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Nanotechnology approaches in the current therapy of skin cancer

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ABSTRACT

Skin cancer is a high burden disease with a high impact on global health. Conventional therapies have several drawbacks; thus, the development of effective therapies is required. In this context, nanotechnology approaches are an attractive strategy for cancer therapy because they enable the efficient delivery of drugs and other bioactive molecules to target tissues with low toxic effects. In this review, nanotechnological tools for skin cancer will be summarized and discussed. First, pathology and conventional therapies will be presented, followed by the challenges of skin cancer therapy. Then, the main features of developing efficient nanosystems will be discussed, and next, the most commonly used nanoparticles (NPs) described in the literature for skin cancer therapy will be presented. Subsequently, the use of NPs to deliver chemotherapeutics, immune and vaccine molecules and nucleic acids will be reviewed and discussed as will the combination of physical methods and NPs. Finally, multifunctional delivery systems to codeliver anticancer therapeutic agents containing or not surface functionalization will be summarized.

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1. Introduction

Skin cancer is classified as melanoma skin cancer (MSC) or nonmelanoma skin cancer (NMSC). NMSC is the most commonly diagnosed type of cancer worldwide [1], and MSC is associated with the highest percentage of deaths [2,3].

Therefore, skin cancer is a disease of global health importance that causes substantial psychosocial impacts and requires considerable investment in terms of treatments and technologies [4,5].

Among the technological advancements in cancer treatments, the use of nanosystems has been an attractive strategy for delivering therapeutic agents. These systems can be developed to overcome biological barriers and to target drug delivery to the tumor sites, thus enabling the use of lower doses, increasing treatment efficacy and decreasing the number and/or severity of the side effects [6–8]. The benefits of using nanosystems for cancer treatments are obvious, although most of the nanotechnology approaches are at the research or development stage, some of them have reached the clinical stage and are already on the market. For instance, Doxil®, a PEGylated liposome containing doxorubicin, is on the market and is indicated mainly for breast and

ovarian cancer, multiple myeloma and Kaposi sarcoma in HIV/AIDS patients [6]. Another nanotechnology product that was approved by the FDA in 2005 is Abraxane®, an albumin-bound paclitaxel nanoparticle that is indicated for the treatment of various cancers, especially metastatic breast cancer and non-small-cell lung carcinoma [7,8].

For skin cancer, to the best of our knowledge, there are no commercially available nanosystems for bioactive molecule topical delivery. Although most nanoparticles (NPs) applications are still in the preclinical stages, some studies have advanced and shown promise, leading these nanotechnology products to be evaluated in clinical trials. For instance, a clinical trial was recently started for cutaneous metastasis. The study (ClinicalTrials.gov Identifier: NCT 03101358) aims to evaluate a topical NP containing paclitaxel for the treatment of cutaneous metastases from nonmelanoma cancer. The study is evaluating the safety, tolerability and preliminary efficacy of three concentrations of this NP [9]. Another phase I study is evaluating the safety, maximum tolerated dose, pharmacokinetics, and clinical activity of a liposomal miR-34a mimic (MRX34) in patients with advanced solid tumors. The introduction of miR-34a mimics into *in vitro* cancer cell lines derived from solid tumors such as skin cancer resulted in an important reduction in cell proliferation, migration and invasion [10].

Nanotechnology approaches represent an opportunity to improve the treatments for different types of cancer, and this review aims to compile and discuss important works using these nanotechnological tools for skin cancer. First, pathology and conventional therapies will be addressed. Then, the challenges to skin cancer treatment will be

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discussed, including the biological barriers that hinder local and systemic delivery of NPs loaded with different kinds of drugs as well as multidrug resistance (MDR). Then, the designs and ideal characteristics of NPs for effective delivery of drugs for skin cancer therapy will be proposed according to the route of administration, followed by the presentation of the main nanocarriers used for drug delivery in skin cancer. Subsequently, treatment strategies for skin cancer using NPs to deliver chemotherapeutics, immune and vaccine molecules and nucleic acids will be reviewed and discussed. Finally, combinations of physical methods and NPs and the use of multifunctional delivery systems will be summarized and examined.

2. Pathology of skin cancer and conventional therapies

2.1. Melanoma skin cancer (MSC)

MSC originates in melanocytes, which are the cells that produce melanin. In this pathological process, several mutations can occur, mainly located in the epidermis, at the basal layer [11,12].

Several risk factors are related to MSC development, but the main risk factor is excessive ultraviolet (UV) light exposure. This exposure may trigger mutations in melanocytes that lead to invasive and metastatic features [13].

The main mutations observed in melanomas involve the BRAF/NRAS/MEK/MAPK pathways, mainly because these signaling pathways are already operating aberrantly, even without mutations, in this type of skin cancer. Thus, they are recurrent targets for therapies aimed at treating melanoma. Unfortunately, current therapies confer MDR, which requires increasingly innovative treatments that act simultaneously and aggressively on the melanoma pathways [13,14]. The MDR effect is discussed in Section 3.2.

The treatments chosen for MSC depend on the stage of the disease and include conventional therapies such as surgical excision and radiotherapy (RT) and innovative treatments such as targeted therapy; immunotherapy; and combinations of systemic, topical and transdermal therapies. When there is no metastatic ability, MSC have a cure rate of 90%; however, in contrast, the survival of patients with metastasis drops to only approximately 10% [1,11,12].

Surgery is indicated for lesions with well-defined borders, but there are risks of lymph node involvement, metastases and relapse [15].

The primary treatment for patients with good prognoses and well-defined lesions is RT. RT has satisfactory tumor reduction rates and is safe and well tolerated [16]. Additionally, RT can be used to treat more advanced stages when associated with other therapies, such as immunotherapy [17].

The molecular mechanisms of MSC have not been entirely elucidated; therefore, treating the disease is difficult. To overcome this challenge, innovative therapies are required that are mainly based on delivery systems using nanotechnology.

2.2. Nonmelanoma skin cancer (NMSC)

NMSC, as opposed to MSC, develops along well-known molecular pathways; hence, therapeutic platforms are more efficient in NMSC than for MSC. Moreover, because it is less aggressive and has lower invasive potential, NMSC is associated with better prognoses and survival rates than MSC [18].

Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) originate in keratinocytes and are known as keratinocyte carcinomas. BCC is more prevalent than SCC, accounting for approximately 70% of the cases, compared with 25% for SCC. Neither type contributes to deaths from skin cancer; nevertheless, they are considered a significant public health problem due to their high incidence, the fact that they are more prevalent than all other types of cancers worldwide, and the burden that they represent [5,19].

NMSC has numerous risk factors that, in addition to genetics, may increase an individual's predisposition to develop this type of neoplasia. The main factors, in addition to genetics, are age; exposure to UV rays and/or ionizing radiation, immunosuppression, genodermatoses, HPV infection, medications such as TNF- α inhibitors, tobacco use, severe skin and/or bone infections, and inflammatory conditions [18–23].

Surgery is an option for the treatment of NMSC; however, depending on the extent of the lesions and their locations, nonsurgical treatments are highly recommended, especially for lesions with well-defined margins and those presenting in relatively confined areas. The main nonsurgical treatments are curettage, electrodesiccation, topical drug administration, cryosurgery and radiation and photodynamic therapies [20,24]. The treatment of choice may vary depending on the lesion area, location, and molecular pathway.

Several kinds of NPs have been widely used for the treatment of NMSC due to their ability to deliver drugs through several molecular pathways simultaneously and to be administered through different routes to prioritize less invasive pathways.

3. Challenges to skin cancer treatment

3.1. Biological barriers

In current skin cancer therapies, NPs loaded with anticancer agents are generally administered through topical/transdermal, intratumoral (local), or systemic (intravenous) routes or through a combination of these. Topical or transdermal applications are noninvasive and self-administered approaches considered for elderly patients, those not considered candidates for surgical treatment, and young patients with extensive lesions in areas of cosmetic importance [25]. These administration routes have been used for most premalignant lesions, such as those of AK; superficial skin cancer, such as noninvasive SCC; and BCC and melanoma in the initial stages [26].

The outermost epidermal layers of the skin, constituting the stratum corneum (SC), are the main cutaneous barrier and influence the uptake of anticancer agents into target cells, consequently affecting the response to topical or transdermal treatments. Typically, in human skin, the SC is composed of approximately 15–30 corneocyte cell layers, which provide a thickness of approximately 10–20 μm . The layers are formed as the product of long epidermal maturation, differentiation and keratinization processes. These processes are induced in the deepest epidermal layers that rest on the basal lamina above the dermis to constitute the basal layer [27]. Additionally, several types of lipids surround corneocytes, such as ceramides, triglycerides, cholesterol, and free fatty acids, to form a complex network of lipids and corneocytes that can absorb water and control the penetration of macromolecules and micromolecules through the skin.

The viable epidermis is another epidermal layer that is localized below the SC and reaches the dermal-epidermal junction formed by the thin basal layer [28]. In contrast to the dermis, the viable epidermis is vascularized, and although it is composed mainly of keratinocytes at different stages of maturation, the viable epidermis is also populated by melanocytes, Langerhans cells and Merkel cells and ranges in thickness from 50 to 100 μm [29].

The dermis is vascularized and responsible for providing nutritional support to the viable epidermis. Lymphatic vessels, sebaceous glands, collagen, elastin, sensory nerves, and hair follicles are localized in the dermis and are arranged within an area that is approximately 0.1 to 0.4 cm thick [30].

Skin cancer with cutaneous morphology presents an over expression of keratin, high levels of lipids, and the presence of erythematous plaques, keratotic papules or nodules compared to healthy tissue [31,32]. Several skin cancer subtypes present with an increased amount of keratin, which leads to diffusion-resistant tissue and results in a significant barrier to the passive transport and retention of NPs in the target cells [31,33]. Generally, AK lesions produce excessive keratin,

making the SC layer thicker and consequently a more difficult barrier to penetrate. Therefore, NPs used against AK need to be properly designed to pass through the SC and reach the deeper epidermis layer, where the Langerhans cells are located, to exert immune responses [34].

Considering the most advanced stage of skin cancer, in which tumor cells extend throughout the epidermis and dermis, several associated treatments have shown better success rates than topical application alone, for example, the combination of topical application and physical methods and the combination of topical and systemic applications. As discussed later (Section 6.4), the enhanced penetration of NPs through the skin by physical methods occurs due to disorganization of the skin barrier such that channels are formed between the corneocytes and/or the keratinocytes. The combination of NPs and physical methods is convenient for the patient because topical drug delivery reduces the first-pass effects and side effects that can occur with systemic administration [35].

However, when topical and systemic applications are combined, NP biodistribution is governed not only by the skin barrier but also by other biological barriers [33]. For instance, the first biological barriers encountered after systemic NP administration are related to the hepatic and renal clearance that removes NPs from the circulation. The structure of the blood vessels under ordinary conditions prevents NP penetration and decreases their retention at the tumor site [36]. In contrast, in cancer, the blood vessels in tumors present fenestrations and endothelial imperfections that create heterogeneous canals with openings of 200 to 2,000 nm that facilitate the penetration of NPs across blood vessels and promote NP delivery to target cells [36]. The combination of these mechanisms, such as poor lymphatic drainage and imperfect blood vessels in tumors, creates an abnormal microenvironment that increases drug delivery into and through the skin [37,38].

Another biological barrier consists of the dense tumor interstitial space composed of collagen, proteins, elastic fibers, and glycosaminoglycans. Because the interstitial fluid pressure is elevated in this condition, the transport of drugs to the interior of tumors is affected. Additionally, the extracellular matrix, which in the tumor environment is excessively rigid and has an elevated collagen content, acts as a barrier to the transport of the NPs to the cancer cells [33,39]. Moreover, features of the NPs influence their interaction with tumor cells, their connection to the components of the cells and the physiological characteristics of the cells. The main interactions between the NPs and tumor cells are adsorption, cellular uptake, and endosomal transport, followed by endosomal escape, metabolism, and degradation [36]. Donahue *et al.* (2019) discussed the cellular internalization pathways of NPs and how the cellular interactions, trafficking and kinetics may be affected by the physicochemical properties of the NPs [40]. Overall, the cell plasma membrane is a cellular uptake barrier to NPs due to its negative surface charge, resulting in selective permeability to biomolecules and NPs. This cellular barrier must be overcome via different routes (e.g., direct cellular entry of the NPs or entry via endocytosis pathways) to see a biological response. For passive entrance into the cell, these delivery systems are translocated across the cell plasma membrane through the lipid bilayer, and then, the NPs are delivered into the cytoplasm, thus overcoming the endosomal entrapment and energy-dependent transport mechanism. During uptake in endocytosis-based pathways, the NPs are restricted within intracellular vesicles until they are released into the cellular cytoplasm.

For transport via endocytosis, different mechanisms may be involved, such as micropinocytosis, phagocytosis, caveolin-independent endocytosis, caveolin-dependent endocytosis, clathrin-independent endocytosis and clathrin-dependent endocytosis [40–42]. NPs after uptake are captured by endosomes, and NPs can be designed to escape and be readily available to act at the specific target site [40,43].

For intratumoral application, NPs can be injected at high concentrations, thus avoiding possible side effects compared to other administration routes. This administration route is minimally invasive, enables the use of low doses, decreases immunostimulation reactions and is less

painful than subcutaneous injection [44]. Moreover, the tumor itself is a potential site of vaccination through direct intratumoral immunization, enabling an increase in vaccine magnitude and enhancing the control of metastasis [45,46]. Injectable gel generated *in situ* has also gained considerable attention from researchers in the past few years for intratumoral administration [47,48]. The gel formed *in situ* from a precursor fluid can absorb water from the environment and swells as a result, and after self-assembling in the gel, the anticancer agent is released in a sustained manner [48].

3.2. Multidrug resistance (MDR)

Nanotechnology is an exciting and promising alternative for overcoming multidrug resistance (MDR), since through this tool, it is possible to provide treatment in a specific, orderly way, with smaller doses of therapeutic agents and fewer toxic effects. The use of nanocarriers enables the effective, targeted delivery of several therapeutic agents made from combinations of molecules aimed at acting synergistically and effectively against cancer types that are resistant and no longer responsive to conventional therapies [49–54].

MDR occurs when tumor cells acquire resistance to various drugs used in therapy. This MDR situation is illustrated by the significantly reduced therapeutic potency of a drug that culminates in the progression of the disease. MDR is considered an obstacle to the effective therapy of several types of cancer, including skin cancer, especially melanoma [55–57].

The MDR effect may arise due to intrinsic and/or acquired factors. Intrinsic factors are related to drug degradation, alteration of prodrugs, alteration of drug targets and receptors, and reduction in drug-receptor interactions. Additionally, membrane changes, metabolic process alterations, cell cycle changes, repairs to DNA damage, and changes in efflux pumps may contribute to MDR. The acquired factors are, in turn, usually associated with epigenetics. Patients who present with intrinsic factors do not respond to conventional treatments; patients who acquire resistance present with decreased effectiveness of treatments over time [55,56].

The molecular mechanisms that lead to this resistance can be defined as noncellular or cellular mechanisms. Noncellular mechanisms are those inherent to the tumor and its characteristics, such as pH, which makes the tumor environment hostile to the otherwise appropriate action of the drugs. Cellular mechanisms are caused by biochemical changes in the tumor cells. The main known mechanisms involved in skin cancer are changes in drug uptake, efflux pumps, and enzymatic activation; alterations in DNA repair; avoidance of apoptosis pathways; and P53 alterations (Fig. 2).

NPs have been used to overcome the MDR effects [54]. For example, changes in efflux pumps can lead to the uptake of active agents in tumor cells. NPs are widely employed to overcome these MDR effects on efflux pumps. For example, a hybrid micelle containing a prodrug of doxorubicin was developed to overcome this MDR mechanism. The MDR effect on efflux was overcome by this NP, and the drug was efficiently delivered in tumor cells, where as previously, there were problems in uptake [58]. Drugs, such as temozolomide, have been encapsulated in NPs to overcome the difficulty in them being uptake in tumor cells. Dendrimers containing temozolomide had improved uptake in melanoma cells [59].

Liu *et al.* (2012) used silver NPs decorated with penetrating peptide (TAT) to overcome the MDR effects that transpired during the NP uptake process. Through this functionalization, the uptake was improved, and consequently, the antitumor activity was more effective [60]. Guo *et al.* (2018), in turn, overcame MDR effects using mesoporous titanium dioxide NPs that combined the following functions: target, drug delivery, and PDT. The active compounds were CD44, N-cadherin, and doxorubicin [61].

Aiming for the apoptotic cascade, Chen *et al.* (2009) chose to target the BCL-2 protein to overcome MDR effects. The authors developed mesoporous silica nanoparticles to co-deliver doxorubicin and Bcl-2

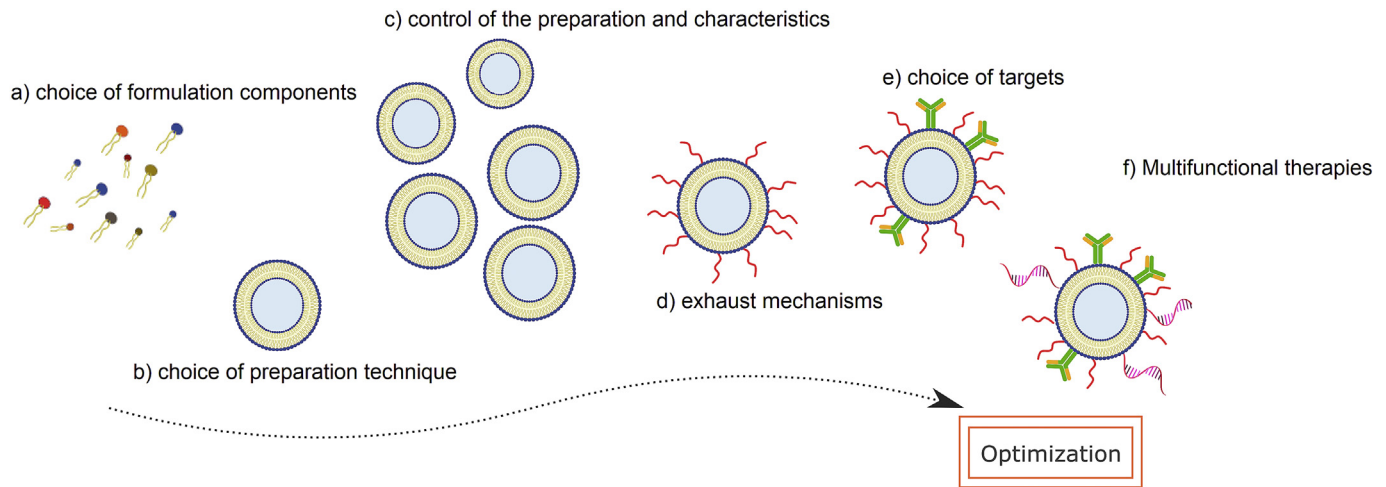


Fig. 1. Main steps of NP development, (a) Choice of formulation components; (b) Choice of preparation technique; (c) Control of the preparation and NP characteristics; (d) exhaust mechanisms; (e) Choice of targets and (f) multifunctional therapies as example: target and specific therapy such as the use of siRNA.

siRNA, and the results indicated that BCL-2 was an important target for melanoma treatment, and inhibition of BCL-2 was crucial for overcoming the effects of MDR on the apoptotic cascade [62].

4. Design and characteristics of the NPs

4.1. Design

The design and characterization of NPs carrying anticancer drugs have reached the first stages of application for effective skin cancer

treatment with few adverse effects. The development of NPs needs to follow specific steps, and the final design is reached according to the nature of the NP chosen, target location and type of therapy desired, as illustrated in Fig. 1. Each administration route (topical, transdermal, intratumoral or systemic) has its own biological obstacles and characteristics; therefore, the therapeutic target tissue and the ideal administration route are important factors to be considered. Thus, this coordinated approach has great potential to optimize NP design and create a new frontier for skin cancer therapy [39,40].

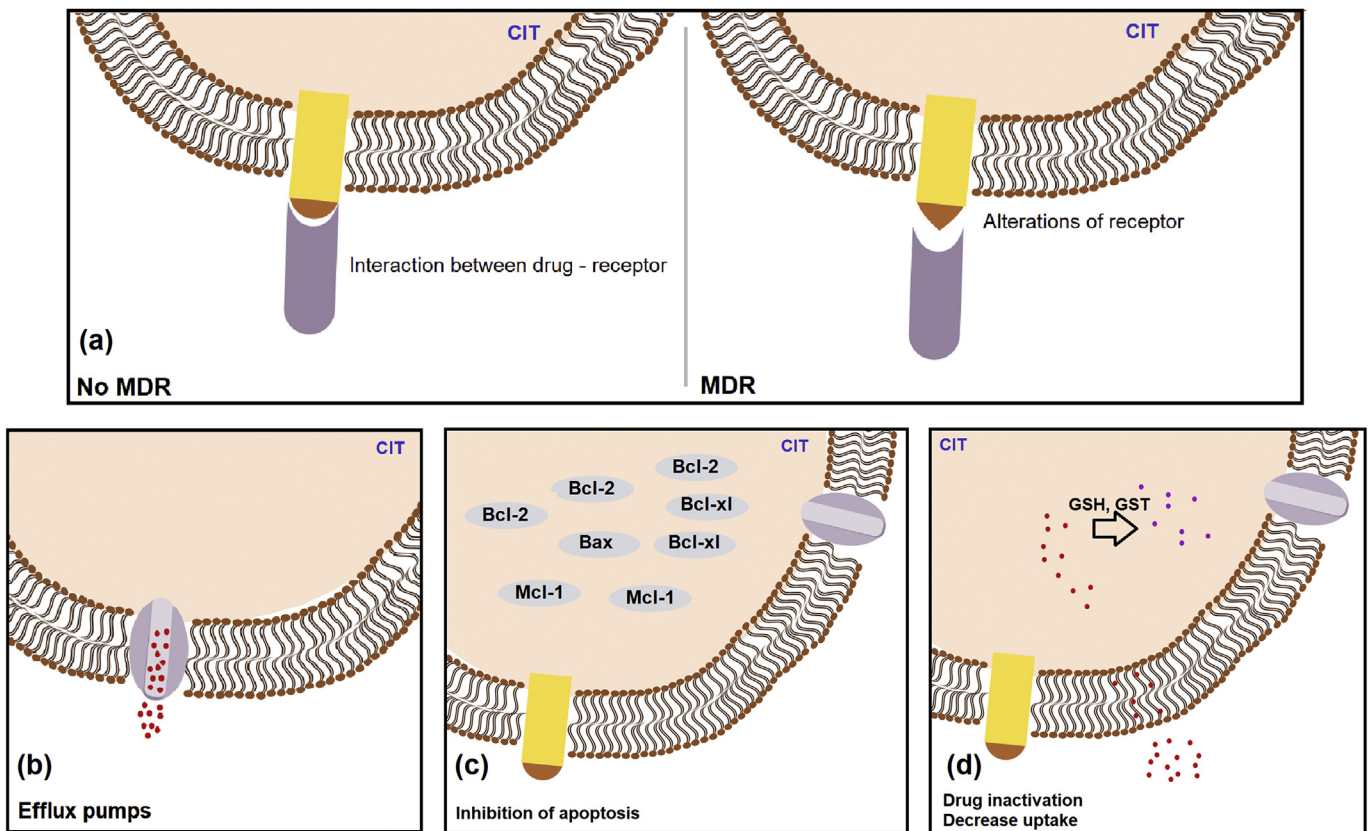


Fig. 2. The main known mechanisms for skin cancer that promote multidrug resistance (MDR), (a) Alterations of receptor sites for drugs [55,57]; (b) Changes in efflux pumps through the interaction between nucleotide binding domains and drug [51,55,57,351,352]; (c) Inhibition of the caspase cascade through the overexpression of anti-apoptotic molecules (Bcl-2, Bcl-xl, Mcl-1) and down expression of pro-apoptotic molecules (Bax) [50,351]; (d) Reduction of cellular uptake of drugs by modification of membrane lipids and inactivation of drugs by glutathione conjugation (GSH) catalyzed by glutathione S-transferase enzyme (GST) [56,57]. CIT = cytosol.

For topical delivery, the major NP challenges are focused in two areas: effective penetration through the SC barrier and accumulation in the epidermal layer at therapeutic concentrations. It has already been shown that small molecules can penetrate the skin by one of three possible pathways: the intercellular route (through the lipid matrix that surrounds the corneocytes), the transcellular route (across the corneocytes and into the lipid matrix) and the transappendageal route, represented by sweat ducts, hair follicles and sebaceous glands [63]. However, for hydrophilic molecules greater than 500 Daltons, such as peptides, and nucleic acids, topical and transdermal delivery are very difficult [64]. Alternatively, NPs can overcome the SC and target tumor cells, whether combined with physical methods or not, by SC barrier disruption and the increase in the drug diffusion due to altered membranes.

NP features, such as particle size and particle size distribution, shape, surface charge, surface function, and chemical nature, can be designed to fit the therapeutic approaches [65]. Thus, well-designed systems should be able to (i) solve drug solubility issues, mainly those related to highly hydrophobic drugs, (ii) encapsulate the anticancer agent in its internal structure to improve drug stability by chemical or physical means, (iii) provide delivery of encapsulated drug by controlled or sustained release rates and (iv) reduce skin irritation by avoiding direct drug-skin contact [34]. Additionally, when systemically administered, NPs should have the ability to (i) overcome the lymphatic drainage system, (ii) remain for a long time in the bloodstream and (iii) reach the therapeutic site at effective doses. Target molecules, such as antibodies, can be used to increase the accumulation of NPs at desired sites [33,64].

However, the process for manufacturing NPs presents several challenges that have made it difficult to scale up production and to translate the NPs created at the bench into clinical practice [66]. In turn, predicting the morphology, particle size, polydispersion index, surface changes (in chemistry, adhesion and charge), encapsulation or complexation efficiency, drug release kinetics, hemodynamic activity and steric stabilization level of the NPs during the early pharmaceutical development process has been the key to achieving a better correlation between use *in vitro* and in clinical trials [67].

4.2. Particle size features

In general, the particle size predicts much about the ability of an NP to penetrate the skin as well as its systemic biodistribution, and therefore, the size has a significant impact on drug pharmacokinetics, particularly drug biodistribution and cellular uptake, and safety [68]. NPs from 6 to 30 nm were able to penetrate the skin via intracellular/intercellular routes and aqueous pores. Particles ≤ 30 nm penetrated the skin in a manner that depended on their surface polarity, hydrophilicity, shape, and morphology. Other particles ≤ 70 nm were deposited in both epidermal and dermal layers, with preferential accumulation in cutaneous appendages, while particles ≥ 600 nm were retained on the SC to form an occlusive film on the skin [69,70]. To NPs fully penetrate the skin and reach the bloodstream under stable conditions, despite the cutaneous appendages to be present in only small portions of the skin, they seem to be the main contributors to NPs directly penetrating the skin and reaching the bloodstream under stable conditions [71], and flexible liposomes can also be carried across the intact skin through the corneocyte bricks [72].

When applied systemically, particles ≤ 30 nm were rapidly cleared from circulating blood by the renal excretion system; particles equal to 100 nm had an increased blood circulation time compared to smaller particles; large particles, between 200 and 300 nm, were retained mainly in the spleen and liver; and those ≤ 400 nm underwent rapid hepatic clearance [68,73]. It is worth noting that particles smaller than 200 nm benefited from the EPR effect, thus favoring targeting of the tumor [68].

Nonetheless, after direct intratumoral injection, particles of approximately 65 nm penetrated the tumor environment until reaching the

cancer cells, while particles between 85 and 120 nm were easily extravasated to the venous outflow immediately after intratumoral injection. Others particle sizes of approximately 178 nm were retained in the tumor interstitium [74,75]. Rigorous control of particle size is an important task when transitioning from lab-scale preparation to a larger-scale manufacturing process. NP size distribution needs to be well controlled in the manufacturing process because it influences the penetration and retention behavior of these systems. However, although simultaneous cutaneous penetration through different routes may favor heterogeneity, the overall process is more unstable [76].

4.3. Shape features

The modulation of the NP shape and surface can predict particle delivery [77]. Particle shape plays an essential role in skin penetration as well as subcellular targeting [33,78–80]. NPs that are rod-shaped, spherical and triangular were investigated for their ability to penetrate different layers of skin. To determine these parameters, follicular and intracellular penetration pathways were considered [81].

Triangular NPs were observed to penetrate the skin more slowly than rod-like and spherical NPs. Furthermore, the rod-like NPs were observed to penetrate and subsequently accumulate at higher levels in the dermal layer, which enhanced their systemic effects [81]. The subcellular trafficking of anticancer agents was most favorable using rod-like NPs, according to a study in which they localized in the nucleus in metastatic cancer cells after 2 h of NP treatment *in vitro* [82]. On the other hand, inhibition of the cellular uptake and subcellular trafficking of discoidal NPs was also observed because they localized in the phospholipid membrane bilayers and thus had a lower tendency to penetrate human cells.

Carbon nanotubes were reportedly compatible with and transportable in biological fluids and entered human cancer cells by endocytosis or needle-like penetration, enabling direct NP cytoplasmic delivery [83]. With this in mind, discoidal and cylindrical shaped NPs are considered potential templates for designing cell membrane-specific and safe theranostic imaging agents for applications in skin cancer treatment [69]. Overall, nonspherical particles demonstrated higher performance than spherical counterparts.

4.4. Surface features

The role of the NP surface is crucial in predicting NP cytotoxic effects, activity, and efficacy when they are intravenously administered, as demonstrated by NPs coated with blood components in the opsonization process to make them targets for clearance by macrophages [68]. In turn, a well-designed particle surface can improve the actions on specific cellular targets, enhance cellular uptake and localization in the target cell of the NPs and prevent serum effects [84,85]. Particle surfaces charged with either positive potential or negative potential improved nanosystem stability by preventing agglomeration and flocculation processes through the enhanced electrostatic repulsion between the particles [86]. Moreover, positively charged particles increase particle binding to the target cells via electrostatic interactions to a greater degree than negatively charged or neutral molecules because of the negative charge of the cellular plasma membrane. Likewise, negatively charged phospholipids are abundantly distributed in the tumor cell membranes [76]. In addition, positive particle surfaces can be engineered based on cationic compounds (lipid or polymer) that favor the complexation of nucleic acids such that they can penetrate the cell membrane and subsequently release the nucleic acid into the cytoplasm or the nucleus to improve the results of the gene therapy used in cancer treatment [87,88].

Regarding systemic administration, one well-known surface modification is the PEGylation coating, which confers a prolonged half-life and superior overall efficacy to NPs compared with that presented by “nonstealth” NPs. This approach decreases NP affinity for the

mononuclear phagocytic system and consequently prevents their early removal from circulation as well as increases their uptake by cancer cells [89]. Other surface-modified NP strategies include the use of antibodies, proteins, aptamers, and folate [90–92], which traffic NPs directly to targeted receptor-positive tumor cells to subsequently penetrate the cell by receptor-mediated endocytosis. However, the effectiveness of NP therapeutic activity is achieved only when the therapeutic agent is localized to the proper target located in the cytoplasm, mitochondria, or nucleus and avoids lysosomal degradation [33].

On the other hand, for topical administration, optimization of the cell uptake and trafficking was further enhanced after the NP surface was decorated with targeted cell-penetrating peptides (CPPs) [93–95]. Among several CPPs, transcriptional activator (TAT) and sensitive matrix metalloproteinases (MMPs), which are over expressed in skin cancer cell membranes and upregulated in the interstitial tumor space, respectively, have been extensively investigated [93,94]. These CPPs can translocate across the cell membrane and deliver both charged and noncharged NPs, which have enhanced accumulation in tumors [69,94].

Finally, in the field of cancer vaccines, inorganic NPs have gained attention due to additionally providing imaging contrast for theranostics or susceptibility to magnetic navigation that is used to increase tissue-specific accumulation [96]. Bocanegra *et al.* (2018) used zinc-doped iron oxide magnetic NPs to deliver a combination of TRL agonists and peptide antigens. The vaccines showed superior efficacy against aggressive B16F10 melanoma cells. Additionally, the researchers could track the vaccine transport from the site of injection to the LNs and tumor by nuclear imaging and magnetic resonance (MR) [97]. Other examples of NP delivery systems include the use of lipid-coated zinc phosphate hybrid NPs [98], liposome-coated gold NPs [99] and multiwalled carbon nanotubes [100].

Some functionalization of NP surfaces aiming to target tumor cells and/or increase the blood circulation time of NPs are discussed later in Section 6.5.

4.5. Intracellular trafficking features

The site of action for several important drugs that are used for the treatment of different diseases is the intracellular organelle. For example, the anticancer drug doxorubicin acts in the nucleus. Therefore, it is essential to design NPs that enable the drug to reach its targeted site of action, surpassing the challenges of intracellular delivery, such as lysosomal degradation and the size restriction of organelle entry [101].

Rationally designed, multiple stimuli-responsive NP approaches were recently explored as promising tools for improving the intracellular trafficking of anticancer agents through rapid cytoplasmic delivery [102]. The most explored stimuli-responsive NPs were designed to target changes in pH, temperature, redox condition, enzyme activity, magnetic field, ultrasonic waves and various types of irradiation [103,104]. For these purposes, biocompatible polyglycerol-based thermoresponsive nanogels showed intracellular localization in lysosomal compartments after 24 and 48 h of treatment, indicating that the lysosomes are the final intracellular fate of the nanogels in cutaneous cells [105]. Polymer-coated CNPs, being nontoxic for stromal cells, showed cytotoxic, proapoptotic, and anti-invasive abilities against cutaneous melanoma cells, with cytosolic distribution [106]. In another approach, CPP-targeted NPs with redox-sensitive coatings accumulated to the highest level in tumor tissue but not in healthy organs [107], resulting in optimized intracellular trafficking, greater antitumor efficacy and improved safety compared to those that already have the approval of the Food and Drug Administration (FDA) [90].

The intracellular trafficking of molecules with different characteristics is distinct. There are few studies in the skin cancer field attempting to complete the understanding of intracellular drug delivery. An appreciation and description of these pathways is essential to the proposal of effective NPs.

5. Nanoparticles for therapeutic agent delivery

It is well known that nanotechnology is an important tool for therapeutic agent delivery in cancer therapies. The rational development of nanocarriers for anticancer drugs has led in advanced systems that can act to target cancerous tissues and cells [108]. Given the magnitude of events and barriers involved in skin cancer, several types of NPs have been proposed and can be initially classified according to the main formulation component [109]: for instance, lipid-based nanoparticles such as liposomes, transfersomes, niosomes, ethosomes, solid lipid NPs, and liquid crystalline nanodispersions; polymeric-based carriers such as polymer NPs, polymeric micelles, and dendrimers [110]; and inorganic nanostructures (Fig. 3). In this section, the main nanocarriers used for drug delivery in skin cancer will be presented.

The combination of nanostructured therapeutic agents in NPs with dosage forms is yet a new and little-explored alternative for delivering NPs [111]. These NPs can be incorporated into dosage forms such as creams [112,113] ointments and hydrogels [114,115], patches [113,116] for topical penetration [115,117,118].

5.1. Lipid-based NPs

Lipid NPs are the most conventional nanocarriers and are mainly represented by lipid vesicles, known as liposomes, which are able to carry lipophilic and hydrophilic drugs [119,120]. These colloidal carriers are composed of lipids and phospholipids that have the advantage of being identical to physiological compounds [110]. Most liposomes are phospholipid-based vesicles that bear a long hydrophobic chain and a hydrophilic head and have the ability to self-assemble [120]. These NPs improve the penetration of hydrophilic molecules, which are encapsulated in the aqueous core, or hydrophobic molecules in the membrane bilayers [121]. In addition, amphiphilic molecules can also be loaded into a liposome core using specific preparation methods [122].

The composition, flexibility, and deformability of such nanocarriers can be adjusted according to the need [120]. Therefore, since large liposomes are excluded from the deep skin layers, small and mostly monodisperse liposomes with a diameter of approximately 30–40 nm can be obtained by a microfluidic procedure to enhance the ability of these lipid NPs to penetrate the skin [123]. Moreover, the mixture of liposomes with biocompatible copolymers led to the formation of vesicles with an increased ability to permeate into the deep skin layers in studies *in vitro* [124].

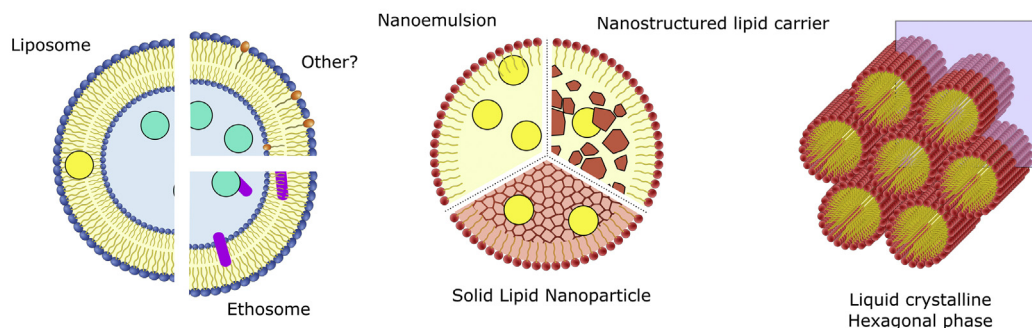
Transfersomes, deformable vesicles used for drug delivery to the skin, penetrate through the SC pores by squeezing themselves along an intracellular sealing lipid [110,125]. Hydrating synthetic nonionic surfactants in the presence or absence of cholesterol or other lipids leads to the formation of niosomes [108,110].

Noninvasive nanocarriers mainly formulated with phospholipids, ethanol, and water are called ethosomes and are found in variable proportions in ethanol [113]. Improving the penetration of drugs into deep skin layers using these NPs was first described by Tuitou *et al.* (2000) [126].

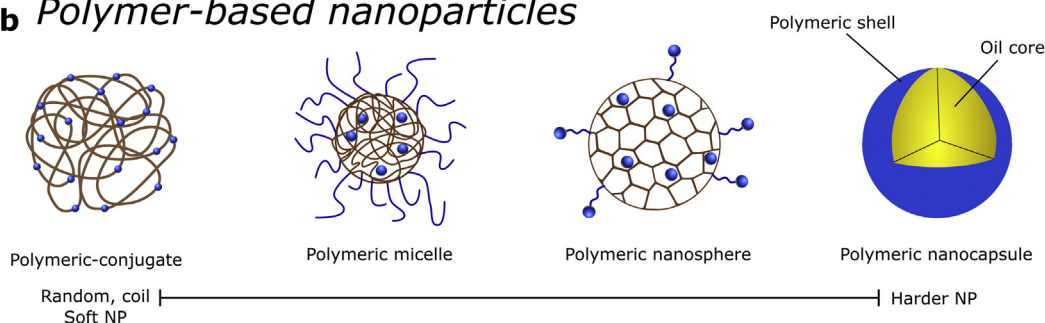
Small-sized carriers in the lipid matrix release drugs based on the influence of the matrix and nanoparticle components [127]. Among these particles are solid lipid NPs, which range from 50 to 1,000 nm and are composed of physiological lipids that can organize themselves and form a dispersion in water or aqueous surfactant solutions [110]. Nanostructured lipid carriers are second-generation lipid NPs tailored with a solid matrix including liquid lipids at a ratio ranging from 70:30 up to 99.9:0.1 [120,128].

Among the lipid-based delivery systems, the liquid crystalline systems also have the capacity to incorporate hydrophilic, lipophilic and amphiphilic molecules as well as to modulate the release of these molecules based on the phase in which NPs are produced. These liquid crystalline NPs are formed from lyotropic liquid crystals that can be classified in mesophases, such as lamellar, hexagonal and cubic phases (11). Each mesophase will generate nanosystems with specific

a Lipid-based nanoparticles



b Polymer-based nanoparticles



c Inorganic nanoparticles

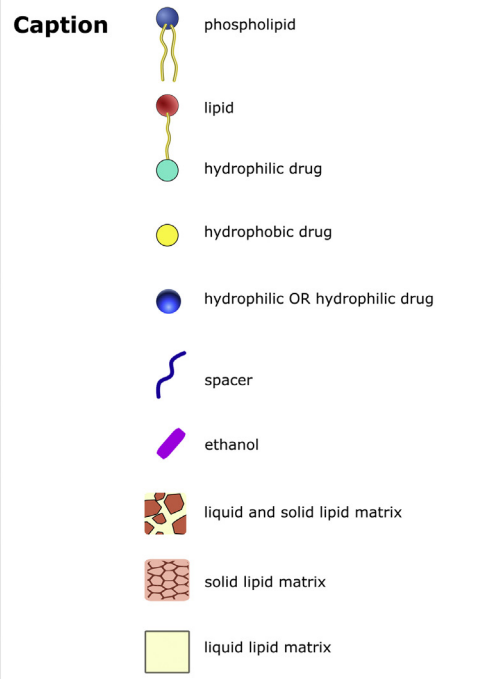
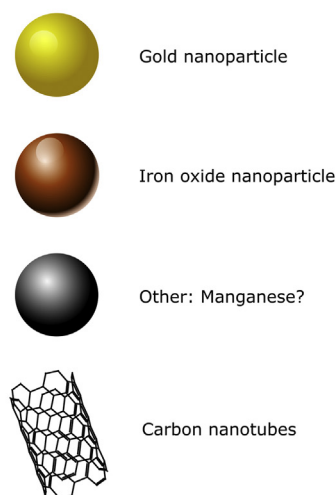


Fig. 3. Main types of nanoparticles used in skin cancer therapy: (a) lipid-based NPs, (b) polymer-based NPs and (c) inorganics.

characteristics, such as i) different patterns of molecule release; ii) different behaviors in the incorporation of molecules; iii) physical stabilities; and iv) degree of tissue penetration [48,129,130].

5.2. Polymer-based NPs

Polymeric NPs have several attractive properties as drug delivery systems, such as ease of manipulation, the potential for

functionalization and surface modification with different ligands, control over behavior *in vivo*, biodegradability and biocompatibility [131,132]. Polymeric NPs are most commonly administered by systemic, intravenous injection, although the use of such nanocarriers with many therapeutic agents that can be delivered across biological membranes is currently in development [132]. Protein NPs provide diverse interactions with drugs and three-dimensional networks to offer several reversible drug molecule assemblies [133].

For example, Pluronic® consists of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) [134]. The PPO unit is a hydrophobic segment that contributes 30% of the copolymer content, and the PEO unit is the hydrophilic segment of the polymer. Pluronic F127® is a copolymer that has gained attention due to its vast therapeutic applications [134]. The hydrophobic core PPO, which creates the environment for lipophilic drug incorporation, is formed from Pluronic F127®. Therefore, the PEO segment prevents adsorption and aggregation of an incorporated protein [134].

Matsumura *et al.* (2004) was the first to develop polymeric nanocarriers, polymeric micelles of PEG-poly(amino acid), which successfully and efficiently accumulated in solid tumors in mice and advanced to clinical trials [132,135,136]. The incorporation of drugs into polymeric micelles can occur by physical, chemical, or electrostatic interactions [137,138]. An example of a polymeric micelle formulation is Genexol-PM (PEG-poly(D,L-lactide)-paclitaxel), a polymeric micelle-formulated paclitaxel [122,138]. In contrast, polymer-drug conjugates are macromolecular agglomerates formed by one or more therapeutic agents, such as small drug molecules, proteins, and peptides, attached by covalent bonds to the polymeric carrier structure [132].

Since proteins are natural polymers with heterogeneous polymer sizes and a range of molecular weights, heterogeneous NP size distributions can exhibit batch-to-batch variations [139]. Suitable raw materials include animal proteins such as gelatin, collagen, albumin, silk proteins and elastin since they have absorbability and their degradation products induce low toxicity compared to synthetic polymers [133]. Moreover, the presence of amino and carboxylic functional groups makes them easy to bind with, which is useful for targeting ligands and surface modifications [122].

Elastin-like polypeptide NPs are also an interesting drug delivery system for several applications [133,140]. These particles are excellent candidates due to their combined amphiphilic behavior, biodegradability, and biocompatibility in a single system. Recently, elastin-like polypeptide NPs were synthesized because of their ability to penetrate deep into tumor tissue after temperature-induced coassembly with CPPs such as octa-arginine [140] and to reversibly self-assemble into micellar structures for photodynamic therapy [141]. Additionally, NPs obtained from plant proteins such as zein and gliadin present a new approach for NP production since they have some advantages [142], although NPs created with them may need chemical modification or physical treatment due to the high hydrophobicity of these proteins.

Other polymer-based NPs, such as polymersomes, which are composed of liposomal membranes from amphiphilic polymers, have also gained increased attention due to the structural variability of such membranes [143]. A hybrid polymeric NP/hydrogel system was developed and evaluated for the permeation of benzocaine *in vitro* and for tracking the NPs in the artificial membrane [144] as a strategy for skin drug delivery. Wakabayashi *et al.* (2018) developed a solid-in-oil (S/O) nanodispersion for transcutaneously delivering hydrophilic molecules, which was an attractive system [145]. Nanoscale hydrogels in the submicrometer range are often called nanogels and are prepared by chemically or physically cross-linking polymers [146]. This polymer network may range in size up to a few hundred nanometers when swollen in water [146] and has been recently been exploited for cancer imaging and drug delivery vehicles [122].

The conjugation of therapeutic agents to polymeric carriers also offers several advantages. The rational design and recently revealed advantages of this strategy is discussed in [132].

5.3. Inorganic NPs

Inorganic nanocarriers have been investigated for imaging and treatment due to their large surface area, improved drug loading capacity with decreased toxic side effects [122,147], high biocompatibility and size-dependent magnetic properties [148]. Gold nanorods have been explored for biomedical applications [149,150] and photothermic

therapy (PTT) due to their high performing photothermal conversion [151]. However, gold nanostructure-based plasmonic hybrids have been developed to have superior performance in PTT [152,153]. Manganese dioxide (MnO₂) NPs have been used due to their high reactivity with endogenous hydrogen peroxide (H₂O₂) within the tumor microenvironment to generate O₂, thus overcoming tumor photodynamic resistance by generating O₂ *in situ* [154,155].

The proliferation rate of cancer cells is high and is also regulated by the cell cycle and DNA replication, and ruthenium-based anticancer drug candidates are used in one strategy to address DNA damage as one of the many metallotherapeutics approved by the US Food and Drug Administration [156,157]. Therefore, the metallopolymer prodrug formed by covalent conjugation of the ruthenium complex to the polymer can be converted into an active drug at a controllable rate [156]. A combination of photodynamic therapy (PDT) and PTT is currently providing promising applications in cancer therapy and is overcoming the inherent limitation of PDT, such that it has achieved attention for malignant tumor treatment, even surpassing conventional treatment technologies such as chemotherapy and radiotherapy, due to its minimally invasive nature, precise application and localized scope of treatment [151].

Carbon nanotubes are synthetic nanomaterials that are well known for the ideal near-infrared PDT potential that gives them the capability to increase the temperature within tumors [158,159]. Water-soluble carbon nanotubes are being investigated for gene and drug delivery due to their nontoxicity, ease in crossing biological barriers and ability to transport molecules to the cytoplasm [160]. Moreover, the association of the dual plasmonic agents in such a hybrid construct demonstrates the enhanced physicochemical properties of both agents and shows outstanding functionality by combining the effects of two different nanostructures [161,162].

In the case of inorganic NPs, their drug delivery potential and imaging capabilities have grown quickly in recent years. These "nanotheranostic" NPs have been broadly used for treatment and diagnosis in breast and liver cancer; however, for skin cancer, few studies have been published [131,163]. Although there are limited studies on skin cancer, the published works showed interesting theranostic NPs for melanoma treatment and detection, which justifies more research in this area. For instance, gold NPs functionalized with anti-nucleolin aptamer (AS1411) and iron oxide NPs functionalized with IR80 have proven to be efficient magnetic resonance imaging probes that induce selective cytotoxicity in A375 melanoma cells after irradiation with an NIR laser [164,165]. In another study, cobalt ferrite oxide (Co_{0.5}Fe_{2.5}O₄) nanotubes loaded in nanoemulsions showed intense time-dependent accumulation in a murine melanoma xenograft model, providing MRI imaging potential. Recently, a new approach to tumor monitoring and therapy using photoacoustic and ultrasonic tools was investigated by Li *et al.* (2018). They developed a multifunctional polymeric NP loaded with gold nanorods (Au-NRs) and liquid perfluorocarbon that was conjugated with a monoclonal antibody (MAGE-1 antibody). They observed that the theranostic NPs specifically targeted melanoma tumor cells with high persistence at the site *in vivo* [166]. In another study, elastin-like polypeptide (ELP)-conjugated gold NPs exhibited photothermal properties. Additionally, a single intratumoral injection of ELP-gold NPs provided simultaneous photothermal/photoacoustic/X-ray computed tomographic imaging and PTT of the melanoma [167].

5.4. Other NPs

Therapeutic agents conjugation to polymeric carriers also offers several advantages. Polymer-drug conjugate therapeutics are constructs that comprise one or more therapeutic agent, and among them the first market approved polymer-protein conjugate, Adagen [132]. Ekladius *et al.* (2019) discuss the advances in different classes of polymer-drug conjugates, such as polymer-protein and polymer-

small-molecule drug conjugates, dendrimers and polymer nanoparticles [132]. Moreover, electrostatic interactions of cationic polymers with negatively charged nucleic acids such as plasmid DNA or small interfering RNA (siRNA) led to the so-called polyplexes [168–170]. Since cationic lipids also can provide negative charges, these structures are used for complexation of genetic material. The structure of these so-called lipoplexes depend on cationic lipid composition, helper lipids and the DNA or RNA [171,172]. Lipopolyplexes are constructs that combine cationic liposomes, cationic polymers and the RNA or DNA via non-covalent interactions [173–175].

6. Treatment of skin cancer by NP drug delivery

In this section, different nanocarriers (presented in Section 5) used for the delivery of chemotherapeutics, immunotherapeutic and vaccine molecules and nucleic acids will be discussed.

6.1. Chemotherapies

Chemotherapy with drugs such as dacarbazine, temozolomide, nitrosoureas, vinca alkaloids, taxanes and cisplatin has been used for cases of advanced MSC [176–178]. 5-FU is an actively and widely used drug for the treatment of skin cancers, such as actinic keratosis and basal cell carcinomas [179]. However, 5-FU is highly hydrophilic, which limits it to ability to reach tumor tissues though the SC [180].

Dacarbazine has a short half-life and is a poorly soluble active drug; it is used as a single-agent FDA-approved anticancer drug and is the drug of choice for use in chemotherapy against MSC [112,181]. This drug has been encapsulated in lipid NPs for topical delivery in MSC treatment [112]. More complex NPs were developed by Liu *et al.* (2017), who rationally designed a nanocarrier based on hollow mesoporous silica NPs enveloped with folic acid-grafted liposomes [182].

Carboplatin, a second-generation platinum compound also recommended by the FDA for the treatment of melanoma, was loaded into poly(ϵ -caprolactone) NPs with a chitosan- β -glycerophosphate gel for intratumoral administration [47]. Additionally, an antitumor effect *in vitro* and in a xenograft tumor model *in vivo* were evaluated by Su *et al.* (2017), who produced paclitaxel-loaded copolymer NPs [183]. The antitumor doxorubicin was encapsulated into polymeric NPs, and this, via self-assembly induced with polyphosphazenes, generated a pH-responsive amphiphilic polymer [184].

Solid lipid NPs were used as a delivery system for temozolomide, which was preliminarily investigated for MSC treatment [185]. In the studies of Jian *et al.* (2017), a temozolomide-loaded polyamide-amine dendrimer in a PAMAM delivery system was explored for use in targeting human melanoma cells *in vitro* [59].

6.2. Immunotherapy and therapeutic vaccines

The immune system is of paramount importance in cancer development. In a successful antitumor response, tumor-associated antigens (TAAs) are presented by antigen-presenting cells (APCs) to T cells, such as dendritic cells (DCs), either at tumor sites or in draining lymph nodes (LNs). The activation of T cells occurs when their receptors engage peptides presented by the APCs in major histocompatibility complex (MHC) molecules. Later, these effector cells travel across the tumor vasculature, penetrating the tumor tissue. Inside the tumor, these tumor-infiltrating lymphocytes (TILs) recognize TAAs on tumor cells to kill them, releasing more antigens and thereby enhancing anti-tumor activity [186,187]. However, cancer cells are able to avoid or block immune surveillance by down regulating MHC I molecules, over-expressing the ligands that inhibit T cell detection, or releasing immunosuppressive molecules that limit the function and proliferation of effector T cells and permit the recruitment of immunosuppressive cells, such as regulatory T cells (Tregs), to the tumor site and allow them to proliferate [188,189]. In skin cancer, the immunosuppression

of T cell activity has been linked to an increased risk of SCC and BCC [190,191], Merkel cell carcinoma (MCC) [192], Kaposi sarcoma (KS) and MSC [193], demonstrating the importance of normal cutaneous immunity in eliminating nascent skin tumors. For a deeper understanding of the basis of oncoimmunology, we refer readers to a review [187].

In recent years, research in the field of immunotherapy has resulted in a growing number of clinical trials and the approval of several immunotherapeutic drugs [194,195]. Moreover, the Nobel Prize in Physiology and Medicine of 2018 was awarded to James Allison [196] and Tasuku Honjo [197] for their discovery of the CTLA-4 (cytotoxic T lymphocyte-associated antigen 4) and programmed cell death 1 (PD-1) immune checkpoints [198].

Immune checkpoints are inhibitors in signaling pathways that regulate the immune system to maintain central and peripheral tolerance and control systemic inflammatory responses in the body [199]. Immune checkpoint inhibitors are monoclonal antibodies directed against immune checkpoint proteins expressed on the surface of neoplastic cells. The CTLA-4 receptor is expressed on T cells and downregulates its activation at the early stages of the immune response by competing with CD28 to bind CD80 and CD86 on APCs [200,201]. Moreover, it upregulates Treg activity, which also diminishes the immune response. Ipilimumab, a human IgG1 monoclonal antibody against CTLA-4, improved the survival of patients with advanced malignant melanoma and was approved by the FDA in 2011 [202,203].

Clinical benefits have also been observed for the PD-1 receptor and/or its ligands, such as PD-L1. PD-1 is expressed on many activated immune cells, and PD-L1 is found in many tissues to limit autoimmunity. However, tumor cells that have been induced to overexpress PD-L1 by proinflammatory cytokines inactivate infiltrating T cells after PD-L1 binds to PD-1 [204,205]. Thus far, five FDA-approved drugs are available for the treatment of several types of cancers through systemic administration, including pembrolizumab and nivolumab (anti-PD-1) for melanoma and avelumab (anti-PD-L1) for Merkel cell carcinoma [206], with response rates as high as 50% [207]. Clinical trials with SCC patients have shown promising results [208].

Despite the promising results, systemically administered checkpoint inhibitors present several limitations, such as resistance and low durable response rates [209,210], severe side effects in numerous organs [211], and no response in many treated patients [206,212]. Moreover, different tumor microenvironments have different mechanisms of immunosuppression [213]. Recent studies have explored other immune checkpoints and are reviewed elsewhere [199].

The first immune therapy strategy for the treatment of MSC was the use of cytokines, which resulted in increased overall patient survival. This strategy is aimed at directly promoting the growth and activity of immune cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukins and interferons are the three most essential agents. In 1998, the FDA approved the use of interleukin-2 (aldesleukin), a protein that triggers the proliferation of T, B, and NK cells [214]. Interferon- α (Intron A) was approved in 1996 as an adjuvant treatment for use against malignant MSC after surgical resection. Interferon- α promotes antitumor immunity by inducing the maturation of immune cells and increasing TAA expression on tumor cells [206]. Despite their long-term use in the clinic, the short half-life of cytokines in circulation results in the need for high-dose administration, which causes numerous unwanted side effects, such as potentially fatal capillary leakage and cytokine release syndrome [206,214]. To address these drawbacks, versions of these cytokines that circulate for long periods have been investigated. PEGylated versions of IFN (peginterferon-alfa-2b) [215,216] and IL-2 were investigated but showed no clinical benefits [195,196]. Moreover, the continual administration of either version of cytokine has been associated with the development of neutralizing antibodies for both the native protein and its therapeutic counterpart [186].

Another strategy is adoptive T cell therapy (ACT), which involves expansion and cellular engineering of patient-derived lymphocytes *ex vivo*

and the reinfusion of them back into patients. The three main types of ACT currently in development involve TILs, chimeric antigen receptor (CAR) T cells and T cell receptor (TCR) T cells. Among these, TILs have shown promising results in the control of metastatic melanoma [217–219]. The development of TILs consists of the extraction of suppressed lymphocytes that have recognized and infiltrated the tumor and expansion of them *ex vivo* with the use of cytokines that reactivate these cells. However, one major hurdle of this approach is the high cost and the need for skilled labor. TCR and CAR T cells are developed by genetically engineering T cells by introducing TCR or CAR proteins to confer specificity for tumors [220]. Despite the sustained responses in the control of metastatic melanoma, these TCR-modified cells have induced off-tumor toxicity [221]. CAR T cell technology shows an important advantage over TCR-based methods and their limitations, as there is no need for MHC identification or costimulation by interleukins [220]. However, the therapeutic success of CAR T cell technology is restricted to hematological cancers [220,222]. However, its effect on solid tumors needs to be validated (reviewed elsewhere [200]).

Cancer vaccines are designed to be potential mechanisms that generate a long-lasting response to cancer development by stimulating immune memory of TAAs. These TAAs may be overexpressed tumor-self-antigens or neoantigens generated by a tumor-specific mutation, but they are not present in healthy tissues [186,223]. Cancer vaccination with nucleic acids can be DNA-based or RNA-based. For this strategy to be effective, the mRNA or DNA must be taken up by the APCs and translated to induce antigen expression. Although nucleic acid vaccination has shown interesting results in melanoma animal models, clinical trials showed no significant clinical outcomes, often because of barriers to nuclear delivery and immunogenicity [224]. Vaccination with TAA peptides is another exciting strategy for antitumor treatment. However, the first trials investigated the infusion of free peptides with poor pharmacokinetic profiles, which contributed to a response rate of less than 3% in clinical trials between 1995 and 2004 [186,225]. Recently, patients were treated with two different approaches for personalized vaccines for melanoma: RNA injection into lymph nodes [226] and subcutaneous administration of synthesized peptides [227], with early phase trials showing promising results. However, delivery of antigens to DCs in the absence of concomitant inflammatory stimuli induced immunologic tolerance rather than immunity. This finding indicates that the use of antigens alone weakly induces adaptive immunity [228].

The increasing knowledge of cancer immunology has led to the combination of vaccines with other agents of interest [228]. A phase III trial with 185 patients with stage III or IV melanoma used a melanoma peptide antigen vaccine (gp100) combined with IL-2 and showed a response rate of 16%, compared to 6% with IL-2 treatment alone, and a progression-free survival of approximately 2.2 months, compared to 1.6 months with IL-2 treatment alone. However, the patients experienced toxic effects consistent with high-dose IL-2 administration [229]. Nevertheless, the coadministration of free antigens and adjuvants can lead to differential delivery to different DCs, thereby dampening the immune stimulation.

Despite continuous advances in the field of immunotherapy, clinical applications still encounter a variety of challenges. As discussed above, these setbacks involve the development of resistance or a lack of response to treatments, toxic effects related to therapies such as cytokines or CART cell infusion, and the limited efficacy of cancer vaccines [230–233]. Nanotechnology has been widely used to address similar limitations in several medical fields.

In cancer immunotherapy, NPs can be directed to different cells and tissues related to the immune response, such as APCs, lymphocytes and lymphoid tissues [234], thereby overcoming the limitations of direct cancer cell targeting [235]. Indeed, the use of nanoparticles associated with T cells or natural killer cells has promoted a higher concentration of drugs to be administered in tumor environments than the concentrations when NPs are used alone [236–239]. Exploiting this strategy, Schmid *et al.* (2017) used the FDA-approved polymers PLGA and PEG

to synthesize NPs with antibody fragments conjugated to their surfaces to target PD-1-expressing T cells in the circulation and tumors. The delivery of a TGF β signaling inhibitor to cells expressing PD-1 prolonged the survival of tumor-bearing mice, whereas the free drugs had no effect [240]. To safely deliver a nucleic acid, Li *et al.* (2016) developed 100 nm polymersomes carrying siRNA against CTLA-4 and demonstrated their ability to enter T cells both *in vitro* and *in vivo*. In mice bearing B16 melanoma, the researchers demonstrated NP uptake by TILs at tumor sites after intravenous administration [241]. Researchers have also demonstrated the combination of NPs with microneedles in MSC treatments [242,243]. NPs were formed by covalent ligation of the immunosuppressor indoleamine 2,3-dioxygenase (IDO) to hyaluronic acid to form an amphiphilic structure that self-assembled to encapsulate anti-PD-1. After transcutaneous delivery in a B16F10 mouse melanoma model, tumor growth was significantly delayed, and 70% of the treated group survived, compared to no survival in the untreated mice [243].

NPs can also be used to facilitate the combination of immunotherapies. Effective activation of immune cells often involves multiple signaling pathways [187]. The administration of free immunomodulators usually leads to only a few immune cell encounters and thus does not provide the desired costimulatory effect. Park *et al.* (2012) designed biodegradable core-shell nanoparticles that combined the features of both solid polymers and liposomal systems for a simultaneously sustained release of a hydrophobic drug (β -cyclodextrin) and a hydrophilic cytokine (IL-2). β -Cyclodextrin is an inhibitor of TGF β , a primary negative regulatory signal produced in tumors. After systemic administration of these NPs, tumor growth was significantly delayed, and the survival of tumor-bearing mice was enhanced. Additionally, the activity of natural killer cells and intratumoral-activated CD8⁺ T cell infiltration were increased in both subcutaneous and metastatic melanoma tumor models [244].

The delivery of NPs can also be directed to the tumor microenvironment. Kwong *et al.* (2013) anchored an engineered IL-2Fc fusion protein and anti-CD137 (a costimulatory receptor upregulated on activated T cells) to the surface of PEGylated liposomes. The intratumoral injection of these particles into the B16F10 melanoma model enabled localized stimulation of T cells without inducing systemic toxicity because the size of the liposomes permitted dissemination through the tumor-draining lymph nodes and tumor parenchyma but not into the systemic circulation. This therapy using PEGylated liposomes cured most primary tumors, with the advantage of avoiding the lethal inflammatory toxicities induced by equivalent intratumorally administered doses of soluble agents [245].

Nanotechnology has also been used to address barriers to adoptive immunotherapy. Major limitations include the need for a rapid increase of antigen-specific T cells in culture and the decreased viability of the transplanted cells that requires coadministration of adjuvants to maximize the performance of the cells.

Some approaches have considered the design of artificial APCs (aAPCs), where proteins necessary for T cell activation are conjugated to the particle surface [246]. Perica *et al.* (2014) designed iron-dextran NPs functionalized with anti-CD28 antibodies and MHC-Ig dimers as aAPCs that bind to TCRs. The use of a magnetic field enhanced the TCR clustering at the T cell surface and reduced the threshold of activation for T cells *ex vivo*, demonstrating that NP binding and cellular activation are influenced by spatial organization at the membrane. Later, mice that had been treated with T cells activated by nano-aAPCs had 8-to-10-fold inhibited growth of B16 melanoma lesions compared to untreated mice and to those in which the T cells were not subjected to the magnetic field [247].

In a different approach to enhance ACT, T cell surfaces are functionalized with NPs to influence their function *in vivo*. Stephan *et al.* (2010) manufactured multilamellar lipid NPs encapsulating cytokines and conjugated them *ex vivo* to free thiol groups located on the surface of T cells. This strategy provided pseudoautocrine stimulation to the donor cells,

resulting in an 80-fold increase in T cell expansion *in vivo* and in prolonged survival of a B16F10 tumor mouse model with low doses of adjuvant drugs administered subcutaneously; however, the treatment was unsuccessful when systemic administration was used [217].

Nucleic acid vaccines have been gaining importance as promising alternatives to conventional approaches. However, the DNA vaccines tested in several clinical trials usually fail as a consequence of nuclear delivery barriers and immunogenicity [248]. Although mRNA vaccines are advantageous because they do not integrate into the genome and can be delivered to the cytoplasm, they are quickly degraded by nucleases and are not easily internalized by cells [249]. In this context, the use of NPs can be beneficial [250,251]. A recent phase I trial was conducted in patients treated with an mRNA that expressed four different TAAs in complex with cationic liposomes. The liposomes protected the RNA from extracellular degradation and mediated its efficient uptake by the DC populations in various lymphoid compartments. Three treated patients demonstrated strong immune responses against self-antigens and showed either regression of metastatic lesions or disease stabilization [250]. Another critical study demonstrated the possible usefulness of subcutaneously delivered ionizable liposomes containing mRNAs encoding the TAAs gp100 and TRP2. This strategy led to tumor shrinkage and increased survival in treated melanoma-bearing mice [229]. Other RNA strategies include the use of short hairpin RNAs (shRNAs) and small interfering RNAs (siRNA) [252–255].

An important advantage of using NPs for antigen delivery is their capability to carry several adjuvants or neoantigens, which is ideal for treating different cancers [256]. To avoid inducing immunotolerance, vaccine antigens are administered with an immune adjuvant, such as a toll-like receptor ligand (e.g., cytosine-phosphate-guanine oligodeoxynucleotides (CpGs)) or cytokine (e.g., IL-2 or GM-CSF). Kuai *et al.* (2017) manufactured high-density lipoprotein nanodisks coupled with antigen peptides identified via DNA sequencing of the tumor exome and CpGs as an adjuvant. Strikingly, the NPs elicited up to 47-fold higher frequencies of neoantigen-specific cytotoxic T lymphocytes than free antigens plus CpGs. The combination of vaccination with immune checkpoint inhibitors amplified the therapeutic efficacy and eradicated B16F10 tumors in >90% of the animal models, compared to approximately 38% eradication in the control animals treated with free antigens plus CpGs [257]. More than being useful for codelivering adjuvants, certain materials can transform the NP itself into an adjuvant [258]. PC7A, an ultra-pH-sensitive copolymer, has been shown to induce a strong cytotoxic T lymphocyte response [259]. The derived neoantigen nanovaccine showed a greater ability to stimulate immunity than poly (I:C), a synthetic ligand of toll-like receptor 3, or CpGs in a B16 melanoma mouse model and resulted in 100% survival after 60 days when administered in association with anti-PD-1 [259].

The studies discussed here have demonstrated the potential use of NPs in promoting more robust antitumor effects in cancer immunotherapy in comparison to the administration of free drug. Although the field of cancer immunotherapy has advanced rapidly, the use of nanotechnology is still in its initial stages and represents a wide and exciting field for further consideration and investigation.

6.3. Gene therapy

Since the gene therapy approach was proposed in 1966, the use of nucleic acids to restore or replace a defective or missing gene has been extensively studied due to the benefit of these strategies [260–263]. Disease treatment by gene therapy can be implemented for three different classes of genetic material and enables the knockin or knockdown of a targeted gene depending on the genetic material transfected: plasmid DNA (pDNA), RNA interference (RNAi) molecules or antisense oligonucleotides (AONs) [260].

pDNA is widely employed to fix a nonfunctional gene or to re-express an endogenous gene aiming to treat a genetic disorder; they are used to permanently change gene expression [260]. For instance,

for the treatment of MSC, Heller's group developed a pDNA that encoded interleukin-12 (IL-12) [264,265]. A pDNA is a double-stranded DNA with a size of hundreds to thousands of bases organized in a round shape. pDNA vectors consist of several components, such as a transgene, an enhancer, a promoter, transcription terminal signals, splicing and polyadenylation sites, and antibiotic resistance genes. The transgene is replicated independently of the chromosomal DNA of the host and is divided into each cell that results from cell division. After the vectors are internalized into the cytoplasm, they are imported into the nucleus, and due to the promoter sequence, the specifically encoded mRNA is transcribed. Following transcription, posttranscriptional modifications occur on the mRNA, and then, it is exported into the cytoplasm, where the mRNA is translated into protein [260].

The mechanism of action of RNAi was established in 1998 when Fire, Mello, and colleagues showed that the administration of double-stranded RNA (dsRNA) promoted the silencing of cytoplasmic messenger RNA (mRNA) that contains a complementary sequence [266]. From that point of discovery, RNAi has been widely studied, and dsRNA effector molecules, usually small interfering RNAs (siRNA), are used as tools in the research of gene function [267,268] and for the identification of potential disease-causing genes [269], offering a promising approach for therapy [270–272]. The RNAi process can be split into two phases: the initiation phase and the effector phase. In the first phase, the effector molecules (siRNAs and microRNAs (miRNAs)) are created. The effector phase comprises the real RNAi mechanism, in which the double-stranded siRNAs and miRNAs are linked with cellular proteins to generate an RNA-induced silencing complex (RISC). In the course of RISC assembly, one strand (the passenger) is eliminated, while the other strand (the guide) forms an active RISC. The single-stranded (antisense) siRNA or miRNA of the activated RISC guides it to the complementary target mRNA. The mRNA is cleaved by the protein Argonaute 2 [270,273,274]. This native gene silencing pathway can be used for the downregulation of chosen genes.

AONs are short single-stranded DNA molecules (15–25 nucleotides) that are complementary to the RNA strand and can specifically inhibit a target mRNA. An AON hybridizes premRNA or mRNA, becoming the substrate for the endogenous ribonuclease H (RNase H), which is an endonuclease that recognizes the mRNA oligo complex and cleaves the RNA strand, leaving the AON intact, which can then bind to other target RNA molecules [275]. For example, AONs targeting the c-myc gene retarded the growth rate of melanoma cells [276].

Studies of the molecular mechanism have shown that cancer occurs via the inactivation of cancer-suppressing genes or the activation of oncogenes, which results in a malignant tumor. Targeting cancer via gene therapy can eliminate both problems by (a) inserting a gene that leads to apoptosis or that increases tumor sensitivity to radiation/drug treatments; (b) expressing a tumor suppressor gene to replace the loss/downregulation; (c) using an antisense (RNA/DNA) strategy to stop the expression of an oncogene; or (d) increasing the immunogenicity of the tumor to trigger immune cell recognition [263,277].

The administration of genetic cargo to the skin can be performed using strategies *ex vivo* and *in vivo*. The strategy *ex vivo* comprises taking human cells, fixing them via gene delivery and regrafting these fixed cells into the patient. In the *in vivo* approach, the genetic material is transferred directly to the skin. Although scientists are still struggling to surpass the challenges of delivering genetic material directly to the skin, the use of the *ex vivo* strategy is not feasible due to the costs, pain and time associated with its clinical application [260].

For transdermal gene delivery, the major hurdle is the SC because of its structure, as discussed in Section 3.1. In addition to the SC barrier, epidermal layers are also obstacles to topically applied genetic material. After overcoming skin barriers, genetic material must overcome the challenges inherent to its own physicochemical properties to reach targeted cells and promote therapeutic activity [260]. Due to their high molecular weight, hydrophilic nature, and negative charge, nucleic acids have low membrane permeability, which results in low

transfection efficiency [272,278]. Studies have shown that the transfection efficiency is entirely different between fibroblasts, keratinocytes, dendritic cells, and macrophages. Another challenge to gene therapy is neutralization and destruction of the genetic material provoked by immune response activation [260].

To overcome these barriers, different approaches have been studied that enhance gene delivery into and through the skin and deliver genetic cargo to desired cells. Nonviral NPs (polymers, lipid-based carriers, cell-penetrating peptides, dendrimers, and gold NPs) have been developed to improve macromolecule penetration through the SC barrier and to induce the intracellular delivery of nucleic acids [279–281]. For example, carbon nanotubes have been demonstrated to enhance transdermal drug delivery. Based on this process, Siu *et al.* (2014) developed a single-walled carbon nanotube for local delivery of siRNA. Treatment of MSCs with a version of this system containing siRNAs specific to BRAF (BRAFV-RAF murine sarcoma viral oncogene homolog B, an important gene in the MAPK pathway) decreased tumor growth over 25 days [279]. Spherical NPs conjugated to nucleic acid have gold cores coated by a dense shell of siRNA, which are highly oriented, immobilized and covalently bound. Within hours after application, these NPs spontaneously penetrate almost 100% of the keratinocytes *in vitro*, in mouse skin, and in the human epidermis [280]. A cell-penetrating peptide and cationic poly(ethyleneimine)-conjugated gold NP (AuPT) successfully enabled penetration of pDNA through the intact SC. This NP was generated by compacting the pDNA into cationic nanocomplexes and was highly efficient in the transdermal delivery of pDNA without any required enhancement. The efficient transdermal delivery and transfection of the pDNA encoding the miRNA-221 inhibitor gene (Mi221) by AuPTs in melanoma cells and melanoma xenografts in mice show that these nanocomplexes provide an excellent approach for skin cancer gene therapy seeking to inhibit both the metastasis and progression of advanced MSC [281].

NPs have also been developed to improve genetic material delivery and transfection by local application [48,282,283]. For instance, siRNA in complex with liposomes and administered intratumorally promoted effective gene silencing, making it an interesting therapy for the treatment of MSC [282]. Nanocomplexes formed with PEI and siRNA were also used for delivering siRNA through intratumoral injection. Intratumoral injection of PEI-siRNA complexes has also been used for the inhibition of STAT3 in cancer. In this study, a linear low-molecular-weight PEI was modified by N-acylation with lipidic side chains. Stearoyl modified PEI (PEI-StA) resulted in an enhanced promotion of STAT3 silencing in B16 cells and a reduction in VEGF production in comparison with the unmodified PEI [283]. *In situ* solidified organogels based on monoglycerides, propylene glycol, tris buffer and polyethylenimine were developed for siRNA delivery. The addition of PEI was crucial for the action of the siRNA. The siRNAs were released complexed with PEI and in a controlled manner [284]. This system, which was able to complex high amounts of siRNA, protected the siRNA from degradation, promoted its internalization, and induced gene knockdown *in vitro* in tumor cell lines. Moreover, in a murine xenograft model of an epidermoid carcinoma, an *in situ* gel was formed after intratumoral injection. Therefore, this *in situ* solidified organogel is an interesting strategy to intratumorally deliver siRNA [48].

Although there are promising studies of nonviral carriers for gene therapy to treat skin cancers, there are few examples that show their use alone. Physical methods (described in Section 5.4) have been used to increase the penetration of genes into the skin by the disruption of the SC, increasing the drug penetration in underlying layers [264,285–287]. Many studies have combined the use of NPs and physical methods for gene delivery. For instance, low-frequency ultrasound was used to permeabilize the skin and facilitate liposome permeation. When liposomes composed of DOTAP, DOPE, and DSPE-PEG2000 in a molar ratio of 4.75:4.75:0.5 and siRNA-V600EB-Raf and siRNA-Akt3 at a weight ratio of 10:1 were topically applied after ultrasonic treatment, siRNA delivery into melanoma cells was enhanced. This approach

reduced the expression of the protein targeted by the siRNA sequence, which suppressed melanoma development [285]. Gold NPs (AuNPs) coated layer-by-layer with chitosan (LbL-AuNP) were designed as carriers for the iontophoretic delivery of STAT3 siRNA to treat MSC. The STAT3 siRNA-loaded LbL-AuNPs, through an apoptosis mechanism, decreased cancer cell viability, and the iontophoretic application enhanced the skin penetration of the siRNA-loaded LbL-AuNPs such that they reached the viable epidermis [286]. ApDNA encoding IL-12 has also been administered through physical methods such as a gene gun [287] or electroporation [264] in MSC treatment.

6.4. Nanocarriers combined with physical methods

Physical techniques that enhance topical and transdermal drug delivery have been applied in the clinic since the 1980s and have emerged as potential approaches for the treatment of skin cancers. Modalities based on electrical, mechanical, or material structures and/or velocity are used in combination with or associated with different types of NPs to optimize percutaneous drug distribution or activation through different mechanisms [288,289]. Thus, physical enhancement methods (Fig. 4), especially laser irradiation (phototherapy, thermotherapy and acoustic therapy), iontophoresis, ultrasound, and microneedles, have been the focus of investigations into the treatment of MSC or NMSC cases (Table 1).

6.4.1. Laser irradiation therapy

Photodynamic therapy (PDT) has been a therapeutic approach extensively explored throughout the scientific community and in the medical clinic among other therapies based on laser irradiation sources. This therapeutic approach takes advantage of the combination of a photosensitizer (PS) and a specific light wavelength that can induce the production of highly reactive hydroxyl and singlet oxygen molecules, which causes a series of biological, chemical and physiological reactions and consequent cell death [290–292]. This review shows the main advantages of using NPs as platforms for specifically targeted delivery of PSs to the tumor region. Lipid-based or polymer-based NPs loaded with different PSs have also been the subject of research and have proven to be a potential strategy for improving the penetration and activity of these molecules in skin cancers [130,293–296].

Liquid crystalline nanodispersions (LCNs) capable of incorporating different PSs [297] improved the skin penetration of protoporphyrin IX (PpIX) according to *in vitro* and *in vivo* assays, compared to the PpIX skin penetration seen with control formulations [130]. Encapsulation of chlorin-e6 or meso-tetraphenylporphine-Mn(III) chloride, a third-generation PS in LCNs, resulted in efficient internalization of these PSs into malignant melanoma cells and exhibited significant photodynamic effects after irradiation (10 J/cm²; 570 nm and 630 nm filters) [296].

An SLN-loaded aluminum chloride phthalocyanine presented a 3.2-fold reduction in the cell viability of the B16F10 cell line after irradiation (0.5, 1.0, and 2.0 J/cm²; 670 nm filter) [293]. Another lipid-based NP, a transthesosome loaded with ferrous chlorophyllin (Fe-CHL), induced a gradual decrease in tumor size in a murine model of melanoma, leading to complete regression within 1 month after irradiation (720 J/cm²; 650 nm filter) with or without a kojic acid gel [294].

Fe-CHL-loaded polymeric NPs and polymeric-lipid hybrid NPs conjugated with the cycloRGDyK peptide induced phototoxicity in melanoma cells (B16F10 cell line) that were irradiated (26 J/cm²; 652 nm filter). The results indicated that the selected ligand, cycloRGDyK, possessed a dual and simultaneously acting role as a disease-specific ligand and an ¹O₂ quencher, making this nanoplateform a promising targeted carrier for PDT [295].

Photothermal therapy (PTT), which generates heat in response to applied laser light, has been well documented as a strategy for highly selective cancer treatment and is mainly used with NPs that are embedded within tumors. Hyperthermic effects can be directly achieved by

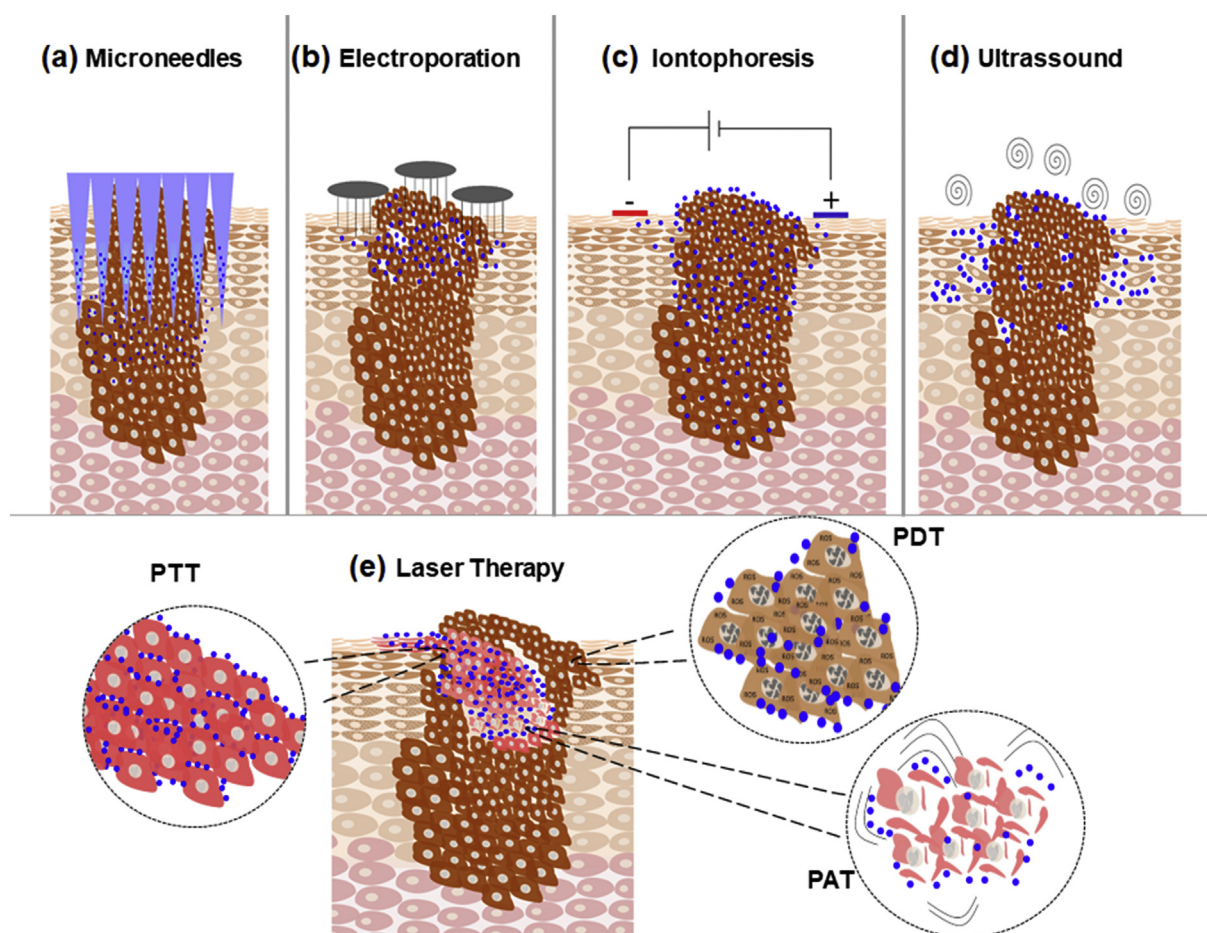


Fig. 4. Physical methods associated with the use of NPs to improve the delivery of therapeutic agents for the treatment of skin cancer: Microneedles, Electroporation, Iontophoresis, Ultrasound and Laser therapy based in PTT (Photothermal therapy), PDT (Photodynamic therapy) and PAT (Photoacoustic therapy).

near infrared (NIR) irradiation that is directed to the tissues and is associated with the different NPs to induce an effective increase in the anti-tumor effects. Then, the stimulation of these NPs by laser irradiation leads to localized heat in the range of 40°C–45°C that causes cancer cell death via apoptosis and necrosis. Another effect of PTT is an increase in the vascular permeability of the tumor, which can facilitate anticancer drug delivery into the tumor [298,299].

In the field of skin cancer therapy, the photothermal properties of different plasmonic NPs have been extensively investigated. Rahimi-Moghaddam *et al.* (2018) found that conjugates of PEG-curcumin-gold NPs potentiated the cytotoxic effects of curcumin in murine melanoma cells after application of low-power NIR, and a considerable reduction in tumor volume was observed *in vivo* after 48 h, with no impact on the physical health of the animal [300]. In the studies of Singh *et al.* (2018), a gold NP coating enabled liposomes to specifically absorb NIR light. Destabilization of the liposomal core occurs via the heat generated by the conversion of NIR light and thus increases the release of encapsulated curcumin. The cytotoxic effects in murine melanoma cells increased more than 80% after laser irradiation for 5 min and caused irreversible cell damage. The addition of curcumin showed improved cytotoxicity in cancer cells [301].

Similarly, cantharidin-loaded liposomes coated with gold NPs showed disruption and release of the contents after exposure to NIR irradiation. Significant differences in the cytotoxic effects were achieved *in vitro* when different irradiation intensities were combined with liposome applications. Additionally, cantharidin improved the efficiency of photothermal therapy by suppressing HSP70 and BAG3 expression and attenuating the antiapoptotic effects on tumor cells [302].

Recently, immunotherapy and plasmonic NPs were combined as an effective therapeutic strategy against tumor growth and for the inhibition of metastases. When irradiated (1.0 W/cm²; 1,064 nm), immunoadjuvant imiquimod (R837) loaded in cetyltrimethylammonium bromide-coated gold nanorods decorated with polyethylene glycol and bovine serum albumin inhibited tumor growth and promoted immune responses against the tumor (increased IL-12, IL-6 and TNF- α). Additionally, treatment with the nanocomplex prevented lung metastasis and tumor recurrence [303]. Chen *et al.* (2018) also used this therapeutic approach for synthesizing aluminum oxide (Al₂O₃) NPs coated with polydopamine. The Al₂O₃ within the NPs, together with CpGs, acted as an adjuvant to trigger cell-mediated immune responses that could eliminate residual tumor cells and reduce the risk of tumor recurrence. The combination of the NPs with laser irradiation (1.18 W/cm²; 808 nm) efficiently killed the cancer cells within 5 min *in vitro*. In trials *in vivo*, the survival was 3.9-to-6.3-fold longer than that in other groups for the animal group that received intratumorally NPs. These results suggest that a combination that inhibits both tumor recurrence and metastasis is a potential therapy for skin cancer [304].

Photoacoustic therapy (PAT) is used to destruct target cancer cells. It is based on the application of an enhanced photoacoustic shockwave using a low-energy laser that damages the target cells by heating but preserves the healthy cells [305,306]. Zang *et al.* (2016) reported a significant antitumor synergistic effect with the application of gold nanorods containing three functional components (gold nanoparticle with photoacoustic effect and used as drug delivery platform, DNA to load doxorubicin (DOX) and, folate functionalized). Treatment resulted in complete healing of tumor lesions and minimal systemic toxicity *in vivo* [307].

Table 1
Summary of main nanocarriers combined with a physical method for skin cancer therapy.

Physical method	Nanocarrier	Anti-cancer agent	Main results	Ref.
Laser irradiation – PDT	Mesoporous silica NPs	Vertoporphyrin	NP treatment promoted a 50 to 70% reduction in cell proliferation after 120 s and 180 s of red-light irradiation.	[290]
	PEGylated gold NPs	Mitoxantrone	LED exposure alone did not cause significant cancer cell death. On the other hand, the maximum PDT efficacy from NP was observed at a radiant exposure of 1.3 J/cm ² and drug concentration of 6 μM	[291]
	Cubic liquid-crystalline nanodispersions	Chlorin-e6 or meso-tetraphenylporphine-Mn (III) chloride	NP containing photosensitizer demonstrated excellent photodynamic properties and similar to free drug at a concentration one order of magnitude larger.	[292]
	Solid lipid NP	Aluminum chloride phthalocyanine	A two-fold increase in laser light radiation at 1.0 J/cm ² promoted greater phototoxicity of NPs, however did not alter the viability of cells exposed to the free photosensitizer.	[293]
	Transethosomes	Ferrous chlorophyllin	Mice having larger tumor volume (252.6 mm ³) that were treated in vivo by PDT, showed in a period of 7 days significant reduction in tumor volume followed by complete tumor regression within 2 months of observation in more than 50% of the mice treated	[294]
	Polymeric NPs and polymeric-lipid hybrid NPs	Ferrous chlorophyllin and cicloRGDyk peptide	Polymeric-lipid hybrid NP produced greater PDT cell death after 1 and 3 h of treatment, while this effect was achieved with polymeric NP after 24 h. Moreover, PDT-mediated cytotoxicity of melanoma cells results demonstrated a time-dependent effects.	[295]
	Polymersomes	Protoporphyrin IX	PDT treatment after 1, 3, and 5 days showed that polymersomes had a sustained anti-melanoma cell effect with significant tumor cell population decrease after 3 and 5 days. Polymersomes containing photosensitizer at 100 μg/mL killed more than 65% of tumor cells and further, more than 63% of normal cells were alive at the same conditions.	[296]
Laser irradiation – PTT	PEGylated carbon nanotubes	-	<i>In vivo</i> PTT treatment using an 808 nm continuous-wave NIR laser diode 808-2 W with the intensity of 8 W/cm ² for 10 minutes showed that the average size of the tumor before and 3 days after the NP treatment was decreased from 566.4 mm ³ to 174.69 mm ³ while in the control group this was increased from 406 mm ³ to 745.31 mm ³ .	[297]
	PEGylated gold NPs	Curcumin	The <i>in vitro</i> effect of gold NP upon irradiation by an 808 nm laser for 10 min on melanoma cell line was clearly observed after laser illumination while laser irradiation alone (without NPs treatment) induced melanoma cell killing effect depending on laser power. <i>In vivo</i> administration of gold NP associated with laser irradiation resulted in effective destruction of melanoma tumor cells.	[298]
	Gold NP coated-liposome	Curcumin	After laser irradiation (780 nm for 5 min), the cytotoxicity of NPs in presence and absence of curcumin was enhanced to 90% and 80%, respectively. These NP exhibited 9-fold reduction in cell viability after laser irradiation in comparison with free curcumin.	[299]
	Gold NP coated-liposome	Cantharidin	The drug released from NP increased the apoptosis of PTT-treated tumor cells. The viability of cells not treated with NP and irradiated showed slight decrease compared to control (non-irradiated). The viability of NP treated cells decreased from 40 to 13% after irradiation ranging from 200 mW/cm ² to 1 W/cm ² .	[300]
	Cetyltrimethylammonium bromide-coated gold nanorods decorated with polyethylene glycol and bovine serum albumin	Immunoadjuvant imiquimod (R837)	The designed NP were efficient in direct tumor destruction through PTT and trigger strong immune response with the help of loaded immunoadjuvant <i>in vitro</i> and <i>In vivo</i> . In addition, NP under laser irradiation inhibited cell metastasis.	[301]
	Polydopamine coated aluminum oxide NPs	Cytosine-phosphate-guanine oligodeoxynucleotide	Tumor eradication from combined photothermal therapy and immunotherapy was observed to 50% of treated animals and the survived for 120 days, which was the end point of the experiment. This investigated strategy efficiently led to maturation of dendritic cell, resulting in the secretion of cytokines and antibodies, just like the proliferation of splenocytes and lymphocytes for anti-tumor immunotherapy.	[302]
	Carbon xerogel NPs	-	The <i>in vivo</i> tumor shrinkage from the combined treatment was strongly increased when compared to non-treated group. An intense necrosis rate was observed when was applied laser irradiation after NPs injection in the tumor. Individual treatment (laser irradiation or NPs alone) exhibited no toxic effect on the tumor area.	[303]
Laser irradiation – PAT	Gold nanorod	Doxorubicin	The photoacoustic effect of NP after 808 nm pulsed laser irradiation exhibited direct physical damage to the cells. This result was attributed to two distinct and complementary mechanisms: the excellent photothermal property and high photothermal conversion efficiency of the NP.	[304]
	Light-responsive nanocapsules	Doxorubicin	The strategy based on the combination pulsed microwave-triggered thermocavitation, gas burst and chemotherapeutic activation exhibited significant tumor reduction. <i>In vivo</i> , the animals demonstrated longer survival with the combined treatment.	[305]
Iontophoresis	Polymers-coated gold NPs	Imatinib mesylate and STAT-3 siRNA	Noninvasive topical iontophoretic NP administration using anodal iontophoresis at 0.5 mA/cm ² during 2 h was compared to	[306]

Table 1 (continued)

Physical method	Nanocarrier	Anti-cancer agent	Main results	Ref.
	Liposome	STAT-3 siRNA and curcumin	intratumoral application. Reduction in tumor weight, tumor volume and suppressed protein expression were increased by the combined nucleic acid and chemotherapeutic drug in NP compared to individual treatments.	[307]
	Nanoemulsions	Zinc phthalocyanine	Anodal iontophoretic application using a current density of 0.47 mA/cm ² during 4 h combined to multifunctional NP resulted in 5-fold greater drug deposition in viable skin compared to non-iontophoretic drug delivery. The codelivery of drug and nucleic acid from combined NP and iontophoresis exhibited an increase in apoptosis events and significant reduction in cancer cell growth compared to treatment of pure drugs and free nucleic acid.	[308]
	Immuneliposomes (functionalized with cetuximab)	5-fluorouracil	The application of anodic iontophoresis at 0.5 mA/cm ² promoted an accumulation of NPs after 30 min of treatment. The results suggest that a longer time of electric current application is necessary to favor the entry and a higher concentration of NP able to overcome the tumor barrier and reach the tumor cells.	[309]
Ultrasound	Polymeric NPs	Docetaxel and chlorin e6	Subcutaneous treatments with NPs combined with anodal iontophoresis at 0.5 mA/cm ² resulted in tumor shrinkage. Similarly, cetuximab-functionalized NPs reduced tumor volume after iontophoresis and subcutaneous injection.	[310]
	Nanocapsule nanoconjugate and microbubble	Curcumin and topotecan	Sonodynamic therapy combined with NPs exhibited higher rates of apoptosis and necrosis compared with the individual and dark treatments. In addition, when NP was exposed to ultrasound, a large amount of ROS was found, leading to mitochondrial damage followed by cellular apoptosis.	[311]
Microneedle	PEGylated gold nanorod and micelles	Docetaxel	Reduced tumor growth rate was observed to the ultrasound exposed when compared to left tumor (not exposed with ultrasound) suggesting that the ultrasound exposure leads to localized mild hyperthermia and that would have also contributed in reducing tumor size through non-coagulative necrosis. Also, the tumor growth was reduced by 3.5 and 14.8 times to the ultrasound exposure treatment as compared to the unexposed and group treated with physical mixture of unloaded drug respectively. Further, survival analysis showed that the 26% of the animals were alive in untreated group whereas 93% were alive at the end of treatment with NP and ultrasound.	[312]
	Gold nanocage	Doxorubicin	The tumor treated with associated microneedles and photothermal therapy reached 50 °C within 1 min, which promoted an irreversible damaged and improved therapeutic efficacy results compared to non-combined treatments.	[313]
	Liposome	Doxorubicin and celecoxib	Combined microneedle and photothermal treatments have stronger inhibition capability on tumor growth. The final tumor volumes and tumor weights evaluated at 12 days of treatment were relatively lower for combined treatments in presence of chemotherapy drug than in the absence of chemotherapy drug.	[314]
	Mesoporous organosilic NPs	Phthalocyanine and with small molecule inhibitors (dabrafenib and trametinib)	<i>In vivo</i> , topical application of the microneedle and through co-loaded NPs resulted in a significant decrease in mean tumor weight. Treatment with NPs alone showed a smaller tumor reduction compared to combined therapies.	[315]
	pH-responsive lipid NPs	Cisplatin	There was no significant difference of NP amount skin release between the untreated and microneedle-treated skin samples at the first 10 minutes. Then, one hour later, the microneedle treatment increased the NP skin penetration rates dramatically which were 63% compared to 27.2% for treatment without microneedle. For <i>in vivo</i> treatment in xenografted tumor mouse model, the tumor growth curve of untreated group exhibited exponential growth of tumors, while photodynamic therapy combined with NPs effectively reduced this growth.	[316]
			Treatments with microneedle and NP significantly increased apoptosis and cytotoxicity in tumor cells, exhibiting a significantly reduced tumor weight and volume in xenograft tumor animal model.	

Applications reported in the last three years (2017-2019).

Abbreviations: PDT = Photodynamic Therapy; PTT = Photothermal Therapy; PAT = Photoacoustic Therapy.

In studies conducted by Wang *et al.* (2019), DOX-loaded light-responsive nanocapsules demonstrated significant antitumor activity *in vitro* and *in vivo*. It was reported that after pulsed microwave irradiation, the nanocapsules absorbed energy to generate a large thermoacoustic shockwave that simultaneously decomposed molecules into carbon dioxide and ammonia, causing cavitation and, consequently, cellular damage. The thermoacoustic shockwave and the gas burst also

mechanically disrupted intracellular organelles, which resulted in a high ratio of necrotic cells, and DOX was released from the cytosol into the nucleus to initiate cell death [308].

6.4.2. Iontophoretic therapy

Iontophoresis is a type of electrotherapy used to promote the permeation of drugs into the skin and is mainly used for the administration of

charged ionic molecules and macromolecules. Briefly, in this technique, two electrodes (anode and cathode) are placed on the surface of the skin, and a constant or pulsed electric current (0.1–1 mA/cm²) is applied and dispersed through a solution containing the drug. This causes a drug iontophoretic flux through the skin, which refers to the sum of the electromigration and electroosmosis effects [289,309]. Several preclinical and clinical studies have evaluated the transdermal iontophoretic release of skin cancer treatments, including chemotherapy, vaccines, photosensitizers, and oligonucleotides [309]. Moreover, iontophoresis is promising for improving the permeation of NPs loaded with therapeutic agents.

In studies by Labala *et al.* (2015, 2016, 2017), polymer-coated gold NPs (GNPs) were investigated for the topical iontophoretic administration of imatinib mesylate (IM) and STAT-3 siRNA [286,310,311]. The results showed that iontophoresis (0.47 mA/cm²) increased the penetration of the IM-loaded GNPs by 6.2-fold compared to the passive application and promoted greater retention in the viable skin [311]. Similarly, the application of iontophoresis increased the penetration of GNP-siRNA STAT3 into the skin. Treatment with GNP-siRNA STAT3 in murine melanoma cells exhibited a reduction in STAT-3 expression, and events related to early and late apoptosis were observed. The iontophoretic codelivery of STAT-3 siRNA and IM-loaded GNPs in a murine melanoma model showed results similar to those of the intratumoral application: a significant reduction in the percentage of tumor volume and tumor weight and suppressed STAT-3 protein expression compared to that seen with treatment with GNPs with STAT-3 siRNA or IM [312]. Iontophoretic delivery of the STAT-3 siRNA complex into curcumin-loaded liposomes also showed promising results against epidermoid carcinoma cells [310,313]. Iontophoretic application (0.47 mA/cm²) of liposomal curcumin resulted in the accumulation of curcumin in viable skin at a level that was 5-fold higher than that seen after the four-hour passive application of free curcumin [310].

In other studies, the use of iontophoresis improved the epidermal penetration profile of PAMAM loaded with an antisense oligonucleotide (directed to the Bcl-2 protein) and 5-FU-loaded immunoliposomes functionalized with an anti-EGFR antibody (cetuximab). The results in a murine model of skin cancer confirmed the therapeutic efficacy with a reduction in tumor volume and cellular proliferation [314,315].

6.4.3. Ultrasound and electroporation therapy

Ultrasound and electroporation, electricity-based modalities, have not been extensively explored to determine their combined effects on NPs. The effects of ultrasound on tissues and cells include cavitation, thermal increases, and acoustic creep. After electroporation, a transient increase in the permeability of cell membranes occurs under the influence of an appropriately modulated electric field [289,316,317]. Liu *et al.* (2018) reported antiproliferative and antimetastatic effects *in vitro* and *in vivo* in a murine model of melanoma treated with docetaxel and chlorin e6 (Ce6)-loaded NPs and the application of low-intensity ultrasound (1 W/cm²) [318].

Prasad and Banerjee (2019) were the first to report the development of nanoconjugate and microbubble (MB) nanocapsules that were associated with ultrasound-mediated co-delivery of hydrophobic and hydrophilic drugs (curcumin and topotecan). The results showed that exposure to ultrasound (2 W/cm²) was significant in reducing the toxicity induced by free drug concentrations of curcumin and topotecan *in vitro* that was accompanied by an increase in necrotic and apoptotic cells. In a murine model of MSC, a reduction in tumor growth was observed. These results suggest that the ultrasound application facilitated tumor permeabilization and that insonation catalyzed release on demand and thus facilitated simultaneous release and internalization. Additional factors may have played a role in tumor reduction, such as energy production by cavitation, ROS formation, and localized mild hyperthermia that led to noncoagulative necrosis [319].

6.4.4. Microneedle array therapy

Among the physical methods used to deliver drugs, microneedle (MN) technology has been frequently used to facilitate the intradermal/transdermal delivery of drugs in a minimally invasive manner [289,320]. Briefly, the MN mechanism of action is based on disruption of the skin layer to create micrometer-sized pathways through which the drug is directly applied to the epidermis or the upper dermis region, from where it passes into systemic circulation without facing a barrier [320,321]. The combined use of MNs and NPs has been leveraged to achieve the release of low-weight drug molecules in the dermis and for transcutaneous immunization [289,321].

Hao *et al.* (2017) demonstrated improved antitumor efficacy against epidermoid carcinoma when micelles loaded with docetaxel were delivered by a near-infrared (NIR)-responsive PEGylated gold nanorod (GNR-PEG)-coated poly(L-lactide) microneedle system (GNR-PEG-MN). In the tumor region, the transcutaneous penetration of GNR-PEG increased with the application of MNs. The combination of the MN system with docetaxel culminated in tumor eradication and no recurrence during the study period. Notably, the results demonstrated that the delivery of GNR-PEG and docetaxel via MNs was safe and could be used to reduce the chemotherapeutic dose needed [322]. In the investigations by Ahmed *et al.* (2019), treatment with liposomes loaded with doxorubicin (DOX) and celecoxib via solid MNs promoted superior antitumor results. MN pretreatment increased the penetration of liposomes by approximately 2-fold compared to passive delivery. The application of liposomes coloaded with DOX and celecoxib significantly inhibited tumor growth in melanoma xenograft mouse models compared to the application of liposomes loaded with a single drug. However, in the group in which MNs were preapplied, the antitumor effects were more effective [323].

MNs have been associated with photodynamically active mesoporous organosilica NPs to enhance the effects of PDT. NPs produced with phthalocyanine covalently bound to the silica matrix dramatically increased the quantum yield and photostability. The mesopores of the NPs were further loaded with dabrafenib and trametinib, which target the hyperactive mitogen-activated protein kinase (MAPK) pathway for melanoma treatment. Skin pretreated with a microneedle presented an increased fluorescence signal in both the epidermis and dermis. In tumor regression studies with a xenografted melanoma mouse model, the superior therapeutic efficacy of the NPs was confirmed through a combination of photodynamic therapy and targeted therapy [324]. In another study, Lan *et al.* (2018) investigated the application of MNs and found that their use improved the penetration of pH-responsive lipid NPs loaded with cisplatin that were used for targeted antitumor therapy. In a xenograft animal model, it was demonstrated that MNs loaded with cisplatin NPs significantly increased the cytotoxic effects in and the apoptosis of cancer cells, resulting in a significant reduction in the tumor size. The tumor growth inhibition ratios in the group with MN-delivered cisplatin-loaded NPs were 76%, which is considered a promising antitumor effect. Moreover, no serum platinum levels, nephrotoxicity, pulmonary toxicity or hepatotoxicity were detected *in vivo*, indicating the safety of this technique [325].

6.5. Multifunctional delivery systems

Advances in understanding the progression and survival of cancer have led to increased interest in developing innovative therapeutic interventions. In this context, the design of multifunctional delivery systems that carry at least two bioactive molecules with different pharmacological and physicochemical properties against cancer has been the focus of extensive investigations for potential therapeutic strategies [11,326–328]. For skin cancer therapy, multifunctional delivery systems have been developed for the delivery of at least two molecules and/or the combination of chemotherapeutics, bioactive compounds of natural origin, immunotherapeutic molecules and siRNAs and have been shown to be beneficial compared to single bioactive molecules. This strategy

can also be useful in cancer therapy when metastasis occurs [329]. A summary of the applications of this approach is shown in Table 2.

DOX and paclitaxel (PTX) are chemotherapeutic drugs commonly used in multifunctional nanocarriers and have different pharmacological mechanisms and cellular targets. Solid lipid NPs were used to codeliver paclitaxel (PTX) and ascorbyl palmitate to treat murine melanoma that had metastasized to the lungs of mice and provided synergistic antitumor effects *in vitro* and *in vivo*. It has been reported that this treatment actively suppressed tumor growth and eliminated more cancer cells from the lungs than treatment with lipid NPs loaded with a single drug via a synergistic effect that reduced the Bcl-2/Bax ratio [329]. In addition, PTX-loaded SLN modified with Tyr-3-octreotide has also been shown to reduce tumor volume in murine melanoma models and to induce antimetastatic and immune responses (an increase in IL-12, IFN- γ and TNF- α) [330]. In another study of melanoma models, PTX-loaded hyaluronic acid micelles modified with tocopherol succinate (TOS) were able to accumulate in the tumor to induce the EPR effect, the redox sensitivity was able to control release of the drug, and dissociation of the TOS led to synergistic antitumor effects with PTX treatment [331].

The codelivery of PTX and ceramide C6 in nanoemulsions, developed by Carvalho *et al.* (2017), showed a synergistic antitumor effect in melanoma cells. Coencapsulation of PTX and ceramide decreased the concentration necessary to reduce cell viability by 2.5–4.5-fold to 50% (EC₅₀), indicating a synergistic effect [332]. In other studies, codelivery of ceramide and DOX through lipid nanocarriers was effective in enhancing the antitumor efficacy of DOX *in vitro* and in murine melanoma models [333,334]. Wang *et al.* (2017) reported an antitumor efficacy of $93.94 \pm 2.77\%$ for a multifunctional nanocarrier, a finding that was significantly higher than that of a single drug-loaded nanocarrier and the Duopaifei® control [334]. More recently, a combined therapy of DOX and the autophagy inhibitor wortmannin was improved by Cu (I)-catalyzed click chemistry-triggered aggregation of azide/alkyne-modified micelles. After the micelles reached the tumor *in vivo*, the catalysts were intratumorally injected, resulting in aggregation of the micelles induced by the click reaction. Furthermore, the decreased amount of autophagosomes, the expression of LC3-II, and the increased level of p62 confirmed the inhibition of autophagy and the synergistic effect in suppressing melanoma and breast cancer in mice [335].

Bioactive compounds of natural origin are known for their multiple biological activities, and in antitumor therapy, their effect in combination has been the focus of several studies. Curcumin and chrysin coencapsulated in PLGA-PEG NPs exhibited a significant decrease in the expression of metalloproteinase-2 and -9, which are enzymes associated with the invasion and metastatic ability of cancer cells, in a murine melanoma model [336]. An additive effect on the codelivery of curcumin and topotecan in nanocapsules was demonstrated, and it was reported that the IC₅₀ after ultrasound exposure was reduced by 150-fold and 100-fold compared to that found with treatment with free curcumin and topotecan, respectively [319]. Another example of a natural compound codelivered with a chemotherapeutic compound was reported by Mishra *et al.* (2019). The functionalized hyaluronic acid liposomes coloaded with eugenol and dacarbazine exhibited a 9-fold and 2-fold increase in induced cytotoxicity in melanoma cell lines compared to that induced by free dacarbazine and eugenol-loaded liposomes, respectively. An increase in the number of late apoptotic cells was found (45.16% vs. 8.43%), together with a significant inhibition of cell migration and proliferation. These results indicate that when delivered in combination with eugenol, the dose of dacarbazine needed for chemotherapy is reduced, leading to an implied reduction in undesired toxicity [337].

Using an immunotherapy and nanovaccine approach, Zhuang *et al.* (2016) proposed the synthesis of hybrid lipid-coated zinc phosphate NPs to coencapsulate antigenic peptides (TRP2₁₈₀₋₁₈₈ and HGP100₂₅₋₃₃) and monophosphoric lipid A (MPLA). Data from studies conducted *in vitro* and *in vivo* (metastatic melanoma model) showed that the system coloaded with peptides and MPLA was effectively captured by

dendritic cells with distinct time-dependent accumulation and cytokine enhancement, such as increases in IL-12, IFN- γ and TNF- α , which are related to the efficiency of T cell activation. A reduction in angiogenesis and a minimal number of metastatic lung nodules were also observed [338].

To enhance the antitumor immune response, Lu *et al.* (2018) demonstrated that codelivery of CpGs and OVA in biodegradable glutathione-depleted dendritic mesoporous organosilica NPs produced increased cytotoxic T lymphocyte activity and led to reduced tumor growth in a murine melanoma model [339]. Similar antitumor effects and immunization levels were achieved in the investigations of Zhang *et al.* (2018) through the coloaded of CpGs, tyrosinase-related protein 2 (Trp2) peptides and two mutated epitopes (M27 and M30) in layered double hydroxide NPs. Significant inhibition of tumor growth (approximately 50%) in the mouse melanoma model was observed [340]. Treatment with CpGs and DOX coloaded onto dendrimers has been shown to enhance the immune response induced by DOX and to suppress tumor growth and metastasis synergistically. Additionally, due to the coating of the dendrimers with antimetastatic low-molecular-weight heparin, platelet-induced epithelial-mesenchymal-like transition was prevented, which disrupted the arrangement of tumor cells via changes in the actin cytoskeleton, resulting in suppression of the migration ability of the tumor cells [341].

Immunotherapy combined with chemotherapy has also been proposed as a strategy to improve antitumor effects by distinct mechanisms [342–344]. PTX and IL-2 coloaded on thermosensitive NPs showed significant inhibition of melanoma tumor growth (88%) and metastasis and prolonged overall survival for tumor-bearing mice compared with monotherapies. In addition, activation of innate and adaptive immunity in the tumor microenvironment was observed [342]. In another study, DOX-loaded liposomes were functionalized with a T cell receptor (TCR)-like antibody (scFv G8 and Hyb3) directed against the melanoma antigen A1 (MAGE-A1) that is presented by human leukocyte antigen A1 (M1/A1) [345]. The accumulation of targeted liposomes was 2- to 2.5-fold and 6.6-fold enhanced *in vivo* compared with that of nontargeted liposomes and free drugs, respectively. The superior antitumor activity of the MAGE-A1-targeted DOX-loaded liposomes was observed in tumors with positive M1/A1 expression [345]. The functionalization of the 5-FU-liposomes with cetuximab was also shown to improve antitumor effects with a great reduction in melanoma tumor volume (>60%) compared to that induced by the free 5-FU or control liposomes [346].

Gene therapy, such as that based on siRNA, can be used to suppress the production of cancer target proteins effectively. In combination with chemotherapeutics, it can improve targeting selectivity and counterbalance drug resistance, thereby increasing therapeutic efficacy synergistically. Furthermore, combination therapy can enable the reduction of the required dose of individual chemotherapeutic agents, which consequently reduces potential adverse drug reactions [87,347]. Bcl-2 siRNA and PTX coadministered in pH-sensitive liposomes exhibited a marked antitumor effect in melanoma cells compared with that found when free PTX was applied [348]. In this study, PTX-inducible drug resistance (antiapoptotic response) was observed but was effectively decreased by the parallel silencing of the Bcl-2 gene during the administration of chemotherapy, and significant overexpression of Bax (proapoptotic inducer) and down regulation of pro-caspase-3 were observed, indicating activation of cellular apoptosis [348]. Duan *et al.* (2018) developed liposomes containing an Aurora kinase inhibitor (XY-4) and Bcl-xl siRNA to enhance antitumor effects through distinct mechanisms of cell cycle arrest and induction of apoptosis. In murine melanoma cells, liposomal delivery exhibited strong anticancer effects in a concentration-dependent manner and increased efficacy of the codelivered agents. The authors reported that the combination of XY-4 and Bcl-xl siRNA resulted in cell apoptosis through a mitochondrial-based signaling pathway (via caspase-9 and caspase-3). The multifunctional liposome treatment exhibited a significant reduction in tumor

Table 2
Multifunctional NPs reported in the last three years (2017–2019).

Nanocarrier	Anti-cancer agent	Functionalization	Functionalization: Target /Mechanism of action	Key studies	Ref.
Polymeric NP	Peptide P20 (CSSRTMHHC)	Combined peptide C (CVNHPAFACGYG-HTMYHHYQHHL)	Tumor targeting, mainly in pulmonary metastases.	Development of PLGA-NP for protection and codelivery of bioactive peptides in metastatic melanoma.	[353]
	Curcumin and topotecan	-	-	Development of NP co-loaded with curcumin and topotecan for therapeutic regimen for spatiotemporal delivery of dual drug for treatment of cancer.	[319]
	Paclitaxel and IL-2	mPEG-PLGA	Stabilization of NPs and prolongation of circulation time.	Study of antitumor and immunostimulant effects of codelivery of paclitaxel and IL-2 in thermosensitive NPs.	[342]
	Doxorubicin and IFN- λ	mPEG-PLGA PEG	Prolongation of circulation time.	Development of dual-sensitive NP for co-encapsulating doxorubicin and interferon- γ and to realize the codelivery of immunotherapy and chemotherapy agents against melanoma.	[343]
	Curcumin and chrysin	PEG	Better ability to reach tumor layers and stability.	Investigation of the co-encapsulation of curcumin and chrysin in PLGA-PEG-NP in the treatment of melanoma.	[336]
	All-trans retinoic acid	Anti-CD20 antibody	Specific binder targeting (CD20 is a marker of melanoma-initiating cells).	Investigation of efficiency all-trans retinoic acid -loaded PLGA-NP conjugated to anti-CD20 antibody targeting cancer initiating cells.	[355]
Micelle	Dasatinib	PEG-MMP2-sensitive peptide-phosphoethanolamine and folic acid-PEG-phosphoethanolamine	Prolongation of circulation time, decrease the nonspecific distribution in nontumor tissues, and increase the tumor accumulation.	Micellar NP loaded with dasatinib were produced with functional polymers as a strategy to improve drug efficacy, tumor targetability and decrease drug resistance.	[356]
	Paclitaxel and doxorubicin	mPEG-SS	mPEG-SS conjugated paclitaxel has disulfide bond sensitive to redox capable of releasing a drug in the tumor microenvironment (acid).	Development of micelle system based on redox-sensitive mPEG-SS-paclitaxel and mPEG-SS-doxorubicin conjugate by synchronized and controlled release.	[357]
	Paclitaxel	Tocopherol succinate	Increase the tumor accumulation and dissociation in redox environment and drug release.	Development of paclitaxel-loaded micelles formed by conjugated tocopherol succinate and hyaluronic acid to improve delivery, targeting and anti-tumor synergistic effects.	[331]
	Doxorubicin and wortmannin	-	-	Developed of strategy for the codelivery of doxorubicin and wortmannin in azide/alkyne-modified micelles and Cu (I)-catalyzed click chemistry-triggered aggregation.	[335]
	Doxorubicin	3-aminophenylboronic acid, LMWH and D- α -tocopheryl succinate conjugates	-	Development of multifunctional micelles formed by antimetastatic components as carrier of chemotherapeutic agent (doxorubicin).	[358]
Hybrid micelle	CpG and Trp2 peptide	-	-	Design of polymeric hybrid micelles as a simple and potent antigen/adjuvant codelivery system with highly tunable to overcome intracellular and lymphatic delivery barriers.	[359]
Dendrimers	CpG and doxorubicin	LMWH	Inhibition of tumor cell and platelet interaction and protection of CpG against reticuloendothelial system clearance and potential cationic NP toxicity.	Coated-anti-metastatic low molecular weight heparin dendrimers loaded with doxorubicin and immunoadjuvant CpG as a strategy to promote immune, antitumor and anti-metastatic activation effects.	[341]
Hybrid NP	siRNA Bcl-2	Derivative thiolated hyaluronic acid (redox-sensitive)	Colloidal stability, prolongation of circulation time and dissociation in redox environment and drug release.	Production of calcium phosphate NP complexes complexed with siRNA and coated with disulfide cross-linked hyaluronic acid as an intelligent system for selective release of siRNA through GSH-triggered disassembly and endosomal escape.	[360] [353]
	Salinomycin	Anti-CD20 aptamers	Specific binder targeting (CD20 is a marker of melanoma-initiating cells)	Design of lipid-polymer-NP to improve the delivery of salinomycin and targeting melanoma stem cells through anti-CD20 aptamers.	[361, 354]
	siRNA PD-L1 and mRNA Trp-2	DSPE-PEG ₂₀₀₀ and mannose	Specific binder targeting (preferential uptake by the dendritic cells in the lymph nodes after subcutaneous administration)	Development of lipid-coated calcium phosphate NP and functionalized with PEG-mannose to direct uptake by lymphatic dendritic cells and release of encapsulated immunotherapeutic agents.	[350, 355]
SLN	Paclitaxel and ascorbyl palmitate	-	-	Development of SLNs co-loaded with ascorbyl palmitate and paclitaxel for synergistic therapy in metastatic melanoma.	[329]

Table 2 (continued)

Nanocarrier	Anti-cancer agent	Functionalization	Functionalization: Target /Mechanism of action	Key studies	Ref.
	Paclitaxel	Tyr-3-octreotide	Specific binder targeting and increased immunogenic response.	Design of paclitaxel-loaded SLN functionalized with Tyr-3-octreotide targeting tumor cells with somatostatin receptors.	[319, 356]
	All-trans retinoic acid	2-hydroxyoleic acid	Colloidal stability of liposomes and anti-tumor selectivity.	Design of liposomes composed of 2-hydroxyoleic acid, an antitumor agent, and loaded with a chemotherapeutic agent.	[362, 357]
Liposome	5-fluorouracil	Cetuximab	Specific binder targeting through receptor ligands expressions on tumor cell surface (IgG antibody that has affinity for EGFR).	Conjugation of cetuximab into 5-fluorouracil-loaded liposome to improve targeted delivery in squamous cell carcinoma associated with the iontophoretic application.	[314]
	Eugenol and dacarbazine	Hyaluronic acid	Specific binder targeting through receptor ligands expressions on tumor cell surface (CD44 receptors).	Development of liposomes loaded with eugenol and dacarbazine and coated with hyaluronic acid to confer targeting cancer-initiating cells and overcoming mechanisms of drug resistance.	[337, 358]
	Doxorubicin and C8-ceramide	-	-	Development of ceramide-based liposomes and loaded with doxorubicin as a system to promote enhanced cytotoxic effects on melanoma cells.	[333, 359]
	Aurora-A inhibitor (XY-4) and siRNA Bcl-xl	-	-	Co-administration studies of the newly synthesized Aurora-A kinase inhibitor XY-4 and Bcl-xl siRNA in cationic liposomes in skin cancer therapy.	[349, 360]
	Doxorubicin	Antibody (scFv G8 and Hyb3)	Specific binder targeting through receptor ligands expressions on tumor cell surface (melanoma antigen A1)	Studies of the efficacy of doxorubicin-loaded liposomes directed against melanoma antigen A1 by conjugation with T-cell receptor-like antibody (scFv G8 and Hyb3).	[345, 361]
Liposome and deformable liposome	Curcumin and siRNA STAT-3	-	-	Investigation of the liposomal co-delivery of curcumin and STAT3 siRNA by non-invasive topical iontophoretic application to treat skin cancer.	[310,313]
Transfersomes	Paclitaxel	Cell-penetrating-peptide (R8H3)	Increased penetration into the skin and tumor stroma as well as more efficient transport into tumor cells.	Development of a hydrogel of transfersome-embedded oligopeptide to enhance transdermal delivery of chemotherapeutic drug	[363, 362]
Nano- and micro-emulsion	Paclitaxel, C6-ceramide and tributyrin	-	-	Studies of the efficacy of co-delivery paclitaxel and C6 ceramide in micro and nano-emulsions containing tributyrin (a butyric acid pro-drug included for potentiation of cytotoxicity).	[332, 363]
Lipid-based nanosuspension	Doxorubicin and ceramide	-	-	Development of ceramide-based nanosuspension with docetaxel to exhibit a synergistic therapeutic effect.	[334]
Silica NP	Dacarbazine	Folic acid-grafted liposomes	Specific binder targeting through receptor ligands expressions on tumor cell surface.	Rationally designed of hollow mesoporous silica NP for the encapsulation and targeted (folic acid-grafted liposomes) release of dacarbazine for eradicating melanoma.	[182]
	CpG and OVA antigen	Tetrasulfide bond and Polyethyleneimine	Electrostatic attraction and antigen loading. And, sensitive-redox bonds activate immune system and immunogenic activity against the tumor.	Development of glutathione-depletion mesoporous organosilica NP by codelivery of antigen protein (OVA) and a toll-like receptor 9 agonist into antigen presenting cells and induced endosome escape.	[236]
Gold NP	Mesilato de imatinibandsiRNA STAT-3	-	-	Production of layer-by-layer assembled gold NP containing imatinib mesylate and anti-STAT3 siRNA delivery in melanoma associated with the iontophoretic application.	[312]
	Cas9-sgPlk-1 plasmids	Cationic lipid (DOTAP, DOPE, cholesterol) DSPE-PEG ₂₀₀₀ and TAT peptide	Protection, guidance and specific release.	Design of TAT peptide-modified gold NP condensed with Cas9-sgPlk-1 plasmids and coated lipids to allow cellular delivery and plasmid release via thermic effects.	[354]
Layered double hydroxide NP	CpG, Trp2 peptide e two mutated epitopes (M27 and M30)	-	-	Studies of constructing Layered double hydroxide NP-based multi-target therapeutic cancer vaccine, using peptides antigens and two mutated epitopes.	[238]

Abbreviations: PLGA = polylactic-co-glycolic acid; IL-2 = interleukin-2; IFN- γ = interferon gamma; mPEG = methoxypropylpolyethylene glycol; PEG = polyethylene glycol; MMP2 = matrix metalloproteinase 2; DSPE-PEG2000 = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]; LMWH = Low molecular weight heparin; CpG = cytosine-phosphorothioate-guanine oligodeoxynucleotides; Trp-2 = tyrosinase-related protein 2; siRNA = small interfering; Bcl-2 = B-cell lymphoma 2; PD-L1 = programmed death-ligand 1; mRNA = micro RNA; STAT-3 = signal transducer and activator of transcription 3; OVA = ovalbumin; TAT peptide = HIV cell-penetrating transactivator protein.

weight and no systemic pathological changes *in vivo* [349]. In another approach, the codelivery of programmed cell death protein 1 siRNA and tyrosinase-related protein-2 mRNA in lipid-coated calcium phosphate NPs was shown to increase anticancer immunization, which was accompanied by significant inhibition of tumor growth and metastasis, in a murine melanoma model [350].

In addition to carrying at least two bioactive therapeutic agents, a multifunctional delivery system can also be designed to perform other functions to surpass biological barriers and effectively deliver their loads to the target tissue/organ. Functionalization is a strategy that allows the delivery system to have extra function, for example, to allow longer circulation time and reach the desired location by the EPR effect or through interactions with ligands or to be able to respond to specific stimuli from the pathological microenvironment [104]. A summary of the functionalization of NPs used in skin cancer is also presented in Table 2.

7. Conclusion and future perspective

Nanotechnology has established a science that has changed the way we face the challenges of new disease treatments, and this technology allows us to go beyond what conventional treatments are capable of. Nanomedicine for skin cancer has already become a current scientific practice, and it has demonstrated in recent years a major evolution in the treatment of solid skin tumors, including aggressive and invasive tumors, by various routes of administration, as well as initial tumor lesions by less invasive routes of administration such as topical route.

Regardless of the route of administration, the use of NPs to carry active molecules or their combination is attractive for reducing the tumor mass, and this is a recurrent result in scientific research. Individualized and specific therapies have been made possible with the use of NPs, as have a reduction in drug concentrations, which leads to a lower intensity of adverse effects compared to conventional chemotherapies.

In conclusion, NPs enable therapies such as immunotherapies and gene therapies to be used in more efficient ways, which is important since they are high-cost therapies. The versatility of these nanocarriers grows exponentially because although their use adds to the cost of therapy, it comes with innumerable benefits, which in terms of cost-effectiveness, are worth conceding, as these benefits lead to greater prospects for diagnosis and treatments, in addition to allowing the use of theranostics in cancer patients. As with other strategies, the use of NPs could make both types of therapies feasible for clinical applications.

As discussed in this review, although the nanotechnology field offers several strategies to fight cancer, many efforts are still needed to generate effective and safe therapies.

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