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REVIEW ARTICLE

Smart Nanocarriers for Targeted Cancer Therapy

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Abstract: Cancer is considered one of the most threatening diseases worldwide. Although many therapeutic approaches have been developed and optimized for ameliorating patient's conditions and life expectancy, however, it frequently remains an incurable pathology. Notably, conventional treatments may reveal inefficient in the presence of metastasis development, multidrug resistance and inability to achieve targeted drug delivery.

In the last decades, nanomedicine has gained a prominent role, due to many properties ascribable to nanomaterials. It is worth mentioning their small size, their ability to be loaded with small drugs and bioactive molecules and the possibility to be functionalized for tumor targeting. Natural vehicles have been exploited, such as exosomes, and designed, such as liposomes. Biomimetic nanomaterials have been engineered, by modification with biological membrane coating. Several nanoparticles have already entered clinical trials and some liposomal formulations have been approved for therapeutic applications. In this review, natural and synthetic nanocarriers functionalized for actively targeting cancer cells will be described, focusing on their advantages with respect to conventional treatments. Recent innovations related to biomimetic nanoparticles camouflaged with membranes isolated from different types of cells will be reported, together with their promising applications. Finally, a short overview on the latest advances in carrier-free nanomaterials will be provided.

Keywords: Smart nanocarriers, active targeting, targeted therapy, biomimetic nanomaterials, cancer, clinical trials.

1. INTRODUCTION

Cancer is one of the prominent causes of death worldwide [1]. Current options for clinical intervention rely on tumor resection by surgery and chemotherapy/radiotherapy regimens. Chemotherapy, based on the use of chemotherapeutic drugs and introduced for the first time in 1942 [2], plays a pivotal role. In the last decades, the development of new molecules for cancer therapy has continuously increased becoming a multi-billion-dollar industry and nowadays, many chemotherapeutics have entered clinical practice (*e.g.*, cisplatin, doxorubicin, paclitaxel) [3]. Similarly to chemotherapy, radiotherapy is widely employed for cancer treatment and recent researches have allowed to finely tune radiation doses for reducing several heavy side effects in already debilitated patients [4]. Indeed, both therapies present many drawbacks, such as lack of tissue specificity and induction of Multidrug Resistance (MDR) and only a careful cost-benefit evaluation can determine the best intervention strategy. MDR occurs when tumor cells become resistant to chemotherapeutic agents, leading to inefficacy of the treatment due to inactivation of the administered drug [5]. Many mechanisms are involved in this process, such as i) increased drug efflux from the cells, by means of membrane transport proteins, ii) altered metabolism of the drug, iii) activation of DNA repair mechanisms, iv) modification of the cell cycle and v) dysregulation of apoptosis [5]. An essential point that needs to be further addressed is how to achieve tumor targeting and anti-cancer effects without damaging surrounding healthy tissues. In recent years, the introduction of gene therapy and immunotherapy has demonstrated to be also associated with severe side effects, tumor resistance and recurrence [6]. Even though traditional clinical protocols for cancer therapy have significantly improved patient's survival, however it is clear that continuous challenges remain open for future researches [3].

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In the last decades, nanomedicine has addressed many issues related to conventional cancer therapy [7]. Nanomaterials, due to their small size, high surface-volume ratio, high versatility and stability *in vivo*, have been successfully employed. Nanoparticles have demonstrated passive targeting abilities by exploiting the Enhanced Permeability and Retention (EPR) effect typical of tumor tissues and displaying low systemic toxicity [8]. Moreover, they have been successfully employed for overcoming MDR [9]. Nanocarriers for delivery of chemotherapeutic agents have been designed with improved efficacy, due to their high bioavailability and low amounts required for achieving the desired therapeutic effects [10].

Multifunctional nanoparticles have been developed and functionalized for actively targeting tumor cells and microenvironment, without damaging healthy tissues [11, 12]. Smart nanomaterials, responsive to external stimuli and controlled by tissue microenvironment changes (*e.g.*, pH conditions) have been engineered for triggering precise release of multiple cargoes, obtaining synergistic effects and acting as theragnostic agents (*i.e.* for use in diagnosis and therapy) [13, 14].

Due to the above-mentioned reasons, nanomedicine represents a promising approach for solving many issues related to current cancer therapies and holds great promises for entering clinical trials. In the near future, engineering *ad hoc* nanomaterials for active targeting will help making personalized cancer therapy a real option. In this review, current approaches aimed at active targeting will be discussed with a special focus on natural nanocarriers (exosomes) and synthetic vehicles, such as liposomes and DNA origami. Promising biomimetic nanocarriers coated with bio-derived elements, such as red blood cells, platelets, leukocytes and tumor cell membranes will be described, evidencing their advantages for future clinical applications. Finally, an overview on carrier-free nanomedicine will be reported, underlying their first successes in clinical translation.

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2. ACTIVE TARGETING AND NANOMATERIALS FOR ACTIVE TARGETING

In the last two decades, many passively targeted nanocarriers have been approved for medical application [15]. On the contrary, none of the actively targeted nanomaterials has been evaluated in clinical trials. A lot of efforts have been put into unraveling the mechanisms underlying the interaction between nanomaterials and biological systems. Tumor heterogeneity, microenvironment features, endosomal escape are some of the several reasons that can prevent tissue penetration. Extravasation of nanocarriers is controlled by perfusion, that however varies between the different regions of the tumor [16] and is influenced by size and shape of the nanomaterial [17, 18].

The introduction of active targeting, achieved by functionalization of nanocarriers with ligands that specifically bind overexpressed tumor receptors or antigens, has brought many successes toward increased drug retention times and univocal uptake by target cells, without damaging healthy tissues [19]. For example, folate has been chosen for its high affinity toward folate receptors overexpressed on tumor cell surface and activated macrophages [20]. Additionally, active targeting allows to reach different locations in our body and therefore it can be employed for treating hematological tumors and metastases, that normally are not subjected to EPR effect. Interestingly, functionalized nanocarriers can be designed responsive to external stimuli and specifically releasing their cargo upon external stimulation (*e.g.*, high temperatures, ultrasounds, magnetic stimuli, light, Fig. **1**).

Although the many successes, however some challenges remain to be overcome. Most of the knowledge acquired on solid tumors is based on studies performed on subcutaneous xenografted mouse models, that however do not mimic human solid tumor conditions relative to EPR effect [21]. Further researches are therefore needed in order to design nanomaterials as active drug delivery vehicles for cancer therapy and the introduction of preclinical models that more accurately represent real tumors are demanded.

2.1. Exosomes

Exosomes are defined as small vesicles of an approximate diameter of 30-150nm originated from endosomes and released from cells by fusion of Multivesicular Bodies (MVBs) to the cell membrane [22]. They circulate in extracellular fluids and are present both in physiological and pathological conditions [23]. Exosomes have been recognized to be involved in cancer development and metastatic progression and are responsible for exchanging molecules and cellular messengers between tumor cells and surrounding tissues [24]. Exosomes naturally contain many kinds of molecules, such as proteins, nucleic acids, carbohydrates and lipids, but can also be easily loaded with small drugs and bioactive molecules. Nowadays, many examples have been reported of their successful application in pre-clinical models [25-27]. Exosomes influence many tumor cell properties, such as proliferation, angiogenesis, invasion and metastasis [28] and aggressive cancers release exosomes that can modify the properties of the tumor itself, providing a dynamic behavior that favors metastatic development and progression.

They can be exploited for cancer diagnosis, prognosis and follow up and as nanocarriers for drugs in cancer targeted therapy [29, 30]. Exosomes possess many features that make them ideal candidates for delivery of biomolecules across the body. They are i) specific, ii) safe, iii) stable, iv) biocompatible, and v) slightly cytotoxic, due to the fact that they do not spontaneously accumulate into human tissues. Moreover, they are able to avoid phagocytosis, bypassing lysosomes and causing low immune responses [31]. Finally, their hydrophilic core makes them ideal for encapsulating soluble drugs [32] and for achieving a controlled release of their cargo upon specific stimuli [33].

Doxorubicin has been investigated for exosomal loading both *in vitro* and *in vivo*. These nanocarriers efficiently delivered the chemotherapeutic agent across the Blood Brain Barrier (BBB), *via* receptor mediated endocytosis [26]. Dendritic Cell (DC)-derived exosomes loaded with doxorubicin and targeted by means of RGD peptide, specific for αv integrin expressed on cancer cells, inhibited tumor proliferation in mice much more specifically than the free drug itself and the not targeted exosomes [34]. Similarly to doxorubicin, paclitaxel has been efficiently delivered in multidrug resistant cells by macrophage derived exosomes [35]. In a recent study, it has been reported that isolated exosomes clustered with magnetic nanoparticles and modified with transferrin, efficiently localized in the tumor and inhibited cell proliferation upon magnetic field exposure [25].

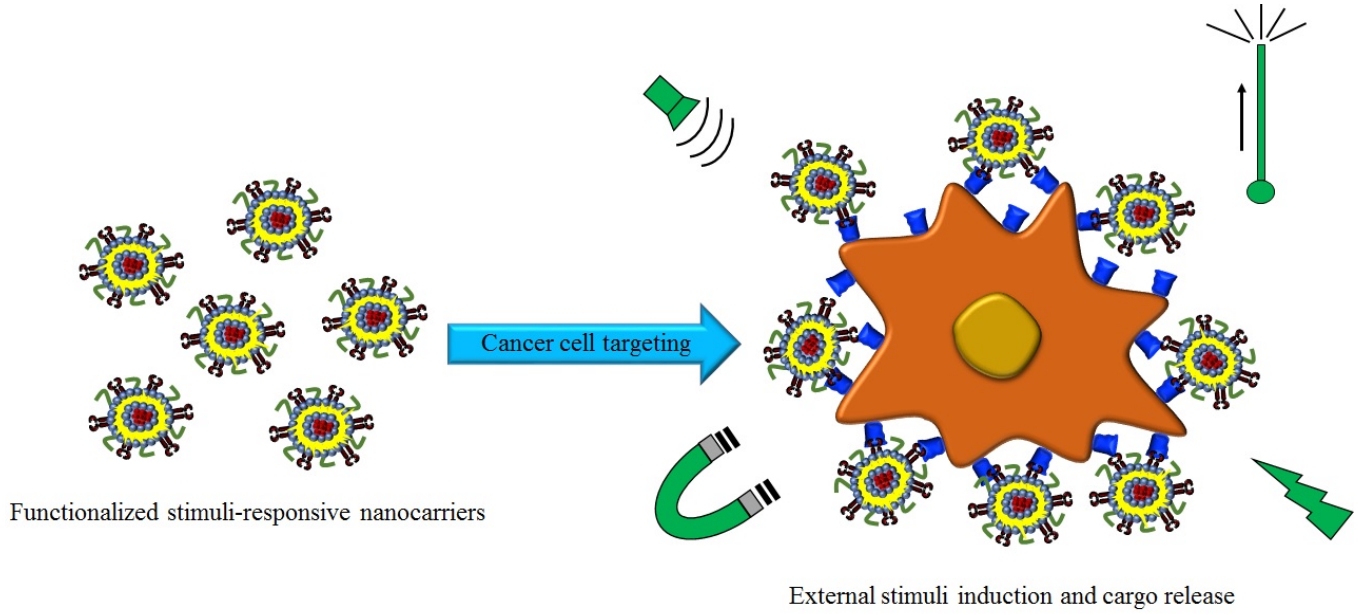

Fig. (1). Active targeting allows univocal delivery of functionalized nanomaterials to cancer cells and triggered release of their cargo upon specific external stimulation. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Exosomes naturally deliver nucleic acids and can also be used as vectors for gene therapy, achieving silencing of oncogenes and genes responsible for multidrug resistance development. Kim *et al.* demonstrated that exosomes directly injected into the tumor were able to deliver CRISPR/Cas9 targeted against PARP-1, making cells sensitive to cisplatin and inhibiting their proliferation *in vivo* [36].

Exosomes can also carry small interfering RNA (siRNA) and micro RNA (miRNA) to cancer cells. BCR/ABL siRNA was delivered to resistant chronic myeloid leukemia tumors *in vivo* [37]. Exosomes were very proficient in targeting *RAS* gene by KRASG12D siRNA [38].

Exosomes can be also exploited for vaccination against cancer: when derived from tumor or dendritic cells, they can stimulate immune responses or, by loading with antigens that are recognized by dendritic cells, they can induce their maturation.

Nowadays, the first strategy is not used anymore due to the low safety and efficiency in gaining a sufficient immune response [39]. Dendritic cells stimulated with poly(I:C) and ovalbumin produced highly immunogenic exosomes. Upon DCs stimulation with a melanoma cell lysate and poly(I:C), they elicited a strong immune response at the tumor site, reducing tumor growth [40]. Very recently, dendritic cells were transduced with heterologous human/rat HER2-neu derived targeting ligand and exosomes isolated from these cells produced a potent immune activation and protection from xenograft implantation, when administered *in vivo* [41]. Unfortunately, during phase I clinical trials, limited efficacy of dendritic cell derived exosomes has been demonstrated, while good toleration has been observed in patients affected by non-small cell lung cancer and melanoma [42, 43].

DCs were exposed to IFNγ or LPS to induce inflammation, while stimulated with IL-4, IL-10, TGF β1 to induce anti-inflammatory responses [44]. Recently, dendritic cells releasing exosomes have been engineered in order to produce immunogenic vesicles [45] that have been tested in a phase II clinical trial in NSCLC patients [46]. IFN-γ-dendritic cell exosomes loaded with MHC class I- and class II-restricted cancer antigens were administered to patients with inoperable NSCLC without tumor progression. They were able to act as immunotherapeutic agents boosting natural killer cell arm of antitumor immunity, providing longer progression-free survival [46].

Some more clinical trials are currently undergoing. A phase I trial is evaluating exosomes as carriers for curcumin in colon cancer patients and will give the first results within 2022 [47]. Moreover, exosomes derived from ascites (Aex) and combined to Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) have been evaluated in phase I clinical trial for immunotherapy of advanced colorectal cancer patients [48]. Forty patients were treated with Aex alone or together with GM-CSF. The therapy revealed safe and well tolerated and, in the case of the combination with GM-CSF, it resulted in a tumor specific antitumor cytotoxic T lymphocyte response [48].

Finally, exosomes carrying KRASG12D siRNA are under clinical evaluation in a Phase I trial enrolling pancreatic cancer patients bearing this gene mutation and will be concluded in 2022 [49].

In spite of the several clinical trials currently undergoing, some issues need to be considered when dealing with exosomes for targeted cancer therapy. Depending on the kind of cell from which they are originated, different quantities of exosomes can be released that may favor (when isolated by tumor cells) [50] or inhibit tumor growth (when isolated from healthy donors) [51].

Importantly, cells producing extracellular vesicles have to be cultured without altering their phenotypes and procedures for exosome isolation need to be standardized in order to get the purest and highest-quality material required for clinical applications [52]. Appropriate techniques for obtaining high throughput and scalable procedures have to be considered [53] and storage practices for preserving their properties and stability have to be set in order to become clinically applicable [54].

2.2. Liposomes

Liposomes are widely diffused as drug delivery vectors for cancer therapy [14]. They are artificial amphiphilic vesicles composed by assembled phospholipids exposing polar phosphate heads, while retaining apolar tails toward the interior. Due to these properties, they are ideal for carrying both hydrophilic and hydrophobic molecules. Liposomes are among the most biocompatible nanomaterials for drug delivery and nowadays there are some formulations under evaluation at the clinical stage or already approved for clinical use by Food and Drug Administration (FDA) and European Medicines Agency (EMA) [55].

Liposomes surface can be modified in order to enhance bioavailability and stability and can be engineered in order to be responsive to specific stimulation [56]. Liposomes functionalized with folate efficiently targeted cancer cells expressing folate receptors, delivering doxorubicin and achieving cytotoxicity [57]. Similarly, liposomes loaded with carboplatin demonstrated their efficacy in *in vivo* models [58].

Another effective approach is based on surface conjugation with transferrin, due to the increased expression of its receptor on tumor cells [59]. Liposomes loaded with doxorubicin were effective in a rat model of liver cancer [60]. Lipid nanoparticles encapsulating etoposide and functionalized with transferrin successfully targeted gastric cancer cells [61].

Another molecule exploited for active targeting by pegylated liposomes is HER2, overexpressed in some breast cancers and other tumors [62]. Vascular Endothelial Growth Factor (VEGF) and Vascular Cell Adhesion Molecule (VCAM) have been similarly considered, due to their high expression levels in actively growing tumors, characterized by enhanced angiogenesis.

Cell Penetrating Peptides (CPPs) and apolipoproteins have been attached to nanoparticles for the treatment of brain tumors, due to their ability to bind receptors present on endothelial cells in the blood brain barrier [63, 64].

Functionalization with antibodies or antibody derived fragments has demonstrated to be highly efficient in targeting cancer cells [65] and immunoliposomes have been employed for tumor growth inhibition both *in vitro* and *in vivo* [66, 67].

The possibility to fabricate nanocarriers responsive to external stimuli has allowed to finely trigger drug release. The first stimuli-responsive nanocarriers for cancer therapy date back to the late 1970s, when liposomes responsive to hyperthermia were created for controlled release [68]. Nanocarriers specifically directed to the tumor microenvironment have been designed either sensitive to intrinsic (*e.g.*, pH, enzymes, reducing agents) or extrinsic stimuli (*e.g.*, light, heat, ultrasound, magnetic fields). Usually, the stimulus induces a change in the nanomaterial's conformation, promoting drug release [69]. Currently, two stimuli responsive nanocarriers have reached clinical trials [70]. Nevertheless, some limitations remain to be overcome: i) external stimuli hardly penetrate tissues, ii) precise localization of nanocarriers needs to be achieved for preventing damage to healthy cells, and iii) increased biocompatibility is required before clinical translation.

Watson *et al.* have demonstrated that the use of an ultrasound based external stimulus was able to disrupt physiological barriers, favoring liposomes extravasation and accumulation into tumor tissue [71].

Table 1. FDA and EMA-approved liposomal formulations for cancer therapy.

Drug	Material	Cancer Type	Year	Refs.
Doxil®/Caelyx®	Liposomal doxorubicin	Kaposi's sarcoma Advanced ovarian cancer Multiple myeloma	1995 2005 2008	[72]
DaunoXome®	Liposomal daunorubicin	Kaposi's sarcoma	1996	[73]
Myocet®	Liposomal doxorubicin citrate	Metastatic breast cancer	2000	[74]
Mepact®	Liposomal mifamurtide	Osteosarcoma	2009	[75]
Marqibo®	Liposomal vincristine	Acute lymphoblastic leukemia	2012	[76]
Onivyde®/MM-398	Liposomal irinotecan	Pancreatic adenocarcinoma	2015	[77]
Vyxeos®	Liposomal daunorubicin + cytarabine	Acute myeloid leukemia	2017	[78]

Regarding FDA and EMA-approved liposomal formulations, Table **1** [72-78] reports the liposomal formulations currently approved for cancer therapy [55]. Doxil®, administered as single agent in patients subjected to stable antiretroviral therapy, demonstrated durable responses in 40% of them [72].

One study, involving women affected by metastatic breast cancer, showed improved disease upon administration of Myocet® respect to doxorubicin, in combination with cyclophosphamide. In a second study, 26% of women receiving either Myocet® alone or doxorubicin alone improved with treatment. In a third study, disease improved in 46% of women administered with Myocet® and cyclophosphamide as compared to 39% of women receiving epirubicin with cyclophosphamide. Notably, fewer heart problems occurred in patients receiving Myocet® than in those administered with doxorubicin [74].

Beneficial effects have also been observed in patients treated with Mepact®, with no disease recurrence in 68% of patients, upon its administration. Notably, death risk was also significantly reduced [75].

2.3. DNA Origami

DNA is a macromolecule with self-assembly properties and has emerged as a nanomaterial that can be engineered for synthetizing multifunctional drug delivery nanocarriers. In particular, DNA origami technology has allowed to fabricate nanostructures with several shapes, able to be loaded with drugs for cancer targeted therapy. Seeman was the first to demonstrate the possibility to obtain DNA nano-constructs, thanks to its intrinsic ability to fold by complementary base pairing [79]. Moreover, due to its biological nature, it is perfectly biocompatible, easily modifiable and can be functionalized with lipids, proteins and inorganic molecules [80, 81]. In 2006, Rothemund *et al.* demonstrated that a long single stranded DNA could be folded creating a scaffold and many short ssDNA could help keeping the structure stable [82]. Different moieties have been attached to DNA origami scaffolds, for example cholesterol, that favors lipid fusion [83].

The most important advancement linked to DNA origami is the possibility to encapsulate drugs, enhancing their efficacy in chemotherapy, reducing drawbacks and overcoming multidrug resistance. DNA origami has been shown to be poorly cytotoxic and stable in physiological environment, probably due to its complex conformation that protects from nuclease degradation [84]. Modifications in DNA origami structure may significantly improve its properties: a lipid bilayer encapsulated DNA origami nanostructure has been demonstrated to be sufficiently stable after intravenous injection [85]. Zhang *et al.* showed high efficacy of DNA triangles carrying doxorubicin in mice [85]. Indeed, DNA origami resulted to be the ideal system for carrying intercalating chemotherapeutic agents, depending on their complexity and relaxation state [86].

In order to obtain targeted nanocarriers, the addition of DNA aptamers has allowed to induce conformational changes in response to target molecules [87]. Notably, DNA origami has also been made responsive to external stimuli for triggered release. A photolabile crosslinker was incorporated and, upon illumination, DNA nanocages released their cargo in a controlled manner [88].

DNA nanostructures have also been modified by attaching targeting ligands such as folate [89], cell penetrating peptides [86] and transferrin [81]. Triangular and tubular DNA origami loaded with doxorubicin have been delivered to breast cancer cells increasing apoptosis, thanks to finely controlled release [90].

Photodynamic Therapy (PDT) is a cancer treatment that exploits the use of photosensitizers to kill cancer cells, even though these molecules are often poorly absorbed and soluble. Therefore, nanocarriers have been employed for enhancing their efficacy [91]. Gold nanorods have been functionalized on DNA origami and injected in mice [92, 93]. Results clearly demonstrated that these nanostructures localized in tumor cells, causing death upon Near Infrared (NIR) irradiation [94]. Very recently, an autonomous DNA origami robot has been designed and functionalized with a DNA aptamer recognizing nucleolin and carrying the blood coagulation protease thrombin. This nanorobot was able to activate coagulation, tumor necrosis and inhibit tumor growth *in vivo* [95].

DNA origami nanocarriers have not yet entered clinical trials. Although they are very stable and not toxic, their pharmacodynamic properties together with their long-term cytotoxicity, need to be further unveiled into animal models. Even though they overcome multidrug resistance, their internalization routes need to be investigated. Additional studies will be performed for definitively demonstrating their cancer targeting abilities and reduced side effects respect to conventional agents and for confirming their therapeutic selectivity and specificity.

3. BIOMIMETIC NANOCARRIERS FOR CANCER THERAPY

Nanoparticles efficacy can be limited by a series of factors linked to physiological mechanisms, such as permeability of endothelial vessels, blood pressure and recognition by the immune system.

In the past few years, bio-inspired nanoparticles highly biocompatible, biodegradable and able to carry specific cargoes across the body with enhanced vascular permeability, have been designed [96]. Multiple functionalizations, by means of antibodies and molecules binding membrane receptors overexpressed on target cells, have been realized in order to finely direct each vector to its target tissue. Recently, new strategies have been introduced for cancer therapy. In particular, camouflage of nanomaterials with membranes derived from different kinds of cells, such as Red Blood Cells (RBCs) [97], platelets [98], leukocytes [99] and tumor cells [100], has been demonstrated to improve their properties, both in

terms of longer circulation times and reduced recognition by immune cells.

3.1. Red Blood Cells

Red blood cells are naturally able to circulate in our body without being recognized by immune system cells and physiologically lack cellular organelles. Importantly, they express transmembrane protein CD47, that acts as an inhibitor of phagocytosis mediated by macrophages [101].

In 2011, polymeric nanoparticles were covered by erythrocyte membranes showing high circulation times in blood and reduced uptake by macrophages, as compared to Polyethylene Glycol (PEG)-functionalized lipid-polymer hybrid nanoparticles. In this study, it was demonstrated that RBCs camouflage imparted increased stability and higher loading efficiencies to the nanoparticles [97].

Luk *et al.* demonstrated that doxorubicin could be efficiently delivered *in vivo* by Poly(Lactic-co-Glycolic Acid) (PLGA) nanoparticles coated with RBC membranes, achieving successful inhibition of tumor cell proliferation [102]. Su *et al.* designed polymeric nanoparticles enclosing paclitaxel and coated with a shell derived from red blood cells. They were administered together with the tumor-penetrating peptide RGD, demonstrating targeting ability in a breast cancer model and capacity to reduce lung metastases [103]. PLGA nanoparticles encapsulating perfluorocarbon and coated with RBC membranes achieved long circulation times and, upon enhanced loading of oxygen, they were able to deliver it to the solid tumor, increasing radiotherapy efficacy [104].

Inorganic nanoparticles have also been enclosed in RBC membranes, preserving their properties while giving biomimetic properties to gold nanoparticles [105].

Intriguingly, intact red blood cells have been used as vehicles for nanoparticles responsive to external stimuli. RBCs attached to iron oxide nanoparticles carrying the photosensitizer chlorine [6] and loaded with doxorubicin showed a synergic effect in an *in vivo* model, combining photodynamic therapy and response to a magnetic field [106]. More recently, red blood cells carrying oxygen and photosensitizers induced *in vivo* tumor death upon stimulation [107].

An interesting approach combined Photothermal Therapy (PTT), PDT and chemotherapy for breast cancer treatment. RBCs-based vesicles, containing oxyhemoglobin (PDT) together with a photosensitizer (PTT) and loaded with doxorubicin, inhibited cell growth, induced cell apoptosis after laser irradiation and suppressed tumor recurrence and metastasis [108].

3.2. Platelets

Platelets have been used as camouflage for nanomaterials because of their biological properties and proven involvement in tumorigenesis [109]. Indeed, cancer cells induce inflammation that retrieves platelets to the tumor site favoring angiogenesis and allowing Circulating Tumor Cells (CTCs) extravasation [110]. Silica nanoparticles coated with platelet derived membranes and functionalized with Tumor Necrosis Factor (TNF)-Related Apoptosis Inducing Ligand (TRAIL), efficiently targeted CTCs in lung vasculature and reduced metastases *in vivo* [111].

Core-shell nanocarriers coated with platelet membranes and carrying TRAIL and doxorubicin in a breast cancer model demonstrated effective in apoptosis induction and chemotherapeutic agent intracellular release, with inhibition of metastatic progression [112].

An interesting approach based on two modules, a nanocarrier functionalized with RGD peptide able to target tumor vessels and induce inflammation, and a second nanovehicle, coated with platelet membranes and carrying paclitaxel, efficiently accumulated at the tumor site amplifying active tumor targeting and releasing the chemotherapeutic agent *in vivo* [113, 114].

3.3. Immune Cells

Immune cell membranes have been exploited as camouflage strategy. Recently, leukocyte derived membranes were used to coat nanoporous silicon particles [99]. Membrane proteins integrity and proper functioning were demonstrated, such as reduction of mononuclear phagocyte system uptake, limitation of immune responses and good tumor targeting ability [115, 116].

Leukosomes, constituted by leukocyte derived membranes and lipidic vesicles, efficiently delivered chemotherapeutics and facilitated tumor locus imaging *in vivo* by preferentially targeting inflamed endothelium much more efficiently than liposomes [117].

Liposomes carrying emtansine were also coated with macrophage derived cell membranes targeting a breast cancer mouse model. These nanocarriers were able to reach metastatic cells inhibiting their proliferation [118]. Kang *et al.* showed that neutrophil-mimetic nanoparticles loaded with proteasome inhibitor carfilzomib, specifically targeted CTCs limiting metastatic progression in a breast cancer model [119].

3.4. Tumor Cells

Due to the capacity of cancer cells to be subjected to homotypic aggregation and to undergo immune escape mechanisms, their membranes have been used for nanomaterials camouflage [100].

PLGA nanoparticles have been coated with melanoma cell derived membranes demonstrating the feasibility of the approach and the presence of cancer cell antigens on the membranes, that clearly favored nanoparticles uptake in tumor cells. By coupling nanoparticles with an FDA approved lipopolysaccharide-derivative binding Tlr-4, Fang *et al.* were able to stimulate dendritic cell maturation, inducing subsequent immune responses directed against the tumor. Homotypic recognition between cancer cells conferred specific tumor targeting properties for drug delivery [120]. Polymeric nanoparticles coated with breast cancer cell membranes and loaded with paclitaxel demonstrated their ability to target homotypic cells and reduce induced immune responses. Nanocarriers were administered *in vivo* and displayed effective accumulation, both at tumor and metastatic loci, more than the free molecule *per se* [121].

Chen *et al.* developed biomimetic PLGA nanoparticles covered with breast cancer derived membranes fused to indocyanine green, showing their photothermal activity and fluorescence/photoacoustic imaging properties *in vivo* (*i.e.* theragnostic application) [122].

The possibility to interfere with tumor microenvironment modifying its physiology and modulating its mechanisms can help inhibiting tumor growth and metastatic progression [123]. Biomimetic nanoparticles have been designed not only as efficient nanocarriers but also as vehicles of precise features present on membrane donor cells. Although very promising, some properties of these nanomaterials need to be further investigated before clinical application. Establishing reliable protocols for isolating membranes from patient's cells is an essential requisite in order obtain sufficient amounts for *in vivo* therapeutic effectiveness.

As reported in Fig. (**2**), current cancer clinical trials involving nanocarriers mainly exploit liposomes and exosomes, that have been already widely investigated in preclinical studies (Fig. **2A**). In the last decade, exosomes gained a prominent role in the overall landscape of cancer clinical trials (Fig. **2B**).

4. CARRIER-FREE NANOMATERIALS FOR CANCER THERAPY

One of the greatest challenges to be faced before translating nanoparticles into the market is upscaling their synthesis procedure

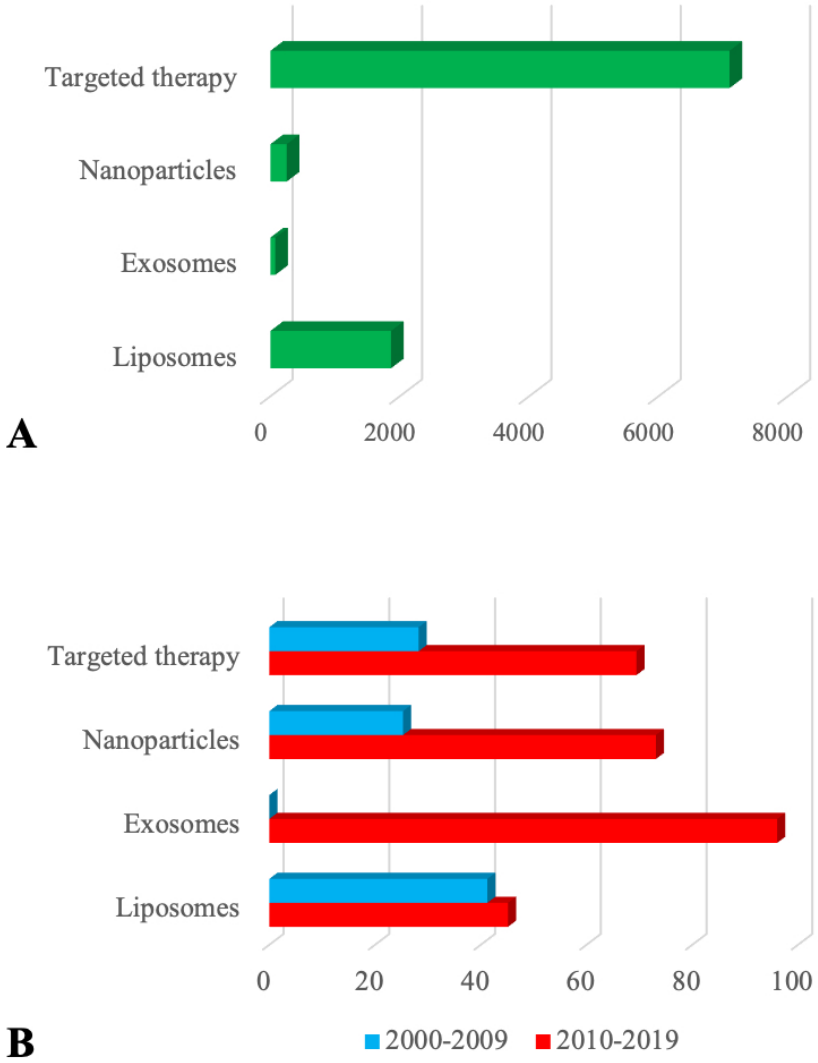

Fig. (2). Cancer clinical trials. (**A**) Total number of clinical trials for nanocarriers applied to cancer therapy currently registered on www.clinicaltrials.gov. (**B**) Number of clinical trials started during the last two decades, 2000-2009 (cyan) and 2010-2019 (red), represented as percentage respect to the total studies shown in **A**. Accessed December 11, 2019. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

[124]. Indeed, often some excipients used during fabrication may remain upon purification and can be released after *in vivo* administration and material biodegradation, potentially inducing undesired adverse effects, such as inflammation, immune responses or long-term toxicity [125, 126].

Thus, important work has to be performed for improving nano-formulations, avoiding not necessary ingredients. An interesting option comes from carrier-free nanomedicine, based on the self-assembly of therapeutic molecules without the need of external nanocarriers. The advantages of this approach are multiple: i) prevention of rapid drug clearance, ii) high loading efficiency, iii) small size and high tumor tissue penetration, iv) large scale fabrication [127, 128]. Carrier-free nanomaterials can be classified into four main categories: drug nanocrystals, prodrug self-assembled nanoparticles, drug-drug conjugates, antibody-drug conjugates. In the following section, a few examples of researches ongoing in this field are reported.

Drug nanocrystals consist of pure molecules aggregated by self-assembly in a dynamic equilibrium that provides internal order and stability. Their aggregation is favored by the introduction of hydrophilic groups. As an example, doxorubicin has been mixed to 10-hydroxycamptothecin and demonstrated enhanced cytotoxicity in breast cancer [129]. Notably, already six nanocrystal compounds have been available on the market and some are under evaluation in clinical trials [130, 131].

Prodrug self-assembled nanoparticles are produced by drugs decorated with small groups, which can become active upon external stimuli or at specific intracellular conditions (*e.g.*, pH) [132]. Amphiphilic prodrugs can assemble into nanoparticles by intermolecular interactions created by hydrophobic regions of insoluble drugs [133]. Stimuli responsive cleavable linkers, that contribute to finely regulate the release of drugs under tumor microenvironment conditions and/or the presence of specific enzymes, can activate prodrugs into drugs able to efficiently inhibit tumor proliferation [132, 134]. For example, matrix metalloproteinases overexpressed in tumor extracellular matrix can be exploited to disassemble prodrugs, providing drug release. Tanaka *et al.*, prepared an MMP7-sensitive precursor of hydrogelation. In absence of MMP7, hydrogelation was inhibited due to the conjugation of the gelator with gelation-preventing moiety. The introduction of peptide linkers sensitive to MMP7 made them cleavable by converting the prodrug into a hydrogel, able to initiate necrosis of cancer cell [135].

Drug-drug conjugates are constituted by hydrophobic and hydrophilic drugs for targeted delivery. Water soluble antitumor drugs

have been conjugated to poorly water-soluble drugs with a hydrolysable linker. In the case of irinotecan-doxorubicin, their aggregation caused fluorescence quenching. Upon linkage break and drugs release, dual-color fluorescence was recovered and allowed tracking their location during cancer therapy [136]. Interestingly, these nanoparticles efficiently overcome multidrug resistance in breast cancer.

Finally, Antibody-Drug Conjugates (ADCs) are constituted by monoclonal antibodies and cytotoxins, linked together by chemical linkers. This kind of carrier-free nanomedicines recognize a specific antigen expressed on cancer cell membrane, thanks to the presence of the antibody. Once the specific binding occurs, the complex is endocytosed and the cytotoxic cargo is carried to lysosomes and endosomes, where it is released killing cancer cells. Several antibody-drug conjugates have been already approved by FDA or are under pre-clinical evaluation [137]. In 2000, gemtuzumab ozogamicin was approved for acute myeloid leukemia treatment. This drug was constituted by humanized IgG4 antibody directed against CD33 surface receptor and conjugated to calicheamicin. Seven years after it was withdrawn [138], it has been approved anew by FDA for administration alone or in combination with chemotherapy in AML newly diagnosed adults and in patients with relapsed/refractory disease [139].

In 2019, ado-trastuzumab emtansine was approved by FDA for the adjuvant treatment of HER2-positive early breast cancer with residual invasive disease after treatment with taxane and trastuzumab-based therapy. The clinical trial demonstrated that the treatment significantly prolonged overall and progression-free survival of patients [140].

Vadastuximab talirine, constituted by a novel synthetic Pyrrolobenzodiazepine (PBD) dimer and a humanized anti-CD33 IgG1 antibody through a maleimidocaproyl valine-alanine dipeptide linker showed positive results in xenotransplanted mice. Its antileukemic activity was also observed in multi-drug resistant animal models, prompting its clinical evaluation in AML patients [141].

In the next future, a precise strategy for controlling drug ratios in multidrug formulations will be necessary for achieving maximized efficacy, minimizing potential side effects of carrier-free nanomedicine.

CONCLUSIONS AND FUTURE PERSPECTIVES

The advent of nanomedicine and the related technological progress has set a new perspective in cancer therapy. Generation of innovative drug delivery systems based on smart nanocarriers has provided efficient strategies to overcome some of the limitations linked to conventional therapy, such as multidrug resistance, cytotoxicity and several undesired severe effects. The introduction of actively targeted nanocarriers has given the possibility to obtain highly selective nanomedicine, reducing potential drawbacks. However, several challenges remain to be faced before definitive introduction of smart nanocarriers into clinical practice. One limitation of nanoparticles production relies on the difficult scalability of the fabrication procedures and in the setting of strict criteria for quality control. Large collaboration between basic research scientists, clinical committees, pharmaceutical companies and governments will be required for successful application of smart nanocarriers for cancer therapy [142]. Many nanomedicines have been already approved by FDA and EMA for clinical practice and several clinical trials are currently undergoing. Nanomaterials made responsive to stimulation of one or more external stimuli are currently being developed for improving safety and efficiency of cancer nanomedicine.

Last but not least, the choice of animal models best mimicking human tumor tissues and tumor microenvironment is also required, in order to prevent inadequate evaluation of nanocarriers therapeutic efficacy.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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