011). Other reagents can be used in a similar strategy to SATA, such as its analogue succinimidyl acetylthiopropionate (SATP) which has an additional methylene carbon and S-acetylmercaptosuccinic anhydride (SAMSA) which has an anhydride moiety at one end in place of the NHS ester. This anhydride moiety undergoes nucleophilic attack by amines forming an amide bond and producing a carboxylate group that provides a negative charge to the final conjugated molecule. This additional charge can affect the conformation and consequently the activity of some proteins (Manjappa et al., 2011).

Upon thiolation, by any of the strategies described above, the mercapto or sulfhydryl group can then be used to conjugate to any crosslinking agents containing sulfhydryl-reactive groups, such as maleimide (for covalent conjugation) or 2-pyridylthiol groups (for reversible conjugation). The reaction with maleimides leads to the formation of conjugates through an irreversible thioether bond (Gordon et al., 2015; Guo et al., 2020; Sandeep et al., 2020). Maleimides, or maleic acid imides, are most used in conjugation reactions due to their greater selectivity to the

sulfhydryl side chain of cysteine and for providing clean, fast, and efficient reactions (Eroğlu & Ibrahim, 2020). They exhibit unique reactivity to Michael additions, and their reaction with free sulfhydryls is 1000 times faster than with lysine ε-amino groups at pH 7.0, which improves selectivity (Ravasco et al., 2019; Renault et al., 2018).

Maleimides undergo alkylation reaction with sulfhydryl groups by the reaction between the thiol group and the C1 carbon of the maleimide, to form stable thioether bonds, in the pH range between 6.5 and 7.5, called Michael thioadditions that characterize the "maleimide chemistry" (Eroğlu & Ibrahim, 2020; Ravasco et al., 2019; Renault et al., 2018). The ring tension imposed by the alkene moiety on the maleimide structure further enhances the electrophilic nature of the conjugated imide, while the solvent type, alkaline environment, and thiol type are known to play an important role in the reaction kinetics and its reaction selectivity (Ravasco et al., 2019). The pKa of the mercapto group can change drastically from one thiol to another, and depending on the chemical structure and intermolecular interactions, the thiol or thiolate can be stabilized or destabilized (Renault et al., 2018). Under alkaline conditions (pH > 8.5), hydrolytic degradation of maleimide can occur to non-sulfhydryl-reactive maleic amides, as well as thioether thiosuccimide (Michael addition product) to stable thioethers of succinamic acid (Ravasco et al., 2019) (Fig. 5.5). Control of reaction conditions should be done by using buffers that exclude thiol-containing compounds, to avoid competition for coupling sites. Furthermore, to avoid reoxidation of sulfhydryls, dissolved oxygen in these buffers must be removed (by vacuum degassing followed by inert gas bubbling), and metal chelating agents that can catalyze oxidation must be added (Manjappa et al., 2011; Marques et al., 2020). Interestingly, the thiosuccinimide conjugates resulting from the maleimide reaction have limited stability, due to their propensity to add retro-Michael. In circulation, the released maleimide payload can then react with plasma

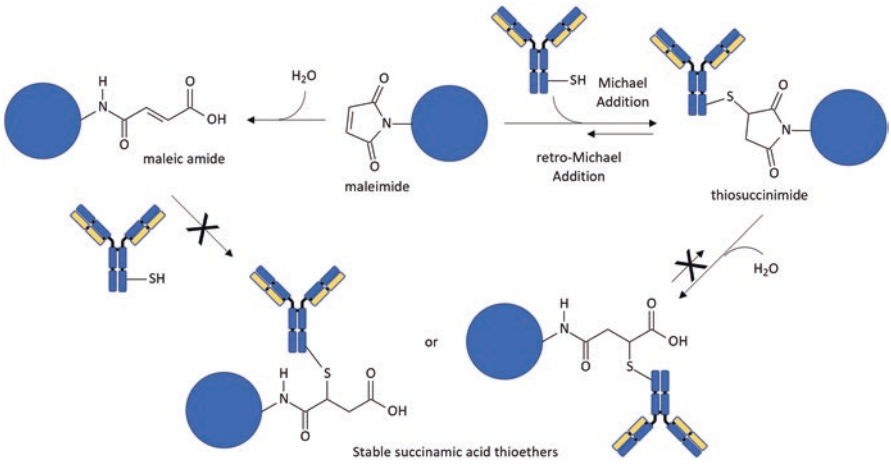

Fig. 5.5 General Michael addition and hydrolytic pathways of maleimides and thiosuccinimides

thiols or diffuse into nearby cells, resulting in reduced efficacy and/or safety of the conjugates formed. This instability can be mitigated by forcing post-conjugation hydrolysis of the thiosuccinimide, creating a stable chemical bond (Walsh et al., 2021; Yao et al., 2016).

Maleimide chemistry is very useful in the functionalization of nanoparticles (NPs) with mAbs. The conjugation of NPs to the maleimide group can be accomplished in several ways, while the modification of mAbs, to expose the -SH group, is done by thiolating agents. And when compared to methods of conjugating carboxyl and amine groups, conjugation via thiol and maleimide can be considered more effective, as it avoids crosslinking between mAbs while maintaining their immunoreactivity (Guo et al., 2020). One of the methods for coupling thiol-containing proteins or antibodies to nanoparticles with amino groups is by using a heterobifunctional crosslinker containing an amine-reactive NHS ester at one end and a thiol-reactive maleimide group at the other end, linked by a hydrophilic PEG spacer arm (NHS-PEG-maleimide). NHS-PEG-maleimide has been the most widely used crosslinking agent in antibody conjugation strategies to polymeric NPs for the treatment of cancer (Juan et al., 2020). This PEG coating strategy promotes a hydrophilic character to the nanoparticles after modification and helps mask any hydrophobic character the nanoparticle surface may have while providing terminal thiol-reactive maleimides for ligand coupling (Hermanson, 2013d). This coupling can be described in three steps, in which the first, the amino groups of the nanoparticle are activated by the crosslinker via the amine-reactive end. In the next step, thiolation of the antibody is performed, and finally, after removing the sulfhydryl addition reagents, the nanoparticles are mixed with the thiol-containing antibodies to form the conjugate. These heterobifunctional crosslinkers can also combine the maleimide group to phospholipids such as 1,2-distearoyl-sn-glycero-3--phosphorylethanolamine (DSPE)-PEG-maleimide (DSPE-PEG-maleimide), a frequently used amphiphilic reagent (Marques et al., 2020). In addition to these, homobifunctional ligands containing the maleimide group at both ends can also be used, and it may be necessary to employ the thiolation of nanoparticles. This strategy can lead to undesirable autopolymerization reactions (Hermanson, 2013c).

3.2.3 Click Chemistry

Click chemistry is a term used to describe thermodynamically favorable chemical reactions, with excellent bioorthogonality, that connect two molecules in a simple way and with high yields (Elzahhar et al., 2019; Freitas et al., 2011). Click chemistry reactions are biocompatible and can occur efficiently at room temperature under physiological conditions, and the bonds in the resulting chemical are irreversible. This type of reaction is widely used for the modification of biomolecules such as nucleic acids, lipids, and proteins with various compounds (Juan et al., 2020; Liu et al., 2017; Takayama et al., 2019). Sharpless, who introduced this term, defined click chemistry as a group of reactions that "…must be modular, broad in scope, give very high yields, generate only harmless by-products that can be removed by

non-chromatographic methods, and be stereospecific (but not necessarily enantioselective)" (Kolb et al., 2001).

[3 + 2] Azide-alkyne cycloaddition reactions (AAC) catalyzed by copper(I) (CuAAC), strain-promoted [3 + 2] azide-alkyne cycloaddition reactions (SPAAC), typical reaction of [4 + 2] Diels-Alder (DA), and inverse electron demand hetero-Diels-Alder (iEDDA) reactions configure the "click chemistry" strategy (Juan et al., 2020).

The copper (Cu)-catalyzed cycloaddition reaction (CuAAC) between azide and alkyne groups, producing a 1,2,3-triazole ring, was the first to be designated as "click chemistry" (Fig. 5.6). Azides and alkynes are almost non-existent in biological systems and inert to most functional groups and biomolecules (Spicer & Davis, 2014). The basic CuAAC process requires only copper ions in the +1 oxidation state. There are several methods to generate active Cu(I) catalyst for the CuAAC reaction. These can be provided by a discrete Cu(I) complex, by metallic copper, or by impregnated copper materials that expose cuprous ions to the reaction solution or, more conveniently, by a mixture of a Cu(II) salt and a reducing agent, with sodium ascorbate by far the most popular (Hein et al., 2008; Presolski et al., 2011).

CuAAC reactions are broad in scope, provide high yields under mild conditions, and react quickly at low temperatures or even under ambient conditions; solvents such as tetrahydrofuran (THF) and dimethylformamide (DMF) are compatible with water and miscible with water, are regiospecific (giving only 1,4-disubstituted derivative), have minimal and/or harmless by-products, and are suitable for microscale synthesis without the need for manipulations using protecting groups (Elzahhar et al., 2019). In addition to these characteristics, the 1,2,3-triazole ligand

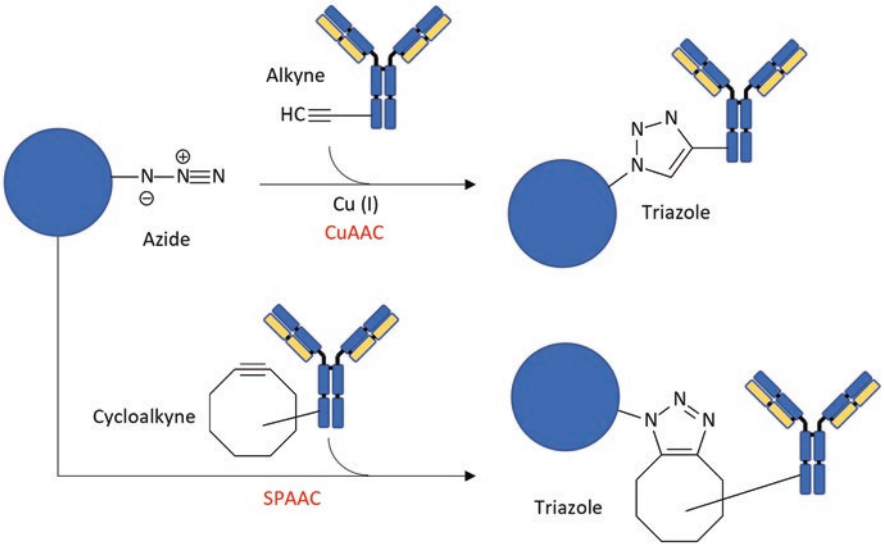

Fig. 5.6 Conjugation via click chemistry, CuAAC and SPAAC reaction. (Adapted from Marques et al. (2020))

is extremely soluble in water and has electronic properties very similar to amide bonds but is not subject to the same hydrolysis reactions (Hein et al., 2008).

There are two common approaches to functionalizing biomolecules with azide or alkyne loops: through N-hydroxysuccinimide (NHS)-mediated amide bond formation using an amine and carboxylic acid or through the reaction of a thiol with a substituted maleimide (Pickens et al., 2018). In this context, each heterobifunctional linker contains an NHS ester or a maleimide at one end and azide or alkyne groups at the other (Juan et al., 2020). NHS esters are among the most popular compounds used to functionalize biomolecules because of their aqueous compatibility, commercial availability, and ability to selectively target primary amines present in lysine or N-terminal residues. There are a variety of commercially available heterobifunctional linkers containing NHS esters or maleimides, which can be conjugated to amines or thiol groups present in the molecule of interest adding the desired azide or alkyne functionality (Pickens et al., 2018). However, commonly used click chemistry requires transformation of amine groups into antibodies to terminal azides or alkynes and still cannot control the orientation of the conjugated antibodies (Jeong et al., 2017).

Furthermore, the biggest challenge in bioconjugation applications involving CuAAC would be the removal of the copper catalyst. Copper is incompatible in vivo and known to cause serious side effects such as hepatitis and neurological and kidney diseases (Elzahhar et al., 2019). The most effective way to improve azide-alkyne cycloaddition biocompatibility has been to eliminate the need for Cu(I) catalysis and accelerate the reaction with alkynes that are activated by ring tension.

An alternative is stress-promoted azide-alkyne cycloaddition (SPAAC), also known as copper-free click chemistry (Mckay & Finn, 2014; Ramil & Lin, 2013). In this approach, the inclusion of an alkyne in an 8-membered cycloalkyl ring would lead to a massive deformation of the acetylene bond angle, increasing the tension in the ring. This destabilizing factor provides an enormous acceleration of the reaction. Reaction with an azide lowers the tension of the alkyne ring within the cyclooctin structure and thus drives the reaction without the addition of cytotoxic copper (Elzahhar et al., 2019) (Fig. 5.6).

The main restriction on the use of SPAAC in bioconjugation derives from the relatively large size and hydrophobic nature of the cyclooctin component, which can affect its distribution and alter the biological properties of the species to which they are attached (Mckay & Finn, 2014). Furthermore, the cost of strained cyclooctin reagents is considerably higher than their terminal alkyne counterparts. Fortunately, alternative synthetic routes are making SPAAC reagents more accessible and less costly to employ (Pickens et al., 2018). Seeking new alternatives to speed up the SPAAC reaction rate, chemists discovered that inverse electron demand Diels-Alder (iEDDA) reactions between a tetrazine diene (Tz) and a dienophile such as trans-cyclooctene (TCO) met the requirements of click chemistry. This third-generation copper-free click chemistry provides a faster second-order reaction rate constant than CuAAC and SPAAC (Kim & Koo, 2019).

3.2.4 Other Covalent Methodologies

The covalent methodologies, previously described in this chapter, can be applied by modifying the surface of preformed nanoparticles, by reacting with the functional groups exposed on the surface of these nanoparticles, or by incorporating the conjugate into the ligand during the preparation of these nanoparticles (called the pre-insertion method) (Marqués-Gallego & De Kroon, 2014; Nag & Awasthi, 2013). These techniques have as main disadvantages the need for an excessive amount of ligands and the possibility of the portion linked to the biomolecule with biological function facing the inner part of the nanoparticle (in the case of the pre-insertion method) (Nag & Awasthi, 2013). In addition, exposure of conjugates to solvents, in the pre-insertion method, can lead to the loss of their active conformation (Marqués-Gallego & De Kroon, 2014). To circumvent some of these problems, another preparation strategy, mainly used in liposomes, is the post-insertion method (Marqués-Gallego & De Kroon, 2014; Marques et al., 2020; Nag & Awasthi, 2013). This method involves coupling antibodies to the terminal portion of lipopolymer derivatives (phospholipids usually linked to polyethylene glycol (PEG) such as 1,2-distearoyl-*sn*-glycero-3-phosphorylethanolamine (DSPE)-PEG-maleimide) and slowly adding the phase micellar, containing the conjugate, to the diluted suspension of preformed drug-loaded liposomes, during a simple incubation step, in which the micelles are transferred to the liposomal bilayer (Eloy et al., 2017c; Iden & Allen, 2001; Jiang et al., 2021; Nag & Awasthi, 2013).

The post-insertion technique is a simple, flexible, and attractive method from a manufacturing point of view because it can prepare targeted liposomal drugs for clinical applications with a wide variety of ligands (Iden & Allen, 2001; Jiang et al., 2021). The insertion of the lipopolymer is a spontaneous process, but it depends on the incubation conditions, mainly temperature and time, on the concentration and nature of the composition, so that the concentration of amphiphilic lipopolymers must be lower than the critical micellar concentration (CMC). The incorporation of PEG lipids on the surface of the liposome is reduced as the length of the PEG chain is increased (Eliasen et al., 2019; Nag & Awasthi, 2013). Optimizing the preparation conditions in this approach is challenging, as the optimal conditions can vary depending on the liposomal layer formulation and the insertion of the modified lipopolymer, when altering the membrane, can cause leakage of the trapped agent (Marqués-Gallego & De Kroon, 2014). Eliasen et al. (2019) evaluated the preparation conditions of the post-insertion method by fluorescence technique, analyzing the conditions of incubation time and temperature, as well as the type of binder, and demonstrated the effect of these variables on the degree of post-insertion (Eliasen et al., 2019).

4 Nanoparticles Functionalized with Antibodies

4.1 Lipid Nanoparticles

Among the variety of nanoparticles applied for cancer treatment, lipid nanoparticles (LN) are efficient drug carriers for targeting tumor tissue; increasing the stability, specificity, and concentration of the encapsulated molecule; and decreasing the side effects (De Mendoza et al., 2009). In addition, they can encapsulate various therapeutic agents such as small soluble or insoluble molecules, proteins, and nucleic acids. They also have the advantage of being biodegradable and biocompatible, being absorbed by the lymphatic system, and improving the bioavailability of the encapsulated agent (Ramishetti et al., 2015; Shukla et al., 2018). The LN have their surface modified through the attachment of monoclonal antibodies, which is very promising for targeting of several types of cancer (Silvestre et al., 2020). Currently, most studies focused on cancer treatment with LN include liposomes (Eloy et al., 2017b), solid lipid nanoparticles (De Mendoza et al., 2009), nanostructured lipid carriers (Zimmermann et al., 2000), nanoemulsions, microemulsion, lipid nanocapsules (Formica et al., 2021), and hybrid nanoparticles at lipid base (Bummer, 2004).

4.1.1 Liposomes

Liposomes are considered the largest class of LN being widely used in the treatment of cancer. They can be defined as a lipid vesicle system with the interior of aqueous core that can contain hydrophilic drugs (Fernandes et al., 2021; Li et al., 2019). Among the advantages of these nanoparticles, one can mention their biocompatibility as they present similarities with the biomembranes, the decrease in toxicity, as well as the improvement of solubility, protection of the encapsulated agent from degradation by external agents, improvement in tissue penetration, increased drug half-life, and ability to transport hydrophilic and lipophilic drugs (Malam et al., 2009; Silvestre et al., 2020).

In the quest to further improve the specificity of liposomes, immunoliposomes were developed, which are formed by the conjugation of antibodies or antibody fragments on the surface of the liposome, before or after preparation of the formulation, covalently or not, thus improving active targeting by the liposome uptake of specific receptors on the membrane of cancer cells (Eloy et al., 2017c; Petrilli et al., 2020).

Monoclonal antibodies (mAb) can be conjugated by the post-insertion method where micelles are prepared containing the antibody and the ligand and only then are incorporated into the liposome. Another way to couple the mAb is using maleimide chemistry, by forming a thioether bond by reactions with cysteine thiols with maleimide groups. This type of anchoring produces stable ligands and a high conjugation efficiency (Paszko & Senge, 2012). Trastuzumab (Tmab), a monoclonal antibody that targets the human epidermal growth factor receptor 2 (HER2),

expressed in between 20 and 30% of breast and ovarian cancers, can be conjugated on the surface of the liposome (Amin et al., 2018; Barrajón-Catalán et al., 2010; Zhang et al., 2019). Combination therapy with chemotherapy and phototherapy has achieved good results when associated with immunoliposomes. Nguyen and collaborators combined phototherapy and immunoliposomes in breast cancer. In vitro studies addressed phototherapy with polypyrrole in the release of rapamycin with targeting via the monoclonal antibody trastuzumab. The formulation presented therapeutic efficacy and strongly affected the proliferation of cancer cells. Cytotoxicity was analyzed by the MTT method (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide). Thus, the cells were treated with the immunoliposome and with the free antibody being irradiated by NIR laser followed by cell incubation. The results showed higher fluorescence intensity in cells treated with the immunoliposome in the BT-474 lineage, but the immunoliposomes showed lower uptake in the MCF-7 and MDA-MB-231 lines, due to the lower expression levels of the HER2 receptor. It was thus demonstrated the ability of chemotherapy combined with the immunoliposome to improve the treatment of breast cancer (Nguyen et al., 2017).

To enhance idarubicin delivery, Amin et al. (2018) developed a Tmab-conjugated immunoliposome coupled directly to the lipid membrane using a lipid anchor via hydrophobic bonds. In the in vitro study, the breast cancer cell line (HER2) with overexpression (SK-BR-3) was used. The antitumor effects of the immunoliposome were significant and superior to all other treatments, improving its effectiveness by decreasing the viability of the SK-BR-3 cells proven by the MTT assay. The study concluded that the increase in cellular uptake was favored by the binding of the immunoliposome with cells that overexpress HER2, which could cause membrane damage from the generation of radicals that caused DNA damage (Amin et al., 2018).

Rodallec et al. (2018) produced an immunoliposome loaded with docetaxel and functionalized with Tmab. In an attempt to conjugate the antibody, two methodologies were tested. In the first methodology, the addition of NaOH was used after the extrusion step of the hydration of the lipid film, increasing the pH by crosslinking the carboxyl groups in acidic functions on the surface of nanocarriers and amines of the ligands. In the second methodology, the Tmab antibody was thiolated, in this way, the terminal group (-SH) can be functionalized to the sulfhydryl groups, and only then added to the PEGylated liposome (Paszko & Senge, 2012; Rodallec et al., 2018). The in vitro study was developed using three human breast cancer cell lines that vary in HER2 expression, with MDA-MB-231, a model with low expression considered canonical triple negative, positive MDA-MB-453, and overexpressing SKBR3. The immunoliposome showed better performance in terms of size, encapsulation rates, stability, and in vitro activity. The study showed better conjugation by the antibody thiolation method; the formulations obtained by this technique showed greater antiproliferative efficiency and drug release and that the greater the expression of HER2, the greater the cellular uptake.

Some drugs have major disadvantages, for example, side effects, as well as unfavorable pharmacokinetics, such as rapamycin. In a study developed by Eloy (2017a), it was shown that the improvement in cytotoxicity, pharmacokinetics, as well as

improvement in the encapsulation of rapamycin encapsulated in loaded immunoliposome conjugated with Tmab used in combination with the lipophilic inhibitor of the mammalian target of rapamycin (mTOR) showed superior therapeutic efficacy to the antibody-free loaded liposome. Tmab was thiolated with the aid of Traut's reagent and incorporated into the liposome prepared for conjugation. Cytotoxicity was evaluated in cells expressing HER2 (MDA-MB-231, SK-BR-3). The authors concluded that rapamycin and trastuzumab exhibited synergistic effect in vitro and via immunoliposomes, the cytotoxicity reduced by the slow release provided by the system (Eloy et al., 2017a). In another study, rapamycin was combined with paclitaxel in an immunoliposome containing trastuzumab for HER2(+) breast cancer cells. In the in vivo study developed using nude mice, the immunoliposome presented better results, compared to the liposome, controlling tumor growth and volume (Eloy et al., 2017b).

Bevacizumab is a humanized anti-VEGF monoclonal antibody fragment designed to inhibit and prevent mainly ocular angiogenesis. Karumanchi et al. (2018) prepared two types of liposomes, conventional and PEGylated. The liposome developed presented a particle size between 100 and 200 nm, and the use of cholesterol (25–30%) with phospholipids (70–75%) provided a prolonged release of the encapsulated antibody. The ARPE-19 cell line was treated with free and entrapped in liposomes, and the treated cells were submitted to the MTT assay. Free antibody is susceptible to degradation and has a short intravitreal half-life and is susceptible to aggregation and denaturation. When the antibody is encapsulated, its stability is improved, causing cytotoxic effects in vitro and in vivo when administered (Karumanchi et al., 2018).

The EGFR that is overexpressed in cancer cells has its activity blocked and inactivated by the monoclonal antibody cetuximab. The cytotoxicity of the drug may be improved when encapsulated in an immunoliposome functionalized with cetuximab. Thus, the drug 5-fluorouracil (5-FU) used in the treatment of skin cancer was encapsulated in an immunoliposome containing 1,2-distearoyl-sn-glycero-3--phosphocholine (DSPS) and CHOL. The liposome was produced by the lipid hydration method; in the study, the antibody was conjugated using the DSPE-mPEG2000-Mal anchor. The IC_{50} result showed a fivefold lower reduction compared to the free drug; the liposome-conjugated antibody showed a better cell viability result. The results demonstrated synergism with a combination index of less than 1, which proves the potential to improve the cytotoxicity of 5-FU and cetuximab (Petrilli et al., 2016).

Associated with the lipid nanoparticle, the iontophoresis technique can be used; this technique increases the retention of the drug in the skin layers in addition to the increase in cytotoxicity due to the greater permeabilization of the drug in the cell membrane by the presence of iontophoresis. The iontophoresis technique assists in the penetration of nanoparticles containing drugs, due to the movement of charges and solvent flow caused in the ionized skin, making it permeable to cations. In this sense, the formulation must contain competitive ions and adequate pH to guarantee electromigration. In this sense, 5-FU-loaded immunoliposomes were combined

with iontophoresis, resulting in better 5-FU skin penetration and better skin cancer tumor control in xenograft model (Petrilli & Lopez, 2018).

Another antibody that has been used to improve the pharmacokinetics and immunogenicity of drugs is mAb2C5. It was employed as a targeting ligand in liposome in cancer cell model An immunoliposome loaded with paclitaxel, salinomycin, and mAb conjugate has been developed to prevent cancer growth and metastases. The antibody was bound to the surface of the liposome by binding to the surface lipid ligands. The produced nanoparticles were stable with a size range between 170 and 220 nm, a polydispersity <0.2, and a zeta potential in the range of −13 mV to −20 mV. In the in vitro study, holographic imaging was followed to evaluate cell death and cell division and to evaluate the inhibition of proliferation caused by the formulation. In the in vitro study, the SK-BR-3 and MDA-MB-231 cell lines were used. The isotype on cell surface-bound nucleosomes in vitro was quantified using flow cytometry analysis; the observed results showed that the combination therapy intensely affected cancer cell proliferation, significantly increasing cell uptake compared to rhodamine-labeled single liposomes. The ex vivo study used nude female mice, where mouse blood was collected and evaluated in order to assess the toxicity of the immunoliposome produced. The results obtained through the holographic images of immunoliposome showed an increase in cell death and cell proliferation and division. In the ex vivo study, it was possible to analyze the reduction correlated with the low toxicity of the immunoliposome (Narayanaswamy & Torchilin, 2021).

4.1.2 Solid Lipid Nanoparticles and Nanostructured Lipid Carriers

Solid lipid nanoparticles (SLNs) are nanospheres formed by a solid lipid core and are a system loaded in a different way from traditional carriers such as emulsions, liposomes, and microemulsions. SLNs have advantages compared to liposomes, particularly the better colloidal stability and ability to prevent drug immediate release (De Mendoza et al., 2011). SLNs have been loaded with mAbs. To this end, Battaglia et al. (2015) developed SLNs with entrapped bevacizumab (BVZ) for glioblastoma. Basically, the antibody was solubilized in the aqueous phase to facilitate loading, the medium was acidified to form lipophilic groups, and the antibody was then covalently conjugated. The results showed 80% cell inhibition for those treated with immunoliposome and 30% inhibition for cells treated with free BVZ. In the in vitro study, it was possible to observe the existence of the antiangiogenic activity of the immunoliposome. Antibody activity increased up to 100–200-fold when loaded on NL (Battaglia et al., 2015).

Nanostructured lipid carriers (NLC) are classified as the second generation of SLNs. They were developed to address the low drug-carrying capacity of the SLN. The difference between the two systems is their lipid composition: in NLC, the solid lipid is homogenized with a liquid lipid to obtain a solid structure and to avoid the crystallization process after the solidification of the particles of the system (Zimmermann et al., 2000).

Guo et al. (2019) developed a NLC loaded with paclitaxel (PTX) functionalized with cetuximab (CET) (CET-PTX/DMN-NLCs) for EGFR targeting. The antibody was thiolated and bound to the surface of the NLC by chemical reaction (EGFR). The NLC showed sizes of about 130 nm, and the zeta potential changed from 20 mV to 30 mV. In this study, in vitro and in vivo assays were performed. The study showed a cellular uptake of 65.8% for CET-PTX/DMN-NLCs in A549 cells. In the in vivo study, tumor xenografts from mice were used, and the study showed that immunoliposomes inhibited tumor growth. The tissue distribution results showed that the nanocarrier containing the antibody led to a greater accumulation of drug in the tumor and the concentrations of free drug present in the kidney and the heart were lower (Guo et al., 2019).

Nanostructured lipid for intravenous administration showed reduced side effects when functionalized with bevacizumab (BVZ) and loaded with docetaxel (DTX) associated with the treatment of malignant brain cells. The antibody was covalently conjugated on the surface of the nanoparticle, for which the antibody was thiolated with Traut's reagent and followed by filtration and lyophilization. In the in vitro study, the assay was performed in cell lines that overexpress VEGF (U87MG and A172), and the immunoliposome caused cell death by apoptosis in cells that overexpress VEGF, which was not observed in healthy cells. The in vivo study was carried out in orthotopic rats using free DTX and DTX immunoliposomes. It was observed for the group containing the immunoliposome reduced tumor growth up to 70% in the treatment for 15 days, whereas in the group with free DTX, no tumor reduction was observed (Di Filippo et al., 2022).

4.2 Polymeric Nanoparticles

Polymeric nanoparticles (PNPs) are formed by a matrix consisting of polymers, which are fundamental materials for the composition of these versatile drug delivery systems, confering some properties, such as size and selective targeting (Juan et al., 2020; Soppimath et al., 2001). They can be designed and prepared with various polymeric components, with different molecular weights, structures, and functions to meet the requirements of a particular drug (Shukla et al., 2019). Antibodies and fragments can be functionalized into polymeric nanoparticles for targeted delivery and are under constant research (Bhattacharya, 2020).

Synthetic polymers are commonly used in the preparation of these nanocarriers, including polyesters, poly(glycolic acid) (PGA), poly(lactide-co-glycolide) (PLGA), poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL), polyacrylic acid, and others, which are biocompatible materials approved by the Food and Drug Administration (Bapat et al., 2018; Juan et al., 2020; Masood, 2015). Preparation methodologies consist of nanoprecipitation, emulsification and solvent evaporation, solvent displacement, salting out, nanoencapsulation, and spray drying (Li et al., 2020; Sousa et al., 2018).

Poly(lactic acid) is a hydrophobic aliphatic polymer widely explored due to its easy degradation and versatility in the controlled delivery of bioactive molecules. Known as polylactides, this class of polymers consists of four different compounds: poly(L-lactic acid) or PLLA, poly(D-lactic acid) or PDLA, poly(D,L-lactic acid) or PDLLA, or meso-PLA. These materials are widely used in the preparation and development of nanoparticles as delivery systems for various therapeutic substances, including vaccines. The association of PLA with its various isomers can be used as an innovative polymer in the delivery of anticancer drugs. In addition to being a promising biomaterial and offering flexible physical properties, nanoparticles demonstrate in vivo biodegradability and biocompatibility for the effective delivery of molecules to the therapeutic target (Kumar & Kumar, 2019; Peres et al., 2017).

Polymeric nanoparticles can undergo functionalization as a strategy in oncology for targeted and controlled therapy, reducing toxicity, through conjugation with antibodies. The process consists of covalent conjugation through the linkage of the amine or sulfhydryl groups of antibodies, by the chemistry of carbodiimide and maleimide, respectively, in the presence of auxiliary components in the crosslinking N-hydroxysuccinimide or NHS, N-hydroxysulfosuccinimide or sulfo-NHS, NHS/maleimide, PEGylated analogues (NHS-PEG-maleimide), succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), and sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) under adjusted pH conditions. In this way, antibody groups can be linked to polymers arranged on the surface of the nanoparticle (Juan et al., 2020; Son et al., 2009). In this sense, Niza and colleagues developed polymeric PLA nanoparticles conjugated with trastuzumab for the enhanced delivery of dasatinib to HER2-positive tumors. The functionalized nanocarrier formulation induced greater cytotoxicity in HER2 overexpressing cell lines, inducing apoptosis when compared to the pure drug, showing to be a more efficient alternative than unconjugated nanocarriers (Niza et al., 2019).

Furthermore, Sun and Feng also developed polymeric nanoparticles consisting of PLA and D-α-tocopheryl polyethylene glycol succinate (TPGS) and copolymer of carboxyl-terminated TPGS (TPGS-COOH) conjugated with trastuzumab antibody, and the nanoparticle was loaded with docetaxel for specific delivery to breast cancer cells. It was shown the increased targeting of functionalized carriers to breast cancer cells after 24, 48, and 72 h of treatment compared to non-functionalized nanoparticles. And the targeting effects of the conjugation showed a high mortality rate in breast cancer cells that show high overexpression of HER2 compared to cells with moderate overexpression of this receptor (Sun & Feng, 2009).

The ability of polymer micelles made up of PLA and polyethylene glycol (PEG) to deliver doxorubicin (DOX) and functionalized with anti-VEGF antibody was evaluated by Chang et al. The size of the PEG-PLA nanoparticles was about 100 nm. However, when loaded with doxorubicin, this value changed to 200 nm, and even when functionalized with the antibody, the hydrodynamic diameter of the conjugates (VEGF-PEG-PLA-DOX) changed to 220 nm. The targeting efficiency of the nanocarriers (VEGF-PEG-PLA-DOX, PEG-PLA-DOX, DOX) was evaluated

in vitro by cell viability in the lung adenocarcinoma lineage (A549). The micelles conjugated with VEGF showed a higher inhibitory effect indicating a more potent antitumor effect. In vivo study evaluated the antitumor efficacy of polymeric micelles (VEGF-PEG-PLA-DOX) in nude mice, where the tumor sizes of the functionalized polymer micelle treatment group were significantly smaller than those of PEG-PLA-DOX micelles or the free DOX group, corroborating in vitro analysis (Chang et al., 2020).

Poly(ε-caprolactone) or PCL nanoparticles are among the polymers that have a potential effect on the controlled release of drugs in anticancer therapy (Shukla et al., 2019). PCL is one of the synthetic polymers used as a strategy for biomedical application; unlike other polyesters, it has several unique properties of biodegradability and miscibility with other polymers, it is non-toxic and has high crystallinity, in addition, the slow degradation of this material stands out as an attractive property to improve the prolongation of drug delivery in specific therapeutic situations (Gan et al., 2018; Peng et al., 2019).

In this sense, Peng and collaborators designed polymeric micelles made of PCL-PEG and conjugated with Herceptin (trastuzumab) loaded with paclitaxel for controlled drug release in HER2+ breast cancer cells. Thus, a new non-biodegradable conjugation method was proposed, based on the thiol-ether bond (-C-S-C-) to covalently link the antibody to the functional groups available on the surface of the nanocarrier. The hydrodynamic size of the blank micelles (PCL-PEG) was 111 nm; when encapsulated with paclitaxel (PTX-PCL-PEG), this value increased significantly to 135 nm; however, the conjugation with the mAb (PTX-PCL-PEG-Herceptin) changed the value slightly to 144 nm. Circular dichroism provided results for both free and conjugated Herceptin, which indicated that the antibody was successfully conjugated to the nanoparticles via the thiol-ether binding strategy. Biological assays in breast cancer cell line (SKBR-3) showed that the nanocarriers (PTX-PCL-PEG and PTX-PCL-PEG-Herceptin) entered cells through endocytosis. Complementary confocal laser scanning microscopy study showed accumulated fluorescent signals from the nanoparticle (PTX-PCL-PEG-Herceptin) surrounding SKBR-3 (HER2+) cells compared to MDA-MB-231 (triple-negative) cells, indicating specific delivery and targeted to HER2+ tumor cells. The in vivo assays evaluated in subcutaneous SKBR-3 xenograft model mice tested the formulations (TAXOL®, PTX-PCL-PEG, and PTX-PCL-PEG-Herceptin) and showed that PTX-PCL-PEG-Herceptin had improved targeting ability to tumor tissues reducing non-specific toxicity; furthermore, the PTX-PCL-PEG-Herceptin nanocarrier was two- to threefold more effective in suppressing tumor growth in relation to the free drug and the unconjugated nanocarrier (Peng et al., 2019).

With a polymeric constitution similar to the previous study, Xu and collaborators developed polymeric nanoparticle based on PEG (polyethylene glycol) and PCL (poly-ε-caprolactone) conjugated with monoclonal antibody PD-L1 and synergized with docetaxel (DTX). The mAb antibody was conjugated through an amidation reaction resulting from the binding of primary amine groups on the surface of the nanoparticle with the carboxylic group of the mAb. In vitro assays performed in MGC803, MKN45, and HGC27 gastric cells showed that mAb-DTX-PEG-PCL

nanoparticles exhibited greater cytotoxicity in cancer cells that express the PD-L1 protein, with a significant increase in cell apoptosis and inhibition of cell proliferation in the G2/M phase of the cell cycle (Xu et al., 2019).

Gan and collaborators have developed polymeric nanoparticles made up of the copolymers TPGS-b-poly(caprolactone) (TPGS-b-PCL) and Pluronic (P123) conjugated with anti-glypican-3 antibody (GPC3), for the delivery of sorafenib (SFB) to hepatocellular carcinoma (HCC). The modification of the anti-GPC3 antibody with sulfhydryl was carried out through a thiolation reaction (Ab-SH) resulting from the mixture of the antibody with Traut's reagent, and then it was mixed with the NP-SFB nanoparticle for bioconjugation. In this study, in vitro assays showed that the ability of NP-SFB-GPC3 to kill HepG2 cancer cells is significantly superior to that of free SFB and NP-SFB, as well as the in vivo assay evaluated in nude mice with HepG2 xenograft, confirmed the inhibition of tumor growth when functionalized polymeric nanoparticles (PNPs) are administered in HCC (Gan et al., 2018).

PLGA is a hydrophobic copolymer of poly(lactic acid) or PLA and poly(glycolic acid) or PGA; it can be produced by melt copolymerization processes of cyclic lactic acid and cyclic glycolic acid in the presence of catalysts under specific temperature and pressure (Erbetta et al., 2011; Kumar & Kumar, 2019; Shukla et al., 2019). PLGA nanoparticles provide potential advantages to anticancer drug delivery systems, being able to encapsulate hydrophilic or hydrophobic substances, reduce the administered dose, are non-toxic and biodegradable, and facilitate drug delivery to the tumor by the EPR effect (Choudhury et al., 2019; Zhong et al., 2020). In addition to its advantages, PLGA is a biomaterial commonly used in targeted delivery with monoclonal antibodies (Bregoli et al., 2016; Son et al., 2009; Varshochian et al., 2013).

In this context, Acharya and collaborators prepared and characterized PLGA nanoparticles (NPs) loaded with rampamycin (RAPA) and conjugated with an anti-EGFR antibody for delivery to breast cancer. The conjugation was based on carbodiimide chemistry, where the nanoparticle was activated with 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide(EDC)andN-hydroxysuccinimide (NHS). Then the nanoparticle was incubated with the anti-EGRF antibody and finally kept under overnight incubation at 4 °C, followed by ultracentrifugation to remove the unconjugated antibody. The developed nanoconjugates (EGFR-RAPA-NPs) showed a size of 274 nm with a monodisperse distribution. Biological cell uptake assays revealed that the immunonanoparticles had an uptake rate almost 13 times higher than the unconjugated nanoparticles. In vitro cytotoxicity studies in MCF-7, a breast cancer cell line, indicated that nanoparticles (EGFR-RAPA-NPs) had greater antiproliferative activity even at very low doses than unconjugated nanoparticles (Rapa-NPs), resulting in an enhanced cytotoxic effect of the loaded drug (Acharya et al., 2009).

The association of polymers in a formulation can be performed as an alternative to reverse or circumvent possible undesirable effects, such as toxicity and others. In this sense, Lee and collaborators have developed polymeric nanoparticles made of PLGA and PEG; the latter is also a polymer that can be used both to assist in the solubility of hydrophobic drugs and to perform surface modifications of the

nanoparticle, conferring advantages of prolonged circulation, increased active solubility, decreased immunogenicity, and specific targeting by the effect of increased permeability (EPR) (Lee & Chang, 2017; Shukla et al., 2019). Copolymer nanoparticles (PLGA and PEG) encapsulated with doxorubicin and indocyanine green were conjugated with anti-HER2 antibody through reaction with carbodiimide with EDC/NHS. In vitro assays showed that the viability of MDA-MB-453 cells overexpressing HER2, when treated with the conjugated polymeric nanoparticles and laser irradiation, suffered significant cell death. Moreover, the presence of the PEG polymer allowed the nanoparticles to have an increased circulation time due to their low immunogenicity (Lee & Chang, 2017).

Polymeric nanoparticles can acquire structural forms that facilitate and help the targeting and controlled release of treatment through physical or chemical stimuli. In this scenario, Zhong and collaborators produced polymeric nanoparticles in nanobubble format made up of poly(lactic-co-glycolic) PLGA acid loaded with paclitaxel and conjugated with Herceptin (PTX-NBs-HER) for targeting breast cancer in association with ultrasound (US). The antibody was conjugated using the carbodiimide method, where the nanoparticles were activated using activator coupling (EDC)/(NHS), incubated with Herceptin antibody (20 mg/ml) during 2 h at room temperature, and centrifuged and washed with 2-(N-morpholino)ethanesulfonic acid (MES) to remove unbound antibodies. In vitro studies in MCF-7 cells, overexpressing HER2, showed that NPs (PTX-NBs-HER) targeted cells bound more when compared to NPs (PTX-NBs). In addition, both (PTX-NBs-HER) and (PTX-NBs-HER+US) had the best cell death results, corroborating in vivo assays in models of tumor xenograft of MCF-7 cells in nude BALB/c mice, which showed that the formulation (PTX-NBs-HER+US) had increased drug content in cancer cells, revealing the synergistic effect of paclitaxel, Herceptin antibody, and nanobubbles generated by the ultrasound irradiation, facilitating and directing the accumulation of drug to the tumor (Zhong et al., 2020). Other studies of antibody conjugates using PLGA polymer are described in Table 5.3.

4.3 Inorganic Nanoparticles

The use of nanoparticles for drug delivery is described in the literature as a promising tool that can be applied to monitor, treat, and diagnose cancer (Taleghani et al., 2021). Nanoparticles can be divided into organic and inorganic nanoparticles (NPs), which are made up of metals and metallic oxides. The stability of inorganic nanoparticles in high temperature is better than organic particles, showing an advantage in cancer treatment and provides materials with gold and iron oxide under test in cancer patients (Pugazhendhi et al., 2018). For pancreatic cancer, Feraheme® iron oxide NPs are being studied in clinical trial (Liu et al., 2022).

Although inorganic NPs have properties such as free radical generation and heating that generates controlled tissue destruction for the treatment of neoplasms, active targeting, through the attachment of antibodies (Abs) in these NPs, can be

Table 5.3 Biological studies using PLGA nanoparticles conjugated with antibody for cancer treatment

Cancer type	Formulation	In vitro/in vivo *assays*	Main findings	Ref.
Human glioblastoma Adenocarcinoma Human melanoma	Polymeric nanoparticles of poly(lactic-co-glycolic acid), loaded with temozolomide (TMZ) and conjugated with cetuximab (Cmab-TMZ-PLGA-NPs)	U-87MG, SW480, and SK-Mel 28 cells were used to evaluate cell viability and cell uptake by confocal laser scanning microscopy and live/dead assay for cell effect treatments (calcein-AM/ethidium homodimer-1)	The polymeric nanoparticles presented characterization of particle size of 162.26 ± 5.09 nm, polydispersivity index of 0.16 ± 0.07, and zeta negative potential, showing stability. The effects of polymeric nanoconjugates on treatment significantly reduced the viability of the three strains that overexpress EGFR. The live/dead assay showed that treatment with Cmab-TMZ-PLGA-NP showed a reduction in cell viability, with greater absorption in U-87MG cells than in SW480 and SK-Mel 28 cells, showing to be a versatile drug delivery strategy in the treatment of cancer where there is overexpression of EGFR	Duwa et al. (2020)
Human glioblastoma cells (with and without EGFR vIII overexpression)	PLGA-PEG (D,L-lactic-co-glycolic acid)-(polyethylene glycol)- polymeric nanoparticles loaded with doxorubicin (DOX) conjugated anti-EGFR vIII antibody (mAb-DXR-PLGA-NPs)	The study used U87MG and U87MG vIII cell lines, employing. Fluorescence microscopy and flow cytometry, to evaluate in vitro cell uptake and apoptosis	The nanoparticles developed showed a size of 196.5 ± 14.6 nm, polydispersivity index of 0.64 ± 0.1, and zeta potential of −5.05 ± 2.05. Functionalized nanoparticles (mAb-DXR-PLGA-NPs) showed a more pronounced decrease in cell viability than non-functionalized nanoparticles, with more pronounced effects on U87MG vIII cells than U87MG cells, showing that conjugation with antibody can improve treatment efficacy	Eivazi et al. (2020)

(continued)

Table 5.3 (continued)

Cancer type	Formulation	In vitro/in vivo *assays*	Main findings	Ref.
Colon cancer, human lung carcinoma, lung cancer, and pancreatic tumor cells	Camptothecin encapsulated in polymeric nanoparticles of poly(lactic-co-glycolic acid) (PLGA) conjugated with cetuximab (CTX) (CTX-CPT-NPs)	Cell viability assessment and cell death were performed in vitro on HCT116, A549, HKH-2, HCC827, and PANC-1 cells. In vivo studies were performed in female mice with severe combined immunodeficiency, where PANC-1 cells were implanted subcutaneously in the flanks of the animals	The nanoparticles (CTX-CPT-NPs) exerted a more pronounced apoptosis effect with cell death than the formulation not conjugated with cetuximab. In vivo studies showed a 30% difference in tumor volume reduction compared to the non-antibody conjugated control group, where the body weight of the animals tested remained constant due to the effect of drug nanoencapsulation and specific targeting to cells	McDaid et al. (2019)
Human breast cancer	Polymeric nanoparticles (PLGA-DSPE-PEG-maleimide) encapsulated with paclitaxel and functionalized with herceptin	Cell lines BT474 and MCF7 were applied to assays of cell uptake by flow cytometry, cell uptake by confocal microscopy, and cell death through in vitro cytotoxicity	The nanoparticles prepared by pre-conjugation strategy (PLGA-DSPE-PEG2000-PTX-her) showed pronounced cytotoxicity in relation to the post-conjugation strategy in BT474 cells with HER2 overexpression and higher percentage of cellular internalization, revealed by flow cytometry. The mechanisms of clathrin-mediated and caveolae-dependent endocytosis were evidenced as the main mechanism of internalization of nanoconjugates in cells	Yu et al. (2016)

useful in several applications in the field of in vivo diagnosis. The use in human therapy provides targeted drug delivery, gene therapy, cell tagging, and magnetic or optical hyperthermia treatments, among others. Targeting is achieved by attaching ligands to the surface of inorganic NPs with high affinity for overexpressed receptors on tumor cells. The binding of this system (Abs-NPs) in the tumor results in the specific and efficient internalization of drugs via receptor-mediated endocytosis (Montenegro et al., 2013; Pugazhendhi et al., 2018; Taleghani et al., 2021).

4.3.1 Iron

Magnetic nanoparticles are widely used, for example, in targeted drug delivery, for magnetic hyperthermia therapy, cell labeling, and magnetic resonance imaging (MRI) as substance for contrast. Interest in magnetic nanoparticles for biomedical applications has increased over time due, in part, to their ability to react to magnetic fields (AMF). It is known that magnetic nanoparticles are able to convert the energy of an AMF into both thermal (in the case of high-frequency fields) and mechanical (low-frequency fields). Particularly, superparamagnetic nanoparticles (SPIONs) formed by iron oxide (Fe_3O_4) due to their excellent magnetic properties and chemical stability can be used for studies applying components for targeting. In addition, to their own ability to accumulate in tissues under an external magnetic field, the detection of tumor cells mediated by such antibodies coupled to magnetic nanoparticles in combination with hyperthermia represents a significant advance in cancer treatment and a substantial improvement in the survival of cancer patients (Antal et al., 2021; Ivanova et al., 2021; Khaniabadi et al., 2020; Montenegro et al., 2013).

In this sense, Antal and collaborators used physical adsorption to immobilize antibodies on the surface of the developed nanoparticle (Montenegro et al., 2013). Thus, the methodology used to bind Ab to the surface of the nanoparticles by physical adsorption consisted of using the lyophilized monoclonal antibody VII/20 specific for carbonic anhydrase IX (CA IX). In the later step, a colloidal solution of Gly5-MNPs had an Ab solution added. After the formation of the pellet, Ab0,5-Gly5-MNPs were used for in vitro experiments. After conjugation of Ab to Gly5-MNPs, the diameter was shifted from 48.0 nm to 78.6 nm. Assessment of cytotoxic potential was verified in melanoma cell lines and survived and even proliferated in the presence of unconjugated Gly5-MNPs or antibody-conjugated Ab0,5-Gly5-MNPs; in addition, the cells did not show reduced metabolism or cytostatic effect. The immunofluorescence study showed that Ab0,5-Gly5-MNPs specifically bind to the CA IX antigen found on the cell surface of B16-FL-CA IX cells. Thus, the antibody-conjugated nanoparticles showed efficient internalization capacity. This important phenomenon of cellular internalization may have implications for selective partitioning of MNPs in tumors, and internalization may also be useful for therapeutic applications where efficacy is modulated by intracellular localization (e.g., magnetic hyperthermia) (Antal et al., 2021).

Studies combining targeted magnetic nanoparticles with antibodies are also used with theranostic purpose: the combination of therapeutic and diagnostic agent for

treatment and monitoring as well as diagnosis. Thus, theranostic agents combine photosensitizers and contrast agents and use optical or magnetic resonance imaging techniques that are non-invasive, in a single unit for photothermal therapy (PTT) and magnetic resonance imaging (MRI). These nanoparticles have greater permeability and retention effect and the ability to exit the body via urinary excretion without long-term toxicity (Khaniabadi et al., 2020; Moradi et al., 2021).

Retention capacity was observed by Moradi et al. (2021), who conjugated anti-CD3 monoclonal antibody to iron oxide (Fe_3O_4) after coating by carboxymethyl dextran (CMD) using cyanogen bromide (CNBr) generating the nanomaterial MPN-CD3. This conjugation is accessible and fast up to 4 h as it is stable under physiological conditions, providing great magnetization, and covalent bonds occur on the metal surface. The physicochemical characteristics of the material showed an MNP size distribution prior to 18 nm conjugation, and after conjugation, the size of the MNPs obtained was 40–100 nm, indicating the increase in the size of the MNPs after the conjugation, in other words after antibody binding to MNPs. In vivo studies were performed staining the tumor portions, and MRI scans were conducted to assess the specificity of the bioconjugated material (MNP-CD3). As a result, the signal-to-noise and contrast-to-noise ratio showed that CD3+ cells were colocalized when stained in the joints of mice with the antibody-containing material compared to the control that was not bioconjugated (Moradi et al., 2021). Similar behavior was observed by Khaniabadi et al. (2020) that, through the excellent performance of T2 magnetic resonance imaging at low concentration of the developed nanosystem (SPION-porphyrin conjugated with traztuzumab), support the applicability of ION-PP-TZ as a new contrast agent (Khaniabadi et al., 2020).

4.3.2 Gold

Gold has been applied in various medical treatments over the years and has no harmful effects. Inorganic gold nanoparticles (AuNPs) are currently being extensively studied due to their unique versatile properties and can be applied in diagnostics through the provision of bioimages enabling early detection and cancer therapy. The high surface-to-volume ratio of AuNPs makes possible their application of drug delivery, as well as functionalization to enhance target specificity. AuNPs have different shapes – nanospheres, nanorods, nanoshells, nanotriangle nanostar, nanohexagon, nanobranched, and hollow spheres, depending on their size and physical characteristics. AuNPs have already been tested with results as contrast agents, biosensors, therapeutics, as well as theranostic potential (Aldahhan et al., 2021; Andrade et al., 2020; Medici et al., 2021).

In addition to drug incorporation, AuNPs as nanosystems for drug delivery may combine functionalization with molecules that allow targeting (such as antibodies) to achieve a level of target specificity. The functionalization process can generally be carried out through the reduction of gold salts in the presence of thiols or thiol-derived molecules, so that strong, non-labile covalent bonds are formed through adsorption of the thiol surface, which are stable even when exposed to pH changes.

This process also contributes to the assessment of the toxicity of AuNPs because, if not properly functionalized, they can accumulate in the spleen, liver, and lymph nodes, preventing filtration by the reticuloendothelial system (Aldahhan et al., 2021; Medici et al., 2021). In this sense, Liszbinski and collaborators developed a strategy of combining the drug 5-fluorouracil and anti-EGFR (epidermal growth factor receptors) antibody loaded on the nanoparticle of AuNPs to increase the specific delivery of 5-fluorouracil against colorectal cells that overexpress EGFR. The nanoformulation demonstrated the induction of apoptosis in addition to necrosis in a dose-dependent manner; inhibition of proliferation was also observed in a non-dependent manner (Liszbinski et al., 2020). Comparatively, Shabbir et al. also developed anti-EGFR nanoparticles with lutetium (177Lu-AuNPs) for colorectal and breast cells, showing that the strains that overexpressed the receptor were sensitive to the nanosystem as well as the biodistribution in mice with xenografts demonstrated the greater uptake of [177Lu]-AuNPs with cetuximab after 4 h of injection via the liver and spleen (Shabbir et al., 2021). Other biological studies applying gold nanoparticle conjugated with antibodies can be seen in Table 5.4.

High antibody coverage in AuNPs is important for biosensor function, but it must be stable with accessibility to conjugated antibodies ensuring the ability to recognize the antigen. Physical adsorption is a method that requires little knowledge of surface chemistry, being a simple and direct method of attraction between AuNPs and the antibody. Byzova et al. (2017) and Ruiz et al. (2019) verified the parameters that govern the preparation of this bioconjugate, such as the isoelectric point of the antibody, the amount of antibody added, and the pH, making it possible to adjust the orientation of the antibody molecules on the surface of citrate-coated AuNPs. In this way, the work results in the statement that the antibody conjugation process on the surface of the gold nanoparticle involves several factors that must be correctly analyzed to ensure greater and more stable conjugation. Computer simulation assays can provide support in understanding these interactions between the antibody and gold (Byzova et al., 2017; Ruiz et al., 2019).

The ability of AuNPs as diagnostic contrast agents was evaluated by Aston and colleagues where a poly(ethylene glycol) (PEG) molecule containing a reactive NHS ester group (succinimidyl valerate) forms covalent bonds with the antibody (VHH-122 OR C225) through the primary amines. And the thiol group contained at the other end of the PEG molecule binds to the surface of the gold nanoparticle. The hydrodynamic diameter had a more pronounced increase with PEGylation, going from 28 nm to 42 nm, and after conjugation with VHH-122 antibody, the diameter increased from 42 nm to 45 nm, with no significant increase. However, after the addition of C225 antibody, the diameter increased from 42 nm to 68 nm. The targeting efficacy of the nanosystems (C225-AuNPs, VHH-AuNPs, and PEG-AuNPs) was evaluated in vitro by EGFR binding assays in squamous cell carcinoma (A431), which overexpress EGFR. C225-AuNPs were found to have a high level of binding, with nanoparticles visibly bright over most of the cell surface under dark-field microscopy. Computed tomography imaging performance was evaluated in C57BL/6 and nude mice; all tested groups had tumor accumulation, but for the cetuximab target group, it was significantly higher (Ashton et al., 2018).

Table 5.4 Biological studies applying gold nanoparticle conjugated with antibodies for cancer therapy

Cancer type	Formulation	In vitro/in vivo *assays*	Main findings	Ref.
Human squamous cell carcinoma	Gold nanoparticle (AuNPs) and cetuximab	Cell internalization of AuNPs and $AuNPCet_{50}$ was conducted using A431 strain as well as immunocytochemistry and fluorescein isothiocyanate (FITC) annexin V/7-AAD assessments	The results found showed that bioconjugations without the need to use ligands such as EDC-NHS to conjugate the antibody to the surfaces of AuNPs were successful and that there was a greater amount of intracellular $AuNPCet_{50}$ than AuNPs without antibody in the assays. And also, in A431 cells, the nanoparticles were internalized through the transport of vesicles, due to a probable endocytic mechanism	Andrade et al. (2020)
Human breast cancer cell	Gold nanoparticle trastuzumab and indium-111 (trastuzumab-AuNP-^{111}In)	The cytotoxicity of trastuzumab-AuNP-^{111}In on SK-BR-3 and MDA-MB-361 human breast cancer cells in vitro and a breast cancer xenograft mouse model into female CD1-athymic mice for evaluate tumor growth inhibition properties and normal tissue toxicity	Trastuzumab-AuNP-^{111}In demonstrated targeting for cell lines SK-BR-3 and MDA-MB-361 being internalized more efficiently than AuNP-^{111}In without antibody and located in a perinuclear region. The absorbed radiation dose deposited in the tumor by trastuzumab-AuNP-^{111}In was 60.5 Gy, and values lower than 0.9 Gy were received for normal organs	Cai et al. (2016)
Murine glioblastoma cell	Citrate-gold nanoparticle (Cit-AuNPs) and anti-mouse VEGF-A monoclonal Ig	The effect of cit-AuNP on growth curve in GL261 cell proliferation was tested. Female C57BL/6 J mice was used for tumor volume, body weight measurements, and also Cit-AuNP in the cerebral parenchyma	The groups selected for study (cit-AuNP or anti-VEGF Ig covered-AuNP) did not demonstrate a reduction in glioblastoma tumor progression in vivo, possibly because the amount that crosses the blood-brain barrier is not sufficient. In the GL261 glioblastoma cell line growth curve assays, it was observed that Cit-AuNP, at different concentrations, did not alter the proliferation state of GL261	Silva et al. (2020)

Human colon carcinoma and cervix carcinoma	Gold nanoparticle, cyanine 5, and mAb198.3 antibody (AuCOOH(Cy5)_ mAb198.3)	Colo 205 and HeLa cells were used to assess AuNP cytotoxicity on cell viability and proliferation, flow cytometry, and confocal microscopy analysis of FAT1 expression. In vivo bioimaging studies were conducted in BALB/c athymic nude mice	The toxicity of Au compounds in the Colo 205 cell showed no cytotoxic effects at the highest concentration of 5 μM. It was observed by confocal microscopy that AuCOOH(Cy5)_ mAb198.3 after 4 h of incubation could bind to the cell membrane, thus captured or surrounded by the cytoplasm and nucleus. The images obtained from tumor mice show intense fluorescence at the tumor site due to the targeting effect of mAb198.3 and EPR effect, almost 90% of AuCOOH_mAb198.3	Fan et al. (2015)
Human gastric cancer cell	Gold nanoparticle and trastuzumab (T-AuNPs)	MKN7, MKN74, NCI-N87, and NHLF cells were applied in assays like lipid hydroperoxide and confocal fluorescent microscopy. For the antitumor effects of T-AuNPs, female BALB/c nude mice and female NOD/SCID mice were used in tumor models	Generated by induction of autophagy, the cytotoxic activity was quite pronounced not only in the trastuzumab-sensitive NCI-N87 gastric cancer lineage but also in the MKN7-resistant lineage. In the in vivo study, the biodistribution of T-AuNPs after systemic administration for NCI-N87 showed accumulation of the nanoparticle in the liver and few in the tumor tissue	Kubota et al. (2018)

4.3.3 Silica

Among the materials that improve the drug delivery profile to cancer cells, reducing toxic effects, are mesoporous silica nanoparticles (MSNPs) which are solids formed by several empty channels that are arranged in a two-dimensional porous structure with physicochemical characteristics such as nanometer size, high surface area and ordered porous arrangement. MSNPs have corrosion resistance, biocompatibility, and mechanical, chemical, and thermal stability. In general, pore volume and surface area are adjustable as well as pore diameter which can vary in the range of 2 to 50 nm. To functionalize the surface of MSNPs in a specific and efficient way, antibodies are used through covalent bonding or electrostatic interaction. In order to avoid immune responses and increase dispersivity and stability, PEG and other hydrophilic polymers can be used forming a bond between antibody and nanoparticle (Taleghani et al., 2021).

In this sense, Zhang and collaborators developed the MSN material functionalized with carboxyl groups for conjugation of bevacizumab (BVZ) on the surface of the nanoparticle for targeting to ovarian cancer. The nanoparticles had a size without antibody of approximately 135 nm and after incorporation of the antibody the size of 166 nm (Zhang et al., 2015). This size range was also verified by Wang et al. (2017) with 135 nm for conjugation of the cetuximab antibody using the antibody thiolation method with MEA reagent (β-mercaptoethylamine) (Wang et al., 2017). Despite the increase in size, it still allows targeting and passive accumulation in tumor tissues by the EPR (enhanced permeation and retention) effect, suggesting its suitability for the delivery of cancer drugs. Thus, the nanoparticles developed were small enough to avoid detection and destruction by the reticuloendothelial system (RES), prolonging the time of systemic circulation.

The antibody was conjugated to the MSN material through the interaction between the amine groups of the Abs and the carboxyl groups of the MSN. About 100 mg of acid-terminated MSN in MES buffer was activated by reacting with EDC/NHS for 2 h. The MSN is then incubated with the antibody followed by centrifugal separation of the free antibody. Biological assays conducted initially by flow cytometry showed a threefold increase in cellular uptake for the antibody-conjugated material (BMSN/Ab) compared to the antibody-free material (BMSN) for the 2 h and 4 h assays. It can be seen that BMSN/Ab exhibited a threefold increase in cell uptake at the end of 2 h and 4 h. A higher uptake of BMSN/Ab in cancer cells was also observed, being primarily attributed to the specific affinity of BMSN/AB for the receptor overexpressed in ovarian cancer cells (OVCAR-5). Complementary colony formation assay was performed to evaluate the cytotoxic potential of BMSN/Ab where the control (no treatment) had no effect on cell proliferation and although BVZ and BMSN generated control in colony formation, the combination of antibody with the inorganic silica nanoparticle inhibited about ten times compared to the control (Zhang et al., 2015).

Another possible modification for antibody conjugation was described by Mozafarinia et al. (2021) modifying mesoporous silica with amine (MSNs-NH2) to conjugate the Herceptin antibody (trastuzumab) which is made up of two carboxylic

functional groups. In this study, EDC/NHS and a Herceptin concentration of 0.01 mg/ml were also used. The in vitro studies conducted showed the preference of the developed nanoparticle (MSN-NH2@Her) for HER2+ cell lines, considering that the studied lines SKBR3 (HER2+) and MDA-MB-231 (HER-) showed a difference in binding with the developed nanoparticle. Since SKBR3 cells had high cellular uptake of MSN-NH2@Her, due to the targeting generated by their HER2 receptors, MDA-MB-231 cells were not able to absorb the nanoparticle due to the lack of receptors, thus not occurring binding to the plasma membrane (Mozafarinia et al., 2021).

4.3.4 Quantum Dots

The quantum dots (QDs) are versatile fluorescent inorganic semiconductors in the form of nanocrystals with optical and electronic properties. The QDs can be adjusted in their photoluminescence emission band; the adjustment takes place from the ultraviolet to the infrared region based on the choice of composition and size of the nanocrystals. The composition may consist of binary (CdS, ZnS) or ternary (AgInS, CuInS) alloys. Due to environmental and biological issues, the synthesis of QDs containing Cd obtained by organometallic route has sought replacements for alternative syntheses such as aqueous. This nanoparticle produced under hydrophilic conditions favors the process of direct chemical conjugation of molecules, generating specificity for cell receptors. In addition to applications for cell imaging, optical bar code due to photostability and strong fluorescence intensity, quantum dots can be used to verify interactions between protein molecules and detect signal transduction in cells through fluorescence resonance energy transfer (FRET), being a promising nanomaterial for the treatment and tracking of cancer cells in vivo (Ruan et al., 2012; Santana et al., 2019).

Santana and collaborators developed quantum dots of ternary alloys: the AgInS2 nanoconjugates (AIS). The synthesis was carried out by aqueous route using chitosan (Chi) as a stabilizing agent and binding agent for anti-vascular endothelial growth factor antibody (abVEGF, Avastin®) and salt precursors (silver, indium, and sulfide). The deposition of ZnS was performed on the nanoparticle, making the semiconductor material (ZnS-AgInS2, ZAIS) coated with a chitosan ligand with a size of approximately 4.4 nm. And the anti-VEGF antibodies were covalently bioconjugated using EDC/NHS producing the immunoconjugates (ZAIS/Chi-abVEGF). The specific targeting of cancer cells was evaluated using cytotoxicity assays for glioma tumor cells (U87). Cell viability compared to normal cells (HEK 293 T) had a significant reduction of 40% when QDs had conjugation with antibody with a concentration of 3 mg/mL. And for higher abVEGF concentrations (30 mg/mL), the reduction of cancer cells was 65%. Thus, the development of a hybrid nanomaterial biofunctionalized with affinity antibodies to target and kill cancer cells was effective (Santana et al., 2019).

Ruan et al. using binary alloy quantum dots (CdTe) conjugated with ribonuclease A (RQDs) verified their application in vivo. The material developed is composed of

a large number of quantum dots; the average size of the RQDs is about 40 nm. The monoclonal antibody HER2 was modified with a sufhydryl group and later conjugated to RQDs through the thiol antibody group. Thus, two steps can be described: the primary amines present in RNase A being coupled to N-succinimidyl iodoacetate (SIA) and the binding of the iodoacetyl group to the thiolated antibody HER2. The evaluation of the HER2-RQDs nanosystem targeting was performed in gastric cancer cells (MGC803). The images obtained by confocal microscopy show strong red fluorescence around the nucleus, indicating the directed entry of HER2-RQDs by the HER2 overexpression in cancer cells. And image of morphology showed apoptotic characteristics like cavity in the endoplasmic reticulum and cell surface containing bubble-shaped protrusions. The distribution of HER2-RQDs in in vivo and in situ nude mouse model was analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The analysis allowed to identify that the amount of the nanoparticle used in the study in the gastric tumor tissues was 10.2 ppm after 24 h of treatment, but the other tissues evaluated (heart, kidney, spleen, lung, liver, and blood) showed values below 5 ppm, indicating the favoring of the distribution of HER2-RQDs in gastric cancer tissues in vivo. Therefore, the material developed may be a type of imaging probe aimed at gastric cancer (Ruan et al., 2012).

5 Conclusion

The development of monoclonal antibodies has offered new opportunities for the treatment of several diseases, including cancer. The recognition ability and its particular reactivity, with the possibility of bioconjugation, have stimulated the development of several therapies. Targeting antibodies for the functionalization of nanoparticles with different approaches have been described demonstrating the versatility and range of possibilities for the development of new therapies. Functionalization using maleimide chemistry has been the most used technique, and new approaches to click chemistry have been developed seeking to improve its safety. The different conjugation techniques employed in the functionalization can alter the physicochemical properties of the nanoparticles and the binding sites of the antibodies resulting in dramatic variations in the stability and in the intended therapeutic effect for the formulation. The biggest challenge is choosing the ideal strategy for a particular type of cancer. The use of antibody-functionalized nanoparticles is still less developed than ADCs, which have approved clinical protocols, and commercial formulations are already available. However, the use of nanoparticles seems promising because, in addition to promoting selective delivery, they have other advantages such as increased drug stability, controlled release, and improved pharmacokinetics and immunogenicity. Different types of nanoparticles and conjugation strategies have been tried and optimized in the search for conjugation efficiency, with adequate conformation and density of the conjugates, in order to improve the circulation time and binding capacity of functionalized nanoparticles with antibodies directed to the different types of cancer. In this chapter, several functionalization

approaches and in vivo and in vitro studies were presented with excellent perspectives in the development of treatments for cancer. Although it is a challenge, drug-loaded nanoparticles functionalized with antibodies are expected to be developed and approved.

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Part II
Strategies for Cancer Therapy Through Nanotechnology

Nanotechnology to Correct Mitochondrial Disorders in Cancer Diseases

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Abbreviations

CPP	Cell-penetrating peptides
CRISPR	Clustered regularly interspaced short palindromic repeats
ENS	Enzymatic noncovalent synthesis
Fe/S	Iron-sulfur
GFP	Green fluorescent protein
LHON	Leber's hereditary optic neuropathy
MELAS	Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes
MERRF	Myoclonic epilepsy with ragged-red fibers
mtDNA	Mitochondrial DNA
MTS	Mitochondrial targeting signal peptide
ND2NADH	Dehydrogenase 2
nDNA	Nuclear DNA
OXPHOS	Oxidative phosphorylation
ROS	Reactive oxygen species
TIM	Mitochondrial inner membrane
TOM	Mitochondrial outer membrane
T-ZnPc	Zinc phthalocyanine

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1 Introduction

Mitochondria are fundamental organelles for the functioning of a eukaryotic cell and, therefore, life and health as we know it. A long-standing question is to understand which processes make mitochondria essential and why. The main source of energy in any metabolic reaction occurring in any cell at any time is glucose. Mitochondria play an important role in cellular bioenergetic pathways, being able to generate energy in the form of ATP from oxidation of glucose through oxidative phosphorylation (OXPHOS). It is estimated that about 90% of all energy generated within a cell is attributed to mitochondria, earning them the title of "powerhouse of the cell" (Herrera et al., 2015).

Energy production by aerobic respiration, however, is just one of the many roles played by mitochondria. Indeed, mitochondrial proteome consists of more than 1000 proteins involved in a wide range of biochemical processes and metabolic pathways, including tricarboxylic acid cycle, fatty acid oxidation, gluconeogenesis, protein synthesis, amino acid metabolism, quinone and steroid biosynthesis, iron-sulfur (Fe/S) cluster biogenesis, generation of reactive oxygen species (ROS), maintenance of calcium homeostasis, initiation of apoptotic cascade, and ion homeostasis, among others (Calvo & Mootha, 2010).

Mitochondrion is also the only organelle (except for plant chloroplasts), outside the nucleus with its own genome, the mitochondrial DNA (mtDNA), giving support to the accepted theory of an endosymbiotic origin of mitochondria, in which a proteobacterium took residence within a eukaryotic cell. Over evolutionary time, most of the bacterial genes encoding components of the present-day organelles were transferred to the nucleus, remaining only a few, but very important, proteins that are encoded by the mitochondrial genome and expressed exclusively in the mitochondrial matrix (Roger et al., 2017). Among these are four enzymatic complexes, components of oxidative phosphorylation (OXPHOS) system. Complex I, the first enzyme of OXPHOS, comprises more than half of all the polypeptide encoded by mitochondria, being the largest but least understood of them all.

On the counter side, although mitochondria generate almost all the energy required for cell survival, they also generate highly reactive oxygen species that undermine cell viability if there is an imbalance between ROS generation and detoxification (Zhou et al., 2016). Moreover, mtDNA inherent protective proteins and repair systems are less robust compared to nuclear DNA which leads mtDNA being more prone to be affected by ROS that are inherently present in high concentration inside the mitochondria. Consequently, mtDNA mutations are a more common phenomenon than the nuclear ones. These series of key discoveries have reawakened the scientific interest in this long-known cell organelle. It has become increasingly evident that molecular lesions to the mtDNA lead to mitochondrial dysfunction and contribute to a variety of human disorders, ranging from severe metabolic diseases, neurodegenerative and neuromuscular diseases, obesity, and diabetes to ischemia-reperfusion injury and cancer (Hahn & Zuryn, 2019; Taylor & Turnbull, 2005).

To overcome mitochondrial loss of function, the treatment of mtDNA-based diseases has been mainly focused on the administration of a "drug cocktail," composed of small molecules or biologic active compounds, to alleviate a variety of symptoms. However, these therapeutic strategies are largely supportive rather than curative, and, in general, they reveal to be ineffective (Pfeffer et al., 2012). Consequently, increasing efforts are being made either to design molecules able to target mitochondria or to develop drug carrier systems for the selective delivery of drugs and DNA into mitochondria. An emerging attractive approach for mitochondrial targeting is gene therapy. The use of a therapeutic vector based on mitochondrial DNA, along with its affinity to the site of mitochondria, can be considered a powerful tool in the reestablishment of normal mitochondrial function. The insertion of functional mtDNA in mutated cells may provide the normal mitochondrial function reestablishment and, consequently, a promising approach to treat mitochondrial disorders (Jang & Lim, 2018).

In this chapter, we discuss the role of mitochondria in cellular metabolism and homeostasis and how their dysfunction is linked to human diseases and pathologies, particularly in cancer and the ones related with complex I mutations. Furthermore current strategies for mitochondria-specific targeting of mitochondrial genes using nanotechnology and their expression, future therapeutic applications, and challenges.

2 The Mitochondrial Genome

Mitochondria contain their own DNA and all the machinery necessary for its replication, transcription, and translation. Structurally, mtDNA is like the one of its antecedent bacteria: compact, circular, and double-stranded DNA, ranging in size depending on species. The number of copies of DNA per cell usually ranges from 10^3 to 10^4 depending on the cell type and tissue origin, yet fertilized cells or cells with diseases, such as cancer, have altered mtDNA copy numbers (Reznik et al., 2016). Throughout evolution, the transition from a proteobacterium to a permanent specialized ATP-producing organelle has been accompanied by major changes including massive gene acquisition and lost and transfer to the nuclear genome, thus affecting mitochondrial proteome. Consequently, additional roles in metabolism and biosynthetic pathways in mitochondria have gradually developed over time (Johnston & Williams, 2016).

Advances in mitochondrial genome sequencing have provided for the identification of approximately 1500 proteins that comprise the mammalian mitochondrial proteome. However, these 16,569 base-pair molecules only code for a relatively small number of proteins and RNAs. The human mitochondrial genome includes 37 genes encoding for 13 mRNA (translated to 13 proteins), 22 tRNA, and 2 rRNA (12S and 16S rRNA) molecules, which are not able to produce all the proteins needed for its functionality. Thus, the majority of mitochondrial proteins are encoded by the nucleus, synthesized on cytosolic ribosomes, and then imported from the cytosol to mitochondrion by a complex importing machinery (Fox, 2012).

All the mitochondrial genome is arranged in two strands of DNA that differ in their base composition. A heavier, guanine-rich, outer strand comprises most information, encoding for 2 rRNAs, 14 tRNAs, and 12 polypeptides. On the inner site, the transcription from the light strand results in eight tRNAs and a single polypeptide. Another interesting feature is the lack of introns, and there is only one longer noncoding region referred to as the regulatory region, essential for both the replication and transcription of the genome, ensuring mRNA processing and protein translation. Located within this noncoding regulatory region (NCR) are L- and H-strand promoters (ITL and ITH, respectively), from which mitochondrial transcription is initiated, as well as two putative origins of replication (OH and OL). The transcription process is driven by DNA-dependent RNA polymerase and depends on a couple of cofactors. The resulted polycistronic transcript undergoes further processing (Mercer et al., 2011).

As simple as the transcription apparatus appears to be, the unique features of mtDNA imply different regulation mechanisms, as complex as it benefits a genome that is a core factor in human health. Besides mtDNA replication and transcription, the maintenance and expression of mtDNA involve other levels of control, such as RNA stability, translation by mitochondrial ribosomes, and the insertion of translated proteins into the mitochondrial inner membrane (Fox, 2012).

Considering this, it can be said that mitochondria are not self-sustained entities within the cell, strongly relying upon the import of hundreds of nuclear encoding factors for their function. The importance of such regulatory processes is highlighted by the identification of numerous mutations in nuclear genes that impair mtDNA expression at different levels and has been associated with human mitochondrial diseases (Rusecka et al., 2018).

2.1 Mitochondrial Genes

All the 13 mtDNA-encoded polypeptides synthesized within the mitochondria are subunits of enzyme complexes that constitute the OXPHOS system, the main metabolic function of mitochondria in all aerobic organisms. The precise regulation of these genes is crucial for maintaining ATP production levels required for cellular metabolism. Approximately 90 proteins take part of OXPHOS system (Saada, 2014). Thus, mitochondrial subunits only account for roughly 15% of the total machinery. Also, the nuclear contribution is not limited to the expression of the respiratory chain. The components of numerous other essential pathways are as well nuclear encoded, such as heme biosynthesis, fatty acid/amino acid oxidation, pyrimidine biosynthesis, calcium homeostasis, and apoptosis (Spinelli & Haigis, 2018).

Besides the minority contribution of mitochondria proteome for OXPHOS subunits, they are nevertheless essential because OXPHOS collapses in the absence of mtDNA expression (Larsson et al., 1998). This system consists of five multimeric

complexes set in the mitochondrial inner membrane. Seven polypeptides (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) are subunits of complex I (NADH-ubiquinone oxidoreductase), one (CYTB) is part of complex III (cytochrome bc1 or ubiquinol-cytochrome c reductase), three (COX1, COX2, COX3) are subunits of complex IV (cytochrome c oxidase), and two (ATP6, ATP8) are part of complex V (ATP synthase). Complex II (succinate dehydrogenase) presents four subunits, all encoded by multiple nuclear genes (Saada, 2014) (Fig. 6.1).

Briefly, NADH and FADH2, major outputs from the Krebs cycle, are oxidized generating electrons that are transferred through the respiratory chain (complexes I–IV) to oxygen. The transfer of the negatively charged particles by these protein complexes generates a proton (H+) gradient creating an unequal electrical charge on either side of the inner mitochondrial membrane. This electrochemical gradient is then used from ATP synthase to synthesize ATP, as the H+ spontaneously diffuses back to cross the inner membrane. The ultimate acceptor of these high-energy electrons is oxygen, and therefore, OXPHOS generates both ATP and water.

ATP is the most important energy supplier in many enzymatic reactions; however, it is a versatile molecule, rather than just an energy storage molecule and supplier to the cell. Cells utilize ATP to carry out cellular functions that are necessary for their survival, such as synthesis of proteins, ion transport, growth, and replication (Bonora et al., 2012). It also regulates cell functions by activating a series of intracellular signaling pathways, namely, in the nervous and vascular systems (Lohman et al., 2012). Additionally, it has been implicated in immune responses to tissue damage and in cell proliferation, differentiation, and apoptosis (Burnstock & Verkhratsky, 2010). A correct expression of mitochondrial genome and OXPHOS function is therefore essential for ATP formation to occur at normal levels.

Different cell types within an organism have diverse physiological requirement and different mitochondrial compositions and energy demands, and therefore, it can affect their mitochondrial metabolism and ATP production. Organs with higher metabolic rate, as the liver, kidney, brain, heart, and skeletal muscle system, are the

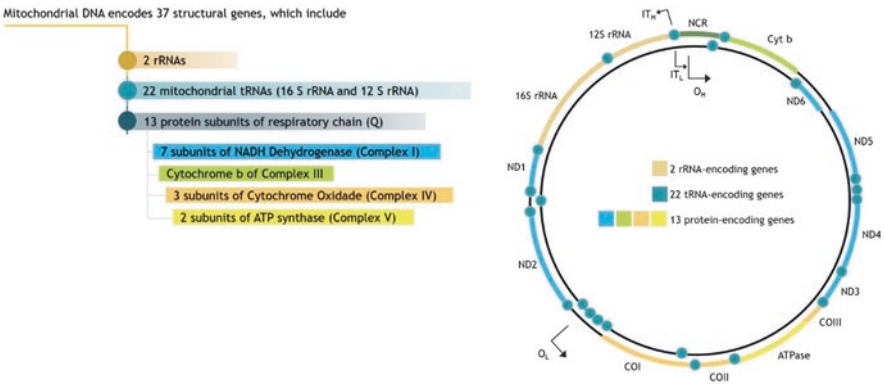

Fig. 6.1 Illustrative scheme of the mitochondrial genome and its complexes, subunits, and structural genes

ones that require more energy supply (Veltri et al., 1990). When cellular respiration is compromised, these are also the first to be affected, ultimately resulting in progressive metabolic disorders and contributing to a distinctive class of conditions known as mitochondrial diseases.

2.2 Mitochondrial Gene Mutations and Mitochondrial DNA Diseases

Efficient mitochondrial function depends upon a series of factors, for instance, (a) coordinated interaction between nuclear and mitochondrial genome; (b) quality control mechanisms of processes such as replication, transcription, and translation; (c) correct assembly of the components of OXPHOS system; (d) control of protein import, folding, and degradation; and (e) DNA repair systems (Saki & Prakash, 2017). In line with this, dysregulation of these pathways is an emerging key in understanding many current mitochondrial diseases.

A primary cause for mitochondrial dysfunction is nuclear and mitochondrial DNA mutations, which are usually associated with OXPHOS dysfunction, changes in metabolite homeostasis, and a variety of tissue-specific clinical features. Not surprisingly, mutation in genes that encode proteins required for ATP production often causes cell deterioration and a subsequent array of human mitochondrial diseases, which can affect multiple organs in the body at any point of an individual's life (Bhatti et al., 2017).

Mitochondrial disorders can derive from mutations transmitted in maternal mtDNA or arise from both nDNA (nuclear DNA) and mtDNA mutations through spontaneous errors during DNA replication, unrepaired damage to mtDNA, or environmental stress, such as ROS production (Farrar et al., 2013). ROS are produced at very low levels during the OXPHOS and play an important role in physiological functions. Overproduction of ROS along with failure of body's antioxidant enzyme systems might result in lipid, protein, and DNA damage. Due to the close proximity of ROS site production to the respiratory chain and its lack of protective histone proteins, mtDNA is particularly sensitive of ROS-induced mutations. In turn, mutations induced by ROS impair OXPHOS leading to more ROS production exponentially increasing mitochondrial dysfunction. The culminating loss of cellular and tissue function includes reduced energy production, cell communication failure, mitochondrial membrane disruption, and induction of the mitochondrial pathway for apoptosis (Webster, 2012).

Undoubtedly, it is clear that a variety of mutations are associated with human pathology. Mitochondrial-associated disorders are diverse and can be devastating, affecting many tissues including muscles, the heart, and the nervous system. These include encephalomyopathies, such as myoclonic epilepsy with ragged-red fibers (MERRF) (Ban et al., 2018); mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) (Henry et al., 2017); neuropathies, such as Leber's hereditary optic neuropathy (LHON) (Carreño-Gago et al., 2017a);

neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, and Parkinson's disease (Van Horssen et al., 2019); deafness (Kokotas et al., 2007); diabetes mellitus (Yee et al., 2018); obesity (De Mello et al., 2018); and cancer (Kim et al., 2016). The severity and phenotype of the disease depend on the grade of heteroplasmy, that is, the percentage of the mitochondria per cell affected by the mtDNA mutation (Wallace & Chalkia, 2013). Epidemiological studies report the number of adults suffering from all forms of mitochondrial disease to be around 12.5 per 100,000 of the population (Gorman et al., 2015b). During the last decades, evolution on genome sequencing techniques allowed the identification of over 250 pathogenic mtDNA mutations (Gorman et al., 2015b). In Tables 6.1 and 6.2, some of the more frequent mtDNA mutations and associated diseases are presented.

2.3 Mutations of the Mitochondrial ND1 Gene and Associated Diseases

Complex I is the major entry point for electrons to the electron transport chain, being considered as a limiting step in the overall respiration and energy production (Fig. 6.2). In mitochondria, it catalyzes the transfer of electrons from NADH (ubiquinone oxidoreductase chain) to ubiquinone and translocates protons across the inner mitochondrial membrane. Along with complex III, it is also regarded as the main source of reactive oxygen species (Hirst et al., 2008). A deficiency in this complex can be attributed to a mutation affecting either a structural subunit or an assembly protein, to an increase in its ROS production or both (Tretter et al., 2004). Mitochondrial DNA encodes seven subunits of this enzyme complex that are involved in proton translocation and ubiquinone binding. This represents roughly half of all mitochondrial genome. Thus, it is expected that the activity of complex I would be the most affected between other mitochondrial enzyme complexes.

Among these, the mt-ND1 (NADH-1) has been shown to play a crucial role for the assembly of subunits in complex I (Kirby et al., 2004). Mitochondrial point mutations in ND1 have first been described in association with two distinct clinical phenotypes, LHON and MELAS (Spruijt et al., 2007; Blakely et al., 2005; Lin et al., 2014). Indeed, different point mutations occurring in mitochondrial genes might have variable phenotypes, which clinically can be difficult to diagnose. Moreover, mt-ND1 mutations have been associated with a few cases of adult-onset dystonia (Spruijt et al., 2007), hearing loss (Mezghani et al., 2013), fatal infantile mitochondrial encephalopathies such as Leigh's syndrome (Lenaz et al., 2004), and progressive cardiomyopathy (Moslemi et al., 2008), among others. More recently, some studies have indicated a correlation between some mutations in the mtDNA and cancer (Kim et al., 2016; Thapa et al., 2016). Kim et al. demonstrated that mutations associated with mtDNA, namely, the ND1 gene, were found in the four stages of kidney cancer (I to V) (Kim et al., 2016). Another study revealed that mutations in the ND1, ATP6, and ATP8 gene have an influence on the onset of breast cancer. However, the ATP6 gene seems to be more predisposed to mutations in this type of

Table 6.1 List of the most common mtDNA mutations and associated diseases

Mutation(s)	Mutant gene(s)	Associated disease(s)	Ref.
m.3243 A > G	Leucine (UUR) tRNA	Diabetes	Decoux-Poullot et al. (2020)
3316 G > A	ND1	Type 2 diabetes mellitus	Lalrohlui et al. (2016)
m.9053G > A	ATP6		Permana Maksum et al. (2017)
8584 G > A/8860A > G/8701 A > G/10389 A > G	ATP6		Lalrohlui et al. (2020)
10398A > G/10310 G > A	ND3		
3970C > T	ND1		
7444G > A/7471DelG/7496A > G	Serine (UCN) tRNA	Hearing loss	Tang et al. (2015)
c.235delC	Gap junction beta 2 (*GJB2*)		Zheng et al. (2015)
7551A > G	Asp tRNA		Wang et al. (2016)
m.3634ANG	ND1	Leber's hereditary optic neuropathy (LHON)	Carreño-Gago et al. (2017b)
m.3460G > A	ND1		Baertling et al. (2018)
S12F/A213V/L345P/A357P/L370P/D399Y	Desmin (DES)	Skeletal myopathy Desmin myopathies	Smolina et al. (2020)
M337V/I383T	C9orf72/ TARDBP	Amyotrophic lateral sclerosis (ALS) and the related disorder frontotemporal dementia (FTD)	Dafinca et al. (2020)
T4291C/A4295G	Ile tRNA	Hypertensive renal dysfunction	Zhu et al. (2019)
m.8914C > T	ATP6	Mitochondrial encephalomyopathies	Guo et al. (2018)
A10398G	ND3	Parkinson's disease (PD)	Chu et al. (2015)
317–318 ins CCC	GAA	Pompe disease (PD) or glycogen storage disease type II	Bahreini et al. (2016)

11719A/12705 T/15043A/15301A	–	Cancer of the digestive system	Muramatsu et al. (2019)
POLG c.1879C > T; p.R627W	POLG	Autosomal recessive progressive external ophthalmoplegia, ptosis, hearing loss, proximal myopathy, and neuropathy	Paramasivam et al. (2019)
At least one mutation in each mtDNA protein-coding gene, with the exception of ND6 and ATP8	–	Hurthle cell carcinoma (HCC)	Ganly et al. (2018)
m.3861A > C	ND1	Sensorineural hearing loss and neurodevelopmental delay	Ammar et al. (2016)
m.8584G > A	ATP6	Alzheimer's disease	Bi et al. (2015))
C3572ins	ND1	Kidney cancer	Kim et al. (2016)
C3572ins 3331del242bp	ND1	Oncocytic thyroid carcinoma Invasive ductal breast carcinoma	Gasparre et al. (2007)
T12601C A13973T	ND5	Invasive ductal breast carcinoma	Gasparre et al. (2007)
T9903C	COXIII	Invasive lobular breast carcinoma	Gasparre et al. (2007)
C3572ins	ND1	Oncocytic pituitary adenoma	Porcelli et al. (2010)
C3572ins	ND1	Renal oncocytoma	Lang et al. (2015)
8584G > A	ATP6	Breast cancer	Thapa et al. (2016)

Table 6.2 List of the most common nuclear DNA mutations and associated diseases

Mutation(s)	Mutant gene(s)	Mitochondrial protein(s)/function affected	Associated disease(s)	Reference
p.(Gly671Trp)/p.(Tyr689His)	AFG3L2 gene	Protein of the mitochondrial ATPase complex	Progressive external ophthalmoplegia (PEO)	Gorman et al. (2015a)
c.993dupA	FBXL4(F-box and leucine-rich repeat protein 4) gene	F-box protein maintains mtDNA integrity and stability	Mitochondrial DNA depletion syndrome-13 (MTDPS13)	Wang et al. (2020)
D1005G	NPC1 gene	Mitochondrial stress regulator protein MNRR1	Niemann-–Pick type C (NPC)	Erickson et al. (2020)
c.523delC	Adenine nucleotide translocator 1 gene (SLC25A4, ANT1)	Impaired mitochondrial complex I activity Increased oxidative damage Decreased l-Opa Altered mitochondrial morphology Sensitization of the mitochondrial permeability transition pore Augmented somatic mtDNA mutation levels Shortened lifespan	Cardiomyopathy	Mcmanus et al. (2019)
POLG c.1879C > T; p.R627W and c.2341G > A; p.A781T	POLG gene	Mitochondrial DNA polymerase gamma (pol γ)	Encephalopathy, seizures, and stroke-like episodes	Paramasivam et al. (2019)
c.424G > A (p.Val142Ile)/ c.469C > T (p.Arg157*)	RNASEH1 gene	Ragged-red and cytochrome c oxidase (COX)-negative fibers Impaired activity of various mitochondrial respiratory chain complexes	Chronic progressive external ophthalmoplegia (CPEO)	Reyes et al. (2015)
p.K202del and p.T108M	TK2 gene	TK2 activity drastically reduced leading to dramatic decrease in mtDNA copy number in muscle	mtDNA depletion syndrome (MDS)	Cámara et al. (2015)

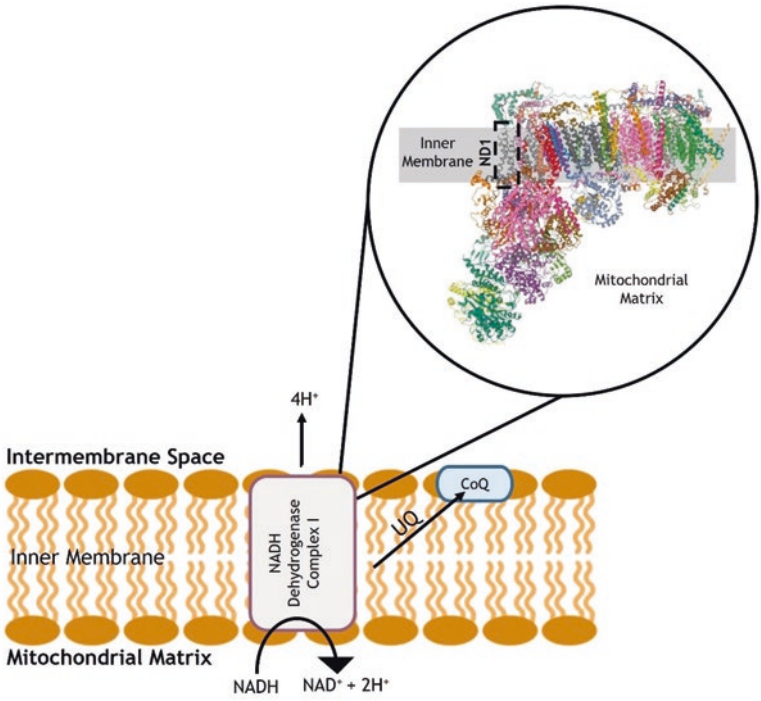

Fig. 6.2 Representation of the mitochondrial matrix where the ND1 subunit of the NADH dehydrogenase I complex is inserted

cases and therefore may play an important role in the prognosis of breast cancer (Thapa et al., 2016).

Conventional clinical practices toward mitochondrial respiratory chain dysfunction are sparse and very limited. Current treatment includes symptom-based management options, such as energy fuel supplements, oxidants, cofactor, and vitamins (Murphy & Hartley, 2018). These focus on improving quality of life and mitigating the set of symptoms, being largely supportive rather than curative. Therefore, there is a clear requirement for innovative therapeutics.

2.4 Mitochondria as a Therapeutic Target in Cancer

As already described above, mutations in the mitochondrial genome can trigger the onset of various diseases. One of the possible consequences of mitochondrial function dysregulation is the development of cancer. These organelles play a key role in regulating metabolism, cell proliferation and death, and signaling pathways. Consequently, alterations in their normal function contribute to the development of tumor cells and their growth, the appearance of metastases, and even resistance to anticancer drugs. Taking into account that they play such an important role,

mitochondria have become a therapeutic target for the development of new mitochondrial therapies that facilitate the treatment of cancer (Kim et al., 2017; Vyas et al., 2016).

The presence of large amounts of ROS can cause mutations in mtDNA, since the DNA repair system in mitochondria is not as efficient as that in the nucleus (Alexeyev et al., 2013). In several types of cancer, these mtDNA mutations are present and play an essential role in the proliferation and maintenance of tumor cells (Lu et al., 2009). One example of the effect of these mutations was described for colorectal cancer, where alterations in subunit 5 of complex I (NADH dehydrogenase 5, (ND5)) showed not only the activation of the Akt signaling pathway, contributing to tumorigenesis, but also an increase in the production lactate and consequent tumor growth (Park et al., 2009; Sharma et al., 2011). Also, mutations in mtDNA induced demethylation of the D-loop region, the region responsible for controlling mtDNA expression. Demethylation of the D-loop region caused an uncontrolled increase in the number of mtDNA copies, promoting the proliferation of cancer cells (Feng et al., 2012). The D-loop region that controls mtDNA replication and transcription was also proved to be very important in gastric cancer. Wen et al. demonstrated that demethylation of the D-loop region leads to a decrease in mtDNA, which is more evident in late stages of gastric cancer. The study further demonstrated that treatment with 5-Aza-2′-deoxycytidine for demethylation led to an increase in the number of mtDNA copies in the treated versus untreated cancer cells (Wen et al., 2013). Lastly, in colorectal cancer, it has also been shown that NADH dehydrogenase 2 (ND2) is overexpressed in the early stage (Wen et al., 2013; Feng et al., 2012).

The CRISPR/Cas9 complex may play a very important role in strategies that aim to minimize or eliminate the effects of mtDNA mutations. This complex is composed of the "clustered regularly interspaced short palindromic repeats" (CRISPR), whose function is to identify specific sequences and the Cas9 enzyme that works as a "cutter" of the sequences identified by CRISPR. Since this complex can be manipulated to find specific mutations in mtDNA, this strategy makes it possible to create a highly specific and personalized therapy for each type of cancer (Fogleman et al., 2016). In addition to this therapeutic function, the CRISPR-Cas9 complex can also be used to identify the role and importance of mitochondrial genes that regulate the function of this organelle (Kim et al., 2017).

Another approach that can be taken for the treatment of cancer using mitochondria as a target is immunotherapy. Immunotherapy sometimes does not offer positive results, and one of the given reasons is the fact that the mitochondria present in cancer cells have a greatly reduced metabolism and function. The mtDNA mutations and consequent dysfunction lead to an ineffective T-cell response, making immunotherapy unfeasible (Kim et al., 2017). Published studies have shown that when it was possible to restore the expression of PGC1-α, mitochondrial activity was restored and consequently the antitumor immunity of T cells was activated, enabling treatment by immunotherapy (Scharping et al., 2016; Bengsch et al., 2016). Immunotherapy through the T-cell response was also tested by the group of Marrache et al. but using vectors at the nanometer scale (Marrache et al., 2013). Zinc phthalocyanine-based systems (T-ZnPc-NPs) were used to stimulate dendritic

cells in order to potentiate an immunotherapeutic response of tumor antigen-specific T cells. T-ZnPc-NPs nanoparticles have characteristics that enable specific targeting to mitochondria, which in turn triggers an immune response. The fact that they are photosensitive allowed the application of these nanocarriers in breast cancer cells, and once stimulated by light, they led to the production of interferon-gamma cytokines, a product of T and natural killer cells (Marrache et al., 2013). These approaches serve as a basis for the development of new types of cancer therapies resulting from mitochondrial dysfunction.

3 Mitochondrial Gene Therapy

Mitochondrial gene therapy emerges as a potential alternative in the treatment of mitochondrial diseases. The treatments available these days only serve to mitigate the symptoms and do not provide a cure. This type of gene therapy allows for a personalized treatment that addresses the problem at its source and can be adjusted for each mutation in the mtDNA that leads to the appearance of diseases. Thus, mitochondrial gene therapy has several advantages compared to conventional drugs. The development of a therapeutic vector that can be produced and distributed on a large scale for the restoration of normal mitochondrial function in mutated cells may be a significant step toward the treatment of mitochondrial diseases. In addition to bringing a new perspective on healing, mitochondrial gene therapy is an economic strategy that can provide localized treatment with long-term action (Coutinho et al., 2017).

Mitochondrial gene therapy can be applied by different strategies. One approach consists in the expression of a gene in the nucleus, which in turn leads to the synthesis of a protein that is later directed and imported to the site of the mitochondria. Viral vectors are commonly used in this indirect mitochondrial transfection, and relevant progresses have been reported in clinical translation, namely, when treating Leber's hereditary optic neuropathy (LHON) (Koilkonda et al., 2014; Wan et al., 2016). This allowed the development of animal models suitable for mutations in mtATP6 (Dunn & Pinkert, 2012). Although a powerful technique, the allotopic expression can have some limitations such as the difficulty of mitochondria imports of more hydrophobic proteins and apparent complementation attributed to forced revertants of the original mtDNA mutations. Therefore, gene-to-gene variability can occur and limit the success of allotopic expression (Perales-Clemente et al., 2011). Additionally, besides the great transfection efficiency achieved by using viral systems, their antigenicity, oncogenic effects, and instability of storage limited this therapy. Direct transfection of mitochondria appears as an alternative to overcome the disadvantages of indirect transfection and thus constitutes a promising tool for mitochondrial gene therapy. However, for the direct delivery of genetic material to mitochondria, it is essential to formulate transporters that allow not only the loading/complexation of DNA but also its targeting to mitochondria (Coutinho et al., 2017).

The delivery of therapeutic DNA depends on the passage through the double mitochondrial membrane, which is impermeable to hydrophilic molecules. So, to overcome this challenge, physical, chemical, and biological approaches to transfer DNA to mitochondria have recently emerged (Jang & Lim, 2018).

Physical methods are used due to their simple and direct way of transferring exogenous genes to cells, since they allow the penetration of cell membranes and intracellular organelles without the need to transport molecules. It has recently been demonstrated that the use of the hydrodynamic injection technique has allowed naked plasmid DNA to be delivered to rat liver mitochondria in vivo (Yasuzaki et al., 2015). Another physical method used to deliver genetic material to mitochondria is biolistic technology, which targets cells with complexes formed by DNA coated with heavy metals. One study demonstrated the delivery of mitochondrial genes using biolistic technology in *Saccharomyces cerevisiae* (Bonnefoy & Fox, 2007). Physical methods, as they do not require carrier molecules, do not induce toxicity associated with this type of molecules. However, the techniques used can damage the target cells in the cell membrane penetration process, and the DNA distribution is uniform throughout the cytoplasm, entering the mitochondrial matrix at random, which makes delivery to the mitochondria difficult (Jang & Lim, 2018).

Chemical methods are the most used and studied for the delivery of genes to the mitochondria, based on specific chemical interactions. Due to the characteristics of the mitochondrial membrane (hydrophobic and negatively charged), it is necessary to encapsulate the negatively charged DNA with carrier molecules that have cationic and amphiphilic properties to mediate the transfection of genetic material to occur. However, these transporters face several obstacles until they reach the mitochondria. In most cases, they must first cross the cellular membrane via endocytosis or translocation. If carrier molecules follow the endocytic pathway, they are not accessible to mitochondria until they escape from the endosome. Accordingly, it is important to find molecules that facilitate the passage of these carriers from the endosome. Furthermore, it is necessary to find strategies to direct these DNA transporters from the cytoplasm to the mitochondria. Thus, mitochondria-targeting molecules are conjugated with DNA-binding motifs or presented on the surface of DNA-enclosing vesicles composed of dendrimers, surfactants, or liposomes (Jang & Lim, 2018).

Mitochondrial proteins expressed in the cytosol as mitochondrial precursor proteins enter the mitochondria via mitochondrial targeting signal peptide (MTS)-mediated translocation. Most MTSs are located at the amino terminus of precursor proteins and are cleaved by proteolysis upon import into mitochondria. The MTS pre-sequences form an amphipathic α-helix that has hydrophobic residues on one side and positively charged residues on the other side. MTS-conjugated proteins enter the mitochondrial matrix through the translocase of the mitochondrial outer membrane (TOM) and that of the mitochondrial inner membrane (TIM). The MTS at the N-terminus of a precursor protein is preferentially recognized by the TOM20-TOM22 receptor subcomplex, which contains clusters of negatively charged residues. The protein passes through the import pore formed by TOM40 (Van Der Laan et al., 2007). Consequently, precursor proteins enter the channel formed by TIM17

and TIM23 across the inner membrane in the presence of a membrane potential. The N-terminal domain of TIM23 encodes an MTS receptor that has negatively charged residues, which interact with the positively charged amphipathic MTS. Through this interaction, MTS conjugates can pass through the mitochondrial double membranes. Accordingly, conjugation of MTS to carrier molecules that have a DNA-binding function has been a strategy to enable specific mitochondrial gene delivery (Esaki et al., 1999; Van Der Laan et al., 2007).

3.1 Mitochondrial Gene/Protein Expression and Mitochondria Targeting Using Nanotechnology

3.1.1 Mitochondrial Gene/Protein Expression

Over the past few years, efforts have been made to develop innovative systems for mitochondrial gene therapy. An example is the use of cationic surfactants with two main hydrophilic groups and two hydrophobic groups as DNA carriers for mitochondrial delivery (Cardoso et al., 2015). These cationic surfactants give rise to micelle-like structures capable of encapsulating the DNA and forming gemini-surfactant-DNA therapeutic nanosystems targeting mitochondria (Wan-Xia et al., 2011). Some researchers have been investigating the development of mitochondrial targeting liposomes with the use of MITO-Porter for therapeutic mitochondrial RNA delivery to diseased cells. These nanoparticles were able to induce a decrease in the expression of target mRNA and its corresponding protein. In addition to this silencing of mitochondrial gene, the fact that they were able to deliver an antisense RNA oligonucleotide (ASO) led to the downregulation of the respiratory chain and consequent decrease in ATP production in cancer cells (Kawamura et al., 2019, 2020).

Other researchers explored a different therapy pathway conjugating *Escherichia coli* and mitochondria to promote DNA transfection in the mitochondrial networks of mammalian cultured cells (Yoon & Koob, 2005).

3.1.2 Mitochondrial-Targeted Delivery Systems

Some other studies have focused on the mitochondrial import pathway (Flierl et al., 2003; Geromel et al., 2001). Lyrawati et al. developed a new strategy for the expression of the reporter green fluorescent protein (GFP) in mammalian mitochondria (Lyrawati et al., 2011). First, they constructed an artificial mini-mitochondrial genome with GFP that was then incorporated in liposome-like vesicles derived from dequalinium, called DQAsomes. DQAsomes were the earliest systems for gene delivery to be reported. They present the advantage of simple preparation and demonstrated the ability to selectively release DNA at the membranes of mitochondrial-like liposomes (D'souza et al., 2005). In this study, DQAsomes could deliver the mitochondrial genome construct to the mitochondria from a range of mammalian

cell lines, resulting in the expression of GFP mRNA and protein (D'souza et al., 2005).

Furthermore, good results were also obtained using delivery systems based on mitochondriotropic molecules with a high degree of mitochondrial affinity (Horobin et al., 2007). One example is rhodamine 123, a fluorescent amphiphile. The use of this fluorescent compound to perform a targeted delivery to the mitochondria comes from the fact that it can easily cross the mitochondrial membrane. This allows its preferential accumulation in the mitochondria and thus enables a better visualization of the nanosystems with rhodamine 123 in their constitution. Salvado et al. formulated mitochondrial-targeted rhodamine plasmid DNA cellulose-based delivery systems with suitable size and morphology that were able to be internalized by mammalian cells and showed targeting ability to mitochondria (Salvado et al., 2015).

Other studies focused on the allotopic expression of proteins encoded by recombinant mtDNA. These therapeutic agents containing mitochondrial targeting sequences demonstrated efficiency of their mitochondrial import by the rescue of the respiratory chain defect observed in fibroblasts harboring mutations in *ND4* or *ND1* genes (Bonnet et al., 2008). The allotopic expression approach has also led to promising results in fibroblasts with mutations in ATP6 (Kaltimbacher et al., 2006). Additionally, Ellouze and co-workers tested a similar approach but in an animal model of LHON. They demonstrated that the allotopic expression of the mitochondrial protein ND4 prevented blindness in rats with mitochondrial dysfunctions that caused LHON (Ellouze et al., 2008).

3.1.3 Mitochondria-Targeted Peptide Delivery Systems

Peri-mitochondrial enzymatic noncovalent synthesis (ENS) of peptides has also been applied in mitochondrial genetic engineering. A research group used this strategy to transfect gene vectors encoding CRISPR/Cas9 into the mitochondria to induce mitophagy and apoptosis and sensitize cancer cells to cisplatin (He et al., 2020).

Faria et al. designed and developed a set of new systems for mitochondrial gene therapy. The nanocarriers created are based on cell-penetrating peptides (CPP) in which a sequence with affinity for mitochondria (MTS) has been added (Faria et al., 2021). The choice of the nanocarrier relied on CPPs as they demonstrate good capacity for cellular internalization while allowing efficient encapsulation of genetic material and present low toxicity. The MTS-CPP vectors demonstrated the ability to complex the mitochondrial gene ND1-encoded plasmid DNA (pND1), mainly due to electrostatic interactions. The pND1 encapsulation efficiency of the developed nano-complexes was tested at different ratios of nitrogen to phosphate groups (N/P). In Fig. 6.3, it can be observed that all formulated systems displayed a high capacity to encapsulate pND1 at N/P $\geq$ 1 ratios and that the introduction of the MTS sequence in the CPPs does not interfere with the pND1 complexation (Faria et al., 2021).

The N/P ratio demonstrated to be an effective parameter controlling the properties of the formulated peptide-based complexes. N/P displayed an effect on the size

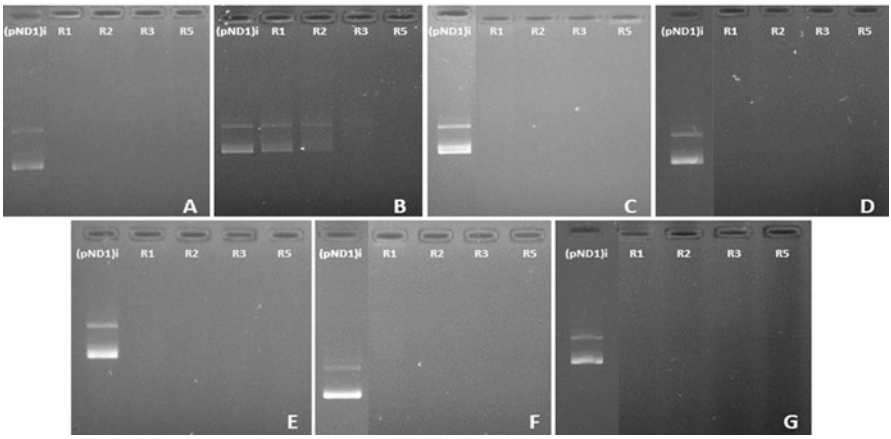

Fig. 6.3 Analysis of pND1 complexation capacity exhibited by CpMTP/pND1 (**a**); WRAP1/pND1 (**b**); WRAP5/pND1 (**c**); (KH)9/pND1 (**d**); MTS-WRAP1/pND1 (**e**); MTS-WRAP5/pND1 (**f**); and MTS-(KH)9/pND1 (**g**) nano-complexes at various N/P ratios, investigated by agarose gel electrophoresis

and zeta potential of the formulated nanoparticles. In general, the increase in the ratio led to a decrease in the average size of formulated complexes, and at the same time, it conferred a greater positive charge on their surface (Table 6.3). These two characteristics are essential for these vectors to be able to cross the cell membrane and consequently introduce pND1 into the mitochondria. All N/P = 1 ratio peptide-pND1 systems showed sizes below 410 nm, reaching sizes below 200 nm for complexes conceived at N/P ratio of 5. The presence of the targeting sequence to mitochondria did not affect the sizes of the prepared vectors. In Table 6.3, the reader can see the sizes and also verify that all complexes have positive zeta potential values, reaching values above +12 mV for MTS-CPP systems with N/P ratios = 5 (Faria et al., 2021). In addition to the low cytotoxicity for cells, MTS-CPP systems demonstrated that they were able to mediate targeted delivery to mitochondria when the MTS sequence is present. Through a fluorescence confocal microscopy study performed on cancer HeLa cells, it was possible to observe that the peptide carriers without MTS demonstrated the ability to cross the cell membrane; however, they accumulated in the cytoplasm of cancer cells, and those that managed to reach the mitochondria were residual. On the contrary, pND1 complexes based on MTS-CPPs revealed an affinity for mitochondria, since there was a clear and intense accumulation of these vectors in the mitochondria. The developed systems demonstrated suitable physicochemical properties for gene delivery applications, namely, pND1 complexation ability, morphology, size, and surface charges. The fact that they have demonstrated efficacy in the specific targeting of mitochondria turns these MTS-CPPs as an excellent nano-platform for future research studies aiming functional mitochondrial protein expression, which may contribute significantly to the progress of mitochondrial gene therapy and cancer treatment (Faria et al., 2021).

Table 6.3 Average zeta potential, mean size, and polydispersity index (PI) for the various peptide/pND1 complexes formulated at various N/P ratios

System	Zeta (mV)	Size (nm)
CpMTP/pND1 N/P 1	+2.00 ± 0.58	402 ± 25
CpMTP/pND1 N/P 2	+3.50 ± 0.50	385 ± 14
CpMTP/pND1 N/P 3	+5.83 ± 0.69	313 ± 10
CpMTP/pND1 N/P 5	+12.67 ± 0.75	236 ± 13
WRAP1/pND1 N/P 3	+25.17 ± 0.69	254 ± 14
WRAP1/pND1 N/P 5	+32.67 ± 0.47	161 ± 9
WRAP5/pND1 N/P 1	+2.00 ± 0.58	388 ± 15
WRAP5/pND1 N/P 2	+10.83 ± 1.21	299 ± 13
WRAP5/pND1 N/P 3	+14.17 ± 0.90	272 ± 11
WRAP5/pND1 N/P 5	+20.67 ± 0.75	186 ± 10
(KH)9/pND1 N/P 1	+4.50 ± 0.50	376 ± 14
(KH)9/pND1 N/P 2	+6.00 ± 0.58	300 ± 11
(KH)9/pND1 N/P 3	+11.83 ± 1.34	261 ± 10
(KH)9/pND1 N/P 5	+22.33 ± 0.75	186 ± 10
MTS-WRAP1/pND1 N/P 1	−1.83 ± 0.90	406 ± 19
MTS-WRAP1/pND1 N/P 2	+1.33 ± 0.75	366 ± 14
MTS-WRAP1/pND1 N/P 3	+6.50 ± 0.76	277 ± 13
MTS-WRAP1/pND1 N/P 5	+11.50 ± 0.76	197 ± 9
MTS-WRAP5/pND1 N/P 1	−2.17 ± 0.90	399 ± 12
MTS-WRAP5/pND1 N/P 2	+7.17 ± 0.90	316 ± 10
MTS-WRAP5/pND1 N/P 3	+10.83 ± 1.07	267 ± 8
MTS-WRAP5/pND1 N/P 5	+19.33 ± 1.60	175 ± 11
MTS-(KH)9/pND1 N/P 1	+3.17 ± 0.69	400 ± 14
MTS-(KH)9/pND1 N/P 2	+5.67 ± 0.75	367 ± 12
MTS-(KH)9/pND1 N/P 3	+8.50 ± 0.50	309 ± 9
MTS-(KH)9/pND1 N/P 5	+14.67 ± 0.75	221 ± 9

3.2 Future Challenges

Mitochondrial gene therapy is a very recent approach to fight mitochondrial dysfunction that can lead to cancer. Due to this fact, the systems that have already been explored for use in this type of therapy are still poorly studied and, certainly, have a great margin for progression. Although there are already valuable and interesting works reporting promising results as the ones mentioned above, the major difficulty encountered is the lack of mitochondria specificity. In this context, future studies should focus on the development of delivery systems that, by their own characteristics or by incorporating targeting sequences and ligands or focusing on other strategies, allow mitochondria recognition and targeting. The presence of additional sequences and/or moieties seems to be essential to ensure effective targeting while preventing their accumulation in the nucleus and cytoplasm of the cell. There are

some sequences already identified with specificity toward this organelle that can be incorporated into previously studied systems or into novel delivery carriers. The constitution of the systems is another relevant aspect that must be considered when designing a vector suitable for mitochondrial gene therapy. As investigated by our team, cationic polymers, based on polyethylenimine, and CPPs appeared to be the most promising materials due to their favorable characteristics for payload complexation, cellular uptake/internalization and endosomes escape, and mitochondria targeting. Polymers, due to their positive charge, facilitate the encapsulation of nucleic acids and their entry into cells, and the addition of ligands to these polymeric systems is already being widely explored. CPPs, because they are easily manipulated and synthesized, facilitate the incorporation of ligands or sequences that can confer mitochondria targeting. During the synthesis of peptides, the addition of these targeting sequences can be explored, which thus become an integral part of CPP. Moreover, the comparison of the therapeutic effectiveness of the use of DNA or RNA for each situation must also be explored. Scientific community should evolve in this path by exploring the many possibilities of genetic material-based platforms toward optimal therapeutic response. Mitochondrial gene therapy is an area with much more to explore and can deeply contribute to the creation of new/advanced treatments for mitochondrial dysfunction and cancer.

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Chronobiology and Nanotechnology for Personalized Cancer Therapy

Tânia Albuquerque, Ana Raquel Neves, Rúben Faria, Telma Quintela, and Diana Costa

Abbreviations

5-FU	5-Fluorouracil
BMAL1	Brain and muscle ARNT-like1
Ccgs	Clock-controlled genes
ChrDD	Chronodrug delivery systems
CLOCK	Circadian locomotor output cycles protein kaput
CRY	Cryptochrome
Doxil	Doxorubicin
DPD	Dihydropyrimidine dehydrogenase activity
FA	Folic acid
GBM	Glioblastoma
ICG	Indocyanine green
MTX	Methotrexate
NA	8-Naphthalimide
OS	Overall survival
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PER	Protein period
PLGA	Poly(lactic-co-glycolic acid)
PTX	Paclitaxel
SCN	Suprachiasmatic nucleus
SLN	Solid lipid nanoparticles

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STAT3 Transcription 3
TMZ Temozolomide

1 Mammalian Circadian Clock

All living organisms on Earth are in constant interaction with their environments, being influenced by external cues which exhibit cyclic patterns. Diurnal periodic variations with the day and night contributed the most for the evolution of species, including humans. These rhythmic changes favored the development of an internal timekeeping system, called biological clock, that keeps track of time allowing the organism to adapt and even anticipate their responses against external stimuli (Bhadra et al., 2017). The field that studies the effect of time on biological rhythms is called chronobiology and has recently gained significant attention with the 2017 Nobel Prize in Physiology or Medicine awarded for discoveries of underlying cellular mechanisms that control circadian rhythms.

Biological rhythms with a recurrence of about 24 h are called circadian, as illustrating by the endogenous secretion of glucocorticoids, characterized by a daily peak around the time of the sleep-wake transition and minimum level in the early part of the night (Oster et al., 2016).

In mammals, including humans, this 24-hour rhythm reflects the activity of a central pacemaker, a master circadian clock located in the suprachiasmatic nucleus (SCN) of the brain's hypothalamus (Hastings et al., 2020); however, the clock machinery can also be found in other brain areas, as well as in most peripheral organs. Neuron cells from SCN form a circadian network that generates rhythmic signals to connect the master clock to secondary peripheral clocks in the body through neuronal and hormonal pathways that regulate many different body functions. In return, these outputs ultimately feed back to the central nervous system and even to the SCN (Grosbellet & Challet, 2017; Mohawk et al., 2012).

To be completely efficient, however, this biological clock needs to be synchronized with external environmental light-dark cycle. Light is the main stimulus for entraining the SCN. Besides light, other environmental signals (zeitgebers), such as feeding-fasting and rest-activity cycles, can entrain circadian rhythms and are dominant zeitgebers (Quante et al., 2019; Pickel & Sung, 2020).

Photic stimuli reach the retina, which contains photoreceptors that transform the photon energy into an electric signal. This information is then transmitted to target neurons in the SCN through retinal ganglion cells (LeGates et al., 2014). At the cellular level, light activates signaling pathways in SCN and induces the expression of a specific set of "clock" genes that comprises the basis of the circadian clock. The key components of the clock include a complex interplay between positive and negative regulators of transcriptional and translational feedback loops (El Cheikh Hussein et al., 2019). The positive elements of circadian clock are the transcription factors circadian locomotor output cycles protein kaput (CLOCK) and brain and muscle ARNT-like1 (BMAL1), whereas the negative elements consist of the protein period (PER1, PER2 and PER3) and cryptochrome (CRY1 and CRY2). CLOCK

and BMAL1 form heterodimers and bind to regulatory element containing E-boxes to promote the expression of PER and CRY. The resulting PER and CRY proteins then dimerize and translocate to the nucleus where they accumulate and finally inhibit their own transcription by interaction with CLOCK/BMAL1, reinitiating another 24-hour cycle. PER and CRY complexes might also be subjected to phosphorylation in the cytoplasm and subsequent degradation. Another autoregulatory feedback loop involves the nuclear orphan receptors REV-ERBα/β and RORα/β/γ which repress and enhance the transcription of Bmal1, respectively. Furthermore, CLOCK/BMAL1 triggers the rhythmic expression of other genes, the so-called clock-controlled genes (ccgs) that regulate essential physiological processes in a tissue-specific manner (Fig. 1) (Korenčič et al., 2014; Buhr & Takahashi, 2013). There is an estimative that about 80% of mammalian protein-coding genes oscillate in their mRNA expression levels, impacting immune system, metabolic processes, cardiovascular function, cognition, memory, and so on (Zhang et al., 2014). Moreover, mutations or deletions of clock genes can affect this entire regulatory network affecting health in general. Misalignment between central and peripheral clocks, as well as between behavioral rhythms and endogenous clock caused by our modern lifestyle, changes environmental conditions and has also proven to increase the incidence of a series of widespread diseases (Baron & Reid, 2014; Finger & Kramer, 2021).

2 Circadian Disruption and Cancer Development

Circadian rhythms can regulate many intracellular pathways related to cancer development such as cell cycle, apoptosis, DNA damage, repair mechanisms, and apoptosis (Albuquerque et al., 2021; Lee et al., 2019; Morgan et al., 2019). Interferences in circadian rhythms can lead to a range of physiological diseases as metabolic syndromes (Ansermet et al., 2021; Kelly et al., 2020), depression (Nguyen et al., 2019), bipolarity (Palagini et al., 2019), cardiovascular diseases (Salazar et al., 2021), sleep associated disorders (Xing et al., 2021; Onat et al., 2020; Patke et al., 2017), neurodegenerative diseases (Obayashi et al., 2021), and autoimmune diseases. Besides, in the past few decades, there has been emerging evidence of a relation between the abnormal expression of circadian clock genes or abnormal circadian rhythmicity and higher cancer risk or cancer initiation and progression (Razi Soofiyani et al., 2021; Kiessling & Cermakian, 2017; Sulli et al., 2019; Li, 2019; Yuan et al., 2019; Reszka & Zienolddiny, 2018; Ye et al., 2018). Recent studies have found that Per1 knockdown significantly decreases the transcript expression levels of p53, a well-known tumor suppressor. Moreover, another study found that at the early G1 phase, the BMAL1/CLOCK heterodimer downregulates the transcription of c-Myc oncogene (involved in G0/G1 transition) to prevent its overexpression (Li, 2019). BMAL1/CLOCK complex also controls the expression of the Wee1 gene, an important G2/M checkpoint kinase. Therefore, alterations in this clock protein expression can lead to several implications in cell cycle and cancer development. Besides, loss of circadian homeostasis is also associated with poor

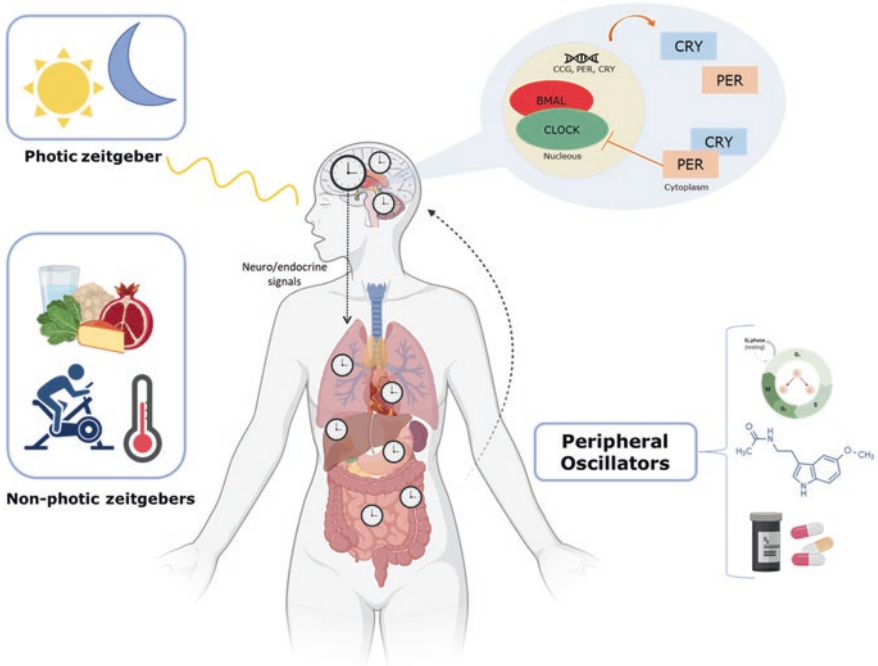

Fig. 1 The circadian clock system. Through evolution, most organisms developed an internal timekeeping system. Circadian clocks consist of a hierarchical network, with a master clock located in the SCN of the brain's hypothalamus, synchronized mainly by the light. Other peripheral clocks were identified in other brain regions and in most peripheral organs. The function of peripheral clocks is regulated through neuronal and hormonal pathways (from the central clock), feeding, and other external cues. In return, these outputs ultimately feed back to the central nervous system and even to the SCN. At a molecular level, this clock is based on an autoregulatory transcription-translation feedback loop comprised by clock genes/proteins that regulate several physiological processes, as hormone secretion, cell division, and drug pharmacokinetics, among others

performance of anticancer treatments and early mortality among cancer patients (Fu & Kettner, 2013). Thus, the International Agency for Research on Cancer, which belongs to the World Health Organization, listed circadian rhythm disorders as possible human carcinogens in 2007 (Group 2A) (Yao et al., 2021). The disruption of regular daily patterns and daily rhythms (in sleep-wake, meal intake, cell cycle, and metabolism) because of the new modern society patterns such as night shift work, jet lag, exposure to bright light at night, sedentarism, and bad food choices is known to contribute to several types of cancer (Damato & Herzog, 2021; Barbosa Vieira et al., 2021) (Fig. 2). Geographic factors or geological cycles like daylight and darkness in different seasons can also affect circadian rhythms (Weissová et al., 2019). Breast (Lesicka et al., 2018; Lellupitiyage Don et al., 2019; Pham et al., 2019), colon (Bishehsari et al., 2020; El-Athman et al., 2018), prostate (Dickerman et al., 2016), lung (Xiang et al., 2018), and colorectal cancer (Liu et al., 2021) are cancer types already documented to be linked to this desynchronization. For example, it

has been shown that there is a highly significant statistical association between polymorphisms of some circadian genes (cry1/2, arntl2, csnk1e, nr1d1/2, per1/2/3,,clock, npas2, and rora/b/c) and the risk of some breast, prostate, and lung cancers (Mocellin et al., 2018). Emerging evidence suggests that tumor hypoxia-induced acidosis inhibits the transcription and alters protein stability of BMAL1 to promote breast cancer metastasis (Kwon et al., 2020). Another study systematically analyzed clock gene alterations in 32 cancer types considering the Cancer Genome Atlas, Cancer Therapeutics Response Portal, and Genomics of Drug Sensitivity in Cancer databases. Researchers found that expression alterations in these genes are linked with key oncogenic pathways, patient survival, tumor stage, and subtype in multiple cancer types (Ye et al., 2018). Recent experiments also show that daily eating patterns as overnight meal intake or time of dinner can influence cancer risk (Sulli et al., 2019; Kogevinas et al., 2018). Finally, a large cohort of study suggests an association between late eaters and circadian perturbations that may lead to breast or prostate cancer development (Srour et al., 2018).

Remarkably, this theme continues to cause some controversy, and the role of the circadian clock machinery in cancer cell development and survival remains elusive (Papantoniou et al., 2018; Leung et al., 2019; Wendeu-Foyet et al., 2018; Cordina-Duverger et al., 2018; Kogevinas et al., 2018). As the most of published results have not always been replicated and some effect sizes have been small (Damato & Herzog, 2021), some researchers consider that the circadian rhythm disruption may be a consequence of cancer progression, but not its driving factor. Some extra factors should be considered, namely, sexes, chronotypes, genetic and/or environmental factors, tumor genotype, type of chronodisruption (e.g., advancing vs. delaying shift schedules), time of sample collection, and exposure duration (Damato & Herzog, 2021).

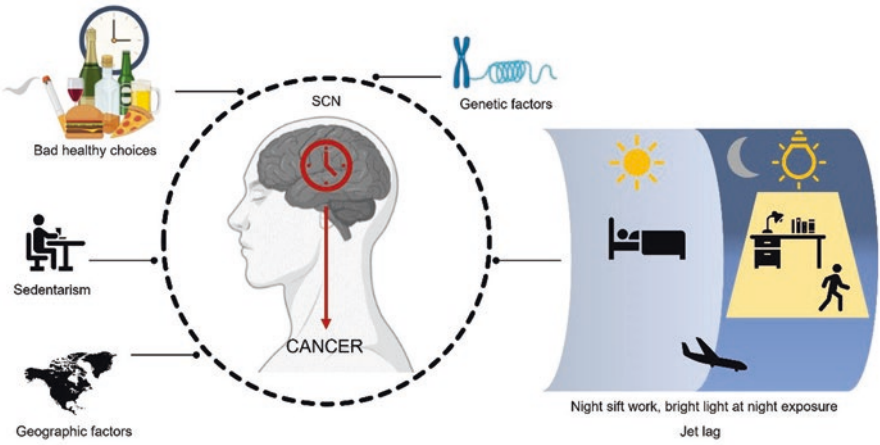

Fig. 2 Circadian rhythmicity disruption and cancer risk. Disruption of regular daily patterns involved in dysregulation of circadian rhythm

3 Chronotherapy for Cancer Treatment

Chronotherapy, also called chronomedicine, is a strategy that coordinates a person's biological rhythms with medical treatment, considering the timing and amount of a medication to optimize the drug desired effect. Besides increasing drug effectiveness, choosing the most suitable time of the day might also reduce undesirable side effects. A great amount of literature supports the importance of timing for treatment outcomes of a broad range of diseases. For instance, bronchodilators to treat asthma are dosed in the evening, since the most severe symptoms occur around 4 a.m. (Lodhi et al., 2019). Some lipid-lowering drugs are most effective when taken before bedtime because the level of its target enzymes peaks at night (Awad et al., 2017). Also, many studies have shown that taking hypertension medications in the evening improved blood pressure dropping, decreasing the risk of heart attack. This fact is related to angiotensin 2 receptor, involved in blood pressure regulation, which is maximally expressed during the nighttime (Carter et al., 2014).

Despite the advances in cancer research, it remains one of the leading causes of death worldwide with limited treatment options. Anticancer drug resistance and overall cytotoxicity of chemotherapy to both normal tissues and malignant cells represent reasons for current treatment failures (Mansoori et al., 2017). Thus, it is important to invest in new approaches to improve efficacy but also reduce toxic side effects in host tissue damage in order to optimize health benefits.

Five decades ago, Franz Halberg, one of the founders of modern chronobiology, envisioned the idea of a circadian-based therapy for cancer, given the differential tolerance to treatment derived from the intrinsic host rhythms (Halberg et al., 2006). Cancer chronotherapy is inciting much attention on medical field as a novel approach to improve current cancer treatment options (Sulli et al., 2018). Accumulating evidence indicates that the inclusion of chronobiology into cancer therapy will likely improve treatment efficacy of many types of cancer, diminishing side effects and enhancing patient survival. The clinical relevance of cancer chronotherapy has been investigated in a considerable number of clinical trials and animal models.

The earlier works on the field demonstrated that the efficacy of over 30 anticancer drugs varies up to 50% as a function of administration time (Innominato et al., 2014).

Recently, a retrospective analysis explored whether timing of temozolomide (TMZ) administration, a standard chemotherapeutic agent for glioblastoma (GBM), could affect GBM patient outcome (Damato et al., 2021). The study reviewed patients with newly diagnosed GBM who had surgery and chemoradiation and were prescribed TMZ to be taken in the morning or evening. Overall survival (OS) was analyzed.

Patients taking morning TMZ exhibited longer OS compared to evening. Interestingly, a previous study in both human and mouse GBM cells had shown that the time of treatment affected TMZ sensitivity of murine GBM tumor cells in vitro (Slat et al., 2017). It was found that the maximum TMZ-induced DNA damage response, apoptosis, and growth inhibition corresponded to the daily peak

expression of the clock gene Bmal1. Additionally, the deletion of Bmal1 abolished TMZ-induced activation of apoptosis. These data suggest that circadian rhythms can regulate TMZ cytotoxicity.

Other clinical trials have indicated significant clinical benefits from specific circadian timing of chemotherapy or radiotherapy (Table 1).

Another recent chronotherapy trial in patients with metastatic colorectal cancer evaluated the safety and efficacy of chronomodulated triple therapy given over 5-day period every 3 weeks (Innominato et al., 2020). Results revealed that timed combination therapy with irinotecan, oxaliplatin, 5-fluorouracil (5-FU), and leucovorin resulted in delayed time to progression and increased overall survival with increased tolerance and safety, compared to routine chemotherapy.

Several mechanisms might be responsible for the benefits of timed delivery of anticancer drugs. Target genes, enzymes, and molecules involved in effectiveness and toxicity of drugs might be expressed rhythmically. In that concern, pharmacokinetics and pharmacodynamics are essential determinants of time-dependent drug effects (Ayyar & Sukumaran, 2021). Today, it is recognized that time-dependent toxicity and efficacy of medications reflect circadian changes in processes of pharmacokinetics. Pharmacokinetics refers to absorption, distribution, metabolism, and excretion (ADME) of a given drug, determining its concentration in target tissues. Moreover, variations in physiology and tissue gene expression can influence pharmacodynamic responses, that is, the effect of drugs on the body (Dong et al., 2020). For example, the variability in 5-FU toxicity is dependent on circadian oscillations in thymidylate synthase activity, its molecular target, and dihydropyrimidine dehydrogenase activity (DPD), the rate-limiting enzyme responsible for its elimination. A clinical study found that DPD activity in peripheral blood mononuclear cells showed circadian rhythmicity, with peak activity during the night modulating the time-dependent bioavailability and efficacy of 5-FU (Jacobs et al., 2016). Besides, most of the anticancer drugs show their cytotoxic effects at specific phases of the cell division cycle, which is also under the influence of circadian rhythms. For example, cells in the S-phase are more susceptible to 5-FU and irinotecan. The number of cells in S-phase and in G2/M phase increases by about 50% in the second half of the darkness period, while G0/G1 cells predominate during the light period in the bone marrow tissues in mice (Lee, 2021; Ozturk et al., 2017). Thus, these findings support for timed chemotherapy approaches to coincide with times of high tumor cell vulnerability and low toxicity to normal tissues. Besides chemotherapy, some studies show that chronoradiotherapy used alone or in combination with chemotherapy could represent a promising approach in clinical practice as well (Bermúdez-Guzmán et al., 2021).

Besides nowadays the relevance of circadian time is well known in pharmacology, and the time of treatment administration is not usually considered in clinical practice. The clinical translation of chronotherapy to diseases such as hypertension, arthritis, and asthma is now standard practice (Baraldo, 2008); nonetheless, the history of cancer chronotherapy includes distinct results and some scepticism. Some reasons for inconsistencies regarding this approach are related with differences in methodology of the studies, differences between sexes, chronotypes, environmental

Table 1 List of cancer chronotherapy studies

Therapy	Cancer type	Chronomodulated schedule	Main findings	References
5-fluorouracil, leucovorin, and oxaliplatin	Metastatic colorectal	Chronomodulated infusions of 5-fluorouracil and leucovorin from 22:15 to 09:45h with a peak at 04:00 h and oxaliplatin from 10:15 to 21:45 h with a peak at 16:00 h	Overall survival was improved in males on chronomodulated schedule when compared with conventional therapy	Giacchetti et al. (2012)
Radiotherapy	Bone metastases	8:00 a.m.–11:00 a.m. 11:01 a.m.–2:00 p.m. 2:01 p.m.–5:00 p.m. group	Females in the 11:01 a.m. to 2:00 p.m. cohort exhibited a significantly higher response rate	Chan et al. (2017)
Paclitaxel-loaded polymeric nanoparticles	Lung	Administration time points: 0, 5, 10, and 15 h after light onset on mice	Enhanced antitumor effect at 15 h after light onset	Hu et al. (2017a)
Cisplatin combined with radiotherapy	Locoregionally advanced nasopharyngeal	Two cycles of chronomodulated infusion (peak delivery of cisplatin at 16:00 p.m.) or flat intermittent infusion synchronized with radical radiotherapy (in both groups)	Better outcomes for adverse reactions and immune functions on study group but no significant difference in 2-year overall survival	Zhang et al. (2018)
Cisplatin, 5-fluorouracil, and radiotherapy	Nasopharyngeal	Chronomodulated chemotherapy of cisplatin from 10:00 to 22:00 h and 5-fluorouracil from 22:00 to 10:00 h each day for 3 days plus Patients in the routine chemotherapy group received cisplatin infusion within 1 h and 5-FU infusion for about 24 h Total irradiation dose was the same for both groups	Higher overall response in chronomodulated group The incidence rates of leukocytopenia, thrombocytopenia, and nausea/vomiting were significantly lower The incidence of radiation-induced complications was similar in the two groups	Gou et al. (2018)
Temozolomide	Glioblastoma	Morning or evening administration	Patients taking morning TMZ exhibited longer overall survival compared to evening	Damato et al. (2021)

factors, interindividual differences, and clock gene polymorphisms that further impact interaction between host, cancer, and treatment outcomes (Selfridge et al., 2016). To overcome some limitations and make a step toward personalized chronotherapy, some strategies might include (a) using mathematical models to predict treatment toxicity and optimize the optimal time of drug administration (Hesse et al., 2021), (b) detailed measures of the individual differences in circadian biology and circadian biomarkers to further optimize the potential benefit of chronotherapy for each patient (Crnko et al., 2021), (c) more phase III trials to firmly establish chronotherapy in medical oncology, and ultimately (d) the development of tumor-specific targeted drug delivery systems to diminish side effect on normal tissues. The addition of the dimension of time in medicine might undoubtedly make a tremendous difference in cancer patients' survival.

4 Nanotechnology Applied for Cancer Treatment

Conventional therapies used to fight cancer cause several adverse effects and do not prevent recurrence and drug resistance (Goldman et al., 2016; Briolay et al., 2021). One of the approaches studied in the recent decades is the application of directional therapies using nanoscale delivery systems. Targeted therapies can be used in place of or simultaneously with radiotherapy and chemotherapy to reduce their adverse effects (Cheng et al., 2019). The most common nanocarriers in this type of therapy are micelles, polymers, liposomes, lipids, and peptides (Mitchell et al., 2021). These materials are among the most used due to their physicochemical properties, which allow them to encapsulate and transport a wide variety of biopharmaceuticals and promote their controlled and targeted release (Tang et al., 2021; Levit et al., 2020). Thus, these systems make it possible to reduce the dose of the therapeutic agent and prevent healthy cells from being affected. Furthermore, these delivery systems show good biocompatibility and lack of immune response (Briolay et al., 2021). Figure 3 schematics the most common developed delivery systems for therapeutic payload release in cancer therapy.

Micelles are a set of amphiphilic molecules that form nanoparticles through a self-assembly process. Micelles have been widely used in cancer therapy due to their ability to transport high drug loads and their capacity to deliver drugs with low solubility (Kapare & Metkar, 2020; Croy & Kwon, 2006). One of the characteristics of micelles is that they are easy to handle and can be modified to provide targeted delivery to target tissues. Recent studies demonstrate the efficacy of several carriers based on polymeric micelles applied to cancer treatment. Jeanbart's group developed a nanosystem using an embedded anticancer drug. Thioguanine-loaded polymeric micelle nanoparticles demonstrated a therapeutic effect superior to the use of the drug thioguanine alone, with a reduction of induced tumors in mice models after 2 days of its injection (Jeanbart et al., 2015). Another group used micelles in the formulation of nanocarriers in order to deliver the inhibitor JSI-124 into tumor cells. JSI-124 is an inhibitor of signal transducer and activator of transcription 3 (STAT3),

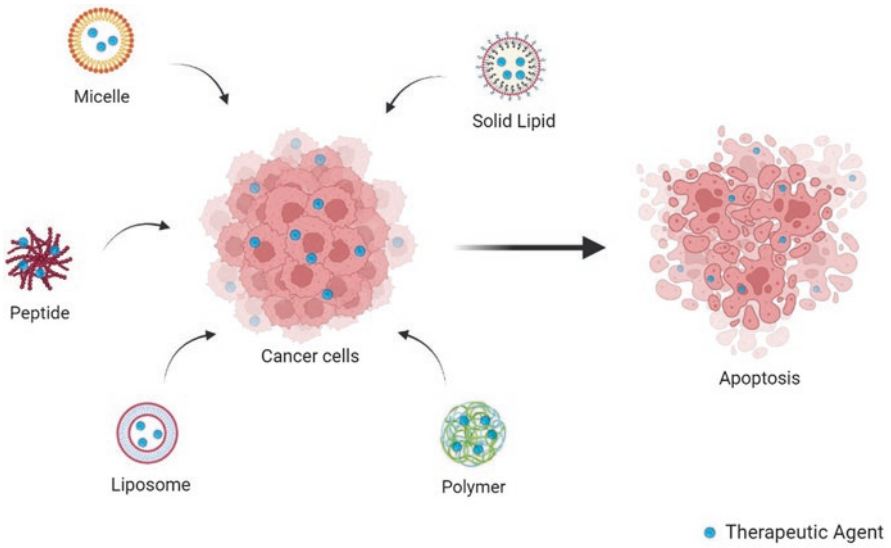

Fig. 3 Nanotechnology in cancer: the most commonly investigated nanosystems for the delivery of therapeutic agents toward cancer cells, to improve cancer therapy

involved in the process of cancer tumor progression. These nanoconjugates demonstrated a greater ability to inhibit STAT3 than the use of free drugs (Garg et al., 2017). Another study revealed that the use of linear polyethyleneimine (PEI)-based nano-micelle vectors was able to internalize in ovarian cancer cells, in vivo and in situ. These nanoparticles thus enabled the release of interfering RNA while transforming these cancer cells into efficient antigen-presenting cells that activated tumor-reactive lymphocytes and tumoricidal activity (Cubillos-Ruiz et al., 2009).

Polymers are a class of materials frequently applied to develop suitable delivery systems for the treatment of cancer, namely, synthetic ones. Polyplexes are formed primarily by electrostatic interactions between the positive charges of amine groups from the polymer and the negative charges of nucleic acids (Faria et al., 2019). There is a wide variety of polymers; however, the most used recently are biodegradable polymers that allow targeted and efficient delivery (Din et al., 2017). Another feature that facilitates the study of this type of material is the fact that it can be modified to improve the release of the therapeutic agents it carries. Polymers such as PEI, dendrimers, or poly(N-isopropyl acrylamide)s have demonstrated this potential in recent studies. One study demonstrated the synergistic effect of using an anticancer drug methotrexate (MTX) together with gene therapy using a DNA plasmid containing the p53 tumor suppressor protein gene. The results showed that the therapeutic effect was greater in cells transfected with the MTX-PEI-pDNA vectors than in cells transfected with the PEI-pDNA systems. The effect of the MTX-PEI-pDNA systems was also compared with the effect of free MTX, again showing a greater therapeutic effect in cells transfected with the systems containing the two therapeutic agents. The group led by Costa demonstrated that systems developed based on

polymers inhibit uncontrolled cell proliferation in cervical cancer cells (Faria et al., 2019). Another study using polymers in the formulation of nanosystems developed a hydrogel capable of functioning as a dual drug carrier. PNIPAM-co-A nanoinjectable hydrogel demonstrated suitable pH responsiveness, allowing for a controlled and sequential release of drugs in tumor tissues (Xu et al., 2021; Wu et al., 2020).

Peptide-based nanocarriers have been studied for specific cancer cell targeting and payload release. The peptides used are generally short, with less than 30 amino acids that are divided into arginine-rich and amphipathic peptides (Ruseska & Zimmer, 2020). Peptides that allow the introduction of biopharmaceuticals into cells are called cell-penetrating peptides. These peptides are easily synthesized using solid-phase methods, providing space for sequence-specific modifications at the molecular level (Rubert Perez et al., 2015). Another advantage is the fact that they can be easily functionalized to ensure specific targeting. The fact that they self-assemble facilitates the formulation of nanoparticles capable of carrying drugs or nucleic acids toward cancer therapy (Habibi et al., 2016; Wang et al., 2017). Several studies have been conducted using peptides to deliver cancer-fighting drugs. The peptides have demonstrated the ability to encapsulate anticancer drugs such as doxorubicin (Doxil), paclitaxel (PTX), curcumin, and FU. Some of these systems have already been tested in preclinical and clinical trials, revealing that they are safe and biodegradable (Habibi et al., 2016). Different studies demonstrate that peptides used as ligands on the surface of other systems can provide targeting to tumor cells of various cancers (Wang et al., 2017; Jiang et al., 2019; Pearce et al., 2012).

Liposomes can be used as nanocarriers as they form lipid vesicles that allow the encapsulation and delivery of both hydrophobic and hydrophilic drugs. These systems have characteristics such as their biodegradability, reduced toxicity, and low immune response (Hashemzadeh et al., 2020). Liposomes are the most characterized and investigated delivery systems for tumor immunotherapy in medicine. There are several FDA-approved liposomal drugs, encapsulating chemical compounds or nucleic acids such as siRNA (Sousa et al., 2018). Liposomal Doxil was one of the first liposomes approved for therapeutic use (Sousa et al., 2018). More recently, liposomes have been developed that respond to temperature stimuli, which allows drug release specifically in tumor tissues. This kind of strategy makes it possible to increase the therapeutic effect and safeguard normal tissue (Swenson et al., 2015). Another study demonstrated that altering the surface of a liposome with a polymer sensitive to pH variations allowed targeted delivery to the tumor, benefiting from the acidic microenvironment of tumor tissues (Yoshizaki et al., 2014). Other liposome-based systems have demonstrated that they can be an excellent strategy in the fight against cancer (Li et al., 2020). Jia and his team used liposomes to create a system targeting the epidermal growth factor receptor. This receptor is overexpressed in cancer cells, being a desirable target for the delivery of anticancer drugs. This study demonstrated that cisplatin-loaded PEGylated liposome vectors revealed the ability to internalize and accumulate in tumor tissues, leading to increased apoptosis and consequent tumor suppression (Jia et al., 2022).

Lipid-based systems have conquered the interest of researchers due to their high affinity for the intracellular space. These lipids can be liquids or solids at room

temperature; however, solid-state systems are the most explored. Easy handling, small size, and cellular absorption are the main properties that make them attractive for therapeutic application (Sezgin-Bayindir et al., 2021; Rehman et al., 2021). Solid lipid nanoparticles (SLN) have been studied for innovative therapies in the treatment of cancer. Studies show that lipid-based systems can be functionalized, namely, with peptides, to display high performance for the treatment of cancer (Hu et al., 2017b; Majidinia et al., 2020). Pearce and coworkers demonstrated that these delivery systems are capable of delivering the drug Doxil to cancer cells (Pearce et al., 2012). Lactoferrin-conjugated lipids were also investigated for the treatment of lung cancer. This conjugated system allowed for a targeted delivery of the drug rifampicin. Lactoferrin-SLN-rifampicin have been shown to be small-sized and surface-charged systems suitable for the transfection of cancer cells (Shilpi et al., 2015). Lipid-based nanosystems have demonstrated their potential and diversity as delivery systems for advanced therapies in cancer treatment (Yang et al., 2020).

In addition to the therapeutic potential, nanotechnology can also be used to investigate diseases. The combination of diagnostic/imaging and therapy approaches is called theranostic (Pant et al., 2017). Nanotheranostics consists of the use of nanoscale systems that allow the simultaneous monitoring of drug release and distribution and the assessment of therapeutic efficacy (Indoria et al., 2020). These nanosystems make it possible to develop individualized therapies suitable for each disease, including cancer (Xie et al., 2010). In recent decades, various systems for theranostics of cancer have emerged (Indoria et al., 2020). Its development arose from the need to find more efficient therapies with fewer adverse effects than conventional therapies, thus realizing a more personalized therapy. Another advantage of these systems is that they allow the identification of metastases, improving their detection in relation to conventional diagnostic techniques (Choi et al., 2012; Ali et al., 2020). Efficient targeting of theranostic nanoparticles to the tumor site is critical for both diagnosis and therapy, with the use of targeting ligands being crucial to recognize and selectively bind to receptors that are overexpressed on certain tumor cells (Chen & Cai, 2014; Chen et al., 2014).

A recent study has shown that the use of HER2-targeted magnetic iron oxide nanoparticles carrying cisplatin made it possible to reduce not only the growth of the primary tumor but also of peritoneal metastases (Satpathy et al., 2019). The nanoparticles created by Satpathy's group also showed good retention in tumor tissues, allowing the identification of residual resistant tumors by in vivo molecular imaging and histological analysis of the tumor tissues (Satpathy et al., 2019). The work developed by Revia's group demonstrated that the formulation of iron oxide and gold core drug delivery vehicles can deliver RNAi with the aim of silencing genes involved in cell proliferation and consequent uncontrolled cell growth. These nanoparticles can also be modified and functionalized with polymers to avoid recognition by the innate immune system, thus allowing greater fixation of these systems in tumors and providing a more efficient delivery of therapeutic agents and optical imaging probes (Revia et al., 2019). The fact that these nanoparticles have metallic cores allows their tracking inside the organism. Systems with iron oxide cores provide contrast enhancement in magnetic resonance imaging, a noninvasive

imaging technique. Gold nanoparticles can be used as a contrast agent in photoacoustic and X-ray CT imaging. The ability to track these systems brings diagnostic information by locating tumor tissues. These systems work as theranostic agents, enabling a tracking of biodistribution and delivery of drugs at the same time that they work as a diagnostic method and therapeutic agent (Revia et al., 2019). Other theranostic systems have been developed to be applied in the treatment and detection of different tumors. For the theranostics of liver and brain cancers, a study reveals that the use of chitosan-Cy-5.5 systems enhances the ability to detect this type of tumors while providing antitumor activity, with low nonspecific uptake by normal cells. The use of near-infrared fluorescent allowed to visualize the location of the tumors, while the encapsulation of the anticancer drug PTX by the chitosan allowed that its toxic effect was less than the drug administered freely (Kim et al., 2010). Several works have focused on the theranostics for breast cancer (Hou et al., 2011; Liu et al., 2012; Zheng et al., 2012). Hou focused on detecting cancer cells that overexpress the folate receptor. Formulated PCMS-NA-FA nanoparticles included folic acid (FA) and 8-naphthalimide (NA). It confirmed that these systems showed greater cellular uptake in cancer cells such as HeLa cells and could function as detection agents (Hou et al., 2011). Peptide-based systems have also been used in breast cancer theranostics. Liu and her group demonstrated that using mPEG-PLGA-PLL nanoparticles was able to encapsulate and deliver an imaging agent (rhodamine) and an anticancer drug mitoxantrone both in cells in vitro and in in vivo models (Liu et al., 2012). Another study showed that the use of lipid-based nanovectors made it possible to overcome difficulties in the use of the fluorescent agent Indocyanine green (ICG), a fluorescent compound with wide biological applications. The systems demonstrated significant stability against photobleaching and long circulation time, superior to free ICG. In vivo studies have demonstrated their specific targeting to cancer cells, being able to be used as a diagnostic agent and targeted imaging (Zheng et al., 2012). Guan and his work group created PLGA-4-arm-PEG-IR-140/Nile red nanoparticles for theranostic pancreas carcinoma. Cells transfected with these systems showed higher levels of p53, Bax, and caspase-3 than non-transfected cells after 48 h, which may mean greater activation of the mitochondrial pathway of apoptosis (Guan et al., 2012). Nanotheranostics is a promising field for the development of new treatment and diagnosis methodologies for various diseases, which may replace or complement conventional methods. This area opens a route of possibilities for the design/development of advanced and high-performance nano delivery systems to be applied in cancer therapy.

5 Chronotherapy and Nanotechnology

As mentioned above, conventional therapeutic approaches could be improved for better bioavailability and target delivery on the tumor site to minimizing adverse effects on healthy noncancerous cells.

Over the last years, new findings on cancer chronotherapy also encouraged the development of novel chronodrug delivery systems (ChrDD) intended for chronotherapy application by different routes of administration (Youan, 2010; Ozturk et al., 2017). Drug delivery systems have the potential of increasing drug bioavailability per se, releasing a bioactive molecule at a specific site with a specific delivery rate (Ketabat et al., 2019), but timing in vivo drug administration to match circadian rhythms of disease is expected to enhance even more therapeutic outcomes. Besides, ChrDD could also reduce treatment duration, nursing intervention, and healthcare expenses.

Innovative technologies for chronomodulated drug delivery used in some clinical trials include external electronic programmable pumps. Melodie™ is a programmable pump that was used before in clinical trials for the delivery of irinotecan, oxaliplatin, and 5-FU into the central vein of metastatic colorectal cancer patients (Ortiz-Tudela et al., 2016). Recently it was used for the delivery of three anticancer drugs into the hepatic artery of colorectal cancer patients with extensive liver metastases (Lévi et al., 2017). However, this system showed some inconsistencies regarding programmed delivery schedules and observed drug concentration within the patient blood. A more attractive approach is the combination of chronobiology with nanotechnology, allowing the delivery of anticancer drugs and/or therapeutic genes to overcome the limitations of current methods. However, very few studies consider the influence of circadian rhythms in the effectiveness of nanocarriers systems. To the best of our knowledge, the first study to consider the effect of time explored the influence of chronobiology on the processes of nanoparticle uptake and transport into the brain (Kreuter, 2015). Nanoparticles were loaded with the analgesic drug dalargin and then injected in mice. The pain reaction of mice was significantly influenced by the time of the day of intravenous administration. The difference between the daytime and nighttime effects was over 20% with best results during the morning time, showing that the outcome of results concerning the drug transport across the blood-brain barrier using nanoparticles significantly depends on the time of day of the experiments.

Concerning cancer treatment, we report two recent studies in this matter. In the first, the antitumor effect of PTX-loaded polymeric nanoparticles (PTX-NPs) combined with circadian chronomodulated chemotherapy in xenografted human lung cancer was tested to screen out to optimum time for the drug delivery (Hu et al., 2017a). PTX-NP cytotoxicity on cancer cells was proven to be affected by the different administration times, which was the best at 15 h after light onset. The expression of two proliferation and angiogenesis markers was lowest at that time point, as the number of apoptotic cells, suggesting a significant antitumor efficacy.

More recently, a study intended to explore the effect of circadian rhythm in the performance of nanoparticles for drug and gene delivery to cancer cells (Albuquerque et al., 2022). The researchers used PEI to encapsulate the anticancer drug MTX and a p53 encoded plasmid DNA (pDNA) and performed transfection at six different time points. In this preliminary study, cell uptake, internalization, and efficacy of transfection were compared during 24 h by means of quantification of cell associated MTX fluorescence and p53 protein expression. High levels of MTX were found

in cancer cells at some specific time points, indicating a possible effect of circadian regulation in nanoparticle internalization. Additionally, high levels of p53 were observed for the same time points at which MTX was more abundant. Moreover, a comparative study was performed on a noncancer cell line, human fibroblasts (Albuquerque et al., 2022). Although poor levels of MTX and p53 protein were found, results also indicated rhythmicity, demonstrating the influence of circadian rhythm on both cancer cells targeting ability and transfection performance. Altogether, these results unravel some mechanisms underlying the effect of circadian rhythm in the performance of nano-based delivery systems. The combination of chronobiology with nanotechnology represents a novel step toward the progress in cancer therapy, and hopefully, these results will stimulate additional investigation on this topic.

6 Conclusions

For centuries, the subject of chronobiology was limited to the study of circadian rhythm in plants. Only during the last decades, the research has been extended to include animals and human beings. Nowadays, chronobiology has emerged as a new, multidisciplinary, and exciting field. Now that the molecular mechanisms of circadian clocks are better understood, the bidirectional relationship between circadian rhythms and heath can no longer be disregarded. In mammals, molecular clocks are present in practically all tissues influencing a wide range of physiological and metabolic functions. Circadian clock genes play roles in the control of the cell cycle, DNA repair mechanisms, and expression of tumor suppressor genes. Thus, the abnormal expression of circadian clock genes and dysregulation of circadian rhythms by environmental factors trigger oncogenic signaling pathways. A long account of research on animal models and human tumor samples revealed that the dysregulation of circadian rhythms is a causing factor for cancer development. Moreover, it was found that some cancers “live” by the clock. The integration of this finding gave rise to a new concept for treating cancer according to biological rhythms, termed chronotherapy. As conventional cancer therapies still hold some limitations, the integration of chronotherapy in clinical practice is seen as a tool to improve therapeutic effect while reducing toxicity. Nanotechnology has also been explored in cancer research. The design and development of suitable drug delivery systems could also improve cancer therapies. In fact, the optimal cancer therapy should depend upon delivery of a drug at the right time, to the right target, and in the right amount. The combination of chronotherapy with nanotechnology could overpass the current limitations of conventional treatment.

Nonetheless, although promising, chronotherapy has still been maintained in the fringes of clinical practice. For chronotherapy to be viable in clinical setting, one must consider the many factors influencing clinical outcomes. Age, genetic variations, sex, and environmental factors are determinant points of an individual’s chronotype and are of extreme importance when discussing personalized medicine.

Additionally, only recently, studies focused on the investigation of the influence of the circadian system on the therapeutic effect of drug delivery systems. Current works still disregard the involvement of circadian system on processes concerning the application of nano delivery systems, such as cell uptake, internalization of the nanocarrier, mechanism of action, and the induced therapeutic effect. More solid data are mandatory to bring more enlightening into this promising field. The bridge between chronobiology and nanotechnology might certainly have a great impact in personalized cancer treatment.

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The Function of DNA and RNA Nanovaccines in the Treatment of Cancer

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Abbreviations

AML	Acute myelogenous leukemia
APCs	Antigen-presenting cells
CTLs	*Cytotoxic T lymphocytes*
DCs	Dendritic cells
DOX	Doxorubicin
EP	Electroporation
FLT3L	Fms-like tyrosine kinase-3 ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ID	Intradermal
IGFBP2	Insulin-like growth factor binding protein-2
IGF-1R	Insulin-like growth factor 1 receptor precursor
IM	Intramuscular
IV	Intravenous
Mam-A	Mammaglobin-A
MHC	Major histocompatibility complex
mRNA	Messenger RNA
mTa_2O_5	Mesoporous Ta_2O_5
NGS	Next-generation sequencing
NPs	Nanoparticles
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
SLPs	Synthetic long peptide
TAA	Tumor-associated antigens
TSA	Tumor-specific antigens
VLPs	Viruslike particles

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1 Introduction

Cancer is a disease caused by genetic changes in some of the body's cells which are involved in abnormal cell growth that invade or spread into surrounding tissues or other parts of the body. The International Agency for Research on Cancer Worldwide reported an update about cancer incidence and mortality which estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020 (Sung et al., 2021).

Female breast cancer, lung, colorectal, prostate, and stomach are known as the most prevalent cancers, and lung, colorectal, liver, stomach, and female breast cancers are discerned as cancer with a high mortality rate (Sung et al., 2021). The current trend in the global diagnosis of cancer and mortality indicates an increase in the survival rate of individuals with cancer, particularly in developed countries (McCormick, 2018).

For a decade, heart disease and cancer have been introduced as the first and second causes of death, respectively, in the world, but according to statistical models estimated by the WHO, after 2030, cancer will become the leading cause of death worldwide (Mattiuzzi & Lippi, 2019).

Cancer care costs about 20% more than heart disease. In 2019, national financial estimates in the USA showed that the economic burden of cancer is $ 21.09 billion, which includes the patient's direct costs and the time spent by the patient. The highest consumption costs are spent on breast, prostate, colorectal, and lung cancers (Yabroff et al., 2021). According to the American Cancer Society, obesity and smoking have been identified as the leading causes of cancer incidence in the USA (Bandi et al., 2021; Caliri et al., 2021; Avgerinos et al., 2019; McCormick, 2018).

Conventional cancer treatments are established mainly by the combination of invasive methods such as surgical procedures, radiotherapy, and chemotherapy. However, the widespread side effects and noneffectiveness of these treatments in all cancer types have persuade interest to the appliance of novel approaches (McCormick, 2018; Li et al., 2018; A Baudino, 2015). Some evidence demonstrated that such invasive surgery prepares a favorable condition for metastasis (McCormick, 2018). Besides, the usage of chemotherapeutic agents has some limitations due to their nonspecific distribution, toxicity, and side effects (Nosrati et al., 2022). Moreover, in some cancer treatments due to recurrence and multidrug resistance, successful eradication is not achieved (Liu et al., 2020).

Due to the complications of conventional treatments, in cancer treatment, it is necessary to design and create new treatment methods that are more efficient and highly accurate with fewer side effects in the treatment of cancer patients.

In this review, a novel approach with a more specific targeting capability of tumor cells based on nanovaccine platforms will be extensively discussed. This review provided a detailed overview of current and emerging nanovaccines against cancer, highlighting their benefits and challenges in cancer treatment.

2 Vaccine in Treatment and Prevention

In recent years, new approaches in cancer therapy have fascinated increasing interest including DNA-based vaccine, immunotherapy-based strategy, and oncolytic virus therapy which showed protective and therapeutic abilities to improve the clinical outcome.

The proficient approaches with lower side effects and high therapeutic efficacy in clinical therapy could be selected in cancer therapy in the near future (Nosrati et al., 2022; Nandedkar, 2009). Cancer treatment vaccines are the vaccines that have protective and therapeutic functions against the existing and the development of new tumors (Emens, 2008).

To develop more potent cancer vaccines, it is necessary to fully understand which kind of vaccine is effective and how cancer vaccines work. Intradermal, subcutaneous, intravenous, and intramuscular administration causes cancer vaccines to be exposed to antigen-presenting cells (APCs). APCs (e.g., dendritic cells (DCs), macrophages, and B cells) uptake vaccine components and present the antigens on major histocompatibility complex (MHC) class I or II. Through the afferent lymph, APCs travel toward the lymph nodes, where priming and activation of T cells occurs. Then, the activated effector T cells migrate to the blood and infiltrate the tumor microenvironment, where effector T cells recognize and destroy specifically targeted cancer cells (Liu et al., 2020).

The presentation of antigen by APCs occurs via two major routes. In the first pathway, in the cytosol of APCs, endogenous antigenic proteins are well degraded by proteasomes. The degraded peptide fragments are presented by MHC-I molecules on the cell surface, which provoke activation of *tumor antigen-specific* CD8+ *cytotoxic T lymphocytes* (*CTLs*) which are the main antitumor cells in the body (Liu et al., 2020). In second pathways, exogenous antigenic proteins are taken up by APCs via endocytosis, and then these molecules are degraded to peptide fragments which are presented on MHC-II molecules to activate CD4+ T cells that encompass helper role and enhance CTL activation and their extended survival. Therefore, the administration and delivery of cancer vaccines in a professional platform that can quickly activate CD4+ T cells and CTLs is a vital step for effective antitumor therapy (Liu et al., 2020; Azadniv et al., 2011).

Cancer-specific antigens are occasionally certain molecules that are presented only on the surface of cancer cells. In cancer treatment vaccines, these molecules are candidate molecules for targeting tumor cells and trigger antitumor immune responses (Zitvogel et al., 2008).

Cancer vaccines boost the host immune system's ability to recognize and consequently attack cancer cells with presented cancer-specific antigen(s) (Vermaelen, 2019).

A decade ago, the first therapeutic cancer vaccine for the treatment of prostate cancer (Sipuleucel-T) was approved in the USA (Liu et al., 2020).

Vaccination prepares the host's immune system against specific antigens, so that it can be beneficial in cancer therapy-associated fields, with poor immunogenicity and some safety concerns. The broad potential of vaccines for cancer prevention

and treatment has been less noticed, and fewer cancer vaccines have been permitted up to now (Liu et al., 2020).

Some nanovaccines that are tested in clinical trials of cancer therapy will be discussed below. Effective cancer vaccines must meet the subsequent requirements: (i) to stimulate effective T cells, a selection of the proper tumor-associated antigen is crucial; (ii) sufficient antigen uptake by APCs (mainly by DCs) is essential to prevent immune tolerance; and (iii) CTLs are key effector cells to destroy all tumor cell types. Due to the influence of CD4 + T cells on the activation of CTL expansion and generation of memory cells, the best vaccine must stimulate both CD4 + and CD8 + immune responses. (iv) To overcome the negative influence of the immunosuppressive condition in the tumor microenvironment, cancer vaccines must be used in combination with other approaches such as chemotherapy and immunosuppressive inhibitors (Liu et al., 2020).

Over the past decades, to overcome the problems of traditional vaccines in cancer vaccine, nanotechnology, via manipulated nanoparticles, shows a hopeful horizon for the treatment of cancer (Liu et al., 2020).

Nanovaccines are a novel approach for the methodology of vaccination by using nanoparticle (NP)-based vaccines as carriers and/or adjuvants, which show numerous competitive advantages over the traditional vaccines (Maina et al., 2020; Gheibi Hayat & Darroudi, 2019; Nandedkar, 2009). Nanovaccines similar to pathogen antigens can be designed to mimic the size and shape of antigens to stimulate the immune system and more efficiently activate antibody and cell-mediated immune responses (Gheibi Hayat & Darroudi, 2019). Nanovaccines are an encouraging approach to hamper uncontrolled cancer-related diseases. The immunologic effect of nanovaccines is not always sufficient to prevent cancer progression but is regularly well tolerated and offers appreciated anticancer properties in some situations (Karimi et al., 2020).

Several types of vaccine may be used to treat cancers, such as adjuvant and DNA vaccines intended for easy recognition by immune cells to induction of a rapid and enhanced secondary response as well (Maina et al., 2020).

The nanovaccines have some advantages such as their better stability, the longer shelf life in blood flow, improved targeted delivery, rubout stimulatory effect on the immune system, no need for a cold chain and booster shots, aptitude to make active targeting, enhanced cross-presentation to induce CTLs, and so on (Gheibi Hayat & Darroudi, 2019; Das & Ali, 2021; Maina et al., 2020). The desired stability of nanovaccines dramatically reduces storage and transport costs and allows rapidly global delivery and access (Maina et al., 2020). Therefore, nanovaccines may be used as a suitable platform for improving vaccine efficacy in cancer therapy.

Due to the size of nanovaccines (average 10–100 nm), it is not necessary for more processing by immune cells to stimulate the immune response, and that works well via plug and play mechanism (Maina et al., 2020; Gheibi Hayat & Darroudi, 2019).

Nanoparticle-based vaccines are found mainly on various nanoscale materials including microemulsions, liposomes, virosomes, micelles, buckminsterfullerene, nanogels, carbon nanotube, dendrimers, and metallic NPs (Gheibi Hayat & Darroudi, 2019; Liu et al., 2020).

The diversity in the component of nanoparticles allows for the designing, manipulation, and optimizing efficacious nanovaccine constructions in particle composition including size, shape, hydrophobicity, surface charge, and self-adjuvanting properties (Maina et al., 2020). Primary nanoparticle-based systems (liposomes) have been elucidated to make available adjuvant function, and their immunological character has innovative capacity for gene delivery. This character supports the booster effect of nanovaccines by enhancing their uptake via APCs in the lymphoid tissues (Karimi et al., 2020).

For example, high-Z mesoporous Ta_2O_5 (mTa_2O_5) nanoparticles developed by Nosrati et al. are a radiosensitizer and doxorubicin (DOX) delivery system that exhibited notable anticancer effects via effective chemoradiotherapy (Nosrati et al., 2022). However, biological and immunological characterization of certain lipopolysaccharides (e.g., *Brucella abortus* RB51 and S19) as an adjuvant enhanced the therapeutic efficacy of DNA vaccines against cervical cancer models (Shirmohammadi et al., 2021; Kianmehr et al., 2015).

In chemotherapy approaches, low molecular weight drugs are removed quickly from the tumor site. To keep the duration of their effect and overcome these problems, nanocarrier systems can be proposed to enclose anticancer drugs and increase the anticancer effects and preserve their accumulation and distribution in tumor environments. However, chemotherapy effectiveness in treating cancer cells is severely reduced by anatomical and physiological barriers, and rapid clearance of drugs by the reticuloendothelial system occurred, and the poor penetrability of the tumor mass is one of the challenges that lead to treatment failure and drug resistance. The utilization of nanocarriers for drug delivery is one of the recent approaches to overcome this problem (Nowroozi et al., 2018).

Alongside with nanocarriers for drug delivery, nanovaccines have promising hope for disease prevention such as AIDS, cancer, malaria, rheumatoid arthritis, chronic autoimmune diseases, bacterial infection, and so on (Gheibi Hayat & Darroudi, 2019; Das & Ali, 2021).

Nanovaccines induce long-lasting cellular and humoral immunity against cancer via administration of candidate vaccine through several routes including intravenous (IV), intranasal (by inhalation), oral (by nebulization), patches of microprojections to the skin, and so on (Nandedkar, 2009; Maina et al., 2020).

3 Tumor Antigen

Tumor antigens are antigenic molecules expressed mainly in tumor cells that can trigger the host immune response. Tumor-shared antigens were broadly classified into two distinct categories based on their pattern of expression: tumor-associated antigens (TAA), which have overexpressed on tumor cells and at lower levels expressed on healthy cells, and tumor-specific antigens (TSA) that express on cancer cells only (Table 1). These tumor markers are potential candidates for use in

Table 1 Different types of tumor antigens

Class of tumor antigen	Description	Examples
Tumor-specific Antigens (TSA) (mutated antigens)	Products of mutated oncogenes	P53, KRAS, RAS, BCR-ABL translocation, ETV6, NPM/ALK
	Neoantigens Tumor specific mutated antigens Mutations resulting in the generation of a neoepitopes	Case-specific mutations
Tumor-associated Antigens (TAA) (unmutated proteins)	Embryonic/differentiation genes	Gp100, tyrosinase, Melan A/ MART-1, gp75/TRP1/− 2, PSA, CEA, PAP, PSMA
	Overexpressed antigens	Her2/neu, hTERT, MUC1, WT1, cyclin B, survivin
	Products of silent genes Expression on healthy cells limited to germline	Cancer/testis antigens (a-Fetoprotein, MAGE-1, GAGE, BAGE, NY-ESO1)
	Oncogenic viral antigens	HPV E6/E7, EBV LMP-1/LMP-2A, HTLV-1 Tax, KSHV/HHV8

diagnostic tests, vaccine designing, and cancer therapy (Janelle et al., 2020; Paston et al., 2021; Lopes et al., 2019; Poorebrahim et al., 2020).

4 Nanovaccines Properties

4.1 DNA Vaccines

DNA vaccines were introduced as a safer alternative to traditional protein-based vaccines including standard live and inactivated vaccines for treating viral diseases. As shown in Fig. 1, DNA vaccines are classified as third-generation vaccines, closed circular DNA plasmids that contain a specific antigen-coding DNA sequence as well as various other immunomodulatory molecules and encode the designed antigens in transfected cells to induce an immune response (Srinivas, 2021; Liu et al., 2020).

DNA vaccines afford many advantages over conventional vaccines including simplicity, ease of production and safety, stability, transport at room temperature, inexpensiveness, decreased likelihood of vaccine-associated hypersensitivity reactions and replication interference, and the possibility of immunization against multiple pathogens in a single vaccination (Liu et al., 2020).

pTVG-HP is a DNA vaccine that is produced in *Escherichia coli* and encodes the cDNA for human prostatic acid phosphatase (prostatic tumor-associated antigen). In prostate cancer treatment, pTVG-HP has been combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant and assessed in phase II clinical trial (Liu et al., 2020; McNeel et al., 2019).

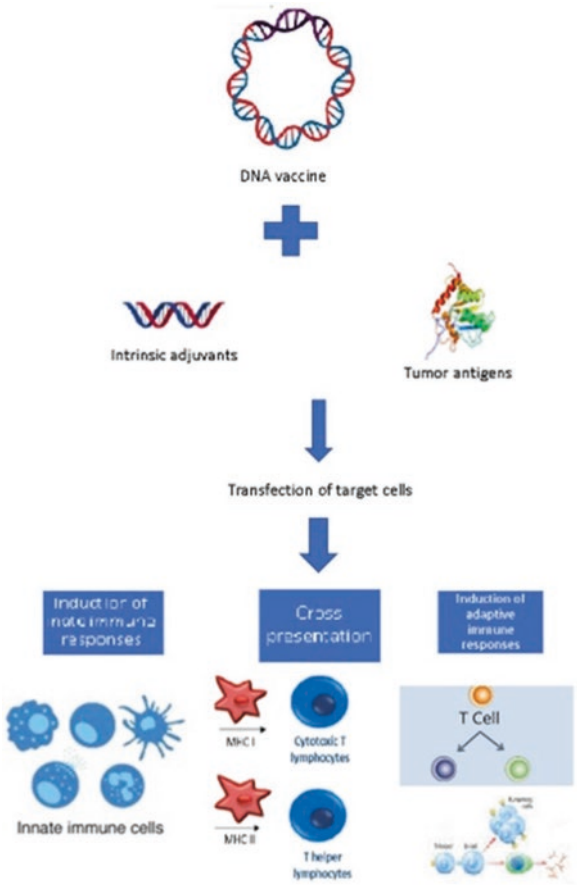

Fig. 1 Mechanism of action with DNA vaccine and suitable stimulatory molecule in the transfected cells

Meanwhile, several other DNA vaccines against other malignancies such as breast carcinoma, bladder carcinoma, medullary thyroid cancer, nasopharyngeal cancer, cervical cancer, and so on are widely being in clinical research (Table 2). Despite the promising results for cancer treatment with DNA vaccines, researchers have reported limited efficacy because the DNA enters the cells by low transfection rates in vivo and requires a more potent delivery system (Karimi et al., 2020). Interestingly, nonviral particulate delivery systems further enhance antitumor immunogenicity compared to viral vectors (Liu et al., 2020; Jazayeri & Poh, 2019).

Table 2 Clinical trials in cancer DNA vaccination (2012–2022)

Cancer type	Backbone/encoded antigen	Combination therapy/adjuvant	Route of administration	Phase	Start year	Trial ID
Anal neoplasm	VGX-3100	/	IM followed by EP	II	2018	NCT03499795
Breast cancer	Personalized polyepitope	/	IM followed by EP	I	2015	NCT02348320
	Mammaglobin-A (Mam-A) antigen	Anastrozole, letrozole, tamoxifen, exemestane, goserelin, endocrine therapy	IM followed by EP	I	2015	NCT02204098
	pUMVC3-CD105/Yb-1/SOX2/CDH3/MDM2-polyepitope: mammalian expression vector pUMVC3 + CD105, Y-box binding protein-1, SRY-box 2, cadherin 3, murine double minute 2	rhuGM-CSF, adjuvant therapy	ID	I	2015	NCT02157051
	pUMVC3-IGFBP2-HER2-IGF1R: pUMVC3 vector + insulin-like growth factor binding protein-2 (IGFBP2), HER2 and insulin-like growth factor 1 receptor precursor (IGF-1R)	GM-CSF (sargramostim), adjuvant therapy	ID	I	2016	NCT02780401
	Neoantigens	Durvalumab (anti-PD-L1 antibody), immune therapy	EP (TDS-IM system)	I	2018	NCT03199040

Cervical cancer	VB10.16: composed by E6/E7 antigen of HPV16 + dimerization entity + APC binding protein	/	Lateral deltoid muscle	I/II	2015	NCT02529930
	GX-188E, encoding E6/E7 fusion protein of HPV 16 and 18, plus the immune-enhancer, Fms-like tyrosine kinase-3 ligand (FLT3L)	/	IM followed by EP	II	2015	NCT02596243
	MEDI0457 = INO3112 = VGX-3100 (encoding E6 and E7 proteins of HPV types 16 and 18) + INO-9012 (hIL-12)	Durvalumab (anti PD-L1 antibody), immune therapy	IM followed by EP	I/II	2017	NCT03162224
	HPV E6 and E7 (VGX-3100)	/	IM followed by EP	III	2017	NCT03185013
	GX-188E	Pembrolizumab	IM followed by EP	I/II	2018	NCT03444376
	HPV E6 and E7 (VGX-3100)	/	IM followed by EP	II	2018	NCT03603808
	HPV E6 and E7 (VGX-3100)	IL-12; durvalumab (anti-PD-L1)	IM followed by EP	II	2018	NCT03439085
	HPV E6 and E7 (VGX-3100)	/	IM followed by EP	III	2019	NCT03721978
Colorectal cancer	Oncoprotein MYB (TetMYB)	Tetanus toxoid peptides and anti-PD1 antibody	ID	I	2017	NCT03287427
Glioblastoma	INO-5401 (separate DNA plasmids targeting WT1, PSMA, and hTERT)	INO-9012 (human IL12), cemiplimab, temozolomide, and radiation	IM followed by EP	I/II	2018	NCT03491683
Melanoma	IFx-Hu2.0 coding for Emm55 streptococcal antigen	/	Intralesion	Early I	2018	NCT03655756
Ovarian cancer	pUMVC3-hIGFBP2 multiepitope: mammalian vector pUMVC3 + human IGFBP2	/	/	I	2012	NCT01322802
	pUMVC3-hIGFBP2 multiepitope	Carboplatin, paclitaxel, chemotherapy	ID	II	2017	NCT03029611
Pancreatic cancer	Personalized neoantigen: pING vector + prioritized neoantigens + mesothelin epitopes	Surgical resection and adjuvant chemotherapy	IM followed by EP	I	2018	NCT03122106

(continued)

Table 2 (continued)

Cancer type	Backbone/encoded antigen	Combination therapy/adjuvant	Route of administration	Phase	Start year	Trial ID
Prostate cancer	pTGV-HP	Sipuleucel-T, autologous peripheral blood mononuclear cells with antigen-presenting dendritic cells that have been activated ex vivo with a recombinant fusion protein (PA2024) consisting of PAP linked to GM-CSF, immune therapy	/	II	2013	NCT01706458
	pTGV-AR	GM-CSF, adjuvant therapy	/	I	2015	NCT02411786
	pTGV-HP	Pembrolizumab (anti-PD1 antibody), immune therapy; rhGM-CSF, adjuvant therapy	ID and IV	I/II	2015	NCT02499835
	pTGV-HP	Nivolumab, GM-CSF	ID	II	2018	NCT03600350
	Neoantigens	Nivolumab (anti-PD-1)/ipilimumab (anti-CTLA-4), and PROSTVAC	IM followed by EP	I	2018	NCT03532217
Renal cell carcinoma	Neoantigens	Durvalumab tremelimumab	IM followed by EP	II	2019	NCT03598816
Solid tumors	hTERT	/	ID EP	I	2014	NCT02301754
Urothelial carcinoma	INO-5401	INO-9012, atezolizumab	IM followed by EP	I/II	2018	NCT03502785

EP electroporation, *IM* intramuscular, *ID* intradermal, *IV* intravenous

4.2 mRNA Vaccines

Similar to DNA vaccines, messenger RNA (mRNA) vaccines are classified as third-generation vaccines. An mRNA vaccine strategy is a next-generation technology that works by introducing a piece of mRNA that corresponds to pathogens/cancer antigen(s), which will produce the target protein and stimulate an immune response (Pardi et al., 2018; Liu et al., 2020). Over the past two decades, many measures have taken mRNA-based technologies for new developing vaccine candidates. According to COVID-19 preclinical and clinical trials, mRNA vaccines not only can make long-term immune responses in both human and animal models but, also, are safe and effective in preventing cancer or diseases. The COVID-19 epidemic triggered more attention to the mRNA-based vaccine approach. The mRNA-based vaccine technologies have made a new era in vaccination against infectious diseases and cancer in the future because they have high potency for rapid improvements and the manufacturing process is relatively cost-effective.

The mRNA vaccines are transcribed in vitro by a bacteriophage RNA polymerase (e.g., T7, a T3, or an Sp6 phage) and template DNA containing the gene encoding the antigen(s) of interest. After, mRNA vaccine will internalize into the *host cell cytoplasm*, where it will be expressed to antigen via the cell translation machinery, and finally that products will trigger an immune response (Rosa et al., 2021; Pardi et al., 2018).

Messenger RNA vaccines have several brilliant advantages over DNA-based vaccines including in vivo delivery of high numbers of antigens and costimulatory signals, inexpensive and rapid manufacturing, rapidly expression and activation tumor-specific T cells, and no reported risk of insertional mutagenesis in the host genome, but its integration concern is under investigation (Table 3) (Rosa et al., 2021).

However, mRNA vaccines are unstable and fragile and require storage and transport at ultracold temperature systems. To solve transportation problems, the encapsulation of mRNA within biocompatible nanomaterials is proposed (Liu et al., 2020).

4.3 Neoantigen-Based Vaccine

Currently, any somatic gene mutations in tumors can be identified by next-generation sequencing (NGS) technologies. Neoantigens are mainly tumor-specific mutated peptides that are only expressed in tumor cells and act as a potent stimulator for antitumor immune responses (Zhang et al., 2021). The discovery of neoantigens is one of the most particular achievements in the field of immuno-oncology research. Neoantigens, a non-self-antigen, can easily be detected by the host immune system and elicit a strong antitumor immune response (Fotakis et al., 2021; Zhang et al., 2021; Liu et al., 2020).

Table 3 Clinical trials with mRNA-based vaccines against cancer

Cancer type	Backbone/encoded antigen	Combination therapy/ adjuvant	Route of administration	Phase	Start year (status)	Trial ID
AML	Autologous WT1 mRNA-electroporated DCs	Chemotherapy	ID	II	2012 (recruiting)	NCT01686334
	GRNVAC1 (autologous mature DCs transfected with mRNA encoding human telomerase)	–	–	II	2007 (completed)	NCT00510133
	TLR7/8-matured DCs electroporated with mRNA encoding WT1, PRAME, and CMVpp65	–	ID	I/II	2013 (completed)	NCT01734304
	Autologous DCs loaded with acute myelogenous leukemia (AML) lysate plus mRNA	–	–	I	2009 (terminated/ slow accrual)	NCT00514189
	Autologous DCs electroporated with WT1 mRNA	–	–	I/II	2016 (active, not recruiting)	NCT03083054
Colorectal cancer	Autologous DCs pulsed with CEA-peptide or electroporated with CEA-RNA	–	ID and IV	I/II	2010 (completed)	NCT00228189
Breast cancer	VRP (alphaviruslike replicon particles) containing self-amplifying replicon RNA for HER2 (AVX901)	Pembrolizumab (anti-PD-1)	IV	II	2018 (recruiting)	NCT03632941
	Shared tumor antigens and patient-specific mutated neoantigens	Surgery and adjuvant chemotherapy	IV as nanoparticulate lipoplex	I	2016 (active, not recruiting)	NCT02316457
	DCs transfected with survivin, hTERT and p53 mRNA	Cyclophosphamide	ID	I	2009 (completed)	NCT00978913

Glioblastoma	Autologous WT1 mRNA loaded DCs	Temozolomide	–	I/II	2015 (recruiting)	NCT02649582
	DC vaccine with mRNA from tumor stem cells	–	ID	I/II	2013 (completed)	NCT00846456
	pp65-shLAMP mRNA DCs with GM-CSF	Tetanus and diphtheria toxoid	ID	II	2016 (recruiting)	NCT02465268
	Human CMV pp65-LAMP mRNA-pulsed autologous DCs	Temozolomide/ basiliximab	ID	II	2015 (completed)	NCT02366728
Multiple myeloma	CT7/MAGE-A3/WT1 mRNA-electroporated autologous Langerhans-type DCs	–	ID	I	2014 (active, not recruiting)	NCT01995708
Melanoma cancer	RBL001/RBL002 (recombinant vaccines: two TAAs of melanoma)	–	Intranodal	I	2012 (completed)	NCT01684241
	Neoantigens [poly-epitopic RNA vaccine (IVAC MUTANOME®)]	RBL001/RBL002	Intranodal	I	2013 (completed)	NCT02035956
	Four TAAs [RBL001.1, RBL002.2, RBL003.1, and RBL004 (Lipo-MERIT)]	–	IV	I	2015 (active, not recruiting)	NCT02410733
	Autologous Langerhans-type DCs electroporated with mRNA encoding a TAA	–	ID	I	2011 (active, not recruiting)	NCT01456104
	mRNA-transfected DCs	IL-2	ID or intranodal	I/II	2002 (completed)	NCT01278940
	Autologous DCs loaded with autologous tumor RNA	–	IV	III	2014 (recruiting)	NCT01983748
	mRNA coding for the corresponding antigen. (Melan-A, Mage-A1, Mage-A3, survivin, GP100, and tyrosinase)	GM-CSF	ID	I/II	2004 (completed)	NCT00204607

(continued)

Table 3 (continued)

Cancer type	Backbone/encoded antigen	Combination therapy/ adjuvant	Route of administration	Phase	Start year (status)	Trial ID
Prostate cancer	RNActive®-derived cancer vaccine (CV9104) (6RNActive®-based compounds)	–	ID	I/II	2012 (terminated)	NCT01817738
	RNActive®-derived prostate cancer vaccine (CV9103)	–	ID	I/II	2009 (completed)	NCT00831467
	DCs are transfected with PSA, PAP, survivin, and hTERT mRNA	Docetaxel	ID	II	2011 (completed)	NCT01446731
	Autologous DCs loaded with mRNA from primary prostate cancer tissue, hTERT, and survivin	–	ID	I/II	2010 (active, not recruiting)	NCT01197625
	RNActive®-derived therapeutic vaccine (CV9103)	–	ID	I	2009 (terminated)	NCT00906243
Non-small cell lung cancer	TAAs (six formulated mRNAs) (CV9202)	Durvalumab/ Tremelimumab Local radiation	ID	I/II	2017 (completed)	NCT03164772
	RNActive®-derived cancer vaccine	–	ID	I/II	2009 (completed)	NCT00923312
Renal cell carcinoma	Autologous ribonucleic acid (RNA) electroporated DCs (AGS-003)	Sunitinib	ID	III	2012 (terminated)	NCT01582672

IM intramuscular, *ID* intradermal, *IV* intravenous

The advent of high-throughput genomic sequencing and neoantigen prediction technologies has made it possible to produce personalized vaccines based on neoantigens (Peng et al., 2019; Zhang et al., 2021; Fotakis et al., 2021). Hence, the pursuit for immunogenic neoantigens with stronger immunogenicity in tumor cells has become a key issue in cancer immunotherapy (Peng et al., 2019; De Mattos-Arruda et al., 2020). Based on numerous reports, neoantigen-based vaccine therapies have revealed remarkable results in a variety of cancer types. Therefore, the application of neoantigen-based vaccine therapies can be promising in the field of cancer immunotherapy (Table 4). Neoantigen-based personalized therapeutic vaccines have some advantages compared to common cancer vaccines including limiting the risk of immune tolerance, no toxicity to normal tissue cells, and enhanced antitumor immune response (Liu et al., 2020; Blass & Ott, 2021).

Table 4 Clinical trial of personalized neoantigen-based vaccine

Vaccine platform	Tumor type	Combination therapy/adjuvant	Phase	Strat Year (status)	Trial ID
IVAC MUTANOME, RBL001/RBL002	Melanoma	–	I	2013 (completed)	NCT02035956
APVAC1 vaccine	Glioblastoma	Immunomodulator (Poly-ICLC and GM-CSF)	I	2014 (completed)	NCT02149225
NeoVax	Glioblastoma	Radiation therapy plus pembrolizumab/ temozolomide	I	2014 (recruiting)	NCT02287428
NeoVax (peptides + poly-ICLC)	Melanoma	–	I	2014 (completed)	NCT01970358
Autologous dendritic cell vaccine matured with melanoma peptides (G209-2M and G280-9V) plus melanoma tumor-specific peptides	Advanced melanoma	Cyclophosphamide	I	2008 (completed)	NCT00683670
NeoVax (peptides + poly-ICLC)	High-risk renal cell carcinoma	Ipilimumab	I	2019 (recruiting)	NCT02950766
NeoVax	Ovarian cancer	Nivolumab	I	2020 (recruiting)	NCT04024878
iNeo-Vac-P01 (peptides)	Malignant tumor	GM-CSF	I	2018 (active, not recruiting)	NCT03662815

(continued)

Table 4 (continued)

Vaccine platform	Tumor type	Combination therapy/adjuvant	Phase	Strat Year (status)	Trial ID
GRT-C903/ GRT-R904	Advanced solid tumors	Immune checkpoint blockade (nivolumab/ ipilimumab)	I/II	2019 (recruiting)	NCT03953235
Neoantigen-targeted vaccine	Colon and pancreatic ductal cancer	Retifanlimab (anti-PD-1 antibody) plus poly-ICLC	I	2022 (not recruiting)	NCT04799431
Neoantigen-expanded autologous DC-CIK cells	Advanced solid tumor	–	I	2021 (recruiting)	NCT05020119
Personalized neoantigen peptide vaccine	Non-small cell lung cancer	Antiangiogenesis drug/EGFR-TKI	I	2020 (recruiting)	NCT04487093
GRT-C901/ GRT-R902	Metastatic colorectal cancer	Immune checkpoint blockade (atezolizumab/ ipilimumab/ fluoropyrimidine/ bevacizumab, oxaliplatin)	II/III	2022 (not recruiting)	NCT05141721
Neoantigen dendritic cell vaccine	Resected hepatocellular carcinoma (HCC) (group A) and liver metastases from colorectal cancer	Anti-PD1 (nivolumab)	II	2020 (recruiting)	NCT04912765
GEN-009 adjuvanted vaccine with Hiltonol (poly-ICLC)	Selected solid tumors	PD-1 inhibitor therapy (nivolumab or pembrolizumab)	I/II	2018 (recruiting)	NCT03633110

However, the clinical efficacy of neoantigen-based cancer vaccines requires a defined optimization and standardization process for the neoantigen identification and its delivery mechanisms (De Mattos-Arruda et al., 2020).

There are currently two kinds of personalized vaccines designed, mRNA vaccine and synthetic long peptide (SLPs) vaccine. Shahin et al. developed personalized RNA mutanome vaccines, and personalized peptide vaccines were developed by Ott et al. (Sahin & Tureci, 2018; Ott et al., 2017). The liposome was chosen for improvement of targeted delivery of personalized RNAs and synthetic peptides into DCs

because of its distinct properties such as biological safety, biocompatibility, simple manufacturing process, and the protection of vaccines from in vivo degradation (Sahin & Tureci, 2018; Ott et al., 2017; Liu et al., 2020).

In this case, bioinformatics and molecular computational tools can be useful in the prediction of neoantigens; therefore, it can support the precise designing of personalized neoantigen-based vaccines (Kardani et al., 2020; Fotakis et al., 2021; Peng et al., 2019). Up to March 2022, more than 88 clinical trials of neoantigen-based cancer vaccines have been registered in www.clinicaltrial.gov. The majority of clinical studies have been performed in recent years.

5 Nanocarrier Design and Development: Critical Points to Induce the Immune Response

An efficient delivery system is required to deliver antigen to APCs and prime T cells to T effector cells with low toxicity. In the selection of delivery system, the distinct physical properties of different types of antigens (i.e., synthetic peptide, mRNA) must be noticed.

For example, delivery systems for subunit peptide vaccines must be designed to overcome their low immunogenicity and fast clearance. Hence, nano-sized delivery systems along with the inclusion of adjuvants are used to elongate the retention of peptide vaccines and boost their immunogenicity, respectively (Tornesello et al., 2020). In the selection of desired delivery systems for DNA/RNA vaccines, efficient internalization, stability, and transcription must be regarded to induce a strong immune response.

To design the best nanocarriers for cancer vaccine delivery with low systemic toxicity and robust antitumor immune response, an extensive understanding of the interaction between the nanocarrier-based delivery system and APCs is essential. The physicochemical properties of nanocarrier-based delivery systems like nanoparticle size, rigidity, surface charge, ligand, and intrinsic immunogenicity must be subjected in the construction (Liu et al., 2020; Tornesello et al., 2020).

The NP size has a significant role in the intracellular fate of NPs in the body which can influence the biodistribution and cellular uptake mechanism and play a major role in eliciting a combined response of humoral and cell-mediated immunity. Following administration of NPs in non-intravenous route (intramuscular, intradermal, or subcutaneous) based on particle size, three scenarios will happen:

I. The majority of small particles (<20 nm) rapidly will be entered into the blood flow and subsequently filtered out by the kidneys and removed through urine.
II. Particles between 20 and 100 nm will be moved into the lymphatic nodes via lymph flow, and then they will be taken up by APCs and presented to them.
III. Larger NPs (>100 nm) will stay at the injection sites until they are gradually captured and moved to lymphatic nodes by APCs (Jia et al., 2018; Liu et al., 2020).

The cellular uptake mechanism is directly dependent on particle size; particles with size 20–200 nm are generally taken up by cells through two classic receptor-mediated endocytosis pathways (clathrin-dependent or caveolae−/lipid raft-dependent). Conversely, particles above 200 nm are preferentially captured by the phagocytosis process. Both distribution manner and cellular uptake mechanism may affect the induced immune response. Previous studies displayed that smaller particle often induced Th2 immune responses while larger particles trigger typical Th1 responses. Accordingly, particle size indeed is one of the main role players in regulating vaccine functions (Liu et al., 2020; Wu et al., 2019).

The results of numerous investigations were performed by separate researchers that revealed rigid NPs compared to soft NPs significantly enhanced immune cell uptake, facilitating immune cell activation and the immune response (Liu et al., 2020).

The surface charge of NPs is another important factor that affects the fate of nanovaccine administration in biological systems (Liu et al., 2020; Jo et al., 2015). Henriksen-Lacey et al. evaluated the effect of surface charge in the distribution and immunogenicity of injected antigen-loaded liposomes (85B-ESAT-6) by comparing natural liposomes against cationic liposomes (CAF01) (Henriksen-Lacey et al., 2011). Their results showed the remaining antigen is traceable only in cationic synthetic liposome platform for up to 2 weeks at the injection site. This result suggested that the aggregation of cationic liposomes causes antigen trapping/retaining and slow release in an extended period of time at the site of injection. This constant stimulation thru depot effect (mechanism of action of adjuvants) may produce an enhanced immune response (Awate et al., 2013; Liu et al., 2020). The results of Henriksen-Lacey et al. study have demonstrated cationic liposomes induced a more prolonged antigen presentation compared to natural liposomes; thus, it can activate the pro-inflammatory signals to trigger the secretion of several cytokines and chemokines. Furthermore, the cell membranes of APCs similar to other cells are negatively charged; thus, using cationic NPs in nanovaccine construction can promote potency of uptake and presentation of antigen (Liu et al., 2020).

The efficient DC-specific delivery can enhance the potency of cancer nanovaccines by using appropriate delivery ligands, which improve both concentrated antigen delivery to lymphatic tissues and APCs (Liu et al., 2020).

Adjuvants are necessary for augmenting immune responses, so they must be included in designing nanovaccines to increase vaccine immunogenicity. Toll-like receptor (TLR) agonists are one of the well-known adjuvants that have been widely investigated in modern vaccines (Liu et al., 2020). TLRs are pattern recognition receptors (PRRs) that initiate the innate immune response by sensing conserved pathogen-associated molecular patterns (PAMPs) which recognize pathogens and then activate multiple steps. The approved adjuvant delivery system for cancer vaccines is listed in Table 5.

TLRs coordinate systemic defenses by induction of both innate and adaptive immune responses to eliminate the invading pathogens via activating inflammatory reactions and DC maturation, enhance cross-presentation, express costimulatory molecules, and expand cytotoxic T-cell responses (Medzhitov, 2001; Liu et al., 2020). Multiple TLR agonists such as polyinosinic-polycytidylic acid (Poly I:C) and

Table 5 Approved/adjuvant delivery system for cancer vaccines

Adjuvant	Backbone	Mechanisms	Types of response	vaccine	Reference
Alum	Mineral salt	Depot effect (antigen stability); inflammasome activation; facilitate humoral immunity via Th2-type immune responses; safe	Ab, Th2 (require other adjuvant induced CTL response)	Human papilloma virus (HPV), hepatitis B virus (HBV), CA-125-targeted vaccines	Oleszycka & Lavelle (2014), De Gregorio et al. (2008), Cuzzubbo et al. (2021)
MF59	Oil-in-water emulsion	Improves humoral and cell-mediated immunity, antigen delivery	Th1, B cell, mucosal response, enhanced APC recruitment	Combination with CpG ODN (TLR9 agonist) in several murine cancer models	O'Hagan et al. (1997), O'Hagan et al. (2013), Tsai (2013), Cuzzubbo et al. (2021)
Virosome Doxil Lipo-Dox Onivyde	Liposome	Improves humoral and cell-mediated immunity, antigen dose sparing, and enhancement of antibody titer; nontoxic	Th1, Th2, B cell, cross-priming, mucosal response	MUC1-based vaccine, hepatitis A virus, Kaposi sarcoma, breast and ovarian cancer, pancreatic cancer	Schwendener (2014), Moser et al. (2013), Wang et al. (2019)
AS04 (Glaxo Smith-Kline)	Aluminum hydroxide-adsorbed TLR4 agonist (alum +MPL)	Improves humoral and cell-mediated immunity	B-cell response, Th1 < Th2	HPV vaccine (Cervarix), hepatitis B virus (HBV)	Garcon et al. (2011), Garcon et al. (2007)

CpG oligodeoxynucleotides (CpG-ODNs) as a synthetic TLR3 and TLR9 ligand, respectively, have also been reported as a potent adjuvant in cancer clinical trials. For example, Gabeleh et al. have specified that administration of α-GalCer with DNA vaccines performed adjuvant effects against tumor in animal model (Gableh et al., 2016). In fact, priming with a DNA vaccine in the presence of α-GalCer and boosting with specific pulsed DC directed an enhanced specific response.

Lee and Nguyen and Liu et al. have reported that combining CpG and poly I:C into polyester NP construction significantly could activate synergetic immune response by improved Th1 response and facilitated anticancer treatment (Lee & Nguyen, 2015; Liu et al., 2020).

Nanoparticle albumin-bound paclitaxel is used as an adjuvant commonly in combination with chemotherapy in numerous cancer clinical trials (Table 5) https://clinicaltrials.gov/ct2/results?cond=Cancer&term=nanoparticle+adjuvant&cntry=&state=&city=&dist=.

6 Viruslike Particles as a Nanocarrier

One opportunity for enhancing the performance of various nanovaccine platforms is the use of cell-targeting approaches. Viruslike particles (VLPs) are one of the advanced recombinant systems engaged in the expression of large quantities of target proteins and used as a carrier for cell-targeting approaches. Knowing the suitable baculovirus-based system, it is possible to engineer vaccines with multiple target epitopes in order to elicit protective immunity (Kianmehr et al., 2015). The immunogenicity of VLPs is possibly due to their interaction with DCs and has been a useful approach for immunity against infections or cancers. The antigens inserted in the VLP play a major role in eliciting a combined response of humoral and cell-mediated immunity. For the generation of vaccines against a range of epitopes or a combination of epitopes, the VLP may be the selective solution for inducing both B- and T-cell responses. VLP nanoparticles are used as carriers of specific antigens inducing an appropriate immune response, without adjuvants.

7 Conclusion

For the generation of vaccines against a range of infections, a combination of DNA or peptides conjugated to nano-beads or nanoparticles may be the superlative solution as they induce both B- and T-cell responses; this approach can improve immunogenicity. But, the scenario for human vaccines is different and complicated. Conventional methods such as chemotherapy, radiotherapy, and surgery are the three main available treatments. In recent years, scientists and clinicians have been encouraged to study more about cancer cells that are predominantly effective at suppressing the individual's natural immune response. These findings helped in developing therapies to prevent some kinds of cancers. Vaccine development and targeted therapy are the most attractive areas in cancer therapy. Cancer vaccine is an emerging therapy that can help us to prevent cancers. Recent developments in the formulation of nanovaccines as a cutting-edge area of research for targeted therapy appear as a chance and challenge in designing and delivering these intentions.

Although several of the new cancer vaccines have shown ability in animal models and extending patients' survival, future vaccine developments are probably needed to sustain improvement to endorse administration efficacy and eliminate suppressive cell responses for prolonging the duration of antitumor immune responses at tumor microenvironments.

In summary, nanovaccines offer quite a lot of benefits over traditional vaccines and have the potential for boosting immunogenicity and efficacy in candidate vaccines. A low dose need of antigen and effectual processing by antigen-presenting cells are the main advantages for tomorrow's nanovaccines. Further studies are needed for approving the use of nanovaccines for disease and cancer prevention or therapies.

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Messenger RNA Nanovaccine in Cancer Immunotherapy

Mengyun Li and Hongxia Zhang

Abbreviations

ADAR	Adenosine deaminase acting on RNA
APOBEC	Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide
hATTR	Hereditary transthyretin amyloidosis
BMP	Bone morphogenetic protein
CAR	Chimeric antigen receptor
COVID	Coronavirus disease
cGAS	Cyclic GMP-AMP synthase
CTLA4	Cytotoxic T lymphocyte associate protein-4
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
eIF4e	Eukaryotic transcription initiation factor 4e
IFN	Interferon
IVT	In vitro transcription
LNP	Lipid nanoparticle
mRNA	Messenger ribonucleic acid
nrRNA	Nonreplicating RNA

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OAS	Oligoadenylate synthetase
PBAE	Poly (β-amino ester)
PD-1	Programmed cell death-1
PEG	Polyethylene glycol
Poly(A)	Polyadenylation
PKR	Protein kinase R
PRR	Pathogen recognition receptor
RIG	Retinoic acid-inducible gene
saRNA	Self-amplifying RNA
SARS	Severe acute respiratory syndrome
STING	Stimulator of interferon genes
TAAs	Tumor-associated antigens
TLR	Toll-like receptors
TRIM	Tripartite motif

1 Introduction

Cancer vaccine, different from other prophylactic vaccine for infectious disease, usually plays a role for therapeutic strategy. The principle of cancer vaccine is to induce potent antitumor responses by vaccinating patients with tumor-specific antigen(s) and immune-stimulating adjuvant (Melero et al., 2014; Coffman et al., 2010). Major types of vaccines include inactivated, attenuated, and recombinant subunit vaccine as well as nucleic acid vaccine. Among these vaccines, nucleic acid vaccines were less well developed in commercial distribution due to immature technology. But in the past few years, messenger RNA (mRNA) vaccine technology has been quickly developed as a novel approach to infectious disease control and cancer therapeutics. mRNA-based cancer vaccine shares some common feature in technology with nucleoside-based drugs and antiviral vaccines. The idea of using RNA in vaccine has been around for nearly three decades (Dolgin, 2021). In vitro transcribed (IVT) RNA was delivered into various types of cells by lipid-based transfection system in 1989 (Malone et al., 1989), and then mRNA vaccine was first proposed and tested on animal model in 1993 (Martinon et al., 1993). With the feature of rapid generic production, noninfectious and non-genome-integrating feature, mRNA vaccine technology has advantages over conventional vaccines and DNA vaccine. Moreover, RNA vaccine also circumvents the problem in extensive production of antigen and avoids potential regulatory barrier in gene therapy.

However, the progress of mRNA vaccine development in past decades was slow (Verbeke et al., 2019). A number of important issues must be considered for a successful mRNA vaccine production and in vivo administration. The in vivo intracellular nucleoside stability and translation efficacy as well as efficient targeted delivery play a key role in the final mRNA vaccine effect. Notably, cancer vaccine is faced with additional challenge in antigen selection and inducing maximum antitumor immunity in a generally immunosuppressive tumor microenvironment. How

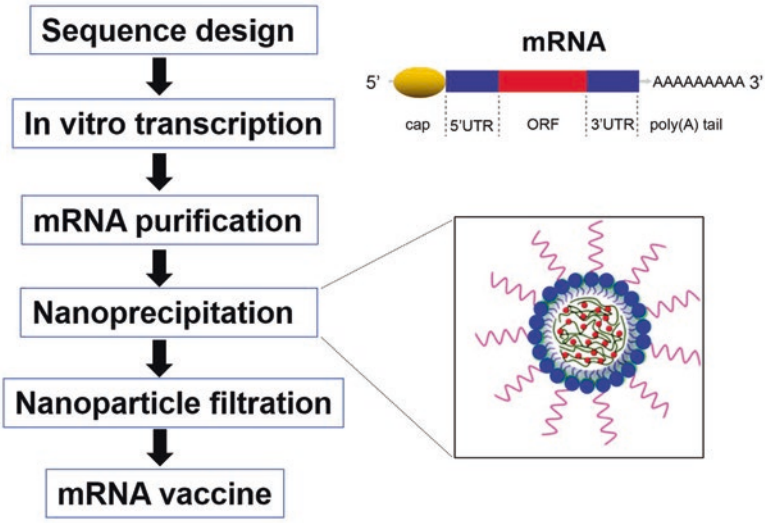

Fig. 1 The synthesis flow of a typical mRNA nanovaccine

to keep a balance between immune stimulation and unwanted toxicity is also another important issue for designing safe and effective mRNA vaccines.

At present, mRNA cancer vaccine is mainly composed of mRNA encoding tumor antigens, delivery vehicles, and immune adjuvants. As shown in Fig. 1, a typical workflow for mRNA vaccine development consists of the following steps: (i) target protein or antigen sequencing selection, (ii)DNA cloning and expanding of antigen coding sequence, (iii) in vitro transcription of linearized DNA into mRNA, (iv) carrier-mRNA formulation, and (v) in vitro efficacy evaluation (Fig. 1).

In this chapter, we first discuss the major components of mRNA nanovaccine, mainly antigen-encoding mRNA with chemical modification and delivery systems with self-adjuvant feature. Tumor antigen sequence selection would be introduced at the end of this chapter. We also review the emerging field of personalized cancer vaccine and discuss recent development and future directions for this promising cancer immunotherapy strategy.

2 mRNA Modification

Generally, the mRNA component of an mRNA vaccine is prepared by in vitro transcription reaction with a linear DNA template using bacteriophage RNA polymerase. The linear DNA template consists of the target protein (antigen) sequence and a promoter. This in vitro transcribed mRNA usually has a 5′ cap structure, untranslated regions (UTRs), and a 3′ poly(A) tail flanking the open reading frame (ORF) to enhance mRNA stability and translational efficiency (Fig. 1) (Kwon et al.,

2018; Van Hoecke & Roose, 2019). Additional modifications are often included to improve mRNA stability and modulate host immune response to foreign mRNA.

2.1 5′ Cap

The 5′ cap structure presents generally in eukaryotic mRNAs (Furuichi, 2015). Initially 7-methylguanosine binds to the 5′ end of mRNA via 5′-5′ triphosphate to form a cap 0 structure (m^7GpppN). The extra methyl group is added to the 2′ hydroxyl of adjacent first and second nucleoside form cap1 (m^7GpppNm) and cap2 (m^7GpppNmpNm), respectively. IVT mRNAs typically use cap1 structures. Cap structure blocks the 5′ end of RNA to avoid exonuclease degradation and promote mRNA stability. Besides, eukaryotic transcription initiation factor 4e (eIF4e) binds to 5′ cap, facilitating ribosome binding and transcription initiation (Sonenberg et al., 1978). There are two major capping methods for IVT mRNA, including posttranscriptional enzymatic capping and co-transcriptional capping by adding cap analogs in transcription mixture. Compared with enzymatic capping, co-transcriptional capping achieves at one step, thereby avoiding additional reaction process of enzymatic capping (Yisraeli & Melton, 1989). In addition, chemical modifications on cap analogs like oxygen substitution into sulfur and modification of 3′-OH into the 3′O-Me can block reverse transcription and enhance translation efficiency (Jemielity et al., 2003).

2.2 UTRs

UTRs, including 5′-UTR and 3′-UTR, regulate posttranscriptional expression efficiency and half-life of mRNA. At transcription initiation, 43S preinitiation complex scans 5′-UTR elements for start codon site. The efficiency of ribosome recruitment to 5′-UTR directly affects the translation efficiency (Hajj & Whitehead, 2017).

There are three major strategies for choosing UTR sequence and optimization in developing mRNA vaccine. The first is to choose UTRs from highly expressed human genes such as human α/β-globin. The second is to use the native virus and target gene UTRs. And the third one is by an in vitro cell-based selection approach by adapting systematic evolution of ligands by exponential enrichment (SELEX) method (Orlandini von Niessen et al., 2019). So far , the first two strategies are commonly used. In fact, BNT162b2, a COVID-19 mRNA nanovaccine developed by Pfizer, takes approach of the first strategy by incorporating 5'-UTR of human α-globin with minor modification in Kozak sequence (Xia, 2021). At present, UTRs used in mRNA vaccine are often from α/β-globin, human heat shock protein, and virus-derived UTRs. Considering the starting site of translation in 5′ end, the presence of internal ribosome entry site (IRES) in the 5′-UTR followed 5′-cap enables a

universal translation process and enhanced antigen protein expression (Tan & Wan, 2008).

Compared to the role of 5′-UTR in initiating protein translation, 3′-UTR is more inclined to maintain the stability of mRNA. The length of 3′-UTR affects mRNA half-life. In general, longer sequence of 3′-UTR would shorten the half-life of mRNA, yet shorter 3′-UTR hampers translation, and optimal natural occurring 3′-UTRs have been screened for mRNA vaccine and cell reprograming (Orlandini von Niessen et al., 2019; Kenyon, 1975).

2.3 Poly(A) Tail

Poly(A) tail at 3′ end of mRNA consists of repeated adenine nucleotides. This polyadenylation signal is important for RNA stability and translational efficiency. The length of poly(A) sequence affects translation efficiency (Passmore & Coller, 2022). In a dendritic cell-based mRNA vaccine, Holtkamp et al. firstly demonstrated the poly(A) tail, and 3′-UTR modification increased IVT RNA stability, translational efficacy, and T-cell stimulatory capacity of mRNA-loaded dendritic cells (Holtkamp et al., 2006).

2.4 Nucleotide Modification

Chemical modification on nucleosides regulates mRNA-mediated innate immune activation and mRNA stability (Kariko et al., 2005, 2008). The delivered mRNA-associated immunogenicity plays dual role in therapy. On one hand, exogenous nucleoside delivery could stimulate innate immune response through intracellular Toll-like receptors (TLRs) to promote dendritic cells (DCs) maturation and enhance antigen presentation. On the other hand, intracellular delivery of exogenous mRNA could elicit host antiviral response. Antiviral response exerts anti-mRNA property by RNA cleavage and translation inhibition (Pollard et al., 2013).

Intrinsic mechanisms to prevent virus replication include double-stranded RNA-dependent protein kinase (PKR) activation and phosphorylation of translation initiation factor (eIF) α subunit, leading to the inhibition of translation, RNase activation through 2′-5′OAS, adenosine deaminase-induced mRNA deamination and ensuing translation stop, and so on. Especially, type I interferon (IFN-I) pathway is the hallmark of antiviral response (Pollard et al., 2013). IFN-I clearly interferes with mRNA-based therapy. Activated IFN-I pathway as an antiviral response to exogenous nucleosides inhibits mRNA expression independent of its specific sequence (Pichlmair & e Sousa, 2007). IFNAR$^{-/-}$ mice, which lack intrinsic IFN-I response, showed an enhance lipid-complexed mRNA expression and antigen response in vivo compared with WT mice (Pollard et al., 2013).

Nucleoside modification enables the host to distinguish host and invader RNA molecules. Unmodified RNA stimulated TLR3, TLR7, and TLR8 , leading to the induction of potent IFNα/β as well as IL-12 (Kariko et al., 2005). Various modifications have been assessed for reduction in TLR activation, including pseudouridine (ψ), N1-methylpseudouridine (m1ψ), 5-methoxyuridine (5moU), 5-methyluridine (m5U), 2-thiouridine (s2U), 5-methylcytidine (m5C), N1-methyladenosine (m1A), and N6-methyladenosine (m6A) (Hajj & Whitehead, 2017). It was reported that mRNA bearing s2U and m5C modification dedicated a higher expression level in vivo compared with unmodified RNA (Kormann et al., 2011). And enzymatic dimerization of uridine as well as pseudouridine can avoid pathogen recognition receptor (PRR) recognition and downstream PKR activation (Kariko et al., 2008). In addition to nucleoside modification, IVT mRNA purification by fast protein liquid chromatography (FPLC) or high pressure liquid chromatography (HPLC) to remove dsRNA impurity is also critical for reducing the immunogenicity of synthesized mRNA (Weissman et al., 2013; Baiersdorfer et al., 2019; Nelson et al., 2020).

Two major types of mRNA are tested in vaccine application. First one is traditional nonreplicating mRNA (nrRNA). For nonreplicating mRNA, the expression level of encoded antigens directly depends on the copy number of mRNA, and the final immune response is closely related with injection dose and frequency. Another one is self-amplifying RNA (saRNA). This kind of RNA has a virus replicase sequence before ORF (Bloom et al., 2021). The replicase element is often derived from alphaviruses, flaviviruses, measles viruses, or rhabdoviruses, which makes mRNA to self-amplify (Lundstrom, 2018). Therefore, saRNA could reach a similar immune response at a lower dosage than nrRNA (Beissert et al., 2020). On the other hand, the larger size of saRNA makes the in vivo delivery more challenging.

3 mRNA Delivery System

mRNA is a single-stranded macromolecule with a negative charge. Electrostatic repulsion makes it difficult for mRNA molecules to pass through the cell membrane and enter the cell. Moreover, mRNA molecule is susceptible to degradation by a variety of enzymes in the body. Therefore, special delivery system is required for in vivo mRNA delivery (Sahin et al., 2014).

Generally, there are two major vectors of nucleoside delivery, including viral vectors and nonviral vectors. Here, we mainly introduce nonviral vectors in detail. Nonviral vectors include lipid, polymer, and peptide-based vectors, among which lipid vector is the utmost investigated. Decades ago, lipid has been explored as a vector for small interfering RNA delivery (Rettig & Behlke, 2012). The mainstream delivery systems currently on the market include protamine carrier technology, polymer carrier technology (polyplexes), and lipid nanoparticle technology (LNPs).

3.1 Protamine-Based Technology

Protamine is a natural cationic protein that can complex negatively charged mRNA molecules into nanoscale nucleic acid particles, thereby protecting mRNA from degradation by RNase in the serum. However, the combination of protamine and mRNA is too tight for mRNA release, and the protein expression efficiency of mRNA vaccines using this preparation was limited, and the expression of antigens is also largely affected by the ratio of protamine and mRNA. The RNActive® platform developed by CureVac in Germany successfully solved this problem (Rauch et al., 2017). Among the protamine-mRNA complexes obtained through the RNActive® platform, protamine as a TLR7/8 antagonist can induce Th1 cell responses, and mRNA can express the target protein to induce specific immune response (Kallen et al., 2013). RNActive® platform-based cancer vaccines against various cancer types are currently under clinical trials (Papachristofilou et al., 2019).

3.2 Polymer Carrier Technology

The clinical application of polymer carrier technology is not as broad as liposomal carriers, but it shows excellent characteristics as a carrier for nucleic acid drugs. Among various polymers, polyethyleneimine (PEI), polyamidoamine (PAMAM) dendrimer, and polysaccharide are often used as mRNA carriers (Moghimi et al., 2005; Chahal et al., 2016). Cationic polymers shuttle nucleic acids across cellular and subcellular membranes by condensing them into nanocomplexes. When designing a multimeric vector, additional consideration should be given to the molecular weight and the amount of charge of the multimeric vector. Excessive carrier molecules and carrying capacity usually cause the tight combination of the vector and the mRNA molecule, leading to a lower expression efficiency. Polymer carrier development is also hampered by its in vivo toxicities.

3.3 Lipid Nanoparticle Technology (LNP)

In 2018, the FDA approved Onpattro therapy in hATTR induced polyneuropathy (Akinc et al., 2019). Onpattro is a small interfering RNA for silencing hATTR protein expression. Clinical application of Onpattro fully takes advantages of the lipid nanoparticles (LNP). Besides siRNA, mRNA can be formulated and efficiently delivered via LNP as well. Commonly used mRNA-LNP prescriptions generally contain four components – cationic lipids, phospholipids, cholesterol, and polyethylene glycol (PEG) – to achieve mRNA encapsulation.

3.4 Ionizable Cationic Lipid

Cationic lipid was first used in RNA delivery in 1989 (Malone et al., 1989). Besides cationic lipid, other cationic polymers including poly(L-lysine), PEI, DEAD-dextran, PBAE, and chitosan were also explored in the delivery system. However, the cytotoxicity of cationic lipid is obvious. The cytotoxicity of cationic lipids is mainly related to its structure and charge. Cationic lipids are amphiphilic molecules with polar heads, linkages, and hydrophobic tails. According to the type of head, they can be divided into quaternary ammonium salt linked cationic lipids, carbamate linked cationic lipids, amino acid, or the cationic lipid of the polypeptide head. In contrast, quaternary ammonium salt linked cationic lipids are more cytotoxic. The carbamate linked cationic lipids are usually neutral or have a low surface charge, and their cytotoxicity will also be reduced. Cationic lipids with amino acids or polypeptide heads have good biocompatibility and strong biodegradability. The cationic lipid DOTMA, the first cationic lipid used for nucleic acid delivery in 1987, is a quaternary ammonium salt cationic substance (Felgner et al., 1987) and is still used in cell transfection.

3.5 Phospholipids

Phospholipids used in LNP formulation play a helper role. For example, dioleoylphosphatidylethanolamine (DOPE) is cationic synthetic phospholipid, belonging to carbamate linked cationic lipid. As an auxiliary lipid in cationic liposomes, it can stabilize the bilayer membrane and reduce the toxicity of positive components, interfere with the lipid membrane and make the endosomal membrane unstable, improve transmembrane efficiency, and help nucleic acid release. DOPE is the first lipid tested in clinical trials (Bibel et al., 1992). Similar to DOPE, DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) was also used in LNP formulation. As another commonly used helper lipid, cholesterol has strong membrane fusibility and promotes the intracellular uptake of mRNA and cytoplasmic entry.

In the chemical synthesis process of LNP, PEG lipids in the ethanol loading formulation play a key role in LNP size and surface characteristics (Cullis & Hope, 2017). Neutral lipid used in cationic lipid complex could reduce cationic cytotoxicity and help to destabilize cell membrane, thus promoting fusion between LNP and cell membrane. PEGylated lipids are located on the surface of LNP to improve their hydrophilicity, avoid rapid clearance by the immune system, prevent particle aggregation, and increase stability. Typical commercial PEGylated lipids include DMPE-PEG2000 (phosphatidylethanolamine-polyethylene glycol 2000), distearoylphosphatidylethanolamine-polyethylene glycol (DSPE-PEG), and PEG2000-DMG.

3.6 LNP Application in COVID-19 mRNA Vaccine

In the end of 2019, the outbreak of the COVID-2019 pandemic demands rapid vaccine development, and mRNA vaccine technology fulfilled the urgent medical needs. The first two mRNA vaccines that passed through phase III clinical trials for SARS-CoV-2 are BNT162b2 developed by Pfizer and BioNTech and mRNA-1273 developed by Moderna, Inc. (Dolgin, 2021). Both vaccines target the pre-fusion stabilized spike glycoprotein of SARS-CoV-2 virus and are encapsulated in LNPs, which are composed of ionizable lipid ALC-0315 (SM-102 for mRNA-1273), helper lipid DSPC and cholesterol, and PEG lipid (Xia, 2021). Based on the remarkable protection efficacy, the FDA authorized an emergency use license for a new coronavirus vaccine developed using mRNA technology. Nevertheless, there are also reports about the side effect of the mRNA vaccines, mainly derived from the inflammatory response induced by lipid components of LNP vectors (Ndeupen et al., 2021; Tahtinen et al., 2022). Although such immunogenicity of lipids is detrimental for preventative vaccine, it may help potentiate antitumor immunity induced by therapeutic mRNA cancer vaccine. Thus, some lipids of LNPs serve a self-adjuvant function besides mRNA delivery vectors when used for therapeutic cancer vaccine.

4 Adjuvant

An adjuvant is an ingredient used in many vaccines that helps create a stronger immune response in people receiving the vaccine (Coffman et al., 2010). Adjuvant usually helps the body to produce stronger and more durable immune response than non-adjuvanted vaccines. Pathogen recognition receptor (PRR) agonist can trigger intracellular immune response. Many PRRs such as Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG-I)-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, and C-type lectin receptors have been discovered, and their corresponding natural or synthesized agonists have been developed as potent adjuvants in vaccine formulations (Van Herck et al., 2021). Here, we discuss the delivery carriers with self-adjuvant properties in mRNA nanovaccine for cancer immunotherapy.

4.1 Delivery Carriers with Self-Adjuvant Properties

Exogenous mRNA entering cytoplasm can be recognized by intracellular innate immune receptors such as TLR3/7/8, which then elicit antiviral response, mainly type I interferon response (Coffman et al., 2010). Although type I interferon response is often considered an adjuvant signal for robust antitumor T-cell response,

it also suppresses mRNA translation (Pollard et al., 2013). Kariko et al. identified that uridine in IVT mRNA elicited TLR-mediated immune activation, and modified nucleoside such as pseudouridine significantly decreased mRNA immunogenicity and increased mRNA translation efficiency (Kariko et al., 2005, 2008). Recently, in another study from BioNTech, Inc. using neutral liposome for spleen-enriched mRNA cancer vaccine delivery, the authors showed that mRNA molecules indeed activate innate immune response through TLR7/8, and such immune activation contributed to the antitumor efficacy of the vaccine (Kranz et al., 2016). Thus, the immunogenicity of mRNA itself can serve as a double-edged sword depending on the purpose of mRNA vaccine.

As carriers developed for in vivo delivery of mRNA, some carriers also showed immune-stimulating characteristics. For example, protamine, the major component of RNActive platform by CureVac, Inc., can activate TLR7 and TLR8 and ensuing antiviral and pro-inflammatory cytokine expression, which facilitate antitumor effect of such cancer vaccine (Skold et al., 2015). In a polyplex-based mRNA vaccine, the authors used cationic liposome to construct a polyplex for mRNA delivery (Persano et al., 2017). In their report, such polyplex activates TLR4 and downstream IL-1β and IFNβ production, which improved antitumor T-cell activity and overall efficacy. In line with this finding, we also observed TLR4-mediated immune signaling in boosting a lipid-like material-based mRNA cancer vaccine (Zhang et al., 2021). In this study, we identified lipid-like material C1 that efficiently expressed tumor antigen from mRNA translation in dendritic cells and promoted antigen presentation and $CD8^+$ T-cell activation. Interestingly, C1 also elicited pro-inflammatory cytokines such as IL-1β and IL-12 in dendritic cells, and such inflammatory response was dependent on TLR4 expression on dendritic cells. It is currently not clear why the carrier-induced TLR response did not impair mRNA translation efficiency. It is possible that nanovectors serve as mild TLR agonists, and induced TLR response was strong enough for dendritic cell activation, but not for RNA translation-suppressing effect. Another possibility is that vector-induced immune response has less effect on mRNA function. Such possibilities need further experimental validation or disapproval.

Besides TLRs, cGAS-STING pathway is another intracellular DNA-sensing pathway, by which cGAS detect cytoplasmic DNA strand and synthesize cyclic GMP-AMP, which serve as an activating ligand for STING, and activate downstream IFN-I signaling and NF-κB signaling, which promote antiviral and inflammatory response (Zhang et al., 2020). STING agonists have been shown antitumor activity when directly injected into tumor tissues and also showed immune-activating effect when used as adjuvant component in cancer vaccines. Lately, two groups identified cyclic lipids that serve as both mRNA vector and STING agonist. In one study, Luo et al. screen a group of lipids and found one with cyclic head named PC7A was able to form nanoparticle structure with mRNA and activates STING in dendritic cells as well (Luo et al., 2017). Such PC7A-based mRNA vaccine showed superior antitumor efficacy on multiple mouse models. Interestingly, the authors further showed that PC7A as a pH-sensitive polymer induced STING activation by the polymer-induced formation of STING-PC7A condensates in a different way

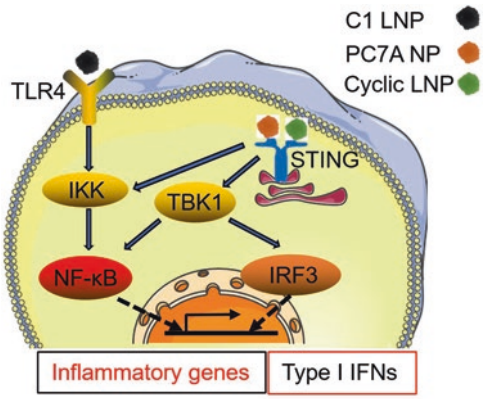

Fig. 2 A cartoon illustration of innate immune signaling pathways activated by different nanoparticles used as mRNA delivery vectors. C1 lipid nanoparticles activated TLR4 and NF-κB signaling (Zhang et al., 2021), while PC7A nanoparticles (Luo et al., 2017) and cyclic lipid nanoparticles (Miao et al., 2019) induced STING activation and downstream NF-κB and IRF3 signaling, together leading to inflammatory gene and type I IFN gene induction

than natural ligand cGAMP (Li et al., 2021). Moreover, PC7A in combination with cGAMP activated stronger STING activation and induced a better antitumor efficacy than individual ligands on several mouse tumor models. On the other hand, Miao et al. performed similar screening using different types of cyclic lipids and identified the lipids with a common structure: an unsaturated lipid tail, a dihydroimidazole linker, and cyclic amine head groups induce intracellular STING pathway activation, which results in limited systemic cytokine expression and enhanced antitumor efficacy (Fig. 2) (Miao et al., 2019). It remains unclear why the nanovector-induced STING activation and ensuing IFN-I promoted antitumor immunity without impairing mRNA translation. Together, using nanovectors with self-adjuvant properties for mRNA vaccine delivery is a popular approach in the recent literature. But before the broad application, further work is warranted to decipher the underlying mechanism for better design of efficient and safe mRNA nanovectors.

5 Antigen Selection for mRNA Cancer Vaccine

The selection of mRNA-encoded cancer antigens is a critical issue in the development of mRNA cancer vaccines. Based on the complex interaction between tumor progression and host immune system , screening for a specific tumor antigen which can be cloned in the mRNA vaccine development is difficult. The prerequisite for these processes is that the encoded antigen can efficiently induce antitumor immunity and ensuing cancer cell killing.

The ideal antigen should enable the immune system to specifically respond to tumor cells while avoiding the attack on normal cells and has a clinical therapeutic effect. Tumor-associated antigens (TAAs) are abnormally expressed on cancer cells and can be used as targets for immunotherapy (Coulie et al., 2014). At present, most cancer vaccines target tumor-associated antigens including cancer/testis antigen, carcinoembryonic antigen, and cell differentiation antigen (Hollingsworth & Jansen, 2019). However, there are two major problems remained in cancer vaccine target TAAs. First is the lower T-cell response because high affinity self-recognizing immune cells are eliminated by central and peripheral tolerance in the process of T- and B-cell differentiation. This kind of vaccine has a requirement on the optimization of immune adjuvants to help improving adaptive immune response. Another problem is the nonspecificity of TAAs. Some normal cells that express these antigens are susceptible to vaccine-induced immune attack, leading to collateral damage.

Neoantigens are another commonly used antigen in cancer vaccines (Schumacher & Schreiber, 2015). Neoantigens or new proteins produced from cancer-specific mutations have very high specificity and immunogenicity. Genomic analysis reveals that patients who burden overall high mutation loads and potential neoantigen numbers benefit more from immune checkpoint blockade therapy such as CTLA4 and PD-1 inhibitor treatment (Van Allen et al., 2015; Hugo et al., 2016). Carreno and colleagues developed a DC vaccine that targets neoantigen and observed increased breadth and diversity of melanoma neoantigen-specific T cells (Carreno et al., 2015). Neoantigens vary between different patients, and accurate neoantigen identification for individual patients was difficult. Thanks to the development of sequencing technology and antigen prediction algorithm, personalized vaccine can be achieved. So far the antigen prediction accuracy is still very low, and multiple antigens are often included in the mRNA vaccine to maximize the chance of producing antitumor immunity. For instance, 34 neoantigens were included in mRNA vaccine mRNA-4157 to induce cancer-specific T-cell response and antitumor effect for treating unresectable solid tumors and resected melanoma (Burris et al., 2019). In addition to CD8 epitope, CD4 epitope also elicits antitumor immunity, but the prediction is even more difficult (Cafri et al., 2020). BNT111, an intravenously administered liposomal RNA vaccine, targets dendritic cells and induces strong antigen-specific T-cell response and antitumor effect in mouse models and is currently under clinical trial for treating melanoma patients in combination with anti-PD1 therapy (Kranz et al., 2016; Sahin et al., 2020).

6 Future Directions of mRNA Cancer Vaccine

Vaccination is essential for inducing antigen-specific T cells response as the initial step, which does not fundamentally solve the common problem of T-cell exhaustion in the process of cancer immunity. Thus, combining cancer vaccines with immune checkpoint blockade therapy can help improve the antitumor efficacy (Curran & Glisson, 2019). Besides encoding antigen, mRNA can also be programmed to

deliver pro-inflammatory cytokines or antibodies into tumor microenvironment to activate antitumor immunity (Van Hoecke et al., 2021). For example, mRNA-2752 from Moderna, Inc. (encoding human OX40L, IL-23, and IL-36γ) is currently under clinical trial to test its effect on T-cell infiltration into tumors and antitumor efficacy (Bauer et al., 2019; Patel et al., 2020). Moreover, mRNA can also be used to express receptors for T-cell reprogramming into CAR-Ts and TCR-Ts, which are promising adoptive T-cell transfer immunotherapeutics for treating blood cancers and solid tumors (Foster et al., 2019). Overall, recent research achievements have demonstrated that mRNA technology hold promise as a therapeutic platform to treat various diseases in addition to tumor immunotherapy and infectious vaccines. The coming years will witness the surge of mRNA drug development as another powerful weapon for treating cancer and other human diseases.

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Part III
Innovative Nanotechnologies for Cancer Diagnostic and Treatment

Nanoparticles for Therapy and Diagnostic Imaging Techniques in Cancer

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Abbreviations

ALP	Alkaline phosphatase
AuNPs	Gold nanoparticles
AuNRs	Gold nanorods
BSA	Bovine serum albumin
cm	Centimeter
CT	Computed tomography
CW	Continuous wave
DC	Downconversion
DFO	Deferoxamine
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
DS	Downshifting
DTPA	Diethylenetriaminepentaacetic acid
EPR	Enhanced permeability and retention (EPR) effect
g	Gram
Gd-DOTA	Gadolinium (III) 1,4,7,10-tetraazacyclododecane-1,4,7, 10-tetraacetate
GRP	Gastrin-releasing peptide
HA	Hydroxyapatite
hMSCs	Human mesenchymal stem cells
IUPAC	International Union of Pure and Applied Chemistry
keV	Kilo electronvolt
MBq	Megabecquerel
MCM-41	Mobil Composition of Matter No. 41
min	Minute

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mL	Milliliter
mPEG-SH	Methoxy poly(ethylene glycol) thiol
MRI	Magnetic resonance image
ms	Millisecond
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaRFA	Nano-radio-frequency ablation
NF-κβ	Nuclear factor-kappa β
NIR	Near-infrared region
NIRF	Near-infrared fluorescence
nm	Nanometer
NMH	Nano-magnetic hyperthermia
NOTA	1,4,7-Triazacyclononane-1,4,7-triacetic acid
NPTT	Nano-photothermal therapy
NUH	Nano-ultrasound hyperthermia
PAMAM	Polyamidoamine
PDMS	Polydimethylsiloxane
PET	Positron emission tomography
PLGA	Poly(lactic-co-glycolic acid)
PTT	Photothermal therapy
QD	Quantum dot
RE	Rare earth
RGD	Arginylglycylaspartic acid
s	Second
SPECT	Single photon emission computed tomography
SPIONs	Superparamagnetic iron oxide nanoparticles
SPR	Surface plasmon resonance
SWIR	Short-wave infrared
SWNTs	Single-walled carbon nanotubes
UC	Upconversion process
UCNs	Upconversion nanoparticles
UV	Ultraviolet radiation
W	Watt
μm	Micrometer

1 Introduction

Recently, nanomaterials are being explored with great optimism by the scientific community for the study of cancer treatment and diagnosis, as they allow for more efficient targeting and delivery of drugs and other important compounds, resulting in greater potency and specificity with decreased adverse side effects. The use of nanoparticles in cancer therapy is attractive for several reasons. These nanomaterials undergo passive and spontaneous accumulation in tumor regions as a result of a phenomenon called the enhanced permeability and retention (EPR) effect (Yhee

et al., 2013). Nanomaterials can also be actively targeted to neoplastic tissues with specific internalization in cancer cells when the surfaces of nanostructures are chemically modified by attaching molecules that bind specifically to receptors overexpressed in these cells (Attia et al., 2019). Furthermore, nanoparticles can be loaded with a great variety of chemotherapeutics, natural molecules, radioisotopes, luminescent agents, and magnetic materials, among other possibilities, acting as specific carriers for controlled delivery of treatment and diagnostic agents. Based on these attractive features of the nanomaterials, new modalities of cancer therapy using nanoparticles have been developed in the last years, such as local photothermal therapies, controlled drug delivery of chemotherapeutics, natural anticancer products such as curcumin, simultaneous treatment, diagnosis with radiolabeled nanocarriers, and a combination of these modalities, among others.

Cancer treatment by local photothermal therapy using noble metal nanoparticles like gold nanoparticles has become a promising strategy to improve the therapeutic efficiency of heat-based treatments of cancer. When combined with a functional nanomaterial, like calcium phosphate-based nanoparticles, photohyperthermia appears as a potential application to directly treat bone cancers by eliminating tumor cells and simultaneously promoting bone repair (Li et al., 2020). The drug delivery was one of the first applications of nanomaterials in biomedical research, and the controlled release of many common chemotherapeutics has been investigated, such as methotrexate, doxorubicin, cisplatin, and natural compounds such as curcumin (Monteiro et al., 2019; Liu et al., 2021). Curcumin has been researched over the years due to its medicinal properties including its anticancer property with low toxicity to healthy cells (Mansouri et al., 2020). Diverse studies point to nanotechnology as a tool to develop efficient delivery systems of curcumin, a natural anticancer, as nanoparticles can prolong its circulation time in the body and increase its bioavailability (Ghalandarlaki et al., 2014). Furthermore, it can be possible to add diagnostic properties to these nanoplatforms and enhance their therapeutic power through the incorporation of radioisotopes and fluorescent probes in the structure or surfaces of the nanoparticle, thus characterizing multifunctional nanomaterials (Bao et al., 2013). Bearing in mind the importance of the nanomaterials in the medical field, it becomes possible to unite all these elements in a single nanoplatform giving rise to the concept of nanotheranostic, which are materials able to act simultaneously in the treatment and diagnosis of specific illnesses through one single formulation (Kalash et al., 2016). Imaging cancer is crucial for guiding decisions about treatment and for monitoring the efficacy of administered therapies. The rare-earth ions are strategic materials for medical imaging and diagnostics due to their optical properties; they have been used in many imaging techniques such as computed tomography scans, magnetic resonance image (MRI), positron emission tomography (PET) imaging, and conventional X-ray systems (Yu et al., 2020). Following the most recent trends in these topics, the purpose of this chapter is to highlight the major impacts of nanotechnology research in therapeutic and diagnostic techniques for cancer management. A comprehensive review of this area of research is beyond the scope of this chapter, and therefore the authors elaborated the topics accordantly to the modality of cancer treatment or diagnosis that they have been investigating recently.

2 Nanoparticles for Therapy

2.1 Hyperthermia

The research and development of materials for use in hyperthermia processes take place in several applications. However, in recent decades, the development of recent technologies for the application of hyperthermia in therapies has been highlighted, especially in the area of cancer treatment. Studies have shown that the association of conventional treatments with hyperthermia processes is more efficient in the lysis of tumor cells, becoming a promising strategy to improve therapeutic efficiency in cancer treatment due to additive or synergistic effects (Ren et al., 2013; Li et al., 2014; Wang et al., 2014). The hyperthermia process is commonly defined as a therapeutic procedure in which tissues are heated to high temperatures in the range of approximately 40–45 °C (Mallory et al., 2016). All cellular components can be damaged by heat, including the cytoskeleton, membranes, and nucleus, but according to studies by Goldstein et al. (2003), the most likely thermal effect with increasing temperature is the denaturation of intercellular proteins, causing permanent irreversible damage, cell degradation, and induction of apoptosis. It is important to note that the degree of damage caused by thermal exposure depends on both the temperature reached and the time of exposure to heat (Hildebrandt et al., 2002; Jaque et al., 2014).

Depending on the location and size of the tumor, different methods and techniques can be applied. Within the field of nanotechnology research, there are several processes for heating a region through hyperthermia such as nano-photothermal therapy (NPTT), nano-magnetic hyperthermia (NMH), nano-radio-frequency ablation (NaRFA), and nano-ultrasound hyperthermia (NUH) (Beik et al., 2016) (Fig. 1). The difference between these processes is how heat is generated. In this chapter, the authors describe the process of heat generation by photothermal therapy (PTT) also called photohyperthermia, one of the treatment modalities that have been most investigated in recent years.

2.1.1 Photothermal Therapy (PTT)

Photothermal therapy (PTT), or photohyperthermia, uses photothermal agents to achieve selective and specific heating of the tumor region in cancer treatment cases. Photothermal agents are heated through interaction with laser light in the near-infrared region (NIR), promoting selective heating of the tumor region. With the development of nanotechnology, several materials with unique optical properties were discovered, such as noble metal nanoparticles, as silver and gold, which can be used as photothermal agents. For these agents to be effective, it is necessary to have improved light absorption and efficient conversion of light energy to heat (Chatterjee et al., 2011; Thakor & Gambhir, 2013; Singh et al., 2015).

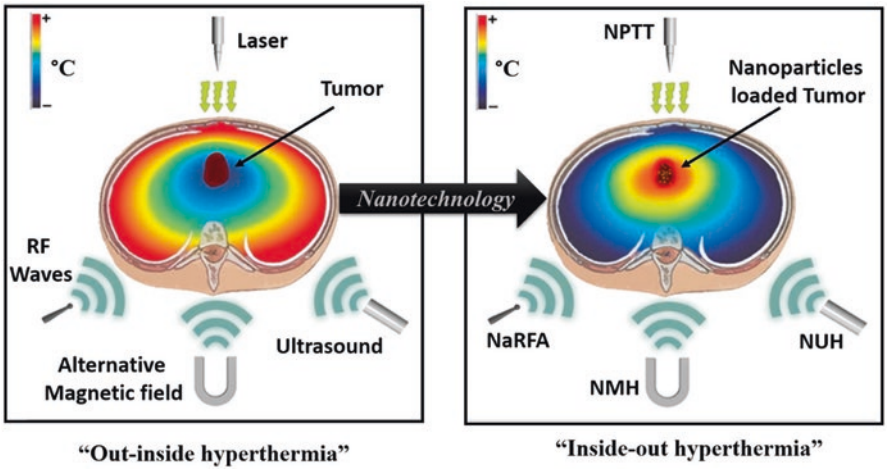

Fig. 1 Scheme of selective heating of tumor region using nanoparticles (Beik et al., 2016)

According to Huang and colleagues (2009), noble metal nanoparticles can confine light photons to induce coherent oscillation in the plasmonic surface of their conduction band electrons, called surface plasmon resonance (SPR). The SPR oscillation induces a strong absorption of light and can be analyzed by the UV-visible spectrum where the regions of the spectrum where the highest light absorption by nanoparticles occur, called SPR bands (Jain et al., 2007; Huang & El-Sayed, 2010). The SPR condition depends on the particle size, shape, and structure but also the dielectric properties of the metal and the surrounding environment, thus imparting unique optical characteristics to the noble metal nanostructures (Link & El-Sayed, 1999; Huang et al., 2009; Huang & El-Sayed, 2010). Photothermal agents show strong absorption of light in the NIR regions of the electromagnetic spectrum, especially at wavelengths from 650 to 900 nm, due to SPR (Thakor & Gambhir, 2013). One of the great advantages of using PTT with thermal agents that absorb light in the NIR range is that most biological tissues exhibit minimal light absorption in this range, which allows greater light penetration without harming healthy tissues. In this way, light in the NIR range can penetrate tissues, such as the skin and blood, noninvasively and deeper, as these tissues scatter and absorb less light at longer wavelengths. Spectral bands in which tissues are partially transparent are due to a simultaneous reduction in absorption and dispersion. The range in the region of 650–900 nm is considered ideal for the use of photothermal agents in cancer treatments, as in this range there is the greatest absorption of light by the agents, the SPR band range, and the least absorption of light by tissues, maximizing the photohyperthermia process (Weissleder, 2001; Smith et al., 2009). Gold nanoparticles, as mentioned above, have great prominence in use as a photothermal agent in photothermal therapy of cancer due to their unique optical properties, and as one of the focuses of this chapter, they will be further detailed in the next section.

2.1.2 Gold Nanoparticles Applied in Photothermal Therapy

The development of light-responsive gold nanoparticles (AuNPs) in the NIR range for photothermal therapy of cancer has received a lot of attention in recent years due to its easy synthesis, surface modification, and greatly enhanced and adjustable optical properties (Jain et al., 2006; Huang & El-Sayed, 2010). For example, gold nanospheres have a single SPR absorption band in the visible spectrum due to their unique oscillation mode. However, the gold nanorods (AuNRs) have two SPR absorption bands, referring to their two oscillation modes in the longitudinal and transverse axes, and the longitudinal SPR band has greater intensity and is located at a higher frequency, within the range of the NIR region. While the transverse band is insensitive to size changes, the longitudinal band moves in the NIR region depending on the length/width ratio, called the aspect ratio, of the nanorods (Nikoobakht & El-Sayed, 2003; Huang et al., 2009). This characteristic promotes the great interest in AuNRs for use in bioapplication, especially in photothermal therapies, as it is possible to develop AuNRs with a specific aspect ratio for each therapy.

Despite the existence of different morphologies of AuNPs, AuNRs are still the most developed and researched structures, mainly in the bioapplication area. According to Huang et al. (2009), of all the possible nanoparticle shapes, gold nanorods are especially intriguing as they offer strong plasmonic fields while exhibiting excellent tunability and biocompatibility. AuNRs have high light absorption efficiency at the same wavelength as the SPR band compared to other morphologies (Chen et al., 2013; Jaque et al., 2014).

Despite the ideal optical properties for application in photohyperthermia, the AuNPs have some limitations, such as instability at high temperatures and to light and high colloidal instability in aqueous systems. Thus, for its use in biological systems, several methodologies are studied, such as functionalization of nanoparticles with biocompatible species and coating with other more stable materials, to ensure the stability of these systems in an aqueous solution ideal for bioapplication (Wu et al., 2011; Dykman & Khlebtsov, 2016; Her et al., 2017) (Fig. 2).

An example of obtaining AuNPs with surface modification to develop stable systems for bioapplication can be seen in the study by Ruttala et al. (2020). In this study, a multifunctional nanoplatform based on AuNP conjugated with lonidamine, an antitumor, and aptamers for cancer treatment was developed. The presence of the aptamer on the surface of AuNPs promoted a significant accumulation of particles in cancer cells due to the specific affinity for mitochondrial receptors. Furthermore, in vitro studies have shown that laser irradiation-based hyperthermia enhanced the chemotherapeutic effects of lonidamine without causing damage to surrounding healthy tissue.

Another alternative that shows promise to overcome the limitations of the biological application of AuNPs is coating the nanoparticles with silica, considered an excellent material for biomedical applications due to its biocompatibility, low toxicity, and high surface area, with silica MCM-41 (Pastoriza-Santos et al., 2006; Giraldo et al., 2007; Chen et al., 2013). Silica tends to favor AuNP dispersion in the liquid, making the surface chemically functional and thermally stable.

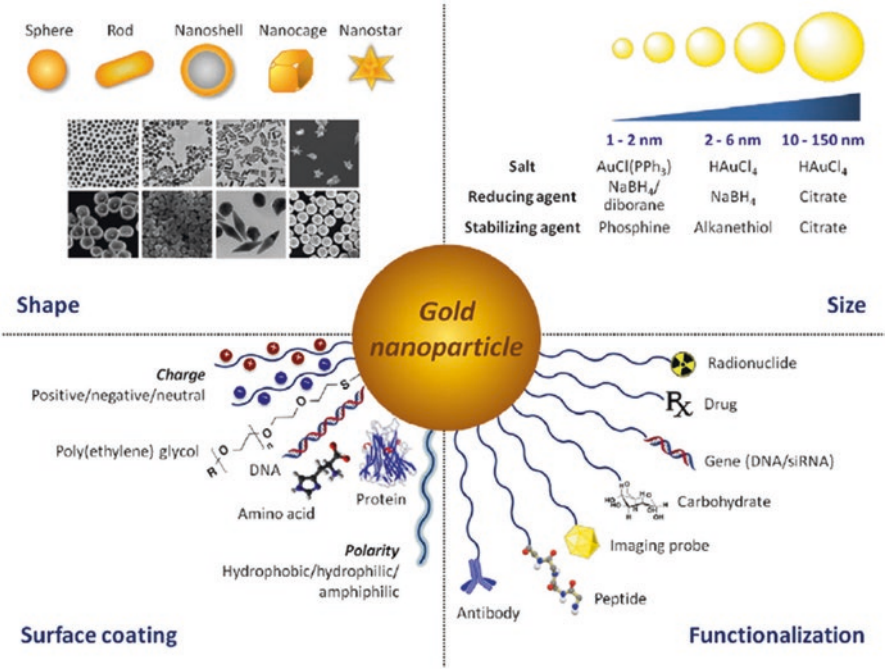

Fig. 2 The synthetic versatility of AuNPs. AuNPs offer a unique platform for straightforward manipulation of particle size, shape, surface coating, and functionalization, enabling fine-tuning (Her et al., 2017)

In the studies by Riva et al. (2017), a nanosystem composed of gold nanorods bonded to silica-coated iron oxide compounds was developed. To prevent the aggregation of iron nanoparticles in aqueous media, these particles were coated with silica and the entire nanosystem, with BSA protein to provide stability to the system in biological media. The developed material presented an SPR absorption band around 840 nm, ideal for application in therapy, and high conversion of laser radiation into thermal energy.

In new research that also addresses the coating of gold nanoparticles with silica, da Meireles et al. (2021) developed a nanocomposite of gold nanorods coated with mesoporous silica MCM-41. The AuNRs obtained presented SPR bands comprised in specific regions of the electromagnetic spectrum in which laser radiation has less interaction with tissues, being ideal for biomedical applications. The material showed high stability and desirable textural and morphological characteristics for bioapplications due to the silicate coating. Furthermore, the nanocomposite showed high biocompatibility and excellent performance in generating therapeutic heating by absorbing laser radiation, making it a promising agent in photohyperthermia.

The association of nanomaterials with different biocompatibilities can assist in the development of nanocomposites for bioapplication. He and coworkers (2020) developed a nanoplatform that associates graphene, which presents low

biocompatibility, with silica, which is widely used in nanocomposites for biomedical applications. In this work, graphene oxide was coated with a layer of mesoporous silica to bind the gold seeds that formed a layer of gold nanosheet. The hybrid of graphene, silica, and gold showed great biocompatibility and high photothermal conversion efficiency leading to a temperature increase of 16.4 °C. And in the in vitro assays, there was cell death, and cancer cells, with the treatment with the hybrid, developed when submitted to NIR irradiation.

The use of gold nanoparticles in therapies may have implications due to the characteristics of these nanoparticles, such as instability in aqueous systems where most biochemical processes occur, thermal instability, and loss of properties with temperature variations, thus decreasing the efficiency of the treatment. Furthermore, gold nanoparticles can form unstable colloidal suspensions, which hinder the transport of nanomaterials to the treatment regions, which can cause adverse effects on biological systems (Zhao et al., 2007; Bohara et al., 2016; Irfan et al., 2018). Thus, the investigation of cytotoxicity, biocompatibility, and photothermal therapy of nanoparticles in contact with the biological system is of paramount importance. Chuang and collaborators (2019) developed a nanosystem of gold nanorods encapsulated with the drug doxorubicin. The results of this work showed that the nanomaterial showed evident photothermal effects with the rapid and repeated release of the drug by near-infrared irradiation. Furthermore, there was significant cellular uptake of the nanosystem by murine colon cancer cells. The development of this work highlights the promising combination of photohyperthermia and chemotherapy in the treatment of cancer, in which the nanocomplex exhibited strong synergistic anticancer effects in vitro and in vivo.

Zhao and collaborators (2022) present the investigation of the biocompatibility in photothermal therapy of gold nanoparticles. In this work, a self-regulating photothermal conversion system was designed for selective photothermal therapy for specific tumor regions. The system was based on gold nanoparticles to investigate the selectivity effect. In the selective in vitro photothermal transformation model, laser irradiation selectively increased the temperature of the indoor microenvironment (pH 5.5) and resulted in a temperature difference of 5 °C from that of the external environment (pH 7.4). Furthermore, in the in vivo skin damage assessment model, the material achieved good tumor inhibition without damaging normal skin tissue compared to conventional photothermal material.

A recently published study shows the effects of the shape of gold nanoparticles in photothermal cancer therapy. In the study by Yang et al. (2021), the performance in photothermal therapy of three gold nanostructures was investigated: gold nanospheres, gold nanorods, and gold nanostars. The gold nanoparticles were surface-modified with mPEG-SH (methoxy poly(ethylene glycol) thiol). Gold nanostars showed greater photothermal conversion efficiency being the most promising agent for photothermal therapy among their counterparts. In vitro cell experiments demonstrated that all PEGylated gold nanoparticles exhibited low cytotoxicity.

Gold nanoparticles can be associated with other materials, even materials with low biocompatibility. Recent work by Zhang and colleagues (2022) has shown the efficiency of photothermal therapy of gold nanorods associated with

L-cysteine-reduced graphene oxide. This work showed the effectiveness of the material in photothermal therapy combined with tumor chemotherapy. The results showed a high therapeutic capacity, due to the high photothermal effect of the gold nanorods and reduced graphene oxide and their synergistic effect. Furthermore, the nontoxicity of L-cysteine ensured the biocompatibility of reduced graphene oxide with a slight side effect on normal tissues. The material produced proved to be a promising photothermal agent with high efficiency of photothermal therapy triggered by NIR laser, excellent stability, and superior biocompatibility.

In view of these works, it was possible to observe the great potential that gold nanoparticles present for their use in the treatment of cancer by thermal therapy. Furthermore, it was interesting to observe that gold nanoparticles present the possibility of association with several other materials, giving rise to multifunctional nanocomposites.

2.1.3 Photothermal Therapy and Bone Regeneration

Bone cancer is one of the biggest diseases that affect the bones, thus becoming a real problem for human health. Osteosarcoma is a primary malignant tumor that usually occurs in children and adolescents. It is the second leading cause of cancer death in teenagers. It can be considered aggressive cancer with a high occurrence of lung metastasis. Symptoms, difficult to differentiate at first, involve spontaneous fractures and local pain (Kansara et al., 2014). Therefore, this disease is not easily diagnosed, resulting in rapid tumor growth. As a result, osteosarcoma promotes cancer metastasis, as well as the formation of large bone defects, causing movement limitations (Ritter & Bielack, 2010; Botter et al., 2014). Traditional therapies such as chemotherapy, radiotherapy, or surgical removal of the tumor, usually combined, have an indecisive prognosis, as they can lead to the proliferation and metastasis of residual tumor cells (Nam et al., 2018). In addition, osteosarcoma affects large areas of the bone, which cannot repair itself, leading to poor quality of life for the patient (Ma et al., 2016b). When malignant tumor cells reach the bone system, the tumor spreads, and bone metabolism becomes unbalanced. This way, healthy tissue is reabsorbed and invaded by the tumor, leading to serious bone defects. After therapy, these defects become a major challenge to be faced (Liao et al., 2021).

With the development of bionanotechnology, new innovative treatments have been pointed out as an option for the therapy of this type of cancer. Photothermal therapy is widely studied and has brought hope for a cure for cancer. Unlike traditional therapies, the use of this type of treatment can avoid side effects, as it can occur through selective targeting.

To combine the complex issue of tumor cell death with bone regeneration, the key is to use functional biomaterials. Bifunctional biomaterials then appear as a potential application to eliminating tumor cells, that is, directly treating the cancer site, as well as promoting bone repair. These bifunctional biomaterials must have specific characteristics for their use. It is common to use calcium phosphate-based materials, once they are biocompatible and osteoconductive (Roberts & Rosenbaum,

2012). Also, the bioactive ions in 3D-printed scaffolds, such as Ca^{2+}, P^{5+}, Si^{4+}, Mg^{2+}, Fe^{3+}, and Mn^{4+}, can improve osteogenic activity; therefore, its application is widely studied (Liao et al., 2021).

The administration can be performed locally or systematically. Figure 3 shows a bone tumor that was killed using bifunctional biomaterials. Locally applied bifunctional biomaterials were inserted into the bone defect area for treatment, photothermal therapy, and subsequent bone repair. The local administration mainly concentrates on 3D-printed scaffolds, scaffolds composed of nano- and microparticles, and hydrogels (Liao et al., 2021).

During the year 2016, Chengtie Wu's group formulated a 3D-printed scaffold modified with a Ca-P/polydopamine nanolayer. The polydopamine nanoparticles used on the surface can cause hyperthermia to kill MDA-MB-231 tumors in nude mice. Additionally, this scaffold could release Ca and P in a sustainable manner to induce femoral defect regeneration (Ma et al., 2016a). In 2018, the same research group designed a high-strength 3D-printed bioscaffold with Fe-$CaSiO_3$ for tumor therapy and bone repair. As a result, the scaffold showed an excellent photothermal effect. The presence of $CaSiO_3$ improved degradation performance and stimulated rat bone marrow mesenchymal stem cell proliferation and differentiation besides promoting bone formation in vivo tests. Therefore, those scaffolds can function as

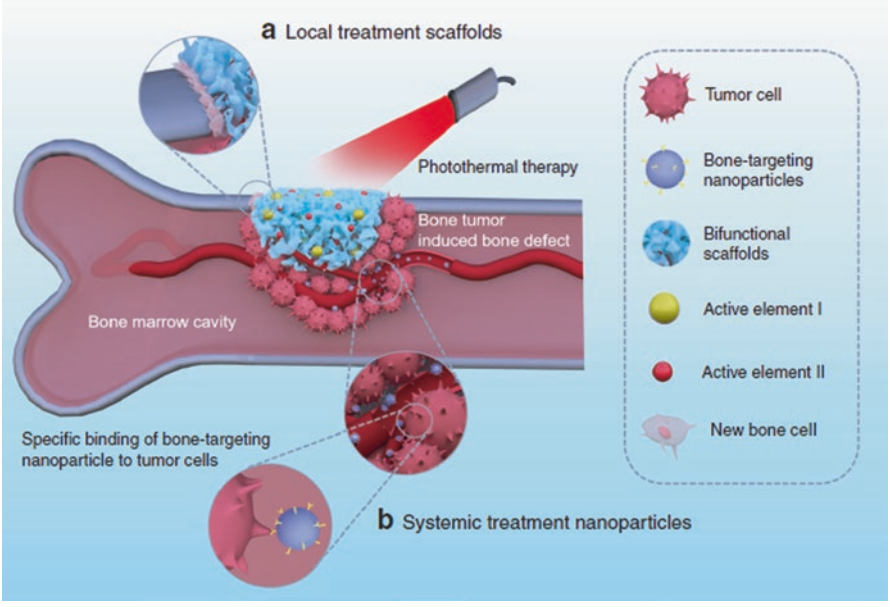

Fig. 3 Bifunctional biomaterials include local treatment scaffolds (such as 3D-printed scaffolds, nano−/microparticle-containing scaffolds, and hydrogels) and systemic treatment nanoparticles (such as bone-targeting nanoparticles) for tumor photothermal therapy and bone regeneration (Liao et al., 2021). "Bifunctional biomaterials" by Liao et al. (2021) is licensed under CC BY 4.0. To view a copy of this license, visit https://creativecommons.org/licenses/by/4.0/

versatile and efficient biomaterials for future bone regeneration and the treatment of cortical bone cancer (Liu et al., 2018b).

In the same year, the Dejian Li group developed a nano-hydroxyapatite and reduced graphene oxide scaffold for photothermal therapy and bone regeneration (Li et al., 2018). The research revealed satisfactory results. The scaffolds showed excellent physicochemical properties, biocompatibility, efficiency in photothermal conversion, and osteoinduction potential. In vitro experiments verified that the photothermal effect of scaffolds could effectively kill MG-63 cells (human osteosarcoma cells). The scaffolds inhibited the growth of the xenografted tumor in mice through infrared laser irradiation. Furthermore, scaffolds were able to simultaneously promote the proliferation and differentiation of bone marrow stem cells as evidenced by ALP (alkaline phosphatase) activity and bone gene expression. In vivo experiments indicated that scaffolds promoted bone repair in rat skull defects, thus concluding that the scaffolds prepared in the work have potential application in cancer therapy and bone repair.

Several studies are linking HA to magnetic materials for different applications, including the treatment of cancer using magnetohyperthermia (Murakami et al., 2008; Tampieri et al., 2012; Iwasaki et al., 2013; Sneha & Sundaram, 2015; Mondal et al., 2017; Campodoni et al., 2021). Mondal and coworkers reported a simple procedure for obtaining a system formed by nano-HA and magnetite nanoparticles. The nanocomposite proved to be hydrophilic and superparamagnetic with good magnetic saturation. Cytotoxicity assays showed that only magnetite nanoparticle samples were toxic to MG-63 cells, thus indicating good cell viability of the system. It was also found that MG-63 cells easily engulfed the nanoparticles, thus contributing to cellular internalization. When compared to magnetite nanoparticles, the HA system registered a smaller increase in temperature but is still efficient for hyperthermia. The authors suggest that HA acts as an insulator surrounding the magnetite particles and, therefore, heat generation was different. Hyperthermia caused necrosis of tumor cells, leading to death, as evidenced in a cytotoxicity assay. The authors then concluded that the system obtained performed well once its biocompatible with great bone regeneration potential in addition to promising material for use in magnetohyperthermia to eliminate residual tumor cells and a potential bone regenerator (Mondal et al., 2017).

The systemic administration, shown in Fig. 3, involves the use of targeted nanoparticles. Systemically administered nanoparticles penetrate blood vessels to reach bone tissue for cancer treatment and to inhibit bone resorption. Bone reabsorption promotes bone destruction and tumor metastasis processes. Moreover, due to the low blood flow in the bone (0.05–0.20 mL min^{-1}/g) (Hirabayashi et al., 2001) and blood-bone marrow barrier, targeted delivery of anticancer agents is highly recommended for bone tumor therapy (Liao et al., 2021).

In 2019, Sun and coworkers prepared gold nanorods encapsulated in mesoporous silica nanoparticles conjugated with zoledronic acid for bone-targeted assisted inhibition of the proliferation of osteoclast-like cells and the promotion of osteogenic differentiation. The targeted photothermal therapy was enhanced to cure breast tumor bone metastasis in the hindlimbs of nude mice. This nanosystem cured the

tumor and inhibited bone reabsorption for breast cancer bone metastasis treatment (Sun et al., 2019).

Zhou and coworkers developed a nanoplatform with platin nanoparticles coated with phytic acid (PA/PtNPs) for bone-targeted photothermal therapy (PTT) of malignant bone tumors. The in vitro assays demonstrated that PA/PtNPs had a high binding affinity to hydroxyapatite and bone fragment and possessed the inherent anticancer ability; therefore, PA significantly enhanced the amount of PA/PtNPs bound on hydroxyapatite and their accumulation at the tumor-bearing bone lesions. Because of PA, PA/PtNPs also exhibited inherent anticancer capability in vitro and in vivo. The in vivo therapeutic study revealed that PA/PtNP-associated combination therapy efficiently regressed the bone tumor growth and tumor-associated osteolysis (Zhou et al., 2019).

All these studies presented biomaterials with potential application to eliminating tumor cells, as well as promoting bone repair. With the benefits of locally administered and systemically administered, the prognosis for patients with bone tumors has great potential to improve. Therefore, these new nanoplatforms may provide new hope for clinical bone tumor therapy while improving patient quality of life and decreasing mortality.

2.2 Curcumin Loaded in Nanoparticles as a Therapeutic Agent

Natural products have been used as traditional medicines for centuries and have shown promise as a source of components for the development of new drugs. *Curcuma longa* Linn is a plant member of the Zingiberaceae family native to Southeast Asia, cultivated in tropical and subtropical regions worldwide (Ravindran et al., 2007). Due to its pharmacological and biological properties, such as antioxidant, anti-inflammatory, antimutagenic, healing, antimicrobial, and anticancer activity, curcumin attracts the interest of researchers worldwide, even though it is present in approximately two centuries of scientific research (Hatcher et al., 2008; Priyadarsini, 2014).

The chemical structure of curcumin, as well as the structure of the other curcuminoids, which are the active components found in the crude extract of turmeric or *Curcuma longa* L., is shown in Fig. 4.

Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione), also called diferuloylmethane, is the main polyphenol responsible for the turmeric properties and yellowish coloration (Priyadarsini, 2014). Its chemical structure consists of two o-methoxyphenolic aromatic ring systems linked by a seven-carbon carbon chain comprising an α β-unsaturated fraction and a β-diketone group (Karthikeyan et al., 2020). The intermolecular interaction of curcumin with its biomolecular targets occurs due to the existence of these three reactive sites. The two o-methoxyphenolic aromatic ring systems act as hydrogen atom donors. The β-diketone portion acts as an excellent chelator of metal ions, and the α,β-unsaturated portion acts as a Michael acceptor in nucleophilic addition reactions. In addition,

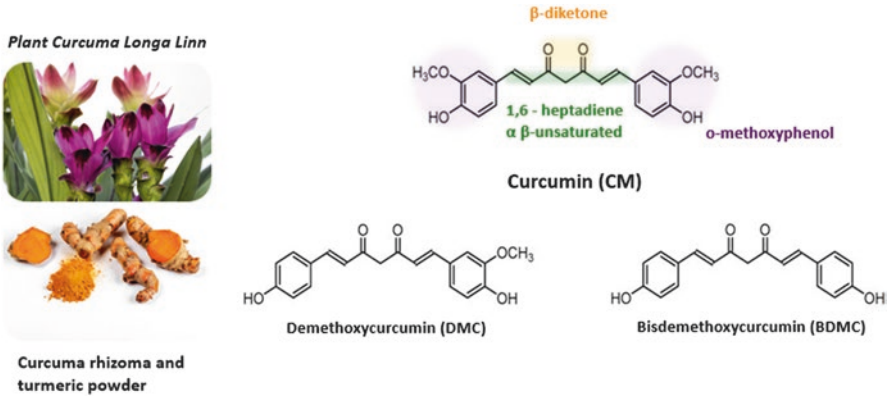

Fig. 4 Chemical structure of curcuminoids extracted from the rhizome of *Curcuma longa* L. "Turmeric and Turmeric Powder on White Background" by Marco Verch is licensed under CC BY 4.0. (To view a copy of this license, visit https://creativecommons.org/licenses/by/4.0/)

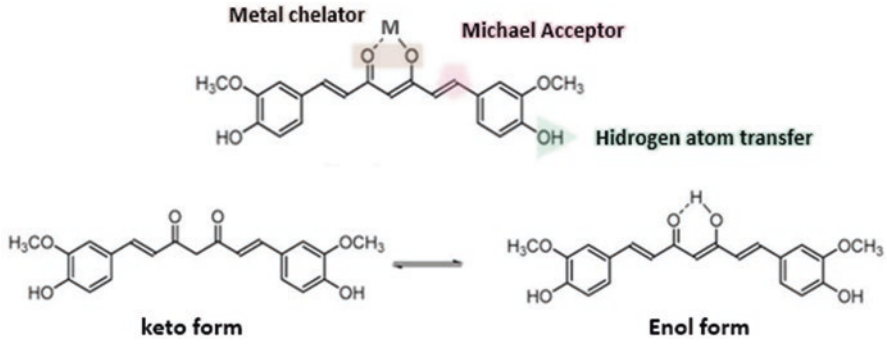

Fig. 5 Curcumin reactive sites and their tautomeric forms

the β-diketone portion has keto-enolic tautomerism that causes curcumin to exhibit different conformations according to the medium. In general, at neutral or acidic pH, the ketone form is predominant, whereas the enol tautomer is exclusively present in alkaline conditions (Fig. 5). In most apolar and moderately polar solvents, the enol form is generally more stabilized than the ketone form.

Over the past 50 years, curcumin has received increasing interest in pharmacological and biological research (Zielińska et al., 2020). Since then, many studies have proven the potentially beneficial properties of curcumin, such as antiproliferative, antimetastatic, antiangiogenic, antithrombotic, immunomodulatory, hepatoprotective, neuroprotective, and especially anticancer properties. At the molecular level, curcumin not only inhibits cell proliferation and metastasis but also induces apoptosis through the modulation of various pro-inflammatory factors, including growth factor receptors, inflammatory cytokines, transcription factors, enzymes, kinases, and adhesion molecules, among others (Zielińska et al., 2020).

However, its clinical application still brings some challenges due to its low solubility, rapid elimination from the body, and low bioavailability (Patra et al., 2018; Mukherjee et al., 2020). For these reasons, researchers around the world have used nanotechnology as a tool to develop delivery systems that can protect curcumin from degradation, prolong its circulation time in the body, and increase its bioavailability (Dang & Guan, 2020). Among the strategies used to increase the bioavailability of curcumin, are substances to improve dispersibility, such as surfactants in obtaining micelles and nanoemulsions, hydrophobic carriers, such as liposomes, and the formation of inclusion complexes, that comprise the most used and successful approaches to obtain curcumin compounds (Tabanelli et al., 2021).

In cancer treatment, chemoresistance is a common problem. In particular, activation of Nuclear factor-kappa β (NF-κβ) is considered an important factor in the development of chemoresistance to the chemotherapeutic drug gemcitabine during breast cancer treatment (Eskandari et al., 2021). Thanks to curcumin's ability to modulate several signaling pathways, it can inhibit NF-κβ activation in addition to inhibiting the development of metastasis and acting in the fight against cancer. To increase the bioavailability of curcumin in the tumor region, Eskandari and colleagues (Eskandari et al., 2021) developed poly(lactic-co-glycolic acid) (PLGA) nanomicelles with a natural fructose homopolymer (levan), for use in the treatment of breast cancer. In vivo assays with mouse models of breast cancer show successful targeting of the nanoformulations to the cancer tissue. They were able to inhibit NF-κβ activation, and joint use with gemcitabine increased the inhibitory activity. These results demonstrate the potential use of curcumin nanoformulations with chemotherapy (Eskandari et al., 2021).

In order to investigate the therapeutic efficacy of curcumin in the treatment of squamous cell carcinoma, Malathi's colleagues (Malathi et al., 2020) developed nanopatterned films. Curcumin-loaded poly(lactic-co-glycolic acid) (PLGA) films were obtained using the polydimethylsiloxane (PDMS) fused molding technique. The films showed good cytotoxicity against human skin cancer cell lines (A431). In addition, they were able to inhibit the progression of skin cancer, induced in albino mice. These results demonstrate the potential use of these films as an alternative skin cancer treatment (Malathi et al., 2020).

Hybrid nanostructures have also been developed to ensure combined drug delivery, with active targeting, mediated by ligands with targeting function (Sharma et al., 2018). Ghanbari and colleagues (2021) developed a nanostructured targeted drug delivery system. The study aimed to evaluate its cytotoxic effect on breast cancer cells overexpressing glucosamine receptors. Nanostructures fabricated with glucosamine-conjugated graphene quantum dots loaded with curcumin showed pH-responsive sustained release and low toxicity to normal fibroblast cells. Furthermore, the results of in vitro cellular uptake studies exhibited stronger fluorescence for the targeted nanoporter against MCF-7 cells compared to the untargeted one. These results demonstrate the enhanced cellular internalization via endocytosis mediated by the glucosamine receptor and confirm the importance of the targeting agent (Ghanbari et al., 2021).

In a promising study, polyamidoamine dendrimers (PAMAM) were used as nanocarriers functionalized with antiapoptotic gene Bcl-2 for targeted co-delivery

of curcumin and siRNA. The nanoparticles not only allowed greater cellular uptake but also promoted greater inhibition of human cervical tumor cell (HeLa) proliferation (Ghaffari et al., 2020). In vitro and in vivo assays using mice have also been performed to investigate the potential of PAMAM dendrimers for the treatment of hepatocellular cancer. PAMAM dendrimers were conjugated with triphenylphosphonium for targeted delivery of curcumin to the mitochondria of liver cancer cells. The results showed that the framework was able to successfully deliver curcumin to mitochondria, selectively and efficiently inducing apoptosis of cancer cells without causing significant damage to healthy cells. They also altered cancer-related genes at the cellular level and promoted cell cycle arrest in G2/M. In addition, they significantly reduced tumor growth in the mouse model and increased survival time. According to the researchers, these results demonstrate the potential of the structure in targeted drug delivery, to mitochondria, not only for the treatment of liver cancer but also for other types of cancer since cancer cells have higher mitochondrial activity (Kianamiri et al., 2020).

Following this trend, a novel study is being developed to obtain a nanostructured system based on hydroxyapatite nanorods, loaded with curcumin and functionalized with folic acid (Marinho et al., n.d.). The objective of the work is to obtain a carrier system for active and targeted delivery of curcumin, with potential use in the treatment of osteosarcoma. In general, it is expected that the nanostructure will be able to help in the treatment of cancer and the recovery of the bone tissue, due to the presence of the hydroxyapatite nanorods.

These researches indicate that curcumin may be a future safe chemotherapeutic agent for the treatment of various types of cancer.

3 Nanomaterials Applied in Diagnostic Imaging Techniques

The emergence of nanomaterials offers a promising strategy for the development of devices for imaging diagnosis applications (Zhang et al., 2019; Ge et al., 2020; Wang et al., 2020; Chen et al., 2021). This procedure is essential for understanding the extent of tumor progression and can mean a relatively more effective way to treat the disease at an early stage (Hou et al., 2017; Ge et al., 2020).

The complexity of the tumor environment requires careful selection of light wavelength for medical use. Excitation of ultraviolet and visible light can cause photodamage to biological tissues and has limited tissue penetration. Therefore, the chosen wavelength should have limited photon scattering, minimized absorption, and negligible autofluorescence to permeate through skin barriers and penetrate deep tissue (Ang et al., 2021).

In this context, optical imaging in the near-infrared (NIR) region, called the "biological window," has gained much attention from the scientific community. The imaging agents that operate in the first window, NIR-I (700–900 nm), can present large photon scattering losses in biological samples, and their penetration depth in tissue is still limited, reaching less than 1 cm. This characteristic restricts their use in biomedical diagnostics and therapy. In contrast, materials that act in the NIR-II

window (1000–1700 nm) have deeper penetration, around 1.8 cm, providing a better resolution for application in biological imaging (Yu et al., 2020).

This characteristic has contributed to the increased use of these materials in both scientific research and clinical practice as biosensors and for real-time imaging (Yu et al., 2020). Wang and colleagues (2019a) developed a nanomaterial in the second biological NIR-II window (1500–1700 nm) for high-resolution fluorescence imaging in vivo. The authors achieved a spatial resolution of over 1550 nm and a penetration depth of 3.5 mm in a simulator. The obtained values were much higher when compared to previous studies (Wang et al., 2019a).

Currently, several fluorescent agents with emission in the NIR-II region such as single-walled carbon nanotubes (SWNTs), organic dyes, conjugated polymers, quantum dots, and rare earth-doped nanoparticles have been explored (Yu et al., 2020). The latter stands out due to the prominent optical, magnetic, and X-ray attenuation properties of rare-earth (RE) elements, as well as the radioactivity of their isotopes, and is re-becoming increasingly popular in the biomedical field (Hong et al., 2019).

3.1 Exploring the Intrinsic Properties of Rare-Earth Elements

The RE elements correspond to a group of 17 chemical elements, including yttrium (Y), scandium (Sc), and 15 metal elements of the lanthanide series, as declared by the International Union of Pure and Applied Chemistry (IUPAC) (Jordens et al., 2013).

An interesting advantage of these elements is the characteristics of high and clear luminescence and fluorescence (Al-Shehri et al., 2019). This property gives the material the ability to absorb energy from some kind of exciter source and emit radiation with a characteristic wavelength (de Lucena et al., 2004). According to the different excitation modes, luminescence can be categorized as photoluminescence, X-ray luminescence, electroluminescence, chemiluminescence, cathodoluminescence, triboluminescence, and bioluminescence (Lei et al., 2020). Photoluminescence, generated by photon excitation, is the most studied and widely used type. The phenomena involved in this property are represented by fluorescence (decay time < 10 ms) and phosphorescence (decay time > 0.1 s) (Neacsu et al., 2019).

3.1.1 Luminescence of Rare-Earth Elements

The luminescent property of rare-earth elements originates from the electronic configurations characteristic of these materials. The electrons transit between the various energy levels and generate rich absorption and emission spectra, allowing efficient photon emission in a wide ultraviolet (UV) to visible and near-infrared spectral region (Huang et al., 2019).

The orbitals 4f are highly protected by sub-layers $5s^2$ $5p^6$ and are not directly involved in chemical bonding. This causes a low variation of the energy of these

levels with the chemical environment in which the ions are inserted. Therefore, the emission wavelengths of Ln^{3+} ions are minimally disturbed by the surrounding ligand field and matrix, resulting in clear emission bands with the same wavelength as the salt Ln^{3+} (Lei et al., 2020).

Despite this, the optical transition, energy transfer, photoluminescent life, and photoluminescent efficiency of dopants are significantly influenced by the structure, site symmetry, crystal field strength, and phonon energy of the host matrices. This fact demonstrates that the image quality depends on the selection of the matrix. We highlight the materials germanate, galogermanate, silicate, and aluminosilicate plicated as a matrix and the lanthanide ions Eu^{2+}, Ce^{3+}, Sm^{3+}, Dy^{3+}, Nd^{3+}, and Pr^{3+} (Lei et al., 2020).

Luminescence related to Ln^{3+} ions can be distinguished by several energy transfer pathways and is divided into three categories: downshifting (DS), downconversion (DC), and upconversion process (UC). This classification is performed according to the relation between the excitation wavelength and the emission wavelength and the selection of the excitation source (Sarkar et al., 2019).

In terms of energy, DS luminescence primarily emits low-energy light through the excitation of high-energy photons. In this process, the electrons are first excited from the ground state to the excited state followed by non-radiative relaxation to the lower excited state and then return to the ground state by emitting light. This method has the advantages of long-term emission, high quantum yield, large displacements of apparent Stokes, and clear emission bands. Most current studies on DS images of rare-earth materials are based on the luminescence of the Eu^{3+}, Tb^{3+}, Er^{3+}, Yb^{3+}, and Nd^{3+} ions. Although some progress has been made in the use of visible emission DS nanoparticles for optical images, the use of UV excitation is still inevitable, which is harmful to cells in some respects and produces strong autofluorescence in biological cells and tissues. In addition, due to strong tissue absorption and low light penetration depth of short wavelength below 600 nm, many DS nanoparticles are limited in bioimaging applications (Sarkar et al., 2019; Lei et al., 2020).

The DC process occurs similarly to DS, where electrons are first excited to the high-energy state and then radiated back to the ground state. The difference is that in this process occurs the conversion of a high-energy photon into two or more low-energy photons, with a high quantum efficiency. The DC process usually occurs between two lanthanide ions. When compared to the DS, reports on the DC process are relatively rare (Wegh et al., 2000; Yu et al., 2015; Lei et al., 2020).

Luminescence UC is the conversion of low-energy radiation into high-energy light through multiphoton absorption processes. The quantum efficiency of this class is dependent on the different energy transfer processes between the energy levels of lanthanide ions. In this process, the commonly employed UC excitation source is the near-infrared lasers, which are very favorable for bioimaging (Chen et al., 2014; Zhou et al., 2015).

According to the demonstration by Liu and coworkers (2018a), a new type of nanoparticles with emissions in the NIR-II region can be obtained with Er^{3+}. This system assisted in the analysis of an in vivo inflammation dynamically at high resolution (200 × 200 μm) due to the large anti-Stokes shift, low autofluorescence, and

tissue scattering of the NIR-II upconversion luminescence (Liu et al., 2018a). Another study showed the imaging and hyperthermia potential of a theranostic agent composed of lanthanides. The erbium (Er^{3+}) enriched nanocrystals ($NaErF_4$) enabled luminescence emission (1525 nm) in the second biological window (NIR-II, 1000–1700 nm). The researchers obtained high-resolution optical images of blood vessels and tumors under excitation of ~980 nm. High concentrations of neodymium (Nd^{3+}) introduced maximum cross-relaxation processes that converted absorbed NIR light (~808 nm) into heat with high efficiency, thus providing abilities for photothermal therapy (PTT). In this study, PTT-treated tumors shrank ~12 times compared to untreated tumors (Wang et al., 2019b).

Zhao and colleagues (2018) explored a novel gene therapy strategy. In this study, a material composed of NIR II-emitting rare earth-doped nanoparticles was developed. The system was designed to deliver gene cargo in an in vitro cancer model and detected tumor lesions in a breast cancer lung metastasis model. The material has been shown to be capable of delivering genetic loading and performing real-time response monitoring (Zhao et al., 2018).

In particular, the luminescence efficiency of most UC materials is still less than 1% due to the low absorption coefficient of lanthanide ions. Many efforts have been devoted to improving the quantum efficiency of these materials. However, a high laser excitation power with overheating side effects is often required in this process, and, according to the American National Standard for Safe Use of Lasers, only a low irradiance of about 0.1 W cm^2 (for 980 nm CW laser diode) can be applied to the human skin (Lei et al., 2020).

3.1.2 Magnetic Properties of Rare-Earth Elements

The lanthanide elements also attracted great interest in magnetic resonance imaging (MRI) because most of them have lone pairs of 4f electrons. Of the lanthanide ions, La^{3+} (the f orbitals are empty) and Lu^{3+} (the f orbitals are fully occupied) have unpaired electrons, which can effectively induce spin-orbit coupling and significant paramagnetic properties (Dong et al., 2015). The magnetic properties of the Eu^{3+}, Gd^{3+}, Tb^{3+}, Dy^{3+}, Er^{3+}, Ho^{3+}, Tm^{3+}, and Yb^{3+} ions allow these ion-containing materials to be used in magnetic resonance imaging (Lei et al., 2020).

Currently, bioimaging applications of most reported materials containing RE are still limited to cellular studies and preliminary animals, and clinical translation is still in its infancy. Some biomaterials containing rare-earth elements such as gadolinium (III) 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (Gd-DOTA, contrast agent for magnetic resonance imaging) and ^{177}Lu-Dotatate (clinical medicine for radiation therapy) were marketed, but most are still in laboratories and preclinical trials (Lei et al., 2020).

Gadolinium is the most widely used rare-earth element in medical diagnosis by magnetic resonance imaging and in contrast agents to improve the sensitivity and specificity of diagnostic images. In this technique, it is possible to visualize body

morphology with a very high resolution, since Gd(III) ions are the best paramagnetic compounds in the periodic table (Mitsumori et al., 2014).

Ma and colleagues (2018) had explored nanoparticle emitting short-wave infrared (SWIR), activated simultaneously by an 808 nm laser and a magnetic resonance imaging (MRI). The system is shown to have the potential to accurately determine the position of tumors (Ma et al., 2018).

Shi and collaborators (2010) developed hybrid Gd_2O_3 nanoparticles doped with Eu^{3+}. The results showed that the nanoparticles were internalized into human mesenchymal stem cells (hMSCs), confirmed by confocal laser scan microscopy and inductively coupled plasma-mass spectrometry. The MTT assay showed that nanoparticles did not present significant cytotoxicity. In addition, the osteogenic, adipogenic, and chondrogenic differentiation of hMSCs was not influenced by the labeling process. With magnetic resonance images, the in vitro detection limit of cells after incubation was estimated at about 10,000 cells. These results are an indication that nanoparticles are promising for luminescent application and as a contrast agent (Shi et al., 2010).

Another study explored upconversion nanoparticles (UCNs) with multimodal fluorescence imaging, magnetic resonance imaging (MRI), and dual-energy computed tomography (CT). The UCNs marked stem cells efficiently and in a long term (at least 14 days), with almost no influence on the viability, cell cycle, apoptosis, and multilineage differentiation. The researchers demonstrated stem cell assembly at the site of the cortical bone defect, and in vivo images were well correlated with in vitro fluorescence observation. Therefore, these results demonstrate that the system was potentially developed for noninvasive real-time cell screening, which may be significant to understanding bone regeneration in a clinic (Chen et al., 2018).

The promising results presented by nanomaterials doped with rare-earth elements led to an increase in the research and development of new materials in biomedical areas (Fricker, 2006), such as biometrics (Bünzli, 2010), bioimaging (Liu et al., 2013), drug delivery (Yang et al., 2015), theranostics systems (Gai et al., 2014), and others.

4 Radiolabeled Nanoparticles for Treatment, Diagnosis, and Theranostics

4.1 Overview of Radiation in Medicine

Since the discovery of X-rays and radioactive emissions in the late nineteenth century and at the beginning of the twentieth century, radiation has been added to the arsenal of under-developing techniques designed to diagnose and treat diseases like cancer (Reed, 2011; Vinet & Zhedanov, 2014). High-energy electromagnetic radiations, like X-rays and gamma rays, were first used in medicine for imaging the inner human body due to their ability to penetrate organic tissues with distinct absorption

characteristics according to the density, thickness, and effective atomic number of the different organs, which makes it possible to convert the differential intensities of the radiation bean that go through the human body into a visible image by capturing the passing radiation with specific radiographic films or detectors. The conventional X-ray systems and the computer tomography systems are based on these concepts. In the 1920s, Hermann L. Blumgart and Otto C. Yens worked on a revolutionary insight to use radionuclides as diagnostic agents. Their approach was based on tracking the path of radioactive iodine in the bloodstream to measure the blood flow or circulation time, being the first diagnostic radiotracer procedure done on a human (Patton, 2003) and the birth of the nuclear medicine and radiopharmacy.

A few years after the discovery of the phenomenon of radioactivity, Becquerel and Curie described some physiologic effects of radioactive emissions of a radium radionuclide (Becquerel & Curie, 1901). By that time, the use of radiation to treat most skin cancers had been reported, followed by a great advance in radiotherapy in the next decades provided by the increasing number of published studies on the use of radioisotopes to treat other diseases as well as the development of more sophisticated techniques and types of equipment (Gianfaldoni et al., 2017; Do Huh & Kim, 2020). Despite the great progress in radiodiagnosis and radiotherapies in the last century, the lack of specificity for radiations to attack only tumors or pathogenic tissues, or to have confined action preferably in cancerous cells or damaged cells, remains a big concern to the use of radiation in medicine. However, the considerable breakthroughs on active targeting nanocarriers by multifunctional nanoparticles research bring to light some to overcome to this problem.

The radiolabeling of nanomaterials has been reported in a great variety of scientific research (Goins, 2008; Xing et al., 2014; Koziorowski et al., 2017; Gholami et al., 2019) aiming for the selective delivery of radiation doses to provide specific diagnosis or specific treatments to many diseases and precise theranostics which means simultaneous therapy and diagnosis (Lim et al., 2015). In scientific research, the radiolabeling techniques are valorous tools that help scientists to elucidate the biodistribution of promisor nanocarriers that are designed for diverse bioapplications, from vaccines to other approaches (Guerrero et al., 2012; Sánchez-Martínez et al., 2013), likewise, the monitoring of functional processes at molecular and cellular levels to probe the molecular abnormalities that are the basis of some diseases including cancer (Morales-Avila et al., 2012).

4.2 Diagnosis of Cancer with Radiolabeled Multifunctional Nanomaterials

Each one of the distinct imaging modalities presents unique features that determine the advantages and disadvantages of a single radiodiagnostic technique above the others. Some of these techniques provide better functional data, while others provide better anatomic or morphological information. Furthermore, even between the

functional imaging modalities, like magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), and positron emission tomography (PET), the unique physical principles that govern the obtaining of each type of image provide distinct information. Considering the advances and peculiarities among the imaging modalities, a combination of these techniques is usually required to detect elusive lesions (Fernández & Cueto, 2009). Therefore, multifunctional nanosystems are capable to reunite two or more distinct imaging agents in one single platform to provide simultaneously morphological, functional, and molecular information, offering the possibility to improve the diagnostic and therapeutic monitoring abilities (Xing et al., 2014). Taking advantage of these possibilities, many scientific works have been demonstrating how nanoparticles can be used to improve imaging diagnosis from multimodal systems.

Cai and coworkers (2007) develop a dual probe for specific tumor vasculature imaging through PET and NIRF (near-infrared fluorescence) systems. The authors modified the amine-functionalized surface of quantum dots with a DOTA chelator for copper-64 radiolabeling and RGD peptides for integrin $\alpha_v\beta_3$ targeting. This multifunctional system was radiolabeled with copper-64, and biological assays were performed to test the binding specificity and quantify the probe uptake in the tumor and major organs. The results show that the radiolabeled dual probe exhibited integrin $\alpha_v\beta_3$ specific binding and RGD peptides improved the uptaking in tumor cells. Furthermore, the authors obtained a linear correlation between the results measured by in vivo PET imaging and those measured by ex vivo NIRF imaging, demonstrating that this dual probe can be an important tool for improved and specific diagnosis of cancer (Fig. 6). In another study, Xie and colleagues (2010) modified the surface of iron oxide nanoparticles with dopamine and encapsulated these functionalized nanoparticles into human serum albumin matrices dually labeled with ^{64}Cu-DOTA and Cy5.5. The authors studied the biological behavior of this system by in vivo PET/NIRF/MRI trimodality imaging and ex vivo analyses for histological examinations. The results show that this multifunctional system manifested prolonged bloodstream circulation with massive accumulation in lesions (Fig. 7) with also low uptake of the particles by macrophages at the tumor area, demonstrating that this system presents promisor features for the cancer multimodal diagnosis approach.

4.3 Radiolabeled Nanomaterials for Cancer Theranostics

The cancer theranostics represent another possibility that the multifunctional nanoparticles brought to science. Recently, scientists are paying attention to the possibility to monitor cancer treatment response through radioactive probes that can provide specific diagnostic imaging simultaneously with selective therapeutic approaches. Fang and coauthors wrote a newborn review with massive information about contemporary preclinical advances in breast cancer theranostics (Fang et al., 2021). According to the authors, some of the theranostic agents highlighted in their work exhibit high potential to be translated to the clinic. In one of these works,

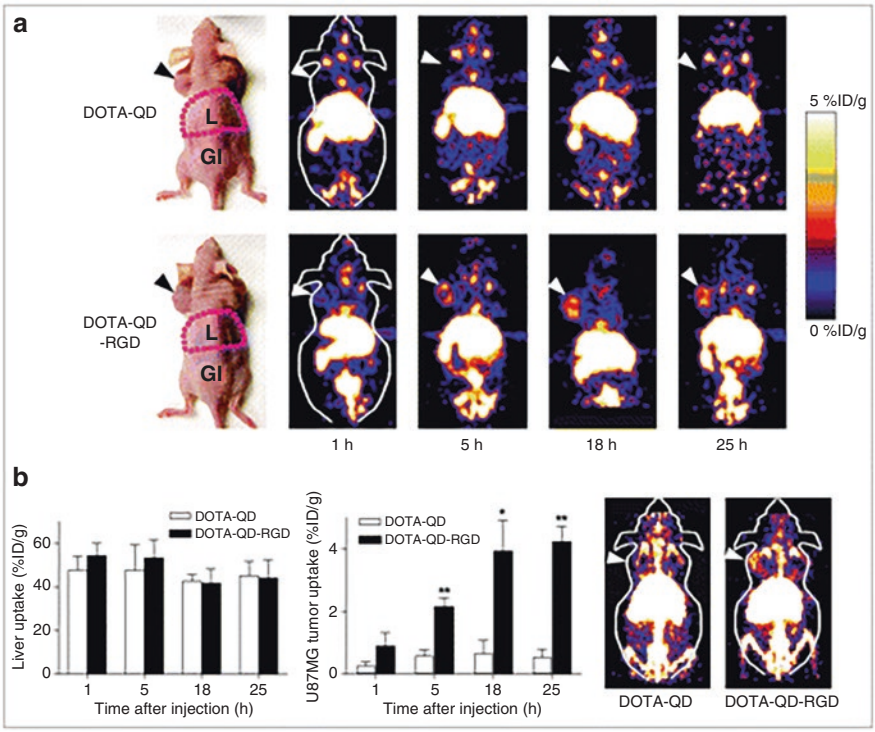

Fig. 6 In vivo PET of U87MG tumor-bearing mice with dual-function PET/NIRF probe. (**a**) Whole-body coronal PET images of mice at 1, 5, 18, and 25 h after injection of 7–14 MBq of ^{64}Cu-labeled DOTA-QD or DOTA-QD-RGD. Arrowheads indicate tumors. The images shown are for slices that were 1 mm thick. GI = gastrointestinal tract; L = liver. (**b**) Liver uptake of ^{64}Cu-labeled DOTA-QD and DOTA-QD-RGD over time, as quantified by ROI analysis of small-animal PET scans (n = three per group). (**c**) U87MG tumor uptake of ^{64}Cu-labeled DOTA-QD and DOTA-QD-RGD over time, as quantified by ROI analysis of small-animal PET scans (n = three per group). (**d**) Two-dimensional whole-body projection of the two mice shown in A at 5 h after injection. Arrowheads indicate tumors. *$p < 0.05$. **$p < 0.01$ (Cai et al., 2007)

Pascual and colleagues (Pascual et al., 2017) described the preparation of a nanoparticulate system constituted of aminopropyl functionalized mesoporous silica nanoparticles gated with MUC1 aptamer, loaded with safranin O and radiolabeled with ^{99m}Tc, designed for specific targeting and cargo release in breast cancer cells. In vitro assays with MDA-MB-231, a breast cancer cell lineage that overexpressed MUC1 receptor, showed nanoparticle uptake with local drug release and a significant reduction in cell viability, while the in vivo studies with MDA-MB-231 tumor-bearing Balb/c mice demonstrated a remarkable tumor targeting (Fig. 8). In another work, indium-111 labeled superparamagnetic iron oxide nanoparticles (SPIONs) conjugated with trastuzumab-doxorubicin and coated with APTES-PEG were designed by Zolata and coworkers (2015) for tumor targeting, drug delivery, controlled drug release, and dual-modal tumor imaging. The APTES-PEG

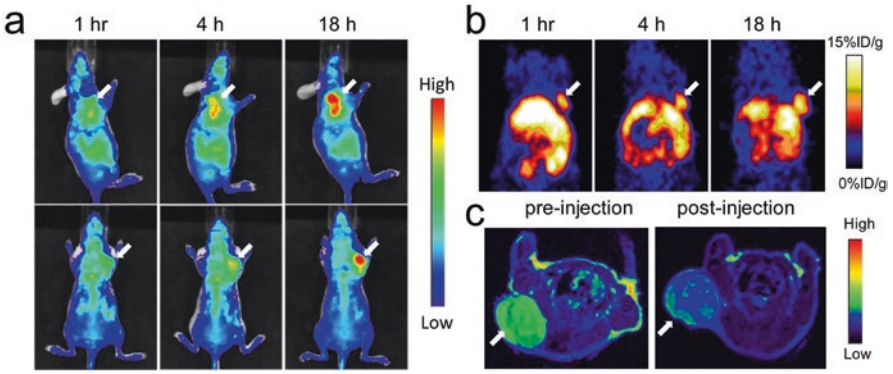

Fig. 7 (**a**) Representative in vivo NIRF images of a mouse injected with HSA-IONPs. Images were acquired 1 h, 4 h, and 18 h postinjection. (**b**) In vivo PET imaging results of a mouse injected with HSA-IONPs. Images were acquired 1 h, 4 h, and 18 h postinjection. (**c**) MRI images acquired before and 18 h postinjection (Xie et al., 2010)

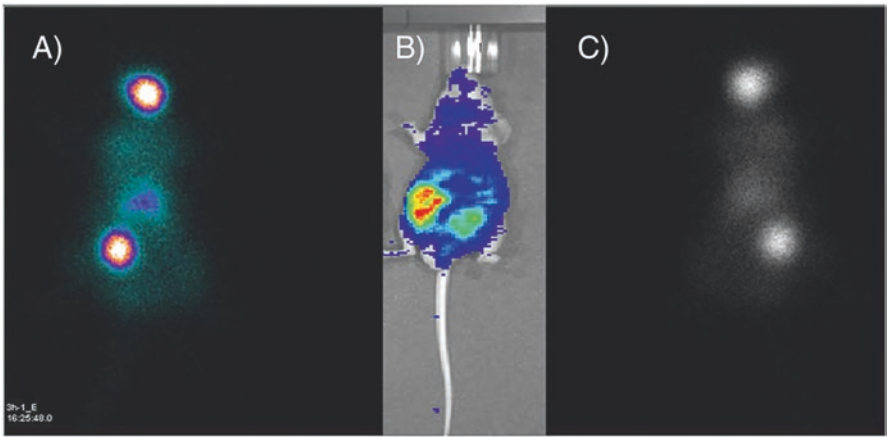

Fig. 8 (**a**) Static planar SPECT image of the MDA-MB-231 tumor-bearing Balb/c mice model after administering the S1-apMUC1-Tc nanoparticles (3.7 MBq/0.2 mL) at 90 min postinjection. (**b**) Bioluminescence images from mice on day 21 after the intraventricular injection of the MDA-MB-231 cancer cells that reveal tumoral lesions. (**c**) Static planar SPECT inverse image of the MDA-MB-231 tumor-bearing Balb/c mice model after administering the S1-apMUC1-Tc nanoparticles (3.7 MBq/0.2 mL) at 90 min postinjection (Pascual et al., 2017)

functionalization aimed at increasing the circulating time in the blood, while trastuzumab aimed at providing active targeting to breast tumor cells. The authors also evaluated the theranostic effects of this system through in vitro assessments in SKBR3 cell lines and in vivo assay with breast tumor-bearing BALB/c mice via biodistribution study, dual-modal molecular imaging, and tumor diameter measurements. Their results showed that the multifunctional nanosystems retain magnetic properties with significant accumulation in tumors sites, being able to generate

dual-modal images from SPECT and MRI systems with high potential for theranostics, considering the promising results from the therapeutic evaluation that were probably achieved under the combined effects from controlled doxorubicin release, Auger electrons, and gamma rays of ^{111}In radionuclide. In another work, Cipreste and coworkers (Cipreste et al., 2020b) developed a nanoplatform with hydroxyapatite functionalized with a new compound, the folate-medronate, and radiolabeled with copper-64. It is known that folic acid receptors are overexpressed in the osteosarcoma, transforming folic acid into a potential targeting molecule for cancer sites. In this work, the ^{64}Cu radioisotope was complexed in the medronate portion of the folate-medronate molecule, acting as a theranostic agent due to the dual positron and beta emissions that allow the PET diagnosis of many kinds of cancers simultaneously to the treatment due to the beta emission. The authors suggest that the complexation route of ^{64}Cu can be improved to achieve increasing radiolabeling rates. Despite that, the authors conclude that the system is significantly potential as a theranostic alternative material.

Great advances have been achieved in cancer treatment and diagnosis on behalf of molecular imaging science. Thanks to the reduced dimensions, nanoparticles can act as carrier vehicles that can penetrate through the endothelial cell layers and interact with various cell structures allowing to record spatiotemporal events at the cellular and subcellular levels (Mirshojaei et al., 2016). Many authors revised the recent progress on radiolabeled nanoparticles for molecular imaging and cancer therapy (Hong et al., 2009; Morales-Avila et al., 2012; Mirshojaei et al., 2016; Kim et al., 2017; Mushtaq et al., 2021). These works reunite the state-of-the-art of molecular imaging researches around the world, bringing to light the importance of the field of science that comprehends the visualization, characterization, and measurement of biological processes at the molecular and cellular levels (Mankoff, 2007). Acting through a distinct approach when compared to conventional diagnostic imaging systems, the molecular imaging trends probe the molecular abnormalities that cause diseases rather than capture images from the areas affected by these alterations (Morales-Avila et al., 2012). For cancer theranostics, the molecular imaging approach brings the advantage of active targeting in specific molecular markers of cancer cells, as demonstrated by Hajiramezanali and coworkers (Hajiramezanali et al., 2019). The authors demonstrated that chitosan coated SPIONS functionalized with DOTA, as a ^{68}Ga radioisotope chelator, and bombesin, as a targeting peptide for specific binding to gastrin-releasing peptide (GRP) receptors that are overexpressed in breast cancer cells, showed insignificant toxicity and high affinity for GRP in a breast cancer cell linage. Moreover, PET and MRI images obtained from in vivo assay showed visible uptake of the radiolabeled nanoparticles in tumor induced xenograft mouse models, indicating that nanoparticles can be modulated for targeting specific molecules that are associated with cancer activities.

4.4 Curcumin-Based Multifunctional Nanomaterials for Theranostic Purposes

Currently, in addition to the various possibilities of application of curcumin in the therapy of various types of diseases and its potential use as a chemotherapeutic agent, the metal complexes of curcumin have enormous potential in the development of new tracers for application in diagnostic medicine. Thanks to these characteristics, it is also possible to use curcumin to obtain theranostic structures, especially in cancer therapy and diagnosis.

In a new research, Tian et al. (2021) developed mesoporous silica nanospheres, doped with gadolinium, loaded with curcumin, and conjugated with carboxymethyl dextran, for theranostic purposes. The nanoplatform was not only able to improve T1-weighted MRI contrast but also produced good synergistic effects in inhibiting tumor growth, due to the joint use of curcumin with sonodynamic chemotherapy (Tian et al., 2021).

A new theranostic molecule was developed by Freidus and colleagues (2020). The new molecule was produced from the reaction between curcumin and leisone giving rise to curcumin naphthoquinone. The structure exhibited intrinsic fluorescence, which allows its use in detection imaging. In addition, it exhibited cytotoxicity to ovarian cancer cells, demonstrating its chemotherapeutic property (Freidus et al., 2020).

Multimodal nanoparticles have also been developed for theranostic purposes. Demir and colleagues (2021) synthesized liposomes loaded with curcumin, bioconjugated with anti-CD44 antibodies, and carbon quantum dots. The nanoplatform showed promise in real-time imaging and showed a potentiated effect on cancer cells (Demir et al., 2021).

The development of hybrid nanostructures represents an important step in the use of curcumin, not only for therapeutic purposes but also for its use in disease diagnosis. Its theranostic potential opens new frontiers for its use in medicine, not only limited to treatment with fewer side effects.

4.5 Radiolabeling Techniques for Nanomaterials and Future Perspectives

Considering the great variety of radionuclides that present unique properties and desired features for biomedical applications and the miscellany of possibilities to incorporate these radionuclides in nanomaterials, there is a vastness of possibilities for designing radiolabeled nanoparticles aiming for the treatment and diagnosis of specific kinds of cancer.

When choosing radioisotopes for labeling nanomaterials aiming for theranostic applications, researchers must pay attention to some ideal features that radionuclides should present, such as a half-life of decay compatible for the application,

accessibility of radionuclides, ease of production, type of decay and radioactive emissions, radiation energy, absence or very low toxicity, and compatible chemistry for conjugations, and scientists must remember that also the daughter nuclides must present these ideal properties. The decay half-life influences the flexibility for transportation and labeling procedures (Ni et al., 2018), which means that very short half-life radionuclides demand rapid labeling chemistry and must be produced nearby the hospital or clinic where the radiolabeled nanomaterial will be administrated in the patient. Considering systemic applications, radionuclides with a very long half-life are not desirable due to radiological protection issues.

Farzin and colleagues (2019) reviewed the use of some radioisotopes in combination with nanomaterials for molecular imaging and therapy applications; the authors described the usage of gold-198, indium-111, copper-64, tellurium-125, rhenium-188, holmium-166, and technetium-99m. The type and energy of radiation emissions of each radionuclide dictate the path length or the range of radiation in tissues like tumors; these are crucial features that determine the application of each radioisotope. For imaging purposes, radionuclides must be positron emitters or emit gamma rays with energies compatible with gamma cameras that are designed for technetium-99m energy (140 keV) (Mirshojaei et al., 2016). Considering cancer therapy applications, the great majority of the radionuclides under investigation are β emitters, which deposit their radiation energy within manifold millimeters in the surrounding tissues. However, if shallow tissue penetration is desired, alpha particles and Auger emitting radionuclides provide more appropriate emissions (Farzin et al., 2019). For theranostic applications, the incorporation of radioisotopes that present dual emissions are desirable – like copper-64 which emits β particles and positrons or iodine-131 which emits β particles and gamma rays with approximately 364 keV or a combination of radioisotopes (Laprise-Pelletier et al., 2017; Keinänen et al., 2019).

Nanoparticles can be radiolabeled through a multitude of techniques including radioisotope direct attachment to the surface of nanomaterials through chemisorption, indirect surface coupling using complexing agents (Cipreste et al., 2020a), and structural incorporation through doping process (Cipreste et al., 2016). Although many possibilities are available for radiolabeling nanoparticles, Ni et al. (2018) stated that "an ideal radiolabeling strategy should be easy, fast, robust, and highly efficient and must make only minimal changes to the original properties" of the nanostructured matrix (Ni et al., 2018). Considering these desirable properties for radiolabeling strategies, some reviews are accessible in the literature to help researchers to design their radiolabeling assays. Ge and fellows (Ge et al., 2020) reviewed the techniques researchers have been using for radiolabeling nanomaterials; the authors divided these techniques into three categories: (i) surface coupling, (ii) inner incorporation, and (iii) interface engineering. Accordantly to Ge and coworkers, for ideal surface coupling, the selection of the chelator for a given isotope is critical to achieving stable radiolabeling, since each chelator has a preference for specific radionuclides. The authors cite some of the most common chelators for radiolabeling techniques that could be applied to nanomaterials: diethylenetriaminepentaacetic acid (DTPA) is a common chelator for ^{111}In or ^{99m}Tc;

1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) for ^{177}Lu; 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) for ^{64}Cu and ^{68}Ga; and deferoxamine (DFO) for ^{89}Zr labeling. Other authors gave more attention to reviewing the approaches for ^{99m}Tc labeling to specific classes of nanomaterials such as iron oxide, gold, silica nanoparticles, titanium, and polymeric nanoparticles (Mushtaq et al., 2021), while Cipreste and colleagues described the radiolabeling of hydroxyapatite nanoparticles through the coordination chemistry of copper-64 with a bisphosphonate modified folic acid molecule (Cipreste et al., 2020a).

The radiolabeling of nanomaterials can also be achieved by chelator-free techniques. Ni and coworkers (2018) discussed the coordination chemistry for radiolabeling silica-based nanoparticles by complexation of radioisotopes as a product of a Lewis acid-base reaction between radioisotopes and oxygen from the deprotonated silanol groups located in the mesoporous structure of silica nanoparticles. Furthermore, radioisotopes can also be incorporated inside the unit cell of crystalline structures of some classes of nanoparticles such as hydroxyapatite. Rezende and colleagues (2019) reported the synthesis of strontium-doped hydroxyapatite nanorods through the hydrothermal coprecipitation method and a double radioisotope activation by neutron irradiation in a nuclear reactor. The authors described that strontium ions replace the calcium ions in the crystalline structure of hydroxyapatite and the radioisotopes ^{89}Sr and ^{32}P are produced by neutron activation of structural strontium and phosphorous. Likewise, Cipreste and coworkers (2016) described similar results by doping hydroxyapatite structure with gadolinium and activating the structural radioisotopes ^{159}Gd and ^{32}P by neutron irradiation.

Although many advances have been made in the field of radiolabeling techniques for bionanomaterials, there are great challenges that separate the basic research from the clinic. Griffiths et al. (2021) discussed the exhausting processes related to the translation of discovery in molecular imaging from preclinical validation steps, passing phase I, phase II, and phase III studies until the usage approval. The authors state that clinical translation reality is far from simple and requires a lot of effort and financial resources. Considering that the majority of the newly designed radiolabeled nanoparticles do not reach the end of this journey, persistence must be the rule for researchers that aim to evolve this technique.

5 Summary

This chapter describes recent studies on the use of nanoparticles for cancer therapy and diagnosis. Cellular resistance to chemotherapeutic agents and the harmful effects caused to healthy tissues by radiotherapy are a challenge that the medical community has faced for years. Thus, new techniques have been constantly studied and developed to minimize these collateral impacts. Nanomaterials have contributed to medicine by introducing new concepts to therapeutic and diagnostic methods, as they have yielded medical benefits in recent years, especially in the field of oncological diseases.

To summarize, new modalities of cancer therapy and diagnosis using nanoparticles have been developed in recent years, such as local photothermal therapies, controlled delivery of chemotherapy drugs, and natural anticancer products such as curcumin. Simultaneous treatment and diagnosis with radiolabeled nanocarriers and a combination of these modalities are also possible due to their high sensitivity, specificity, and multiplexed measurement capability, which can lead to a better cancer patient survival rate.

Therefore, it can be concluded from the studies presented in this chapter that nanomaterials have great potential to be used in the treatment and diagnosis of cancer, presenting optimistic results for the development of advanced and new techniques, being potentially fascinating candidates for a wide range of applications both in treatments and innovative tools in the field of nanomedicine.

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Polymeric Microneedle-Based Drug Delivery Platforms for Application in Cancer Therapy

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1 Introduction

Cancer remains one of the most challenging diseases from a therapeutic point-of-view, ranking as one of the leading causes of premature mortality worldwide (Bray et al., 2021; Sung et al., 2021). Driven by the "magic bullet" concept, nanomaterials have been under constant evolution intending to provide a tumor-site targeted delivery of the therapeutic agents and allow the real-time monitoring (i.e., imaging) of the cancer cells (de Lázaro & Mooney, 2021; Duncan, 2005). However, a seminal review by Wilhelm and colleagues disclosed that less than 1% of the administered nanomaterials' dose reaches solid tumors (Wilhelm et al., 2016). Such data conjugated with the poor clinical translation of nanomaterials highlights the need for new anticancer therapeutic approaches (Mitchell et al., 2021). Among the different technologies developed so far, macroscale drug delivery devices (e.g., injectable hydrogels and microneedles) are currently a hot topic in cancer therapy due to their capacity to deliver and confine the anticancer agents to the tumor zone (Moreira et al., 2019; Huang et al., 2019; Dellacherie et al., 2019). Additionally, the macroscale drug delivery devices can bypass the reduced bioavailability of the

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nanomaterials as well as the off-target accumulation that induces life-threatening side effects (Moreira et al., 2019).

The microneedle-based drug delivery systems were firstly described at more than 30 years as a highly efficient technology for improving transdermal drug delivery (Kim et al., 2012). Since then, microneedles showed to be an effective drug, gene, protein, and even nanoparticle delivery system (Lopez-Ramirez et al., 2020; Ali et al., 2017; Sullivan et al., 2008; Mönkäre et al., 2018). Such prompted the development of microneedles with different structures (e.g., solid, layered, dissolving, hollow, and tips-detachable microneedles) as well as using various materials from stainless steel to polymers (Moreira et al., 2019; Tarbox et al., 2018). The application of microneedles in cancer therapy has been focused on two main areas: (i) the development of anticancer vaccines and (ii) the direct administration of anticancer agents (e.g., nanomaterials and/or chemotherapeutic drugs) to accessible tumors such as skin and oral cancers. Additionally, significant advances have been made using microneedles as delivery devices, namely, in the study of combinatorial therapies based on the simultaneous administration of two or more therapeutic agents such as chemotherapeutic drugs, genetic material, and photothermal and photodynamic agents (Zhao et al., 2021; Dong et al., 2018; Hao et al., 2020). Moreover, the microneedles can enhance the accumulation of the therapeutic agents in the deeper regions of the tumor tissue as well as tailor both in space and time the release of the therapeutic agents, therefore maximizing the antitumoral effect (Jonas et al., 2015; Moreira et al., 2019).

The following sections will summarize the general properties of the microneedle-based delivery systems, focusing on polymeric microneedles, as well as the latest developments of the microneedle application as an anticancer vaccine and/or delivery system of anticancer agents. Moreover, the utilization of biodegradable and biocompatible polymers for the loading of one or multiple therapeutic agents and promoting a sustained delivery is also highlighted, without ignoring the existing limitations and future perspectives for cancer therapy applications.

2 Microneedles

2.1 General Structure and Properties

Microneedle-based delivery systems have been widely studied in transdermal drug delivery since the presentation of the microneedle concept in the 1970s and particularly from the late 1990s onward. In this field, microneedles arise as simple and safer to use painless delivery systems of drugs, proteins, or genes to the dermal layer of the skin (Prausnitz & Langer, 2008). Such approach explores the presence of the micron-sized tips to create channels in the outer layer of the skin allowing a faster

and more effective delivery of the therapeutic agents to the target area (Prausnitz & Langer, 2008). Moreover, the capacity to load different therapeutic agents (i.e., drug molecules, proteins, genes, antibodies, and/or nanoparticles) in the microneedles makes them suitable for developing synergistic anticancer therapies, such as drug-drug and chemotherapy-photodynamic/photothermal combinations.

Microneedles can present different shapes, sizes, and geometries, being the organization as arrays of microscopic needles the most common one, with a height of 50–2000 μm and a base width of 30–250 μm. Moreover, several tip designs were already described in the literature such as conical, pyramidal, beveled, and arrow-headed (please see Fig. 1b) (Rad et al., 2017; Koyani, 2019; Alimardani et al., 2021). In this regard, the available data demonstrate that the tip design can affect the microneedles' penetration efficacy and depth. Lee et al. observed that for microneedles with a low aspect ratio (ratio between tip length and base width), tip length of 600 μm, and base of 300 μm, the pyramidal geometry has two times higher failure force when compared to conical-shaped tips, which was attributed to the larger cross-sectional area of pyramidal microneedles (Lee et al., 2008). Moreover, sharper microneedles require less force to penetrate the tissue and are prone to present higher penetration depths (Römgens et al., 2014; Chen et al., 2012).

The microneedles can also be classified according to the drug delivery method/strategy: (i) solid, (ii) hollow, (iii) coated, (iv) dissolving, and (v) hydrogel-forming microneedles (Fig. 1c). The solid microneedles work as permeation agents creating channels that enhance the penetration of the therapeutic agents administered subsequently in the form of a solution or gel (Wei-Ze et al., 2010; Hu et al., 2020). In turn, the hollow microneedles present a hollow structure that acts as a reservoir for the loading of the therapeutic agents (Cárcamo-Martínez et al., 2021). In this system, the channels running through the micron-sized needles allow the passage of the therapeutics, usually in the form of a solution, upon the microneedle administration at the target area. The coated microneedles are formed using suspensions of the therapeutic agents to create layers at the microneedle's surface (Saurer et al., 2010; Wang et al., 2018). The loading will be dependent on the thickness and/or the number of coating layers (Saurer et al., 2010). Such organization takes advantage of the microneedles' base to support the insertion and the subsequent release/deposition of the coated materials (i.e., therapeutic agents) at the target area. Similarly, the dissolving microneedles are composed of biodegradable materials that dissolve in contact with the body fluids releasing the therapeutic agents that are homogeneously dispersed in the microneedles' matrix (Permana et al., 2019). The hydrogel-forming microneedles are a recent design that started to capture the researchers' attention (Migdadi et al., 2018). These delivery systems are produced using hydrophilic and swellable polymers that allow the microneedles to absorb water and swell when applied at the targeted area. The microneedle swelling is then explored to allow the drug diffusion to the surrounding tissues.

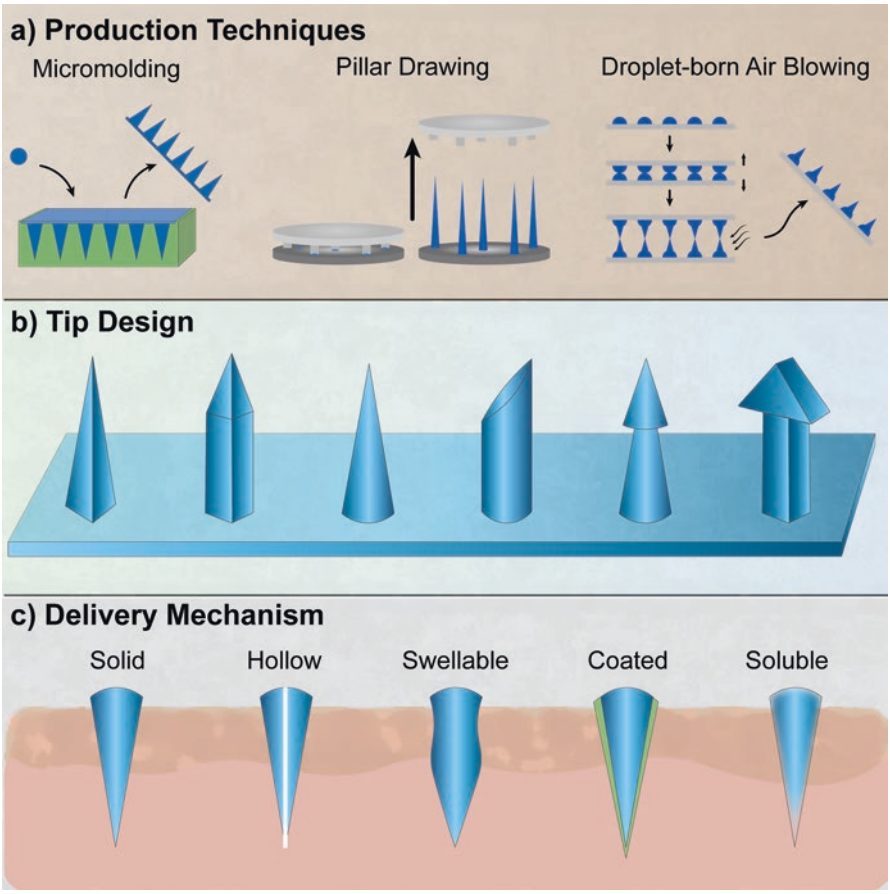

Fig. 1 Schematic illustration of the microneedle fabrication methods (**a**), different tip designs (**b**), and drug delivery strategy (**c**). (**a**) The utilization of molds to direct the production of microneedles by casting polymeric solutions or through polymer melting, micromolding, is one of the most explored processes. Moreover, the pillar drawing and droplet-born air blowing techniques have been showing promising results in the reproducible and large-scale production of microneedles. (**b**) The different tip designs, ranging from pyramidal to arrow-headed, are optimized focusing on parameters such as tissue penetration efficacy and depth as well as the microneedle retention at the target site. (**c**) Solid microneedles are explored to increase tissue permeability by creating microchannels. Hollow microneedles have channels running through the tips allowing the administration of liquid drug formulations. Swellable/hydrogel-forming strategies release the loaded therapeutics in response to the intake of interstitial fluids and swelling of the tips. Coated microneedles explore the loading of therapeutics in a layer-by-layer organization created at its surface. Lastly, soluble microneedle patches promote a rapid or controlled release according to the properties of the polymeric structure (e.g., degradation profile and polymer solubility)

2.2 Production Techniques

The microneedles can be produced using various materials such as metals, ceramics, and polymers (Tarbox et al., 2018). However, the polymers present physicochemical and mechanical properties that make them highly promising for biomedical applications and cancer therapy. The utilization of biocompatible and biodegradable materials allows the optimal delivery of therapeutic agents by tailoring the polymeric microneedles' degradation profile, dissolution, and/or response to specific stimuli (Cheung & Das, 2016; Park et al., 2006). Moreover, such also decreases the production of contaminated medical waste and increases the safety of health professionals. The high manipulability of polymers offers to researchers a wide range of fabrication techniques to produce microneedles (Park et al., 2005; Tarbox et al., 2018). Micromolding is one of the most common production techniques to produce microneedles. In this approach, the microneedles are produced in three major steps: (i) fabrication of a master mold using a strong material, such as metal or silicon; (ii) production of a negative mold of polydimethylsiloxane; and (iii) polymer casting (Rad et al., 2021; Nagarkar et al., 2020). This strategy presents a high precision and reproducibility, which coupled with the simplicity and reusability of the master/negative molds supports the industrialization of the microneedle production process (Liu et al., 2020; Tarbox et al., 2018). Alternatively, the droplet-born air blowing technique is a mold independent production methodology. For that purpose, polymer droplets are dispensed in two different plates that are subsequently stacked with the droplets facing each other and in contact (Huh et al., 2018; Lee et al., 2018). Then, the plates are separated with air flowing through the face of the plates containing the polymer droplets, forming elongated polymeric structures. At a critical distance, the polymeric structures are separated, and a microneedle array is formed in each plate (Lee et al., 2018). Similarly, in the electro-drawing lithography, polymer droplets are also placed on a surface, and the microneedles are produced using a heated polar dielectric crystal to draw the formation of the elongated needle-like structures (Ruggiero et al., 2018). The microneedles are completely formed upon the solvent evaporation that can be further optimized using heat treatments. In turn, drawing lithography uses a plate with an array of pillars to direct the growth of elongated polymeric structures from a base containing a melted polymer (Lee et al., 2010). The plate with the array of pillars is separated at a controlled speed after contact with the melted polymer, and such results in the formation of the microneedles upon cooling and detachment from de pillars.

Apart from the production of microneedle arrays, different techniques can also be used to create layers at the microneedles' surface and encapsulate the therapeutic agents, a strategy usually followed to create the coated microneedles (Saurer et al., 2010; Wang et al., 2018). One of the most explored approaches is the alternate deposition of polyelectrolytes via dip-and-dry cycles. In this technique, the microneedles are immersed in solutions containing charged polymers and/or therapeutic agents to create thin films at their surface (He et al., 2018). Therefore, a multilayered structure is created via the establishment of ionic interactions between the materials with opposite charges at each layer (Skirtach et al., 2011). This design allows controlling

the release profile of the therapeutic agents through the characteristics of the materials used to produce the microneedles, the layer thickness, the number of layers, and the position of the payload regarding the most external layer (Skirtach et al., 2011). Spray coating and inkjet printing are alternative technological procedures to the most conventional dip coating processes that allow the creation of multilayers at the surface of the microneedles with high accuracy, reproducibility, and scalability (Hsu et al., 2016; Akagi et al., 2014; Choi et al., 2017).

3 Microneedles as Anticancer Vaccines

In recent years, the concept of developing anticancer vaccines has captured a great interest of the scientific community showing promising results in preclinical assays of breast, cervical, lung, ovarian, and skin cancers. This approach aims to activate the patient immune system and induce its action against the cancer cells, achieving systemic tumor suppression and long-lasting immune memory and reducing the adverse effects of conventional anticancer chemotherapy (Singh & Kesharwani, 2021; Amani et al., 2021). Moreover, the establishment of the host immune response also allows the eradication of tumor metastasis due to its systemic action (Duong et al., 2018; Guo et al., 2021).

The most common design of anticancer vaccines based on microneedles is based on the administration of immune adjuvants or antigens to the dermis layer of the skin due to a large number of antigen-presenting cells (e.g., macrophages and dendritic cells) in this tissue (Fig. 2) (Zhao et al., 2018). The antigen-presenting cells will then mediate the activation of T and B cells that are responsible for the establishment of the systemic and tumor-specific immune responses (Rice et al., 2008). For example, Zaric and coworkers observed that the ovalbumin delivery via poly-D,L-lactide-co-glycolide nanoparticles loaded on methylvinylether and maleic anhydride microneedles could successfully mediate the priming of $CD8^+$ T cells and induce the IFN-γ producing effector T cells (Zaric et al., 2013). In turn, this immune response triggered by the microneedles containing ovalbumin successfully stalled the growth of B16 melanoma tumors during 13 days, contrasting with the blank microneedles where the tumors tripled in volume. Similarly, Kim and colleagues reported that sodium hyaluronate microneedle mediated delivery could mediate the delivery of a sodium hyaluronate-cytotoxic T-cell epitope peptide (SIINFEKL) conjugate (Kim et al., 2019). This strategy increased the residence time of the peptide in the dermis layer of the skin, favoring the interaction with antigen-presenting cells. Moreover, an increased infiltration of $CD8^+$ T cells within B16 melanoma tumors was observed, which reduced tumor growth (i.e., the treated tumors presented a volume 3.5 times inferior to the phosphate buffered saline treated control group).

The triggering of the host immune response can also be achieved via gene delivery, commonly plasmid DNA encoding tumor antigens (Rice et al., 2008). In this approach, two routes of antigen presentation can be considered: (i) the indirect route where the genetic material is internalized by “nonprofessional” antigen-presenting

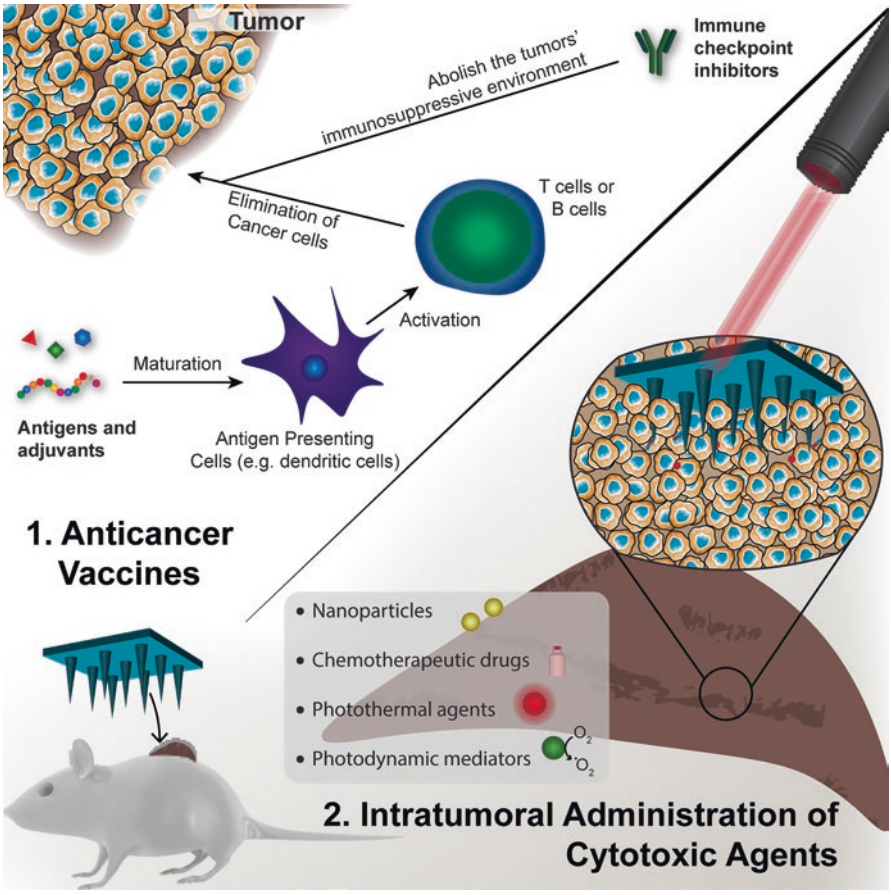

Fig. 2 Representation of the two main approaches to the microneedles' application in cancer therapy. The microneedle-based anticancer vaccines containing tumor antigens, immune adjuvants, and immune checkpoint inhibitors will mediate the activation of the immune cells (cytotoxic T cells) and revert the immunosuppressive environment at the tumor site, triggering the host's antitumoral immune response. In turn, the direct administration of chemotherapeutic drugs, photothermal/photodynamic agents, and nanomaterials aims to promote the eradication of localized tumors

cells, mainly myocytes and keratinocytes, and (ii) the direct route where the transfected cells are antigen-presenting cells, usually dendritic cells (Rice et al., 2008; Riley et al., 2019). In this process, the presentation of the encoded antigen by the major histocompatibility complex class I or class II allows the activation of T cells ($CD4^+$ and $CD8^+$) and indirectly stimulates the humoral immunity (Riley et al., 2019). Ali and coworkers observed that the administration of polyvinylpyrrolidone microneedles containing plasmid DNA/RALA nanoparticles encoding for E6/E7 antigens resulted in a two times higher antibody concentration and increased IFN-γ levels (≈530 pg/mL vs. ≈250 pg/mL of control) (Ali et al., 2017). Moreover, the

priming of the immune response further prevented the establishment and development of cervical tumors in 44% of the mice. In turn, the microneedle application in mice already presenting cervical tumors decreased the area of the tumors and was more effective than the sole administration of E6/E7 plasmid DNA/RALA nanoparticles, final tumor area of 246 mm^2 for microneedles vs. 503.13 mm^2 for nanoparticles. Similarly, Cole et al. explored the priming and activation of the immune system through the utilization of polyvinyl alcohol microneedles loaded with plasmid DNA/RALA nanoparticles encoding for the prostate stem cell antigen (Cole et al., 2019). The microneedle administration induced the local expression of the prostate stem cell antigen and consequently the priming of prostate cancer-specific $CD8^+$ T cells. Moreover, the percentage of prostate cancer-specific $CD8^+$ T cells in the spleen was superior in the group treated with microneedles (21.19%) when comparing the immunization with the free prostate stem cell antigen (12.02%). Moreover, the immune system activation reduced the tumor growth during 70 days, 347.7 mm^3 for the microneedles containing plasmid DNA/RALA nanoparticles and 589.9 mm^3 for the control group.

Apart from the direct activation/stimulation of the immune cells, the microneedle vaccines can also mediate the delivery of agents aimed to revert the immunosuppressive signals present in the tumor microenvironment. This therapeutic approach mainly focuses on the use of immune checkpoint inhibitors, targeting immune regulatory receptors expressed by cancer cells (e.g., monoclonal antibodies against PD-1/PD-L1 and CTLA-4) and metabolic checkpoints (indoleamine-2,3 dioxygenase), to attack the tumor cells' evasion from the immunosurveillance (Ribas & Wolchok, 2018). Ye et al. developed hyaluronic acid-based microneedle patches for delivering 1-methyl-$_{DL}$-tryptophan (1-MT) and anti-PD1 to B16F10 tumors (Ye et al., 2016). The simultaneous delivery of 1-MT and anti-PD1 aims to avoid immunosuppressive signals at the cancer microenvironment, i.e., PD-L1 present at the surface of cancer cells and indoleamine 2,3-dioxygenase immunosuppressive enzyme. This therapeutic strategy was able to slow down the growth of B16F10 tumors, presenting values inferior to 50 mm^2 that contrasts with the 300 mm^2 observed in the control group. Furthermore, the 1-MT and anti-PD1 delivery resulted in a sixfold increase of the recruited $CD8^+$ cells per tumor milligram and reduced the infiltration of regulatory T cells, when compared to the blank microneedles. Lopez-Ramirez and colleagues described that the anti-CTLA4 delivery by a poly(vinylpyrrolidone) microneedle patch containing magnesium microparticles, to control the release profile, mediated the eradication of B16F10 tumors in 60% of the animals and increased the mice survivability rate (Lopez-Ramirez et al., 2020).

4 Intratumoral Administration of Therapeutics

The direct administration of drugs, nanoparticles, and even plasmid DNA/small interfering RNA for mediating the death of cancer cells via microneedle systems is a promising approach to increase the treatment effectiveness by performing a tumor

confined delivery of the therapeutic agents. This approach bypasses the issues associated with systemic circulation and off-target accumulation (Li et al., 2021). Moreover, the microneedles also present several benefits over the intratumoral injection of liquid therapeutics, namely, a superior control (in time and space) over the therapeutics' release and interaction with cancer cells as well as a reduced leakage of the therapeutics to the surrounding healthy tissues (Li et al., 2021; Moreira et al., 2019). Furthermore, the microneedles' structure turns the combination of different therapeutics in one single delivery platform a straightforward process, which is related to the ability to encapsulate simultaneously conventional anticancer drugs, photothermal and photodynamic agents, or even nanomedicines.

Chemotherapy is the gold standard approach in cancer therapy. In this area, the microneedles have been showing promising results in the local and transdermal delivery to cancer cells of both hydrophobic and hydrophilic drugs (Sivaraman & Banga, 2017; Ma & Gill, 2014; Ahmed et al., 2019; Nguyen et al., 2018). Bhatnagar et al. promoted the co-delivery of doxorubicin and docetaxel to breast cancer using polyvinylpyrrolidone/polyvinyl alcohol microneedles (Bhatnagar et al., 2019). The direct administration of both drugs using the microneedles reduced the growth of 4T1 tumors and mitigated the systemic side effects, increasing the mice's survival rate. Huang and coworkers also demonstrated the capacity of dextran methacrylate microneedles to mediate the simultaneous delivery of therapeutic drugs (doxorubicin and trametinib) to melanoma (Huang et al., 2020). The data obtained from B16 tumor-bearing mice showed that the homogeneous dispersion of doxorubicin and trametinib in the microneedles decreased the tumor growth by 70%, whereas the intravenous administration of doxorubicin only achieved a 50% suppression. Moreover, the drug delivery mediated by the microneedles also avoided the systemic side effects associated with the free administration of doxorubicin, namely, cardiotoxicity and neurotoxicity.

Apart from the delivery of conventional anticancer drugs, the microneedles can also be engineered to mediate the action of the emerging photothermal and/or photodynamic agents (Song et al., 2021; Wei et al., 2020; Yang et al., 2021). These therapeutic agents are activated upon irradiation with a light source, generating heat (photothermal therapy) and/or cytotoxic reactive oxygen species (photodynamic therapy) to destroy the cancer cells (Li et al., 2020; Fernandes et al., 2020). The light-triggered therapeutics and their consequently site-specific cytotoxicity have been associated with an overall reduction of the side effects (Hu et al., 2018; Fernandes et al., 2020). Zhu and colleagues described the partial remission (three of the five mice) of subcutaneous 4T1 tumors after treatment with hyaluronic acid microneedles containing 5-aminolevulinic acid for 14 days (Zhu et al., 2019). The data showed that the loading of the photodynamic agent, 5-aminolevulinic acid, in the tips of the microneedles presented a superior penetration and subcutaneous distribution when compared to the conventional topical application of 5-aminolevulinic acid in solution. Therefore, upon irradiation of the tumor site for 5 min with a 635 nm laser (1 $W.cm^{-2}$ for 118 s), an increased generation of reactive oxygen species was achieved followed by an enhanced antitumoral effect, i.e., eradication of

the tumor in three of the five mice, whereas the group treated with 5-aminolevulinic acid solution only slowed the tumor growth during the 14 days.

Moreira and coworkers demonstrated that the production of polyvinylpyrrolidone microneedles with a layer-by-layer organization could be explored to mediate the delivery of doxorubicin and gold-core silica-shell nanorods to cervical cancer cells (Moreira et al., 2020). The authors observed that the alternated deposition of a chitosan layer containing doxorubicin and polyvinyl alcohol enriched with gold-core silica-shell nanorods could determine the sequence of release with a pH and thermal responsiveness, leading to the complete elimination of HeLa cells when irradiated with a near-infrared (NIR – 808 nm, 1.7 $W.cm^{-2}$) laser. Similarly, Hao et al. used hyaluronic acid microneedles containing gold nanorods and doxorubicin to mediate the chemo-photothermal therapy of epidermoid cancer (Hao et al., 2018). The incorporation of gold nanorods conferred to the microneedles the capacity of converting NIR light irradiation (808 nm, 1 $W.cm^{-2}$) into heat, reaching a maximum temperature of 65 °C after 5 min, and a light-triggered doxorubicin release. Moreover, the combinatorial action of the chemo-photothermal therapy led to the eradication of A431 tumors in all five mice, without tumor recurrence for 25 days.

Alternatively, the direct administration of cytotoxic anticancer therapies can also be combined with the vaccine concept to create stronger and systemic antitumoral therapies. Lan and colleagues described the application of polyvinylpyrrolidone microneedles loaded with pH-responsive tumor-targeted lipid nanoparticles containing anti-PD1 and cisplatin for the treatment of head and neck carcinoma (Lan et al., 2020). The response of SCC7 subcutaneous tumors demonstrated that the combinatorial therapy mediated by the microneedles could efficiently reduce both the tumor weight and volume (0.012 g and 18.312 mm^3), whereas the single anti-PD1 therapy mediated by microneedles only slowed the tumor growth (0.05 g and 90.252 mm^3). Moreover, the combinatorial therapy also increased the infiltration of $CD8^+$ T cells to the tumor tissue (75.95% and 47.98% for the combinatorial and single anti-PD1 therapies mediated by the microneedles) and the IFN-γ levels. Alternatively, Chen et al. combined the phototherapies with immunotherapies via microneedle administration to B16 tumors (Chen et al., 2020). For that purpose, chitosan nanoparticles loaded with indocyanine green (photothermal agent) and 1-MT were loaded on polyvinylpyrrolidone/polyvinyl alcohol microneedles. This strategy aimed to revert the immunosuppressive environment at the tumor site by inducing damages in cancer cells through photothermal effect, which also can promote the release of tumor neoantigens and damage-associated molecular patterns. Then, the immune system activation would be potentiated by the 1-MT action. The direct administration of the microneedles could mediate the increase of the topical temperature to values superior to 50 °C after irradiation with a NIR laser (808 nm, 0.35 $W.cm^{-2}$ for 30 s), which combined with the 1-MT action almost eradicated the primary B16 tumors. Contrarily, the single therapies only decreased the growth of the primary tumors during the 14 days. Furthermore, the authors reported that the combinatorial treatment suppressed the establishment of lung metastasis and stalled the progression of the already established secondary tumors, and tumor volume was 4.1-fold smaller than those treated with single photothermal therapy mediated by

the microneedles. This abscopal effect was attributed to a more efficient priming of $CD8^+$ T cells and higher infiltration in secondary tumors (60.57% and 50.37% infiltration for combinatorial and single photothermal therapy, respectively), which strengthens and creates a more durable antitumoral response.

5 Limitations and Future Perspectives

The commonly explored organization of the microneedles as therapeutic patches in anticancer therapy presents some limitations, namely, when the intratumoral administration of therapeutics is envisioned. This approach is often limited to superficial tumors or will require invasive surgeries to implant the microneedles at the desired site. Moreover, the therapeutic focus should shift from treating the main tumor mass to a more integrative approach also encompassing the treatment of tumors' metastasis. Regarding the application of microneedles as anticancer vaccines, additional efforts should be considered for more potent activation of immune cells underlying the skin as well as achieve a deeper diffusion and accuracy in organ distribution of immune checkpoint blockade antibodies or adjuvant cytokines. In this regard, a deeper characterization of bioavailability and pharmacokinetics is also required for a more successful clinical translation of the microneedles. Despite the minimal invasiveness of the microneedle administration, long-term safety risks are poorly explored. In this field, the impact of the recurrent administrations and the frequency of use in normal functions of the skin as well as long-term side effects of both polymers and loaded nanomaterials should also be further evaluated. Nevertheless, the recent preclinical data from the microneedle application in breast, melanoma, oral, cervical, ovarian, and prostate cancers have been paving a path for scientists to continue the development of more effective and personalized therapies.

6 Conclusions

The development of macroscale drug delivery systems for application in cancer therapy has been highly pursued in recent years to bypass the challenges/issues of the systemic administration of anticancer therapeutics. The microneedles arise as a minimally invasive technology that can mediate the delivery of genes, drugs, antibodies, and nanoparticles in a painless and controlled manner. Particularly, in the transdermal delivery of therapeutics, the microneedles can effectively transpose the external impermeable layers of the epidermis and release their payload in the skin's dermis layer. Such has been successfully pushing forward the successful application of microneedles in the single or combinatorial treatment of skin tumors by delivering nucleic acids (e.g., plasmid DNA, small interfering RNA, and oligonucleotides), drugs (e.g., 5-FU, doxorubicin, and docetaxel), immunomodulators (e.g., peptide and protein molecules and antibodies), and nanoparticles. Moreover, the versatility

both in the materials used for the microneedles' production and in the structural arrangements (e.g., solid, layered, dissolving, hollow, and tips-detachable) combined with the loading of nanomaterials (e.g. liposomes, polymeric nanostructures, and gold-based nanoparticles) has been translating in superior therapeutic performances. The combination of microneedle-assisted gene and drug delivery or even photothermal and photodynamic therapies may also show synergistic results for different types of cancer. Nevertheless, despite the increasing popularity of the microneedle application in cancer therapy, there are still obstacles limiting the clinical application of these systems. For example, when combinatorial therapies are envisioned, parameters, such as the time window for combining the action of the different agents, the release sequence, and therapeutics concentration, must be carefully characterized. Moreover, the scale-up of the production to an industrial scale is a major barrier since most of the reported microneedles require multiple preparation steps.

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Clinical Trials Involving Chemotherapy-Based Nanocarriers in Cancer Therapy: State of the Art and Future Directions

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Abbreviations

AIDS	Acquired immune deficiency syndrome;
ALL	Lymphoblastic leukemia;
ARC	International Agency for Research on Cancer;
AUC	Area under the curve;
AuNPs	Gold nanoparticles;
BBB	Blood-brain barrier;
CAFs	Cancer-associated fibroblasts

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CD	Cyclodextrin
CD-PEG	Cyclodextrin-polyethylene glycol
Chol	Cholesterol
DMPC	1,2-Dimyris-toyl-sn-glycero-3-phosphocholine
DMPG	1,2-Dimyristoyl-sn-glycero-3-phospho-(1'-racglycerol)
DNA	Deoxyribonucleic acid
DOPC	Palmitoyloleoylphosphatidylcholine
DOPS	Dioleoylphosphatidylserine
DOTAP	1,2-Dioleoyl-3-trimethylammonium propane
DPPC	Dipalmitoylphosphatidylcholine
DPPG	1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol;
DSPC	Distearoylphosphatidylcholine
DSPE	Distearoyl-sn-glycero-phosphoethanolamine
DSPG	1,2-Distearoyl-sn-glycero-3-phosphoglycerol
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
EPC	Egg phosphatidylcholine
EPR	Enhanced permeability and retention
FDA	US Food and Drug Administration
HCC	Hepatocellular carcinoma
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HSA	Human serum albumin
HSPC	Hydrogenated soy phosphatidylcholine
KRAS	Kirsten rat sarcoma viral oncogene homolog
KS	Kaposi sarcoma
L-MTP-PE	Muramyl tripeptide phosphatidylethanolamine
LNPs	Lipid nanoparticles
LSAM	Large surface area microparticle
MDP	Muramyl dipeptide
MDR	Multidrug resistance
MPEG	Methoxy polyethylene glycol
MPPE	Maleimidated palmitoyl phosphatidylethanolamine
MSPC	1-Myristoyl-2-stearoyl-sn-glycero-3-phosphocholine
NGPE	N-glutaryl phosphatidylethanolamine
NIPAM	Poly(N-isopropylacrylamide)
NPs	Nanoparticles
NSCLC	Non-small cell lung cancer
PC	Phosphatidylcholine
PE	Polyethylene
PEG	Polyethylene glycol
PEG2000-DSPE	PEGylated distearoyl-sn-glycero-phosphoethanolamine
PFS	Progression-free survival
PICN	Paclitaxel injection concentrate for nanodispersion

PLA	Polylactic acid
PLA2	Phospholipase A2
PLGA	Polylactide-co-glycolic acid
POPC	Palmitoyloleoylphosphatidylcholine
PPE	Palmar-plantar erythrodysesthesia
PSMA	Prostate-specific membrane antigen
PVP	Polyvinyl-pyrrolidone
RES	Reticuloendothelial system
RFA	Radiofrequency ablation
RNA	Ribonucleic acid
SM	Sphingomyelin
SPARC	Sun Pharma Advanced Research Company, Ltd.
TAMs	Tumor-associated macrophages

1 Introduction

Cancer is a general term for a large group of diseases, whose causes, characteristics, and occurrence can vary. All of them are characterized by the development of abnormal cells that divide uncontrollably and infiltrate and disrupt normal body tissue. Cancer has a major impact on society across the world, and, in fact, there were 19.3 million new cases in 2020 worldwide (Fig. 1) (https://www.iarc.who.int/). Among these statistics, breast, lung, colorectal, prostate, and stomach highlight as the most common cancer types, with more than 1 million cases each. Moreover, according to the International Agency for Research on Cancer (IARC), the number of new cases per year is expected to rise to 29.5 million by 2040 (https://www.iarc.who.int/).

Current medicine takes advantage of traditional approaches for cancer therapy: surgery, radiotherapy, chemotherapy, phototherapy, immunotherapy, and hormonal therapy (Jabir et al., 2018). Unfortunately, although the available treatments have improved patient survival and treatment outcomes (Ferlay et al., 2021), these clinical approaches can cause nonspecific effects in normal tissues, such as chemical toxicity, radiotoxicity, or phototoxicity, thereby provoking serious issues, namely, nausea, kidney damage, neutropenia, hair loss, loss of appetite, peripheral neuropathy, diarrhea, and skin damage (Koo et al., 2020; Liang et al., 2010). Chemoresistance, and multidrug resistance (MDR) in particular, is another challenge when treating cancer patients. MDR consists on cross-resistance to a wide amount of unrelated chemotherapeutic drugs after exposure to a single anticancer agent (Baguley, 2010; Bukowski et al., 2020). Therefore, cancer research is focused on the discovery and development of biomedical tools to improve the specificity of cancer therapies aiming to achieve therapeutic effect only at the tumor sites.

Although the administration of free chemotherapeutic drugs remains as the gold standard for cancer treatment, this therapeutic strategy still presents inherent challenges (Gonzalez-Valdivieso et al., 2021a, b). One of the most important problems

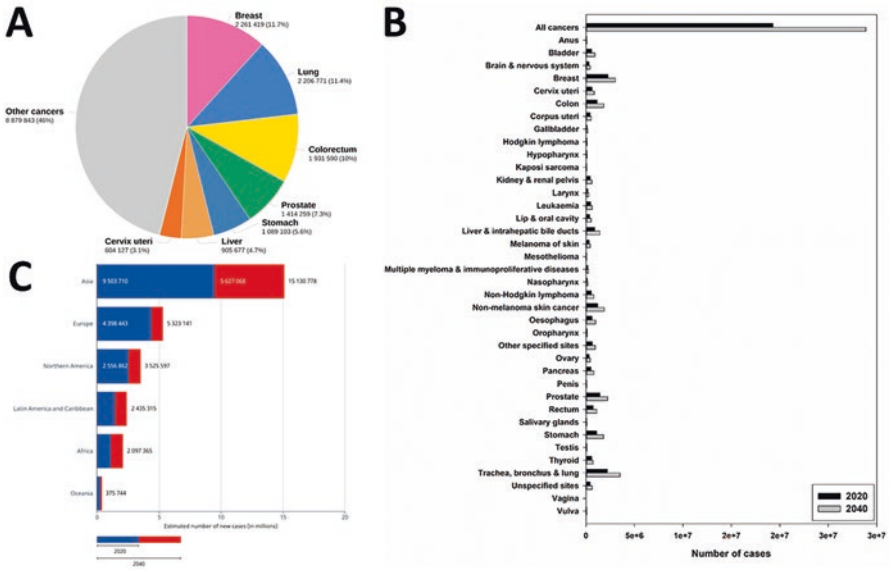

Fig. 1 Cancer statistics across the world. Number of new cases in 2020 for both gender and all ages (**a**). Estimated number of new cases from 2020 to 2040 for both gender and all ages classified by type of cancer (**b**) or geographical continent (**c**). (Data source: GLOBOCAN. Adapted from (https://www.iarc.who.int/))

of current medicine resides in the lack of specific treatments and poor drug accumulation in the tumors (Creixell & Peppas, 2012). As a consequence, undesired side effects in healthy tissues occur, especially in the heart (Octavia et al., 2012), bone marrow (Daniel & Crawford, 2006), gastrointestinal tract (Mitchell, 2006), and nervous system (Grothey, 2003). For this reason, novel approaches are needed to overcome these issues and improve the action of unspecific chemotherapeutic agents.

Nanomedicine is one of these recent strategies for cancer therapy (Awasthi et al., 2018; Bobo et al., 2016; Cao et al., 2020; Shreyash et al., 2021). Nanomedicine has emerged as a new discipline combining biology, engineering, chemistry, and physics, among others, with multiple biomedical applications in the screening, diagnosis, and treatment of diseases (Bayda et al., 2019; Caballero et al., 2022; Gonzalez-Valdivieso et al., 2021a, b; Lammers et al., 2011; Man et al., 2018). The therapeutic potential of nanomedicine aims to use sophisticated systems toward a more personalized medicine, in which each patient could take advantage of tailored approaches (Fenton et al., 2018; Park et al., 2021; Sanchez-Moreno et al., 2018). Thus, recent progress in nanotechnology has achieved the development of novel nanomaterials, whose physicochemical characteristics make them excellent candidates to be applied in the biomedical science, with high impact in the pharmaceutical industry (Norouzi et al., 2020; Park et al., 2021; van der Meel et al., 2019; Wicki et al., 2015). Drug delivery, tissue engineering, viral infections, or pathogenic bacteria are some of the biomedical applications in which nanomedicine highlights as

an effective and promising tool (Das & Ali, 2021; Girotti et al., 2020a; Gonzalez-Valdivieso et al., 2020; Peres et al., 2021; Qiao et al., 2021; Yacoby & Benhar, 2008). In this work, we will focus on nanomedicine for cancer therapy because, even if drug delivery purposes have been explored for diverse diseases, cancer is undoubtedly the main target of drug delivery research (Davis et al., 2008; Shi et al., 2017) and, in fact, multiple drug delivery nanosystems based on these concepts have been translated into clinical products for chemotherapy, such as Abraxane®, DaunoXome®, Doxil®/Caelyx®, Marqibo®, Myocet®, and Onivyde® (Gonzalez-Valdivieso et al., 2021b; Han et al., 2017; Kushwah et al., 2018; Saw et al., 2017).

2 Cancer Physiology

Cancer is characterized by a challenging physiology which is a huge hurdle for biomedical research and demands therapeutic agents to have special features. Therefore, nanomedicine is able to explore multiple features of cancer that provoke low outcome rates and poor drug accumulation. The aberrant proliferation of cancer cells stimulates the fast formation of new blood vessels, also known as angiogenesis, thereby resulting in leaky vasculature with aberrant tortuosity, abnormal basement membrane, poor lymphatic drainage, high interstitial pressure, dense extracellular matrix (ECM) network, or extensive stromal cells, namely, tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) (Matsumoto et al., 2016; Shi et al., 2020). Furthermore, the tumor microenvironment traps many nanocarriers on the tumor vasculature periphery and avoids penetration into the tumor core (Matsumoto et al., 2016).

In addition, cancer cells are characterized by higher expression of multiple proteins, not only cytoplasmic proteins but also anchored receptors to cell membrane (Byrne et al., 2008; Jain & Stylianopoulos, 2010). These cancer markers have huge interest as different targets can be used depending on the type of tumor (Baron, 2012; Sethi et al., 2013). Indeed, cancer markers allow us to even differentiate primary tumors from distance metastasis (Byrne et al., 2008; Quail & Joyce, 2013). Nanocarriers surface can be decorated with molecules (peptides, DNA or RNA aptamers) as targeting systems to specifically drive these devices to cancer cells in specific locations within the body, thereby reducing the amount of drug needed to achieve therapeutic effect and avoiding undesired effects in healthy cells (Agrawal et al., 2020; Girotti et al., 2020a, b; Hwang et al., 2020; Liu et al., 2010; Mitchell et al., 2021). Thus, nanotechnology takes advantages of cancer markers to develop advanced targeted nanocarriers toward personalized biomedical therapeutics (Aguado et al., 2018; Blanco et al., 2015; Cao et al., 2020; Ho et al., 2020).

Beside cancer features and special physiology, the development of accurate systems for controlled release of therapeutics is key when working in drug delivery. Bionanomaterials have been designed for use in advanced drug delivery systems to improve the delivery and efficacy of multiple pharmaceutical agents, such as peptides, antibodies, enzymes, drugs, and vaccines (Caliceti & Matricardi, 2019;

Fenton et al., 2018; Yun et al., 2015). Therefore, designing biomaterials for drug delivery purposes is challenging and has to take into account multiple parameters to achieve the maximum therapeutic benefit: (i) biocompatibility of materials themselves and their degradation products, (ii) physicochemical properties of host materials, (iii) adequate drug for prolonged release, (iv) protection of therapeutic agent from breakdown while maintaining biological activity, (v) predictable release profile, (vi) route of administration, and (vii) cost of material synthesis and production (Helary & Desimone, 2015; Mitchell et al., 2017; Yun et al., 2015).

3 Nanocarriers for Drug Delivery

As a consequence of special tumor physiology, Matsumura and Maeda reported the enhanced permeability and retention (EPR) in 1986 (Matsumura & Maeda, 1986). Their research showed that solid tumors have defective architecture within the blood vessels and enhanced vascular permeability, thereby receiving high amounts of nutrients and oxygen for rapid growth. Thus, the EPR effect considers that this nature of tumor blood vessels facilitates transport of molecules (proteins, drug-polymer conjugates, micelles, liposomes) into tumor tissues: molecules larger than the threshold of renal clearance (40 kDa) showed longer circulation times and slow clearance from the body, thereby being accumulated and retained in tumor tissues for long periods (Fang et al., 2011; Islam et al., 2021; Matsumura & Maeda, 1986; Shi et al., 2020). In contrast, this EPR effect does not occur in normal tissues. Thus, the EPR effect is considered a landmark in tumor-targeted chemotherapy.

As most chemotherapeutic drugs used in clinics are highly hydrophobic, the development of nanomaterials has explored over the past several decades different approaches and origins with different intrinsic and extrinsic properties to achieve better encapsulation and higher concentrations within tumor cells to achieve better therapeutic effect (Figs. 2 and 3) (Howes et al., 2014; Kushwah et al., 2018; Luginbuhl et al., 2017; Minelli et al., 2010; Yousefpour et al., 2019).

3.1 Types of Nanoparticles

3.1.1 Lipid-Based Nanocarriers

Lipid-based nanomaterials offer many advantages, such as simple formulation, self-assembling, biocompatibility, high bioavailability, or the ability to carry large cargo (Sercombe et al., 2015). These advantages make them very attractive for drug delivery purposes, thereby being the most common class of FDA-approved nanomedicines (Anselmo & Mitragotri, 2019; Fenton et al., 2018). There are different types of lipid-based nanomaterials:

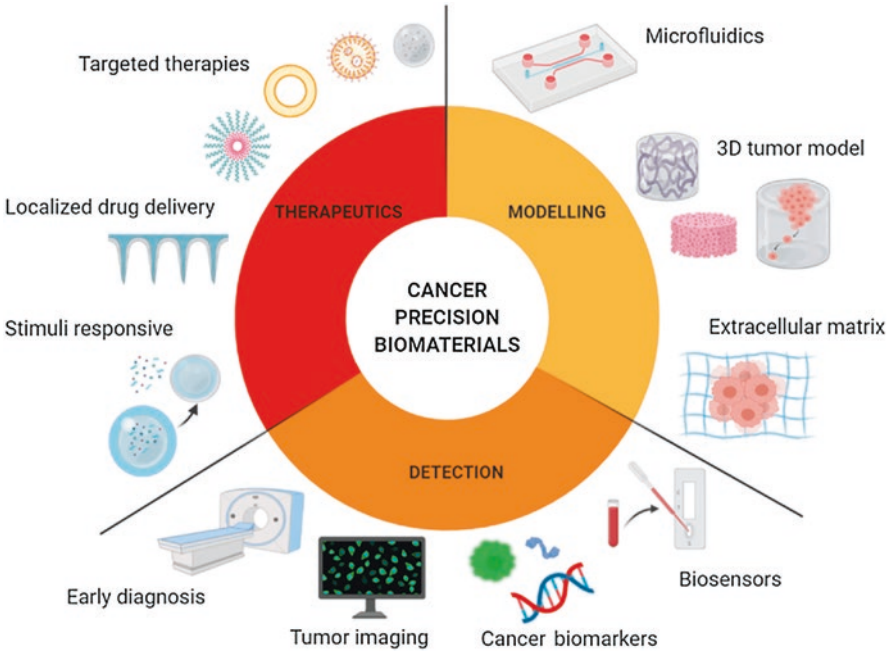

Fig. 2 Application of engineered nanomaterials in cancer. Multidisciplinary research results in a wide pool of tailor-made tools for cancer detection, imaging, and therapy, thereby improving survival rates and treatment outcomes. (Reproduced with permissions from (Caballero et al., 2022))

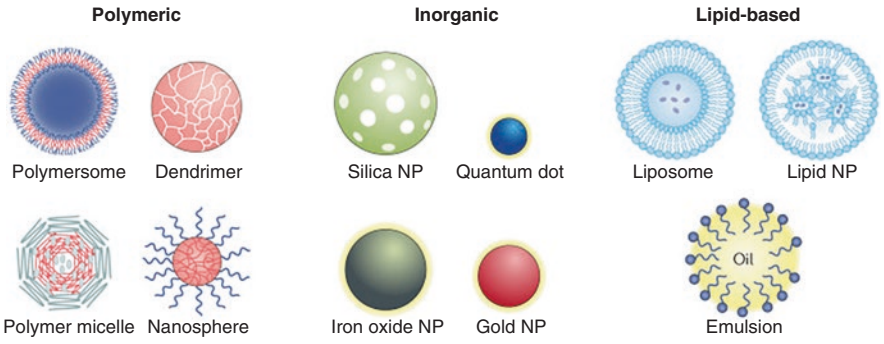

Fig. 3 Types of nanoparticles reviewed in this chapter with different origins: polymeric, inorganic, and lipid-based nanomaterials. (Adapted from (Mitchell et al., 2021))

(i) Liposomes, which are typically composed of phospholipids, thereby allowing the liposome to carry hydrophilic, hydrophobic, and lipophilic drugs (Sarfraz et al., 2018). Liposome' surface is usually modified to extend their circulation times within the body to overcome the fast uptake by the reticuloendothelial system (Alyautdin et al., 2014).

(ii) Lipid nanoparticles (LNPs), which form micellar structures within the particle core. LNPs are typically composed of four major components: phospholipids for particle structure, cationic lipids to complex with negatively charged genetic material, cholesterol for stability and membrane fusion, and PEGylated lipids to enhance longer circulation times (Kulkarni et al., 2019; Leung et al., 2015). LNPs have high efficacy of nucleic acid delivery, simple synthesis, small size, and serum stability as main advantages for gene therapy, but their high uptake in the liver and spleen is an important limitation for translation into the clinics (Cheng et al., 2020; Fenton et al., 2018).

3.1.2 Polymeric Nanocarriers

Polymeric nanocarriers can be synthesized from natural or synthetic materials by emulsification (Brown et al., 2020), nanoprecipitation (Le et al., 2018), ionic gelation (He et al., 2020), or microfluidics (Zhang et al., 2020), among others. Polymeric nanocarriers highlight due to their high biocompatibility, simple formulation, biodegradability, water solubility, stability over time, and wide potential to modify their surfaces for specific targeting (Fenton et al., 2018; Valcourt et al., 2020). Furthermore, this nanomaterial offers many different ways to carry the therapeutic agents, such as binding to the nanoparticle' surface, chemical conjugation to the polymer, entrapping in the polymer matrix, or encapsulation in the core (Mitchell et al., 2021). This wide versatility allows delivery of hydrophobic and hydrophilic compounds, as well as cargos with different molecular weights, ranging from small molecules to proteins and vaccines (Caldorera-Moore et al., 2019; Knight et al., 2019; Liu et al., 2020, 2010; Zhang et al., 2020). However, despite their advantages, polymeric nanocarriers have some limitations, such as particle aggregation and toxicity. There are multiple subtypes of polymeric nanoparticles, such as nanocapsules (cavities surrounded by a polymeric membrane), nanospheres (solid matrix systems), polymersomes (vesicles with membranes composed of amphiphilic block copolymers), micelles (composed of a hydrophilic core and hydrophobic coating), and dendrimers (hyperbranched polymers with complex 3D architecture and active functional groups on the external part to conjugate biomolecules) (Rideau et al., 2018; Shae et al., 2019; Zelmer et al., 2020).

3.1.3 Inorganic Nanocarriers

Inorganic nanomaterials (gold, iron, and silica) have been widely studied for diagnostics, drug delivery, photothermal therapy, and imaging purposes in biomedicine and cancer research due to their physical, electrical, magnetic, and optical properties (Bobo et al., 2016). Therefore, inorganic nanoparticles present the advantage of a great ability to be engineered into tailored nanocarriers with precise physicochemical properties (size, structure, and geometry). Despite their good biocompatibility and stability, inorganic nanoparticles are limited in the clinical application by their

low solubility and toxicity (Bobo et al., 2016; Manshian et al., 2017). There are multiple forms of gold nanoparticles (AuNPs), such as nanospheres, nanorods, nanostars, nanoshells, and nanocages (Quazi et al., 2021). AuNPs can be easily functionalized, thereby allowing researchers to design and develop nanocarriers specifically targeted to different tissues (Bobo et al., 2016; Quazi et al., 2021). Another example of inorganic nanoparticles is magnetic iron oxide NPs, composed of magnetite (Fe_3O_4) or maghemite (Fe_2O_3) (Arias et al., 2018). These nanocarriers present superparamagnetic properties especially useful for various applications as contrast agents, drug delivery vehicles, and thermal-based therapies (Arias et al., 2018; Bobo et al., 2016). Calcium phosphate and mesoporous silica nanoparticles are also inorganic nanocarriers typically used for gene and drug delivery (Huang et al., 2020; Xu et al., 2019), while quantum dots are widely used for in vitro imaging applications (Wagner et al., 2019).

Hence, in this chapter, we will focus on a comprehensive analysis of the clinical application of chemotherapy-based drug delivery nanosystems as advanced tools for cancer treatment.

3.2 Mechanism of Action of Classic Chemotherapeutic Agents

In the mid-1900s, the birth of the chemotherapy entailed a whole revolution in cancer treatment. Before that, the only options available were mainly radical surgical methods, with low success rates, that aimed at the complete eradication of the disease before it could spread and metastasize throughout the organism (Falzone et al., 2018).

Classic chemotherapeutic agents, also referred to as antineoplastic agents, are used to directly or indirectly inhibit the uncontrolled growth and proliferation of cancer cells. Their main disadvantages are related to their low specificity toward cancer cells, generating acute toxicity also to healthy tissues, and the drug resistance mechanisms that lower their efficacy.

In the last decades, new discoveries in the field of immunology, cell biology, and molecular biology allowed researchers to investigate the molecular mechanisms responsible for the neoplastic transformation of cells and to redirect the path toward more specific and personalized therapies, including monoclonal antibodies or immunotherapies, among others. However, the classic chemotherapy, alone or in combination with new treatments, is still a key pharmacological option, despite its notable adverse effects (Falzone et al., 2018; Ferlay et al., 2021).

Classic chemotherapeutic agents are classified according to their mechanism of action and include alkylating agents, antimetabolites, topoisomerase inhibitors, antibiotics, and mitotic inhibitors, among others (Malhotra & Perry, 2003).

Alkylating agents impair cell function by alkylating the DNA molecule. They depend on proliferation for activity, but are not cell phase-specific, and are classified according to their chemical structures and mechanisms (Ralhan & Kaur, 2007). Alkylating agents include nitrogen mustards (More et al., 2019), nitrosoureas

(Mitchell & Schein, 1986), platinum complexes (Bai et al., 2017), oxazaphosphorines (Giraud et al., 2010), imidazotetrazines (temozolomide) (Moody & Wheelhouse, 2014), alkyl sulfates (busulfan, treosulfan, mannosulfan) (Lawson et al., 2021), and hydrazines (procarbazine) (Tweedie et al., 1987), among others.

Oxazaphosphorines (Zhang et al., 2005), such as cyclophosphamide and ifosfamide, are a type of alkylating agent that induce cross-linking at guanine.

Nitrogen mustards are powerful local vesicants. Their metabolites are highly reactive in alkylating the DNA molecule. The hematopoietic system is especially susceptible to these compounds, and dose-limiting toxicity includes myelosuppression. Severe nausea and vomiting are common side effects and, in some cases, alopecia, sterility, diarrhea, and thrombophlebitis. Examples are chlorambucil and melphalan (Diethelm-Varela et al., 2019).

Nitrosoureas (Brandes et al., 2016), for example, carmustine, lomustine, and streptozocin, are very instable and rapidly and spontaneously decompose into highly reactive intermediates. Their lipophilic nature enables free passage across membranes, including the blood-brain barrier. Therefore, these agents are used for a variety of brain tumors, but their dose-limiting toxicity is related to myelosuppression.

Platinum agents that are still widely used as first- and second-line treatments of various tumors produce intra-strand and interstrand DNA cross-links and form DNA adducts that inhibit their replication. Cisplatin, carboplatin, and oxaliplatin are examples of these compounds. Carboplatin shows greater water solubility, slower hydrolysis, and a different toxicity profile. Dose-limiting toxicities for cisplatin are renal insufficiency, peripheral sensory neuropathy, and ototoxicity. For carboplatin, the dose-limiting toxicity is myelosuppression, especially thrombocytopenia (Chen et al., 2013; Dasari & Tchounwou, 2014).

Antimetabolites' major effect is interfering with the building blocks of DNA synthesis, and they are therefore most active in the S phase of the cell cycle and have little effect on the cells in G0. Consequently, these drugs are most effective in tumors that have a high growth fraction. Most of them are structural analogs of the naturally occurring metabolites involved in DNA and RNA synthesis. The antimetabolites can be divided into antifolates, purine antagonists, pyrimidine antagonists, and ribonucleotide reductase inhibitors. These include methotrexate, fluorouracil, cytarabine, gemcitabine, mercaptopurine, pemetrexed, pentostatin, hydroxyurea, fludarabine, and cladribine. They can induce myelosuppression and other severe adverse effects, such as hepatotoxicity or neurotoxicity, among others. Among these, 6-mercaptopurine and 5-fluorouracil, analogs of purines and pyrimidines, respectively, are widely used in clinical practices for the treatment of both hematological malignances and solid tumors (Kaye, 1998; Peters et al., 1993; Peters et al., 2000).

Topoisomerase inhibitors interrupt the DNA unbinding during the S and G2 phases of the cell cycle, by blocking topoisomerases I and II. Irinotecan and topotecan, two water-soluble analogs of the camptothecin, bind to topoisomerase I and are used to treat ovarian, colorectal, and small cell lung cancer. Their main adverse effects include severe myelosuppression and acute diarrhea. In particular, irinotecan

demonstrated to have much more effective antitumor activity than first-generation camptothecins and less renal toxicity. On the other hand, etoposide and teniposide inhibit topoisomerase II, which leads to DNA double-strand breaks and increased DNA degradation. They are used to treat solid tumors, such as testicular and small cell lung cancer, leukemias, and lymphomas, and their adverse effects include myelosuppression and alopecia (Binaschi et al., 1995; Sinha, 1995; Wang & Tse-Dinh, 2019).

Antitumor antibiotics (Galm et al., 2005) can also be used for cancer treatment. First, bleomycin (Froudarakis et al., 2013), which has a cytotoxic effect on nondividing tumor cells, intercalates DNA, resulting in spontaneous oxidation and formation of free oxygen radicals that cause strand breakage. It is effective in the treatment of lymphomas, germ cell tumors, head and neck cancers, and squamous cell carcinoma, but the dose can be limited by the pulmonary toxicity that occurs in 10–40% of the treated patients. Dermatologic toxicity, fever, and anorexia are also frequently seen.

Other antibiotics, such as the anthracyclines doxorubicin, daunorubicin, and idarubicin, do not depend on the cell cycle and have multiple mechanisms of action, including the inhibition of topoisomerase II and the inhibition of DNA and RNA synthesis by intercalation with DNA, DNA strand excision, and generation of free radicals. They are effective in treating leukemias, lymphomas, and breast, ovarian, and bone cancer, and their adverse effects include cardiomyopathy and cardiotoxicity (Bhagat & Kleinerman, 2020; Carvalho et al., 2009; Greene & Hennessy, 2015).

Actinomycin D and mitomycin are also antibiotics with chemotherapeutic activity whose mechanism of action does not depend on the cell cycle. The first one intercalates into DNA and prevents DNA, RNA, and protein synthesis. It is used to treat some childhood cancers and rhabdomyosarcoma, among others, with a dose-limiting myelosuppression and dermatologic toxicity. On the other hand, mitomycin is used to treat gastric and pancreatic cancers. It alkylates DNA and inhibits DNA and RNA synthesis, also causing myelosuppression, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, and fever (Bradner, 2001).

Mitotic inhibitors include vinca alkaloids, taxanes, and nontaxane microtubule inhibitors (Jiang et al., 2006). Vinca alkaloids include vincristine, vinblastine, and vinorelbine. Upon entering the cell, vinca alkaloids bind rapidly to the tubulin and inhibit its assembly, during the S phase. Thus, polymerization of microtubules is blocked, resulting in cell cycle arrest in the M phase. They are used to treat many solid tumors, leukemias, and Hodgkin and non-Hodgkin lymphoma, but peripheral neurotoxicity can limit their dose (Duflos et al., 2002; Martino et al., 2018; Moore & Pinkerton, 2009; Moudi et al., 2013).

Taxanes, paclitaxel, and docetaxel, unlike the vinca alkaloids which cause microtubule disassembly, promote microtubule assembly and stability, therefore blocking the cell cycle in mitosis. Docetaxel is more potent in enhancing microtubule assembly and also induces apoptosis. These compounds have revolutionized the treatment of several solid tumors including metastatic breast cancer, metastatic pancreatic adenocarcinoma (in association with gemcitabine), NSCLC (in association with carboplatin), head and neck cancer, and gastric and prostate cancer. In particular,

these drugs are used when the first-line treatment failed in metastatic patients and therefore represent the only therapeutic option for patients who show drug resistance mechanisms or are not candidates for curative surgical interventions (Mosca et al., 2021; Muggia & Kudlowitz, 2014; Zhang et al., 2019; Zhu & Chen, 2019). Adverse effects include peripheral neuropathy, interstitial pneumonitis, myelosuppression, cardiotoxicity, alopecia, and skin changes (Brewer et al., 2016; Sibaud et al., 2016).

Nontaxane microtubule inhibitors disrupt microtubule stability by blocking mitotic spindles without affecting depolymerization and thus stop the process of cell division at the G_2/M phases. They are commonly used in the treatment of metastatic breast cancer and unresectable liposarcoma. Adverse effects include myelosuppression, peripheral neuropathy, and QT prolongation. Eribulin, ixabepilone, and epothilone are included in this group (Shetty & Gupta, 2014; Swami et al., 2017).

There are other compounds that are also worth mentioning, for example, the L-asparaginase, mostly used in acute lymphoblastic leukemia, an enzyme that breaks down the amino acid L-asparagine to aspartic acid and ammonia, reducing the source of asparagine for leukemic cells and inhibiting protein synthesis in tumor cells. During the treatment, allergic reactions, hepatotoxicity, hyperglycemia, pancreatitis, and blood clotting are frequently observed (Costa-Silva et al., 2020).

3.3 Marketed Chemotherapy-Loaded Nanoparticles for Cancer Treatment

As potent and effective cytotoxic drugs, these classic chemotherapeutic agents would benefit notably from a technology that could improve their specificity toward cancer cells, decreasing their toxicity and adverse effects and thus allowing for the administration of higher doses directed to the tumor. Nanotechnology could be the answer to the specific formulation needs of some of the abovementioned drugs. For example, doxorubicin is known to cause cumulative dose-dependent cardiotoxicity that can be severe, life-threatening, and dose-limiting (Zhao & Zhang, 2017). Changing its pharmacokinetic profile by encapsulating it into nanoparticles has demonstrated to significantly improve this aspect. Meanwhile, the mitotic inhibitor paclitaxel is very insoluble in water and is generally formulated using Cremophor EL, which generates the need for premedication and notably increases its side effects (Gelderblom et al., 2001). Figure 4 summarizes the main formulation problems that can be improved using nanoparticles.

With this idea in mind, for decades, hundreds of scientific groups worldwide have tried to improve the pharmaceutical profile of these antineoplastic agents, encapsulating them in nanoparticles of lipid, polymeric, or even inorganic nature, but it was not until 1995 when this approach finally reached the clinic (Anselmo & Mitragotri, 2019; Kemp & Kwon, 2021; Mitchell et al., 2021) (Table 1).

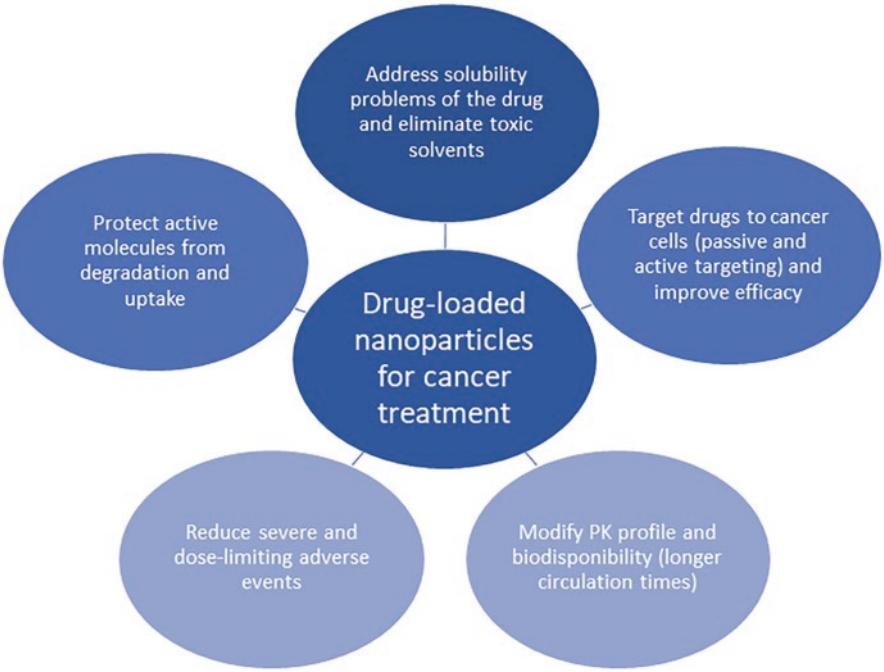

Fig. 4 Main advantages of nanocarriers used for drug delivery purposes against cancer

Doxil® (in Europe Caelyx®), a doxorubicin-loaded liposomal formulation, was the first FDA-/EMA-approved liposomal chemotherapeutic agent (Barenholz, 2012). Its success was based on three key elements: the liposome lipid bilayer was composed of high-T(m) phosphatidylcholine (PC) and cholesterol (in liquid state inside the body), the surface of the liposomes was modified with polyethylene glycol (PEG) to prolong drug circulation time and avoid the uptake by the reticuloendothelial system (RES), and a high drug-loading was achieved with a remote doxorubicin-loading ammonium sulfate-based transmembrane gradient.

With a prolonged circulation time, clearance and volume of distribution are drastically reduced, when compared to free doxorubicin (at least 250-fold and 60-fold, respectively), and the tumor cells are more exposed to the drug, for longer periods. Doxil not only has a better therapeutic effect but also significantly reduces the side effects of doxorubicin, such as myelosuppression, hair loss, vomiting, and diarrhea and, most importantly, the dose-limiting cumulative dose-dependent cardiotoxicity. However, Doxil® causes another characteristic side effect, desquamative dermatitis, which is called palmar-plantar erythrodysesthesia (PPE) or "hand-foot syndrome," and an infusion-related reaction characterized by flushing and shortness of breath (von Moos et al., 2008). This symptom can be alleviated by slowing down the infusion rate and appropriate medication. Moreover, due to the long circulation time of the PEGylated drug, stomatitis (inflammation of mucus lining) became the new dose-limiting toxicity.

Table 1 Commercialized nanoparticle formulations for cancer therapy in the USA and Europe

Name	Particle type	Active molecule	Application	First approval year
Doxil®/Caelyx® (Sequus Pharmaceuticals /Janssen)	PEGylated liposome (HSPC:Chol:PEG2000-DSPE)	Doxorubicin	Ovarian cancer, HIV-associated Kaposi sarcoma, multiple myeloma	1995
DaunoXome® (Galen/ NeXstar Pharmaceuticals)	Non-PEGylated liposome (DSPC:Chol)	Daunorubicin	HIV-associated Kaposi sarcoma	1996
DepoCyt® (Enzon Pharmaceuticals)	Non-PEGylated liposome (DepoFoam technology) (DOPC:DPPG:Chol:triolein)	Cytarabine	Neoplastic meningitis	1999
Myocet® (Teva UK/Elan Pharmaceuticals)	Non-PEGylated liposome (EPC:Chol)	Doxorubicin	Breast cancer	2000
Eligard® (Tolmar)	PLGA	Leuprolide acetate	Prostate cancer	2004
Abraxane® (Celgene)	Albumin particle	Paclitaxel	Advanced non-small cell lung cancer, Metastatic pancreatic cancer, metastatic breast cancer	2005
Oncaspar® (Servier Pharmaceuticals)	PEG	Asparaginase	Acute lymphoblastic leukemia	2006
Mepact® (Millenium/ Takeda Pharmaceutical Limited)	Non-PEGylated liposome (DOPS:POPC)	Mifamurtide	Osteosarcoma	2009
Marqibo® (Spectrum Pharmaceuticals)	Sphingosome (SM:Chol)	Vincristine	Philadelphia chromosome-negative acute lymphoblastic leukemia; Hematologic malignancies and solid tumor	2009
LipoDox® (Sun Pharma Global FZE) (Generic of Doxil®)	PEGylated liposome (HSPC:Chol:DSPE-PEG)	Doxorubicin	Breast neoplasms	2013

Onivyde®/MM-398/ PEP02 (Merrimarck Pharmaceuticals, Inc.)	PEGylated liposome (DSPC:MPEG-2000:DSPE)	Irinotecan	Metastatic pancreatic cancer	2015
VYXEOS®/CPX-351 (Jazz Pharmaceuticals)	Liposome (DSPC:DSPG:Chol)	Cytarabine/ daunorubicin (5:1 molar ratio)	Acute myeloid leukemia	2017
Apealea®/Paclical® (Oasmia Pharmaceutical AB /Elevar Therapeutics)	XR17 micelle platform technology (N-(all-trans-retinoyl)-L-cysteic acid methyl ester sodium salt and N-(13-cis-retinoyl)-L-cysteic acid methyl ester sodium salt)	Paclitaxel	Ovarian cancer	2018

The US FDA approved the first generic version of Doxil® (doxorubicin hydrochloride liposome injection), LipoDox®, made by Sun Pharma Global FZE, in 2013, to ease drug shortage (Pillai & Ceballos-Coronel, 2013).

Just a few months after the approval of Doxil®, DaunoXome®, a liposomal formulation of another anthracycline, daunorubicin, was first licensed in the UK and later approved by the FDA (Petre & Dittmer, 2007). Its liposomes were composed mainly of two lipids, distearoylphosphatidylcholine (DSPC) and cholesterol, with a reduced size and neutral charge that minimized RES uptake, leading to prolonged drug circulation. A citrate salt was used for the active loading of daunorubicin into the nanoparticles.

DaunoXome® was approved for the treatment of AIDS-associated Kaposi sarcoma (KS), in the years where HIV was emerging as a serious threat, and it allowed for the administration of higher cumulative chemotherapeutic doses without significant cardiotoxicity or other adverse effects. Daunorubicin® plasma AUC levels were more than 35fdd greater than those reported for comparable doses of free drug, with responses above 50% for the treatment of KS (Forssen & Ross, 1994; Gill et al., 1996).

There is also a second liposomal doxorubicin approved, in Europe and Canada, for the first-line treatment of metastatic breast cancer, in combination with cyclophosphamide: Myocet® (Batist et al., 2002; Leonard et al., 2009). This formulation consists of doxorubicin encapsulated in non-PEGylated liposomes, made of PC and cholesterol, and its pharmacokinetics differs from both conventional doxorubicin and PEGylated liposomal doxorubicin. The clearance of this formulation is slower than free doxorubicin, with higher plasma levels, but faster than the PEGylated liposomes (Baselga et al., 2014).

Regarding the adverse effects, Myocet® has demonstrated to be substantially less cardiotoxic than doxorubicin and PPE occurs rarely, with an incidence of <0.5% in metastatic breast cancer patients treated in phase III clinical trials. Thus, this formulation has a particular role in patients previously treated with anthracyclines in the adjuvant setting and those with cardiac risk factors (Safra, 2003).

The last anthracycline-based liposomal formulation approved for cancer treatment is actually a combination of daunorubicin with cytarabine, at a cytarabine/daunorubicin 5:1 molar ratio (Blair, 2018). The liposome is composed of DSPC, 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG), and cholesterol. Vyxeos® (CPX-351) efficiently encapsulates both drugs into the same liposome, exploiting the synergies of these two drugs for the treatment of acute myeloid leukemia, providing a survival benefit with acceptable tolerability. In addition, it allows for relatively simple administration versus conventional 7 + 3 chemotherapy. Compared to standard of care treatment, Vyxeos® demonstrated superior median overall survival (3.61 months longer), event-free survival (1.22 months longer), and remission rate (14.4% higher) without increasing treatment-related mortality and toxicities (Lancet et al., 2016; Lancet et al., 2018).

Another sustained-release formulation encapsulating just cytarabine for the treatment of neoplastic meningitis is DepoCyt® (Mantripragada, 2002), prepared by a proprietary technology called DepoFoam®, that comprises

tens-of-microns-in-diameter multivesicular particles formed by compartments separated by lipid bilayers. It is composed of palmitoyloleoylphosphatidylcholine (DOPC) and 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), and its structure allows encapsulation of large quantities of drugs and ensures prolonged release. It is the only liposomal drug for intrathecal administration.

The terminal half-life of the formulation was 40 times longer than that of standard cytarabine (Chamberlain et al., 1995), notably improving its pharmacokinetic profile. The incidence and severity of chemical arachnoiditis, a common adverse event following administration of DepoCyt, can be reduced by the coadministration of dexamethasone.

The first nanoparticulate system for cancer treatment based in polymeric nanoparticles was approved in 2004, with the name of Eligard®. Polymeric nanoparticles represent very versatile vehicles that can be designed to improve the solubility of the encapsulated drug, the release profile, or the specific target, among others. Eligard® is composed of leuprolide (a testosterone inhibiting drug) incorporated into a polylactide-co-glycolic acid (PLGA) nanoparticle and is indicated as an effective treatment for the symptoms of prostate cancer. PLGA (Makadia & Siegel, 2011) is a widely used hydrophobic and biodegradable polymer that slowly decomposes into the constituent monomeric units over time, generating sustained-release profiles of the nanoencapsulated drug.

Oncaspar® (Dinndorf et al., 2007), by Servier Pharmaceuticals, is another approved nanoparticulate polymeric formulation for cancer treatment, which is composed of asparaginase and PEG. By covalently conjugating the native asparaginase to the hydrophilic polymer PEG, it is possible to increase its circulation and retention time, decrease proteolysis, and hide antigenic determinants from the immune system, thus avoiding hypersensitivity associated to the administration of free asparaginase (Jarrar et al., 2006). Oncaspar® was first approved for use in patients with acute lymphoblastic leukemia (ALL) who developed hypersensitivity to asparaginase. Later, it was approved as first-line treatment for ALL, as part of a multiagent thermotherapy regimen.

Abraxane® (Desai, 2016; Green et al., 2006; Lee et al., 2020) by Celgene is an albumin-bound formulation of another chemotherapy, paclitaxel, which is approved by the FDA for the treatment of metastatic breast cancer, NSCLC, and pancreatic adenocarcinoma. Conjugating the drug with albumin eliminated the need for an organic solvent, usually required for the delivery of the highly water-insoluble free paclitaxel, thus notably decreasing medication-associated side effects.

Another Cremophor-free paclitaxel formulation approved by the EMA is Apealea® (Vergote et al., 2020), which is also the newest nanoparticle formulation for cancer treatment in the market (approved in Europe in 2018). It is indicated in adult patients with a first relapse of platinum-sensitive epithelial ovarian cancer, primary peritoneal cancer and fallopian tube cancer, in combination with carboplatin. The formulation is based on the proprietary XR17 micelle platform technology, composed of two novel micelle-forming excipients, N-(all-trans-retinoyl)-L-cysteic acid methyl ester sodium salt and N-(13-cis-retinoyl)-L-cysteic acid methyl ester

sodium salt. Apealea® showed non-inferior efficacy results and improved safety profile in phase III clinical trials against Taxol® (paclitaxel with Cremophor).

Marqibo® (Silverman & Deitcher, 2013), another mitotic inhibitor based formulation, is also approved by the FDA. In this case, vincristine sulfate, a semisynthetic chemotherapeutic agent, was encapsulated in sphingomyelin (SM) and cholesterol liposomes to overcome the dosing, pharmacokinetic, and pharmacodynamic limitations of free vincristine. In clinical trials, alone or in combination, Marqibo® was well tolerated and showed higher activity than standard vincristine treatment, probably due to the pharmacokinetic optimization and enhanced delivery. Currently it is indicated for the treatment of adult patients with Philadelphia chromosome-negative (Ph-) ALL, in second or greater relapse, or whose disease has progressed following two or more antileukemia therapies.

In 2015, based on the encouraging preclinical and clinical data available for the treatment of a variety of solid tumors, Onivyde® (Zhang, 2016), the nanoliposomal formulation of irinotecan, was approved by the FDA, as a combination regimen with 5-fluorouracil (5-FU) and leucovorin, for patients with gemcitabine-based chemotherapy-resistant metastatic pancreatic cancer. In advanced clinical trials, patients who received the combination of this PEGylated liposome formulation and 5-FU/leucovorin gained on average 2 months of survival and showed an average delay in the time to tumor growth of 3.1 months when compared to those who received only 5-FU/leucovorin (FDA Approves Onivyde Combo Regimen for Advanced Pancreatic Cancer, 2015).

Finally, Mepact® was the first drug approved for the management of high-grade, resectable, nonmetastatic bone tumors combined with postoperative combination chemotherapy in children, adolescents, and young adults who have gone through full macroscopic surgical resection. It is made of non-PEGylated liposomes loaded with muramyl tripeptide phosphatidylethanolamine (L-MTP-PE), a fabricated lipophilic derivative of muramyl dipeptide (MDP) (a naturally occurring constituent of bacterial cell walls) that activates monocytes, macrophages, and some cytokines, producing an immune response against osteosarcoma lung metastases. In clinical trials, it demonstrated a very good safety profile, both in patients and healthy volunteers, and given in addition to the usual combination chemotherapy conducted in children and young adults with osteogenic sarcoma showed an increase in 6-year net survival from 70% to 78% (Kager et al., 2010; Meyers et al., 2008).

3.4 Clinical Development of Nanoparticulate Systems for Cancer Treatment

Despite the few nanoparticle-based drugs approved for cancer treatment, many different formulations have reached clinical trials during the last decades. Alkylating agents, antimetabolites, topoisomerase inhibitors, and enzymes, but especially antitumor antibiotics and mitotic inhibitors, have been encapsulated mainly into

PEGylated or non-PEGylated liposomes or polymeric micelles, sometimes functionalized for active targeting, but heavily relying just in the EPR effect (Anselmo & Mitragotri, 2021) (Table 2).

Regarding antitumor antibiotics, doxorubicin is by far the most commonly selected drug for its encapsulation into targeted and nontargeted nanoparticles, and, apart from the already mentioned successfully marketed formulations, many others have been tested in clinical trials. In one example from more than 20 years ago, Mitsubishi Pharma Corporation produced MCC-465, a liposome containing doxorubicin, with PEG and anti-GAH mAb that binds specifically to a molecule on the cell surface of gastric cancer cells. The expectations were high, as the results obtained in xenografts were promising, but the phase I trial in patients with gastric cancer revealed no clinical response, and no more clinical trials were performed with the formulation (Matsumura et al., 2004). HER2-targeted PEGylated liposome MM-302, from Merrimack Pharmaceuticals, experienced a similar fate and, besides the promising safety results obtained in the first phase I clinical trial in breast cancer patients, failed to show improvements in efficacy in more advanced studies (Miller et al., 2016; Munster et al., 2018). The two different formulations of doxorubicin-loaded epidermal growth factor receptor (EGFR)-targeting nanoparticles, from EnGeneIC (Whittle et al., 2015) and the Swiss Group for Clinical Cancer Research of the University Hospital of Basel (Mamot et al., 2012), were not successful in reaching the market either. EnGeneIC is now testing its technology, based on the EDV® Nanocell Platform (bacterially derived minicell) with other cytotoxic drugs, and the Swiss Group for Clinical Cancer Research has just started a new phase I clinical trial with a doxorubicin-loaded PEGylated liposome.

2B3-101 from 2-BBB Therapeutics – that later was sold to Oncology Venture, changing its name to 2X-111 – is a glutathione-containing PEGylated liposome loaded with doxorubicin, for the treatment of solid tumors and especially designed to cross the blood-brain barrier (BBB). The first phase I clinical trial started in 2011, and the results showed a good safety profile (Brandsma et al., 2014). A second phase II clinical trial is registered, but its status is "unknown" since a decade ago.

Worth mentioning is also the case of doxorubicin-loaded ThermoDox® system, the first and only thermosensitive liposome formulation to reach clinical trials, based on lipids that enable the temperature triggered release of their encapsulated content. The initial phase III clinical trial on ThermoDox® (i.e., HEAT trial) evaluating the drug in combination with the interventional oncology technique radiofrequency ablation (RFA), in comparison with RFA alone, for treatment of inoperable hepatocellular carcinoma (HCC) failed to meet its primary endpoint in progression-free survival (PFS). However, analysis of patient subgroups revealed a therapeutic benefit for ThermoDox® in patients who received prolonged RFA treatments, and thus Celsion Corporation decided to start a second phase III clinical trial, OPTIMA, exploring this condition, but it demonstrated that the addition of ThermoDox® to RFA does not provide a measurable survival benefit (Dou et al., 2017; Regenold et al., 2021).

To date, liposomal annamycin (semisynthetic analog of doxorubicin) has been tested in clinical trials, with different formulations, by three companies. The

Table 2 Clinical trials with nanoparticle formulations for cancer therapy

Name (company)	Particle type	Active molecule	Application	Identifiers	Outcomes
Antibiotics					
ThermoDox® (Celsion Corporation)	Lyso-sensitive PEGylated liposome (DPPC:MSPC:DSPE-PEG2000)	Doxorubicin	Various cancers	NCT0093444 (Ph I; Completed) NCT00441376 (Ph I; Completed) NCT00617981 (Ph III; Completed) NCT02850419 (Ph II; Suspended, administrative) NCT01640847 (Ph II; Withdrawn) NCT00441376 (Ph I; Completed) NCT01464593 (Ph II; Terminated, trial design contingent on RFA optimization) NCT02536183 (Ph I; Recruiting) NCT00826085 (Ph I/II; Completed) NCT02112656 (Ph III; Completed) NCT02181075 (Ph I; Completed) NCT03749850 (Ph I; Recruiting) NCT04852367 (Ph I; Recruiting) NCT04791228 (Ph II; Not yet recruiting)	Advanced phase III clinical trials failed to reach primary endpoints. No measurable survival benefit
MM-302 (Merrimack Pharmaceuticals)	HER2-targeted PEGylated liposome (HSPC:Chol:PEG-DSPE)	Doxorubicin	Breast cancer	NCT01304797 (Ph I; Unknown) NCT02213744 (Ph II/III; Terminated) NCT02735798 (Ph I; Withdrawn)	One study terminated because it felt not to show benefit over control per DMC and confirmed via futility analysis. The next study withdrawn before starting due to the sponsor choosing not to fund the trial

EGFR(V)-EDV-Dox (EnGeneIC)	EDV® Nanocell Platform (bacterially derived minicell)	Doxorubicin	Recurrent glioblastoma	NCT02766699 (Ph I; Unknown)	Good safety profile. Three new clinical trials active in Australia with the same technology and highly potent cytotoxic drugs. Carolyn and Inspire trials (recruiting)
Anti-EGFR-IL-dox (Swiss Group for Clinical Cancer Research; University Hospital, Basel, Switzerland)	Anti-EGFR immunoliposome (DSPC:Chol: mPEG-DSPE + anti-EGFR Mab fragments)	Doxorubicin	Advanced triple negative EGFR positive breast cancer. High grade gliomas	NCT02833766 (Ph II; Terminated) NCT03603379 (Ph I; Completed)	The trial was prematurely terminated as per SAKK board decision
TLD-1/Talidox (Swiss Group for Clinical Cancer Research; University Hospital, Basel, Switzerland)	PEGylated liposome (DSPC:Cholesterol: DSPE-PEG 2000)	Doxorubicin	Advanced solid tumors	NCT03387917 (Ph I; Recruiting)	No conclusive results yet
NC-6300 (NanoCarrier)	PEGylated polymeric micelle (epirubicin covalently bound to PEG-polyaspartate block copolymer through an acid-labile hydrazone bond)	Epirubicin	Advanced solid tumors or soft-tissue sarcoma	NCT03168061 (Ph I/II; Recruiting)	Well tolerated with a manageable side effect profile

(continued)

Table 2 (continued)

Name (company)	Particle type	Active molecule	Application	Identifiers	Outcomes
2B3–101 (2-BBB Therapeutics); later the name of the product changed to 2X-111 (Oncology Venture)	Glutathione PEGylated liposome (HSPC:Chol:PEG2000-DSPE)	Doxorubicin	Solid tumors	NCT01386580 (Ph I/II; Completed) NCT01818713 (Ph II; Unknown)	Save and well tolerated in phase I/II clinical trials. Phase II clinical trial in "Unknown" status since 2013
MCC-465 (Mitsubishi Tanabe Pharma Corporation)	PEGylated immunoliposomes (DPPC:Chol:MPPE; tagged with PEG and the F(ab')2 fragment of human monoclonal antibody GAH)	Doxorubicin	Metastatic stomach cancer	Not indexed in ClinicalTrials.gov	Failed to show efficacy in clinical trials
Imx-110 (Immix Biopharma)	PGEylated polymeric micelle (PE-PEG, poly-kinase inhibitor; polyphenol curcuminoid complex, PCC)	Stat3/NF-kB/poly-tyrosine kinase inhibitor and low-dose doxorubicin	Advanced solid tumors	NCT03382340 (Ph I/II; Recruiting)	Encouraging safety results for their ongoing phase Ib/IIa clinical trial
Liposomal Annamycin (Aronex Pharmaceuticals/ Moleculin Biotech)	Liposome (DMPC:DMPG)	Annamycin	Acute myeloid leukemia	NCT03388749 (Ph II; Recruiting) NCT03315039 (Ph II; Completed) NCT04887298 (Ph I/II; Recruiting)	Preliminary results show good safety profile, with no cardiotoxicity and reduced alopecia
Liposomal Annamycin (NYU Langone Health)	Liposome	Annamycin	Breast cancer	NCT00012129 (Ph I/II; Completed)	No detectable antitumor activity in patients with doxorubicin-resistant metastatic breast cancer

Liposomal Annamycin (Callisto Pharmaceuticals)	Liposome	Annamycin	Refractory or relapsed acute lymphocytic leukemia	NCT00271063 (Ph I/II; Unknown) NCT00430443 (Ph I; Terminated)	Well tolerated in phase I/II clinical trials. Second study terminated in 2011
PROMITIL (Lipomedix Pharmaceuticals)	PEGylated liposome (HSPC:Chol:mPEG2000-DSPE)	Mitomycin-C lipidic prodrug	Solid tumors	NCT01705002 (Ph I; Completed) NCT03823989 (Ph Ib; Completed) NCT04729205 (Ph I; Recruiting)	Favorable safety profile and reduced toxicity as compared to equivalent doses of mitomycin-c

(continued)

Table 2 (continued)

Name (company)	Particle type	Active molecule	Application	Identifiers	Outcomes
Mitoxantrone hydrochloride liposome (CSPC ZhongQi Pharmaceutical Technology)	PEGylated liposome (HSPC:Chol:MPEG-DSPE)	Mitoxantrone	Various cancers	NCT02131688 (Ph I; Unknown) NCT02596373 (Ph II; Unknown) NCT05100303 (Ph I; Not yet recruiting) NCT05100329 (Ph II; Not yet recruiting) NCT05089461 (Ph II; Not yet recruiting) NCT04927481 (Ph II; Recruiting) NCT04921878 (Ph I; Not yet recruiting) NCT02856685 (Ph I/II; Unknown) NCT02043756 (Ph I; completed) NCT02597387 (Ph II; Unknown) NCT02595242 (Ph I; Withdrawn) NCT02597153 (Ph II; Terminated, not enough patients enrolled) NCT03776279 (Ph I; Unknown) NCT04668690 (Ph III; Not yet recruiting) NCT04718402 (Ph I; Recruiting) NCT04902027 (Ph I; Not yet recruiting) NCT04719065 (Ph I; Active, not recruiting) NCT04718376 (Ph I; Recruiting) NCT04900766 (Ph I; Not yet recruiting) NCT04548700 (Ph I; Recruiting) NCT04509466 (Ph I; Not yet recruiting) NCT04331743 (Ph I; Not yet recruiting) NCT04352413 (Ph II; Recruiting)	Tested for different cancers (lymphoma, breast cancer, myeloid leukemia, etc.). In general, good safety profile

LEM-ETU (NeoPharm Labs Ltd.)	Liposome (DOPC:Chol:cardiolipin)	Mitoxantrone	Various cancers	NCT00024492 (Ph I; Completed)	Results did not encourage new trials. Last update more than a decade ago
Mitotic inhibitors					
Onco TCS (INEX Pharmaceuticals)	Sphingosome (Chol:SM)	Vincristine	Non-Hodgkin lymphoma	NCT00038207 (Ph II; Completed) NCT00006383 (Ph II; Completed)	Onco TCS changed its name to Marqibo and was approved, in 2012, by the FDA, for Philadelphia chromosome-negative acute lymphoblastic leukemia
INX-0125 (INEX Pharmaceuticals)	Sphingosome (Chol:SM)	Vinorelbine tartrate	Advanced solid tumors	Not indexed in ClinicalTrials.gov	No recent results or news
Alocrest (Spectrum Pharmaceuticals)	Sphingosome (SM:Chol: OPTISOME®)	Vinorelbine	Breast and lung cancer	NCT00364676 (Ph I; Completed)	Generally well tolerated
ABI-011 (NantBioScience), later NTB-011 (in collaboration with Celgene)	Albumin particle	Thiocolchicine analog (IDN 5405)	Solid tumors or lymphomas	NCT02582827 (Ph I; Withdrawn, enrolment not initiated) NCT01163071 (Ph I; Terminated)	One clinical trial terminated and a second one withdrawn
LIPUSU® (Nanjing Luye Sike Pharmaceutical Co., Ltd.)	Liposome (Lecithin:Chol)	Paclitaxel	Advanced solid tumors or gastric and breast cancer	NCT01994031 (Ph IV; Unknown) NCT02142790 (Ph IV; Unknown) NCT02163291 (Ph II; Unknown) NCT02142010 (Ph?; Unknown) NCT02996214 (Ph IV; Active, not recruiting)	Approved in China (2004)

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Table 2 (continued)

Name (company)	Particle type	Active molecule	Application	Identifiers	Outcomes
MM-310 (Merrimack Pharmaceuticals)	Liposome functionalized with antibodies targeted to the EphA2 receptor (Egg-SM:Chol:PEG-lipid functionalized with anti-EphA2 receptor Abs (EphA2 scFv))	Docetaxel	Solid tumors	NCT03076372 (Ph I; Unknown)	Safety update showed Phase I study unable to reach optimal therapeutic index due to continued observation of cumulative peripheral neuropathy
LEP-ETU (NeoPharm Labs Ltd.)	Liposome (DOPC:Chol:cardiolipin)	Paclitaxel	Ovarian cancer	NCT00080418 (Ph I; Completed) NCT00100139 (Ph I; Completed) NCT01190982 (Ph II; Completed)	Last study completed in 2012. Receives Orphan Drug Designation from the FDA in 2015. No new clinical trials since then
Endotag-I (Medigene/ SynCore)	Liposome (DOTAP:DOPC)	Paclitaxel	Breast and pancreatic cancer	NCT01537536 (Ph II; Completed) NCT03126435 (Ph III; Active, not recruiting) NCT00542048 (Ph II; Completed) NCT00448305 (Ph II; Completed) NCT00377936 (Ph II; Completed) NCT03002103 (Ph III; Recruiting)	Phase III clinical trials still ongoing as a second-line treatment for pancreatic cancer

ATI-1123 (Azaya Therapeutics, now acquired by Cytori Therapeutics)	Protein stabilizing liposomes (PSL®) (Phospholipids:Chol, HSA:sucrose)	Docetaxel	Solid tumors	NCT01041235 (Ph I; Completed)	Acceptable tolerability and favorable PK profile in patients with solid tumors. Based on the FDA feedback, the company plans to proceed with a follow-on phase II trial in platinum-sensitive small cell lung cancer who have progressed at least 60 days after initiation of first-line chemotherapy
BIND-014 (BIND Therapeutics)	Prostate-specific membrane antigen (PSMA)-targeted PEG-PLGA or PLA-PEG particle (Accurin® technology)	Docetaxel	Prostate, metastatic, non-small cell lung, cervical, head and neck, or KRAS positive lung cancers	NCT02479178 (Ph II; Terminated) NCT02283320 (Ph II; Completed) NCT01812746 (Ph II; Completed) NCT01792479 (Ph II; Completed) NCT01300533 (Ph I; Completed)	Failed to show efficacy. The company declared bankruptcy in 2016

(continued)

Table 2 (continued)

Name (company)	Particle type	Active molecule	Application	Identifiers	Outcomes
Cynviloq IG-001 (Sorrento Therapeutics; EEUU and Europe)/ Genexol-PM (Samyang Biopharmaceuticals; South Korea)	Polymeric micelle (mPEG-block-D,L-PLA). PEGylated polymeric micelle (mPEG-PDLLA: monomethoxy-PEG-block-poly(D,L-lactide); mPEG-block-D,L-PLA)	Paclitaxel	Head and neck or breast cancer	NCT01023347 (Ph II; Completed) NCT00111904 (Ph II; Completed) NCT01426126 (Ph II; Completed) NCT02739529 (PhI; Unknown) NCT01770795 (Ph II; Completed) NCT01784120 (Ph II; Unknown) NCT00876486 (Ph III; Completed) NCT01169870 (Ph IV; Withdrawn) NCT00877253 (Ph I; Completed) NCT00882973 (Ph I; Completed) NCT01276548 (Ph II; Completed) NCT00886717 (Ph I/II; Unknown) NCT01689194 (Ph II; Completed) NCT00912639 (Ph IV; Unknown) NCT02739633 (Ph II; Unknown) NCT03008512 (Ph II; Terminated, due to poor accrual) NCT02064829 (Ph?; Completed)	Approved for the treatment of metastatic breast cancer and advanced lung cancer in South Korea and other countries (Genexol-PM). There are bioequivalence studies VS Abraxane ongoing to market it in EEUU and Europe, with the name of Cynviloq IG-001, but there is controversy and even legal accusations between companies on the process
Docetaxel-PM/ DOPNP201/ Nanoxel®M (Samyang Biopharmaceuticals)	PEGylated polymeric micelle (docetaxel conjugated to the MPEG-PLA; polymer block via an ester linkage (DTX-PM))	Docetaxel	Head and neck cancer and advanced solid tumors	NCT02639858 (Ph II; Unknown) NCT02274610 (Ph I; Completed) NCT03585673 (Ph II; Unknown) NCT04066335 (Ph?; Recruiting) NCT02982395 (Ph III; Terminated)	Approved in South Korea
Nanoxel® (Dabur Pharma/Fresenius Kabi Oncology Ltd.)	Polymeric micelles (pH-sensitive block copolymer; PVP:NIPAM; PVP-b-PNIPAM)	Paclitaxel	Solid tumors	NCT00915369 (Ph I; Unknown)	Approved in India (2006)

Liporaxel® /DHP107 (Daehwa Pharmaceutical Co., Ltd.)	Lipid nanoparticles for oral administration (monoolein: tricaprylin:Tween 80)	Paclitaxel	Gastric cancer	NCT04046016 (Ph I; Completed) NCT01839773 (Ph III; Completed) NCT03326102 (Ph II; Recruiting) NCT02890511 (Ph I/II; Completed) NCT04675528 (Ph I; Active, not yet recruiting)	Approved in South Korea (2016)
PICN/SPARC1210 (Sun Pharma Advanced Research Company (SPARC Ltd.))	Nanotecton® platform technology (PVP: cholesteryl Sulfate: caprylic acid)	Paclitaxel	Breast cancer	NCT01304303 (Ph I; Completed)	Approved in India (2014)
NK105 (Nippon Kayaku/Nanocarrier)	Micelle (PEG as the hydrophilic segment and modified polyaspartate as the hydrophobic segment. Carboxylic groups of polyaspartate block were modified with 4-phenyl-1-butanol by esterification reaction; consequently, the half of the groups were converted to 4-phenyl-1-butanolate)	Paclitaxel	Breast cancer	NCT01644890 (Ph III; Completed)	The clinical trial missed its primary endpoint
CriPec (Cristal Therapeutics)	Polymeric micelle (proprietary polymers)	Docetaxel	Solid tumors, ovarian cancer	NCT02442531 (Ph I; Completed) NCT03712423 (Ph I; Completed) NCT03742713 (Ph II; Completed)	Phase I clinical trials showed well-tolerated safety profile, but in the phase II clinical trial, the efficacy endpoint was not met

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Table 2 (continued)

Name (company)	Particle type	Active molecule	Application	Identifiers	Outcomes
CRLX301 (Cerulean)	CD-based nanoparticle (covalently conjugated docetaxel to a linear CD-PEG copolymer)	Docetaxel	Advanced solid tumors	NCT02380677 (Ph I/II; Terminated, company decision)	The safety profile was acceptable but the company decided to terminate clinical trials with this formulation
NanoDoce® (NanOlogy)	Large surface area microparticles (nanoparticulates)	Docetaxel	Urothelial carcinoma	NCT03636256 (Ph I/II; Completed) NCT04060628 (Ph?; Available) NCT04260360 (Ph I; Withdrawn, not initiated)	Acceptable safety profile. Encouraging early efficacy results
NanoPac® (NanOlogy)	Large surface area microparticles (nanoparticulates)	Paclitaxel	Pancreatic adenocarcinoma, lung cancer	NCT04314895 (Ph II; Recruiting) NCT03077685 (Ph II; Recruiting) NCT03756311 (Ph?; Available) NCT04221828 (Ph II; Terminated, lack of enrolment) NCT03077659 (Ph II; Completed) NCT03029585 (Ph II; Terminated, business reasons)	Acceptable safety profile. Encouraging early efficacy results
Halaven E7389-LF (Eisai)	PEGylated liposome (HSPC:Chol:MPEG2000-DSPE)	Eribulin mesylate	Solid tumors	NCT01945710 (Ph I; Completed) NCT03207672 (Ph I; Recruiting) NCT04078295 (Ph I/II; Recruiting)	Well tolerated in patients with advanced solid tumors. Phase I and II clinical trials ongoing
Topoisomerase inhibitors					
OSI-211 (OSI Pharmaceuticals)	Liposome (HSPC:Chol)	Lurtotecan	Advanced or metastatic solid tumors; ovarian, head and neck cancer	NCT00006036 (Ph I; Completed) NCT00022594 (Ph II; Completed) NCT00010179 (Ph II; Completed) NCT00003891 (Ph I; Completed) NCT00046787 (Ph II; Completed) NCT00046800 (Ph II; Completed)	Last clinical trials finished more than a decade ago. No news or updates

LE-SN-38 (NeoPharm Labs Ltd.)	Liposome (PC:Chol:cardiolipin)	SN-38 (active metabolite of irinotecan)	Advanced colorectal cancer; small cell lung cancer	NCT00046540 (Ph I; Completed) NCT00104754 (Ph II; Withdrawn) NCT00311610 (Ph II; Completed)	Well tolerated but failed to meet efficacy endpoints
INX-0076 (INEX Pharmaceuticals)	Sphingosome (Chol:SM)	Topotecan	Advanced solid tumors	Not indexed in ClinicalTrials.gov	No recent results or news
IT-141 (Intezyne Technologies)	Polymeric micelle (PEO-poly (glutamic acid) block copolymers through chemical conjugation of SN-38 to the free carboxyl groups present on the poly(glutamic acid backbone))	SN-38 (active metabolite of irinotecan)	Advanced cancer	NCT03096340 (PhI; Terminated by sponsor)	Only clinical trial terminated by sponsor

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Table 2 (continued)

Name (company)	Particle type	Active molecule	Application	Identifiers	Outcomes
CRLX101 (formerly IT-101) (Cerulean)	CD-based nanoparticle (covalently conjugated camptothecin (CPT) to a linear CD-PEG copolymer)	Camptothecin	Ovarian, renal cell, small cell lung, or rectal cancers	NCT02187302 (Ph II; Completed) NCT02010567 (Ph I/II; Terminated, due to funding partner's request) NCT02389985 (Ph I/II; Terminated, company decision) NCT01803269 (Ph II; Terminated, due to lack of activity and slow accrual) NCT01652079 (Ph II; Completed) NCT02769962 (Ph I/II; Recruiting) NCT03531827 (Ph II; Active, not recruiting) NCT02648711 (Ph I; Terminated, company decision) NCT01380769 (Ph II; Completed) NCT01612546 (Ph II; Completed) NCT00333502 (Ph I/II; Completed) NCT01625936 (Ph I; Completed) NCT00753740 (Ph II; Withdrawn, poor trial recruitment) NCT00163319 (Ph III; Completed)	Failed to show efficacy in phase II clinical trials, both alone and in combination
S-CKD602 (Alza Corporation)	PEGylated liposome (PEGylated phospholipids)	CKD602	Advanced malignancies	NCT00177281 (Ph I; Completed)	No news or updates since more than a decade ago
Alkylating agents					
LiPlaCis (LiPlasome Pharma; Oncology Venture)	PEGylated liposome with specific degradation-controlled drug release via phospholipase A2 (PLA2) (DSPC:DSPG:DSPE-PEG2000)	Cisplatin	Advanced or refractory tumors	NCT01861496 (Ph I; Active, not recruiting)	Severe renal toxicity and an acute infusion reaction were observed in patients in phase I study. Thus, LiPlaCis clinical studies were halted

Lipoplatin/Nanoplatin (Regulon, Inc.)	PEGylated liposome (DPPG:soy PC:mPEG-DSPE:Chol)	Cisplatin	Pancreatic cancer; lung cancer	NCT02702700 (Ph I; Terminated, drug supply issues)	Improved safety profile respect reference treatment but modest efficacy benefits. Received Orphan Drug Designation by the EMA in 2007 and clinical trials were ongoing but not results have been published in years and the company has not clarified if the drug is still being evaluated
SPI-77/L-NDDP (ALZA Pharmaceuticals, formerly Sequus Pharmaceuticals)	PEGylated liposome (Chol:HSPC:PEG-DSPE)	Cisplatin or analog	Ovarian cancer; malignant pleural mesothelioma; advanced non-small cell lung cancer	NCT00004083 (Ph II; Completed) NCT00004033 (Ph II; Completed)	Acceptable safety profile but very limited efficacy, probably related to low release from nanoparticles
SLIT® (Transave)/ILC (Insmed Incorporated)	Liposome (DPPC:Chol)	Cisplatin	Metastatic osteosarcoma	NCT00102531 (Ph I/II; Completed)	Well tolerated in heavily treated osteosarcoma patients without the typical toxicities associated to IV cisplatin. More studies are needed
Aroplatin (Liposomal NDDP) (Aronex Pharmaceuticals, now Antigenics)	Liposome (DMPC:DMPG)	NDDP (oxaliplatin analog)	Advanced solid malignancies	NCT00043199 (Ph II; Unknown) NCT00057395 (Ph I/II; Unknown) NCT00081536 (Ph I/II; Unknown) NCT00081549 (Ph I/II; Unknown) NCT00316511 (Ph I; Completed)	Acceptable safety profile but modest efficacy

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Table 2 (continued)

Name (company)	Particle type	Active molecule	Application	Identifiers	Outcomes
MBP-426 (Mebiopharm Co., Ltd.)	Liposome (transferrin-conjugated NGPE liposome)	Oxaliplatin	Advanced or metastatic solid tumors; second-line gastric, gastroesophageal, or esophageal adenocarcinoma	NCT00355888 (Ph I; Completed) NCT00964080 (Ph I/II; Unknown)	No results posted yet
LipoXal® (Regulon, Inc.)	PEGylated liposome (HSPC:DPPG:Chol:DSPE-PEG)	Oxaliplatin	Advanced gastrointestinal cancers	Not indexed in Clinicaltrials.gov (Phase I)	Well-tolerated and reduced oxaliplatin-related side effects, especially myelotoxicity and gastrointestinal tract toxicities
NC-6004 Nanoplatin (Nanocarrier)	Polyamino acid and PEG micelles (PEG poly(glutamic acid) block copolymers)	Cisplatin	Advanced solid tumors; lung, biliary, bladder, or pancreatic cancers	NCT02240238 (Ph I/II; Completed) NCT02043288 (Ph III; Completed) NCT03771820 (Ph II; Recruiting) NCT03109158 (Ph I/II; Completed) NCT02817113 (Ph I; Terminated, due to strategy change)	They are focusing now in the combination treatment of head and neck cancers (phase II clinical trial)
NC-4016 DACH-Platin (Nanocarrier)	Polyamino acid and PEG micelles (PEG poly(glutamic acid) block copolymers)	Oxaliplatin	Advanced solid tumors or lymphomas	NCT03168035 (Ph I; Completed)	Phase I clinical trial completed. No results published yet

Antimetabolites					
FF-10832 (Fujifilm Pharmaceuticals)	PEGylated liposome (Chol:HSPC:N-MPEG-DSPE)	Gemcitabine	Advanced solid tumors	NCT03440450 (Ph I; Recruiting)	A new clinical trial about to start, in collaboration with Merck, for advanced solid tumors, in combination therapy with KEYTRUDA® (pembrolizumab)
Others					
LipoCurc® (SignPath Pharma)	Liposome (patent includes different combinations of PEGylated and non-PEGylated formulations)	Curcumin	Solid tumors	NCT02138955 (Ph I/II; Unknown) NCT01403545 (Ph I; Completed)	Reported very good safety profile, with minimal toxicity despite high blood concentrations. Planning new clinical trials
^{188}Re-BMEDA-liposome (Institute of Nuclear Energy Research, Taiwan)	PEGylated liposome (DSPC:Chol:DSPE2000-PEG)	188Re-N,N-bis(2-mercaptoethyl)-N′,N′-diethylethylenediamine	Advanced solid tumors	NCT02271516 (PhI; Terminated)	Terminated due to concerns of accumulation of radioactivity in both the liver and spleen
Atragen (Aronex Pharmaceuticals, now Antigenics)	Liposome (DMPC:soybean oil)	Tretinoin	Hormone-resistant prostate cancer, renal cell carcinoma, and acute myelogenous leukemia	NCT00003656 (Ph II; Completed) NCT00005969 (Ph II; Withdrawn)	FDA rejected approval

products by NYU Langone Health (Booser et al., 2002) and Callisto Pharmaceuticals (Wetzler et al., 2013) failed to show efficacy in patients and are no longer actively being studied. On the contrary, Moleculin Biotech has just announced updated preliminary safety data for annamycin in its three phase I clinical trials for acute myeloid leukemia and metastases of soft-tissue sarcoma, reporting a promising safety profile, with no cardiotoxicity and reduced alopecia (Gil et al., 2019).

Most of the evaluated antibiotics have been encapsulated in the inner aqueous phase of the liposomes, both by passive or active loading, but there are also examples of lipophilic drugs retained in the lipid bilayer of these nanoparticles. This is the case of Promitil® (Gabizon et al., 2020), a mitomycin-C lipidic prodrug loaded in PEGylated liposomes for the treatment of solid tumors that has already completed two phase I clinical trials showing a favorable safety profile and reduced toxicity as compared to equivalent doses of mitomycin-c. The product is currently being evaluated in a third phase I clinical trial.

Two companies selected mitoxantrone as the drug to be encapsulated into liposomes for the treatment of various cancers. The formulation of NeoPharm Labs Ltd. was evaluated 20 years ago, in a phase I clinical trial, but the results did not encourage the continuation of the studies (Ahmad et al., 2005). The mitoxantrone hydrochloride liposome from CSPC ZhongQi Pharmaceutical Technology has been tested in a total of 23 clinical trials, alone or in combination with other chemotherapeutic drugs, for the treatment of very different cancers, such as malignant lymphoma, metastatic breast cancer, acute myeloid leukemia, advanced pancreatic cancer, etc. In general, the shown safety profile is good, and the technology will continue being evaluated in clinical trials to determine its efficacy (Wang et al., 2021).

Antibiotics have also been encapsulated into polymeric nanoparticles, such as the NC-6300 epirubicin-loaded polymeric micelles that showed to be well tolerated, with a manageable side effect profile, in a phase Ib dose escalation trial in patients with advanced solid tumors or advanced, metastatic, or unresectable soft-tissue sarcoma (Chawla et al., 2020; Riedel et al., 2021). Another example is the PE-PEG-composed IMX-110 system, from Immix Biopharma, presented as monotherapy for soft-tissue sarcoma, that just a few weeks ago announced encouraging safety results for their ongoing phase Ib/IIa clinical trial.

Mitotic inhibitors have also been extensively studied in nanoformulations for cancer treatment, especially docetaxel and paclitaxel. Paclitaxel, very insoluble in water, is generally formulated using Cremophor EL. Docetaxel, more soluble in water, is formulated using Tween 80 and ethanol. Tween 80, albeit less toxic than Cremophor EL, may be responsible of some toxic effects. Thus, nanoparticles are a key technology to eliminate these vehicles and improve the drug's antitumor efficacy.

Merrimack Pharmaceuticals tested a second formulation in a phase I clinical trial – apart from the previously described doxorubicin-loaded MM-302 – the docetaxel-loaded MM-310 anti-EphA2 receptor immunoliposome for the treatment of solid tumors (Kirpotin et al., 2016). The last safety update showed inability to reach optimal therapeutic index due to continued observation of cumulative peripheral neuropathy, and the formulation was discarded (Ernstoff et al., 2018).

The ATI-1123 product from Azaya Therapeutics, now acquired by Cytori Therapeutics, was also tested in a phase I clinical trial with encouraging safety results (Mahalingam et al., 2014). Now, based on the FDA feedback, the company plans to proceed with a follow-on phase II trial in platinum-sensitive small cell lung cancer that have progressed at least 60 days after initiation of first-line chemotherapy. The formulation is composed of phospholipids, cholesterol, human serum albumin (HSA), and sucrose, with the aim of removing the need for solvents, reducing hypersensitivity reactions, eliminating the requirement for premedications, and enhancing systemic docetaxel exposure.

The case of the BIND Therapeutics company is also well known. They developed prostate-specific membrane antigen (PSMA)-targeted polymeric nanoparticles, based on their Accurin® technology, loaded with chemotherapeutics, for the treatment of various cancers (Autio et al., 2018). Specifically, the BIND-014 product was loaded with docetaxel and evaluated in five phase I and II clinical trials for the treatment of prostate, metastatic, non-small cell lung, cervical, head and neck, or Kirsten Rat Sarcoma Viral Oncogene Homologue (KRAS)-positive lung cancers. Despite all the collaborations with the big pharmaceutical companies, the acquired funding, and the high expectations, their products failed to show efficacy in the clinic, and the company declared bankruptcy in 2016.

Cristal Therapeutics relies in polymeric micelles for sustained release of chemotherapeutics too (Braal et al., 2018). Their CriPec® platform is composed of tuneable polymers, biodegradable drug linkers, and optional target motives and has been evaluated, loaded with docetaxel, in three phase I and II clinical trials for the treatment of solid tumors and ovarian cancer. Phase I clinical trials showed well-tolerated safety profile, but in the phase II clinical trial, the efficacy endpoint was not met.

Docetaxel was also one of the chosen molecules for the cyclodextrin-based nanoparticle system of Cerulean, formed by covalently conjugating docetaxel to a linear, cyclodextrin-polyethylene glycol (CD-PEG) copolymer (Piha-Paul et al., 2021). Once again, the safety profile was acceptable, but the company decided to terminate clinical trials fearing lack of efficacy.

Samyang Biopharmaceuticals (South Korea) developed two polymeric micelle formulations loaded with docetaxel and paclitaxel, Docetaxel-PM (also DOPNP201/Nanoxel®) (Lee et al., 2011) and Genexol-PM (Kim et al., 2004; Madamsetty et al., 2019), respectively. These two monomethoxy PEG-b-poly(D,L, lactic acid) (PLA) formulations were specifically designed to improve the solubility of the chemotherapeutic drugs and to avoid the need to use toxic solubilizing agents such as Cremophor EL or Tween 80. Docetaxel-PM is commercialized in South Korea, and it is under clinical evaluation for pharmacokinetic equivalence with docetaxel injection concentrate as well as for safety and antitumor efficacy. Paclitaxel-PM is also available in South Korea and other Asian countries for the treatment of breast, non-small cell lung, and ovarian cancer and is currently undergoing bioequivalence testing to gain marketing approval in the US and European markets, under the name of Cynviloq IG-001, but the process is being long and highly controversial, with even legal accusations between the companies involved.

In addition, there are other four paclitaxel-loaded nanoparticle formulations approved in the Asian market. The first one, called LIPUSU® (Xu et al., 2013; Zhang et al., 2022), is a liposomal formulation, composed of lecithin and cholesterol, that was approved in China for the treatment of non-small cell lung cancer, breast cancer, and ovarian cancer, and it has been administered to over 2 million patients in the last 17 years. The second one is Nanoxel®, by Fresenius Kabi Oncology Ltd., that was approved in India in 2006 (Madaan et al., 2013; Ranade et al., 2013), allowing patients to receive Cremophor and premedication free paclitaxel, with equivalent efficacy. The third, Liporaxel®/DHP107 (Kim et al., 2020; Rugo et al., 2021; Yang et al., 2020), has the peculiarity of being intended for oral administration. The formulation, which is elaborated by mixing up the paclitaxel chemotherapeutic drug with monoolein, tricaprylin, and Tween 80, was approved in South Korea, in 2016, for the treatment of advanced, metastatic, and local recurrent gastric cancer and is currently in clinical trials in patients with other cancers. The last one, the Paclitaxel Injection Concentrate for Nanodispersion (PICN), by Sun Pharma Advanced Research Company Ltd. (SPARC), was approved in India, in 2014, for the treatment of metastatic breast cancer. In a phase II/III clinical study in patients with metastatic breast cancer (Jain et al., 2016; Ma et al., 2021), it was found to be equally effective and safe when compared to Abraxane®. Clinical studies are still ongoing.

Nippon Kayaku and Nanocarrier evaluated a paclitaxel-loaded polymeric micelle, NK105 (Hamaguchi et al., 2005; Hamaguchi et al., 2007; Kato et al., 2012), in a late-stage clinical trial against paclitaxel reference treatment too, but the formulation failed to meet its primary endpoint. Nanocarrier decided to continue clinical trials with a second-generation micelle pipeline in which the drug was chemically conjugated to the polymers inside the nanoparticles. We have already mentioned the epirubicin-loaded NC-6300, and another two, NC-6004 (Subbiah et al., 2018) and NC-4016 (Ueno et al., 2014), encapsulating cisplatin and oxaliplatin, respectively, are also being evaluated in clinical trials. NC-6004, in phase II clinical trials, is administered as a combination therapy, for the treatment of pancreatic, head, or neck cancer, among others. On the other hand, a phase I dose-escalation and pharmacokinetic study of NC-4016 in patients with advanced solid tumors or lymphoma has been completed in 2017, but no results have been published so far.

Finally, two more paclitaxel-loaded liposomal formulations have reached clinical testing: Endotag-I and LEP-ETU. The novelty of Endotag-I, from Medigene, is its positive charge, due to the presence of 1,2-Dioleoyl-3-trimethylammonium propane (DOTAP) in the formulation. It is generally accepted that nanoparticles of neutral or slightly negative charge more efficiently scape removal by the immune system, but positive charges augment the interaction between the nanoparticles and the negatively charged cellular membranes (Mitchell et al., 2021). The hypothesis behind Endotag-I (Fasol et al., 2012) is that because of the positively charged lipids, it interacts with newly developed, negatively charged endothelial cells, which are particularly required for the generation of tumor blood vessels. The nanoparticles attack the endothelial cells as they divide, thus targeting the blood supply to tumors without affecting the blood supply to healthy tissue. However, preclinical studies

and clinical trials conducted on different types of cancer such as breast cancer, adenocarcinoma, or pancreatic cancer have shown limited efficacy and sometimes notable adverse events. There are still phase III clinical trials ongoing, with Endotag-I as a second-line treatment for pancreatic cancer.

On the other hand, the paclitaxel-loaded LEP-ETU (Slingerland et al., 2013), from NeoPharm Labs Ltd., is based on a similar formulation to the already mentioned mitoxantrone-loaded LEM-ETU, and the company evaluated a third composition in clinical trials too: the SN-38-loaded LE-SN-38 (Zhang et al., 2004). The three liposome formulations are based on similar combinations incorporating cholesterol and cardiolipin. LEP-ETU entered clinical evaluation to treat ovarian, breast, and lung cancers and completed its last phase II clinical trial in 2012. Since then, it received the Orphan Drug Designation from the FDA, but no updated information has been released. On the other hand, SN-38 is the active metabolite of irinotecan, and the LE-SN-38 liposomal formulation was tested for the treatment of small cell lung cancer and metastatic colorectal cancer in phase II clinical trials, where the formulation showed to be well tolerated but failed to meet efficacy endpoints.

With a slightly different concept, NanOlogy developed NanoDoce® and NanoPac® (Maulhardt et al., 2021, 2020; Mullany et al., 2020; Verco et al., 2021), two formulations of pure drug, docetaxel and paclitaxel, respectively, composed of large surface area microparticle (LSAM) therapeutic platforms, based on a proprietary supercritical precipitation technology that converts taxane API crystals into stable LSAMs, for tumor-directed therapy and sustained drug release. The administration for both products is local/intratumoral, and they are being tested in phase I and II clinical trials for the treatment of different cancers, such as urothelial carcinoma, pancreatic adenocarcinoma, and lung cancer.

Worth mentioning are two other mitotic inhibitors that have been tested in clinical trials in nanoparticulate formulations for cancer treatment: eribulin mesylate and the thiocolchicine analog IDN 5405. Eribulin mesylate, Halaven®, synthesized by Eisai, got FDA approval in 2010, and the same company is now testing eribulin mesylate-loaded liposomal formulation (Halaven E7389-LF) in clinical trials. Results from the first phase I clinical trial showed the formulation was well tolerated in patients with advanced solid tumors (https://www.annalsofoncology.org/article/S0923-7534(19)58570-2/fulltext#relatedArticles). Two more clinical trials, in phase I and phase Ib/II, are now ongoing in Japan, with the liposomal formulation alone or in combination with nivolumab. On the other hand, IDN 5405, the thiocolchicine analog, was formulated bound to albumin to develop ABI-011 – later NTB-011, in collaboration with Celgene – with cytotoxic and vascular disrupting properties (D'Cruz et al., 2009). The expectations were high as the inventors of Abraxane®, the successful albumin-paclitaxel nanoparticle, were involved in the project; however, the first clinical trial was terminated and the second one withdrawn even before starting patient enrollment.

One of the successful stories that ended up in the commercialization of one of the few approved nanoparticle-based chemotherapeutic formulations started with the testing of various sphingosomes by Inex Pharmaceuticals. The nanoparticles

composed of SM and cholesterol were loaded with vincristine (Onco TCS) (vincristine liposomal-INEX: lipid-encapsulated vincristine, Onco TCS, transmembrane carrier system-vincristine, vincacine, vincristine sulfate liposomes for injection, VSLI, 2004), vinorelbine (INX-0125) (Semple et al., 2005), or topotecan (INX-0076), among others, and evaluated in clinical trials for the treatment of advanced solid tumors and non-Hodgkin lymphoma (Bulbake et al., 2017). A few years later, Onco TCS changed its name to Marqibo® and was approved by the FDA for the treatment of Philadelphia chromosome-negative ALL and commercialized by Spectrum Pharmaceuticals. This company also tested another formulation in a phase I clinical trial, Alocrest, that resulted to be generally well tolerated (Deitcher et al., 2007).

INX-0076 and LE-SN-38 were not the only nanoparticulate formulation based on topoisomerase inhibitors that reached clinical testing. The therapeutic potential of camptothecins (including irinotecan and topotecan) is limited because they rapidly undergo hydrolysis at physiological pH, changing from their active form (lactone ring structure) to their inactive form (carboxylate structure), leading to a short circulation lifetime. Liposomal formulations of these molecules can be designed to overcome these stability issues.

The previously mentioned company, Cerulean, developed a formulation based on camptothecin (apart from the docetaxel-loaded CRLX301), called CRLX101 (Pham et al., 2015; Svenson et al., 2011; Young et al., 2011) (formerly IT-101), developed by covalently conjugating camptothecin to a linear, cyclodextrin-PEG (CD-PEG) copolymer that self-assembles into nanoparticles. The formulation seemed promising at the preclinical level, as it was expected to address solubility, formulation, toxicity, and pharmacokinetic challenges, improving the efficacy. However, in 2013, it failed to show a benefit in lung cancer, causing a strategy change to drug combinations, but 3 years later, the company reported disappointing results for another phase II clinical trial, in combination with bevacizumab, in renal cell carcinoma patients.

Other clinical stage attempts to encapsulate topoisomerase inhibitors in nanoparticles for cancer treatment including OSI-211, IT-141, and S-CKD602. The non-PEGylated liposomal form of lurtotecan, OSI-211 (Duffaud et al., 2004; Tomkinson et al., 2003), from OSI Pharmaceuticals, composed of hydrogenated soy phosphatidylcholine (HSPC) and cholesterol, was evaluated in a total of six clinical trials that finished more than a decade ago, and there are no updates since then. IT-141 (Carie et al., 2011) was composed of SN-38-loaded polymeric micelles and was evaluated in a phase I clinical trial that was terminated by the sponsor. Lastly, the phase I clinical trial testing the PEGylated liposomal formulation S-CKD602 (Zamboni et al., 2009), from Alza Corporation, finished in 2006, and, besides the company qualifying the results as "promising," there have been no news since then.

Regarding the use of alkylating agents, we have already mentioned NC-6004 Nanoplatin and NC-4016 DACH-Platin from Nanocarrier, but there are more examples in clinical trials. The most evaluated drug has been cisplatin, in formulations including lipoplatin/nanoplatin, SPI-77, SLIT®, and LiPlaCis®, among others. Cisplatin is one of the most widely used chemotherapies due to its efficacy against

multiple cancer types but has severe side effects, demonstrating the critical need for specificity and reformulation.

Lipoplatin® (also known as Nanoplatin®) (Boulikas et al., 2005) is a proprietary PEGylated liposome formulation of cisplatin, by Regulon, Inc. The product has been introduced as Lipoplatin® for the treatment of pancreatic cancer and Nanoplatin® for lung cancer. This liposomes, composed of lipids including DPPG, soy PC, MPEG-distearoyl-sn-glycero-phosphoethanolamine (DSPE) lipid conjugate, and cholesterol, have been tested in phase I trials for malignant pleural effusion, phase II trials for breast and gastric cancer, phase II/III trials for pancreatic cancer, and phase III trials for NSCL ((Mylonakis et al., 2010; Stathopoulos et al., 2005; Stathopoulos et al., 2006a, b). In clinical trials, the company announced good safety profiles with reduced adverse effects associated with CPT including renal toxicity, peripheral neuropathy, ototoxicity, and myelotoxicity (Boulikas et al., 2005; Boulikas, 2009). In 2007, the EMA granted Orphan Drug Designation to this product for pancreatic cancer treatment, while clinical trials were still ongoing; however, no results have been published in years, and the company has not clarified if the drug is still being evaluated.

Formulations of cisplatin (SPI-77) (Seetharamu et al., 2010; Vokes et al., 2000; White et al., 2006) or analogs, developed by ALZA Pharmaceuticals, formerly Sequus Pharmaceuticals, were based on stealth liposomes. Results obtained in phase I and II clinical trials demonstrated a good safety profile but very limited efficacy. These findings were attributed to the low loading capacity and insufficient release of the free drug.

LiPlaCis®, developed for treatment of advanced solid tumors, is a liposomal formulation, incorporating cisplatin, which is composed of lipids with degradation properties controlled by the phospholipase A2 (PLA2) enzyme, highly expressed in a multitude of human solid tumors including prostatic, pancreatic, colorectal, gastric, and breast cancers for a tumor-triggered release mechanism. In clinical trials, LiPlaCis® has demonstrated an enhanced therapeutic window compared to cisplatin, with superior PK properties, greater potency, and an increased maximum tolerated dose. However, severe renal toxicity and an acute infusion reaction were observed in patients in phase I study. Thus, LiPlaCis® clinical studies were halted.

SLIT® (Sustained Release Lipid Inhalation Target) (Chou et al., 2013), the liposomal formulation from Transave (later Inhaled Lipid Cisplatin, ILC, from Insmed Incorporated), was composed of dipalmitoylphosphatidylcholine (DPPC) and cholesterol and presented a key novelty: it was an aerosolized formulation for pulmonary administration. In a phase I/II clinical study in patients with osteosarcoma metastatic to the lung, adverse effects associated to the IV administration of cisplatin were not reported, but changes in the pulmonary function were detected in some patients. Major benefits were described in patients with operable and small tumors (<2 cm), but more studies are needed to determine the efficacy and safety of the treatment.

On the other hand, oxaliplatin has also been nanoencapsulated and tested in clinical trials. As a third-generation water-soluble platinum drug, it is different from cisplatin and carboplatin in that it presents free amino groups linked to platinum and

has lower toxicity and tumor resistance. MBP-426 (Sankhala et al., 2009; Senzer et al., 2009) is an oxaliplatin-encapsulated transferrin-conjugated N-glutaryl phosphatidylethanolamine (NGPE)-liposome that targets the transferrin receptor, which is upregulated in many types of cancer. After a phase I clinical trial in patients with advanced or metastatic solid tumors, the formulation entered a phase I/II trial for second-line gastric, gastroesophageal, or esophageal adenocarcinoma in 2009, but results have not been posted yet.

Regulon, Inc., the company that developed the cisplatin-loaded Lipoplatin®, also developed an oxaliplatin-based liposomal formulation, LipoXal® (Stathopoulos et al., 2006a; Tippayamontri et al., 2014). In a phase I study, reduction respect to free oxaliplatin of myelotoxicity, nausea, and peripheral neuropathy was observed, but further clinical tests will be needed to demonstrate the improvement of antitumor activity of LipoXal® over free oxaliplatin.

Aroplatin (L-NDDP) (Dragovich et al., 2006) is a liposome encapsulating a cis-bis-neodecanoato-trans-R,R-1,2-diaminocyclohexane platinum II (NDDP), an oxaliplatin derivative. The multi-lamellar liposomes were formed from 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-racglycerol) (DMPG) and 1,2-dimyris-t oyl-sn-glycero-3-phosphocholine (DMPC) lipids in acidified saline solution. In phase II study, Aroplatin was tested in refractory metastatic colorectal cancer, and, besides the acceptable safety profile, in general the response was modest. To date, there is no report of any ongoing phase III study. Two decades ago, the same company, Aronex Pharmaceuticals (now Antigenics), tried to commercialize another liposomal formulation, loaded with tretinoin and named Atragen (Bernstein et al., 1998), but the FDA rejected the approval.

Apart from the cytarabine-containing marketed formulations, nanomedicines based on antimetabolites for the treatment of cancer have been nearly anecdotic, with only one formulation reaching clinical trials: gemcitabine-loaded FF-10832 (Matsumoto et al., 2021), by Fujifilm Pharmaceuticals. The PEGylated formulation is now being evaluated in a phase I clinical trial, for the treatment of solid tumors, and last year, Fujifilm Pharmaceuticals signed an agreement with Merck to start a new clinical study for advanced solid tumors in combination therapy with KEYTRUDA® (pembrolizumab).

Finally, worth mentioning are two strategies that are not based in traditional chemotherapy: LipoCurc® (Bolger et al., 2019) and ^{188}Re-BMEDA-liposome. LipoCurc®, by SignPath Pharma, is composed of curcumin-loaded nanoparticles. Historically, development of curcumin as a pharmaceutical product has been hampered by its poor absorption and cardiac side effects. Thus, LipoCurc® was designed to improve curcumin bioavailability and toxicological profile. First reported results were encouraging, with a very good safety profile despite the high blood concentrations. They are planning new clinical trials in different cancer types.

^{188}Re-BMEDA-liposome (Chang et al., 2007; Lepareur et al., 2019), from the Institute of Nuclear Energy Research of Taiwan, was the only formulation incorporating radioactive isotopes to reach clinical trials for the treatment of primary solid tumors in advanced or metastatic stage. However, the phase I trial was terminated due to concerns of accumulation of radioactivity in both the liver and spleen

4 Challenges in Nanomedicine Clinical Translation

Despite the uncountable attempts to develop targeted nanoparticulate therapies for drug delivery to tumors, few anticancer nanomedicines have been approved by regulatory agencies, thus generating a debate regarding the real effectiveness of these systems for cancer treatment. Most anticancer medicines follow the same two basic criteria when trying to design effective and safe sustained drug delivery systems based on lipid or polymeric nanoparticles: (1) the EPR effect, caused by the leaky vasculature next to the tumor, increases drug accumulation in the affected area, and (2) long systemic circulation of drug-loaded nanoparticles avoids the uptake by the RES, decreasing drug accumulation in the normal organs and reducing toxicity (Sun et al., 2020). The EPR effect influencing nanomedicines has repeatedly been confirmed, both in animal xenografts and in human cancer patients, using nanoparticle-encapsulated imaging agents (Gaillard et al., 2014; Greish, 2010; Hamaguchi et al., 2004; Koukourakis et al., 2000; Torchilin, 2011), but it is difficult to conclude if this EPR effect is different to the one observed for the free drugs. Free drugs, as small molecules with high plasma protein binding, also accumulate in tumors due to this phenomenon (Tang et al., 2014; Torchilin, 2011), and, due to ethical concerns, clinical trials with a free drug control arm are not possible in most cases; thus, there are very few direct comparisons between the free drug and the nanoparticle formulation.

When Doxil® reached the market, the accumulation of doxorubicin in patient tumors was found to be an order of magnitude higher than with free drug, and pathogenic analysis of KS revealed notably leaky vasculature (Northfelt et al., 1998; Uldrick & Whitby, 2011). However, in a later study, the evaluation of the tumor uptake of radiolabeled liposomes, with the same lipid composition as Doxil®, demonstrated considerable heterogeneity between patients with the same and different cancer types (Harrington et al., 2001). Since then, a few studies have demonstrated significantly higher drug concentrations in the tumors when administering liposomal formulations (Gabizon et al., 1994), but limited improvements have been the reason of failure and cancelation of many clinical trials (Dragovich et al., 2006; Kraut et al., 2005; White et al., 2006).

Recent studies increasingly downplay the EPR effect. An interesting analysis by Wilhelm et al., surveying the literature from the past 10 years, concluded that only 0.7% (median) of the administered nanoparticle dose is found to be delivered to a solid tumor (Wilhelm et al., 2016). Another meta-analysis found no significant difference in clinical anticancer efficacy between liposomal and conventional chemotherapeutics in terms of objective response rate, overall survival, and PFS (Petersen et al., 2016).

Another key aspect is the validity of the animal xenograft models to mimic the biological phenomena observed in human cancers. In the available animal models, the EPR effect is notably exaggerated, resulting in a poor clinical translation (Greish, 2010). Thus, there is an urgent necessity to develop new models for in vivo and in silico testing.

Regarding the long systemic circulation and the high plasma concentration, it can increase tumor accumulation if there is a strong EPR effect or decrease drug accumulation in normal organs to reduce toxicity. However, it can also reduce efficacy or alter drug distribution to different organs, generating new adverse events (Harrington et al., 2001; Ngan & Gupta, 2016; Northfelt et al., 1998).

In addition, even if nanoparticles are able to avoid clearance from blood circulation (by the mononuclear phagocytic systems or the RES, among others) and the shear stress caused by varying flow rates and extravasate next to the tumor, the complex extracellular matrix surrounding malignant cells will notably limit their penetration (Yuan et al., 1994). Furthermore, lack of drug release from the vehicles can significantly decrease drug availability (Laginha et al., 2005; White et al., 2006).

Furthermore, after hundreds of preclinical and a few clinical studies with actively targeted nanoparticles incorporating specific motifs directed to molecules that are usually overexpressed on cancer cells, none of the tested strategies have reached the market (Ernstoff et al., 2018; Mamot et al., 2012; Matsumura et al., 2004). This is probably linked to the fact that actively targeted nanosystems also rely on the same principles as the passive targeting until they reach the microenvironment of the tumor where they can match with the specific molecules on the cancer cell membranes, thus dealing with the same challenges.

In general, most of the marketed nanomedicines failed to show improved efficacy, in comparison with the reference treatment, but they significantly and consistently improved the toxicity profile of classic chemotherapeutic agents, allowing for the administration of higher doses and better patient quality of life (Batist et al., 2002; Drummond et al., 1999; Farokhzad & Langer, 2006).

5 Conclusions

Cancer continues to be unstoppable worldwide, and there will be more than 30 million new cases by 2040, according to the International Agency for Research on Cancer. Thus, novel diagnostic and treatment tools are needed to beat this global challenge. Among the approaches explored by scientists, nanomedicine highlights due to its ability to develop an endless variety of accurate nanomaterials to provide a new landscape in cancer research. Thus, different scientific disciplines, such as engineering, chemistry, physics, nanotechnology, materials science, or medicine, work together to achieve precision systems and also enhance the translation to the clinics and pharmaceutical market. However, even though standardization, stability, and reproducibility are required for this goal, tailored features are mandatory for the successful application of the personalized medicine.

In this chapter, we have evidenced the encouraging potential of advanced nanoparticles as smart drug delivery systems to improve the therapeutic effect of current standard drugs and increased patient survival rates. Undoubtedly, there is still a long journey from the nanocarrier design to translation to the pharmaceutical market as viable products. Although thousands of research articles describe great

outcomes of drug delivery systems with different nature and properties in multiple in vitro and in vivo cancer models, only a small fraction has successfully reached the translation to clinical level. This limited clinical translation of new nanoparticles is mainly due to incomplete therapeutic efficacy and off-target toxicity in vital organs. Nonetheless, results and evidences from previous clinical trials should guide not only the optimization of nanocarrier formulations but also setting clinical studies taking into account the tumor heterogeneity through the introduction of stratified populations instead of broad cancer patients.

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