Drug Targeting Strategies in Cancer Treatment: An Overview

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Abstract: Classic chemotherapy has little or no specificity for cancer cells, normally resulting in low accumulation at the tumor region (inefficacy), and in severe side effects (toxicity). This challenge has resulted in the development of several delivery strategies for chemotherapy agents to improve their concentration at the tumor site, simultaneously increasing their anticancer efficacy, while reducing the associated adverse systemic effects. In this work, the potential of drug delivery strategies involving the use of nanocarriers for controlling the biodistribution of antitumor drugs is deeply revised: passive targeting (through the enhanced permeability and retention effect, EPR effect) and active targeting (including stimuli-sensitive carriers and ligand-mediated delivery). Special attention will be also focussed on the recent approaches for overcoming multi-drug resistance. Finally, a general view of the problem of "nanotoxicity" in cancer treatment is also given.

Keywords: Active targeting, antitumor activity, cancer treatment, chemotherapy agent, colloidal drug delivery systems, controlled release, drug carrier, nanotoxicity; passive targeting.

1. INTRODUCTION

Reseach efforts in cancer have led to an increasing knowledge into its molecular origins that allow to identify new targets and to develop a wide arsenal of therapeutic agents. However, these molecules usually lack from two important requisites: *i*) they do not reach the target site in optimal quantities; and *ii*) they are not effective enough in the tumor microenvironment. For these reasons, treatment failure is frequently encountered even in those cancers that are more sensitive to chemotherapy agents. As an example, the use of the antitumor drug 5-fluorouracil (5-FU) in the treatment of advanced colorectal cancer only induces an overall response of ≈ 10 %. Even more, the combination of this anticancer drug with other chemotherapy agents has merely improved the efficacy to ≈ 45 % [1]. The physiology of the tumor is one of the key factors responsible for chemotherapy failure. Heterogeneous blood supply, interstitial hypertension, relatively long transport distances in the interstitium and cellular heterogeneities are physiologic factors that contribute to the heterogeneous and non-effective delivery of antitumor drugs to the cancer site. For instance, uniform drug diffusion is not possible due to the higher hydrostatic pressure inside the tumor mass. In addition, the non-functional lymphatic system of tumor tissues allows the drug escaping out of them and its dilution in the surroundings [2, 3]. Several important reasons also contribute to this treatment failure: *i*) unfavorable pharmacokinetics of drugs (rapid clearance and rapid biodegradation, determining a short plasma half-life) determines the use of highly toxic doses, and imposes a rigorous treatment schedule for reaching a therapeutic effect; *ii*) large biodistribution *ii*) large biodistribution and non-intended extravasation of chemotherapy agents induce severe toxicity in non-targeted regions; *iii*) poor tumor selectivity; *iv*) susceptibility to induce drug resistance in cancer cells; and *v*) unfavourable physico-chemical properties, such as hydrophobicity, promotes the unsuccessful specific accumulation of drugs at the desired region [4, 5].

With the aim of overcoming these problems, colloidal systems have been associated with chemotherapy drugs in tumor treatment. These associations should result in a specific accumulation at the cancer site and in a prolongation of the exposure of the tumor cells to these active agents. Other benefits of drug concentration at the targeted region is the improvement of its pharmacokinetic profile, the minimization of the associated toxicity, and the reduction of the formation of toxic degradation compounds, due to the *in vitro* and *in vivo* protection of the drug by the carrier [4-6]. Hence, several efforts have been focused on the development of colloidal drug carriers, mainly based on vesicular systems (liposomes and niosomes) and polymers, to effectively transport anticancer drugs to cancer [4, 7]. Special approaches are under intensive investigation: passive targeting strategies (through the enhanced premeability and retention effect) and active targeting strategies (ligand-mediated targeting and stimuli-sensitive carriers) [3, 4]. The focus of this review is to summarize the challenges and opportunities of using these targeting strategies for drug delivery to cancer. Emphasis will be placed on approaches for overcoming multi-drug resistance (MDR), a condition enabling cancer cells to become resistant to multiple different drugs of a wide variety of structure and function.

2. PASSIVE TARGETING STRATEGIES

After administering a conventional drug delivery system in cancer therapy, it displays a very important interaction with the reticuloendothelial system (RES) (mainly, liver and

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spleen). Usually, these colloids are caught in blood by the mononuclear phagocyte system (MPS), depending on their size and surface characteristics. The organs of the RES are already an important but unfavorable site of action for most anticancer drugs, where severe cytotoxicity or acute renal toxicity can occur, as a consequence of drug accumulation [8]. Although toxicity will exist, conventional colloids have a better safety profile than free chemotherapy agents [9]. However, due to the short biological half-life ($t_{1/2}$) of these colloidal carriers ($t_{1/2} \approx 5$ min), achieving other tumor sites is not possible and, thus, the enhancement of the anticancer efficacy of antitumor molecules is limited to targeted tumors in the RES [10].

The development of long-circulating nanoparticles (NPs) has allowed for many nanocolloids to exploit structural irregularities in the tumor vasculature. In fact, passive targeting is based on the unique properties of the cancer microenvironment: *i*) a dysfunctional lymphatic drainage, which results in enhanced fluid retention in the tumor interstitium; and *ii*) leaky vasculature, whose permeability to macromolecules is higher than that of healthy tissues [11]. The biological fate of injected drug delivery colloids can be controlled by adjusting their size and surface characteristics, in order to achive an extensive non-specific accumulation in the tumor (passive targeting) [12, 13]. The size should be large enough to prevent a rapid leakage into capillaries but small enough to escape capture by MPS. This will allow reaching tumor tissues by passing through the gap junction between endothelial cells of the leaky tumor vasculature (100 to 600 nm). Regarding the surface characteristics, any given drug carrier should ideally have a hydrophilic surface to retard the macrophage capture. This can be achieved in two ways: *i*) coating their surface with a hydrophilic polymer, such as poly(ethylene glycol) (PEG), protects them from opsonization by repelling plasma proteins; and, alternatively, *ii*) the drug delivery system can be made of block copolymers with hydrophilic and hydrophobic domains [7, 14]. Drug carriers with both properties (very small size and hydrophilicity) are expected to present selective extravasation in pathological sites, prolonged $t_{1/2}$ and could also directly target tumors located outside the MPS regions. This tumor specific disposition, known as the enhanced permeability and retention (EPR) effect (Fig. **1**), is based on the "leaky" vasculature of cancer tissues which allows the non-specific extravasation and accumulation of these nanoplatforms [7, 8, 12, 15, 16].

As previously commented, the formulation of long-circulating carriers by surface-coating with hydrophilic polymers (generally, PEG) will enhance their accumulation into the tumor site. A shell of hydrophilic and neutral chains will be provided at the particle surface, mainly by physical adsorption or chemical conjugation, that is able to repel plasma proteins (opsonins) and, as a consequence, retard the opsonization process that determines macrophage capture [7, 15]. As an example, solid lipid nanoparticles (SLNs) were surface functionalized with hydrophilic polymers, demonstrating a very long-circulation: $t_{1/2} > 48$ h in humans [17, 18]. Doxorubicin (DOX)-loaded SLNs (≈ 100 nm) were surface decorated with pre-conjugated stearic acid-PEG 2000. Compared to conventional SLNs, this PEGylated colloid presented shielded surface charge and increased hydrodynamic volumes, thus minimizing the uptake by murine macrophages. It was observed that after intravenous (i.v.) injection to rats, these sterically stabilized DOX-SLNs showed large plasma $t_{1/2}$ (lower uptake by the MPS) in comparison to conventional DOX-loaded SLNs and free DOX [19]. Increasing plasma $t_{1/2}$ of liposomal systems can be also achieved by using synthetic phospholipids (which are conjugated to gangliosides), and by grafting PEG [20]. PEGylated liposomes developed a 200-fold decrease in plasma clearance (from 22 to 0.1 L/h), a nearly 100-fold increase in the area under the time-concentration curve (AUC) and, due to their minimal interaction with healthy tissues after systemic administration, up to 50-fold decrease in the volume of distribution (from 200 to 4.5 L) [21]. This enhancement in the biodistribution and pharmacokinetic properties has been described after the administration of DOX-loaded liposomes in patients bearing recurrent high-grade gliomas [22].

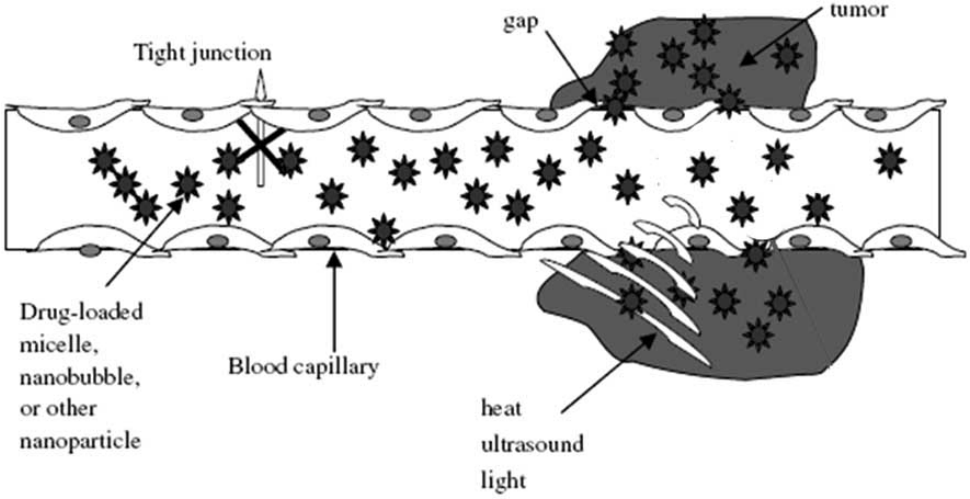

Fig. (1). Passive tumor targeting of drug-loaded nanoparticles through the defective tumor microvasculature. The drug delivery systems are only able to leave the blood when reaching vascular abnormalities. Heat, light, or ultrasound can be also used to trigger drug release locally in the tumor. Reprinted with permission from Ref. [16]. Copyright Elsevier (2007).

Long-circulating polymeric NPs have also been developed for drug passive targeting to cancer. For example, tamoxifen was loaded to long-circulating poly(ε-caprolactone) (PCL) NPs that were surface-modified with pluronic® F-68 or pluronic® F-108 by physical adsorption [23]. After i.v. injection in mice bearing MDA-MB-231 xenografts, a human breast cancer cell line, the surface-modified NPs induced a higher drug accumulation into the tumor (≈ 26 % at 6 h post injection) compared to the free drug in solution. From these results, we can conclude that the presence of poly(ethylene oxide) (PEO) chains on the NP surface very efficiently enhanced blood circulation time and tumor targeting.

In general, it can be said that current surface modification methods require tedious and inefficient synthesis and purification steps, and are not easily amenable to incorporating multiple functionalities on a single surface. Recently, a versatile, single-step surface functionalizing technique for polymeric NPs has been developed. The technique is based on the fact that when a diblock copolymer like poly(D,L-lactide)-PEG (PLA-PEG) is added to an oil/water emulsion (a common medium for the formulation of polymeric NPs), the PLA block partitions into the polymer containing organic phase, and PEG block partitions into the aqueous phase. The final removal of the organic solvent results in the formation of NPs with PEG on the surface (Fig. **2**) [24].

3. ACTIVE TARGETING STRATEGIES

A drug delivery system generally comprising a drug-loaded polymer or liposome that depends only on passive targeting strategies inexorably faces intrinsic limitations due to its low specificity for the tumor tissue. Specific targeting (active targeting) of drugs to cancer tissues can be obtained by several strategies that allow a selective delivery to the target region. Typically through both local and systemic administration of drug carriers surface functionalized with a specific recognition mechanism (ligand- or receptor-mediated targeting: targeting molecules conjugated on their surface that can bind to specific ligands that are unique to tumor cells) or by means of stimuli-sensitive drug carriers (colloids engineered to experience modifications in their structure and physical properties under small changes in the environment, leading to triggered drug release specifically at the tumor site) [3, 7, 14].

3.1. Ligand- or Receptor-Mediated Targeting

This drug delivery strategy has emerged as a valuable approach to target the specific site of interest, while simultaneously avoiding the associated systemic adverse effects. The specific recognition of the biological target through molecular recognition processes (ligand-receptor or antibody-antigene interactions) can be made possible by chemical conjugation of the surface of the drug delivery system to targeting ligands which are tissue-, organ- or cell-specific. Typically this approach leads to receptor-mediated cell internalization [10, 15]. For example, paclitaxel targeting to liver cancer cells was investigated by using PEGylated poly(γ-benzyl-L-glutamate) particles endcapped with galactose moieties which specifically binds asialoglycoprotein (ASGP) receptors in hepatocytes. This formulation showed greater toxicity in cells expressing ASGP receptors (HepG2) than the free chemotherapy agent [25]. Potential methods for active targeting has been extensively investigated, including colloids coupled to specific monoclonal antibodies (MAb) [26, 27], as well as ligand-coated particles targeting proteins expressed on endothelial cells forming the neovasculature of growing tumors (e.g., integrin surface receptor) [28], or on cancer cell membranes (e.g., folate receptor) [29].

3.1.1. Monoclonal Antibodies (MAbs)-Mediated Targeting

FDA-approved MAbs can be directed against several surface antigens or receptors of tumor cells [30]. Immunoliposomes (liposomes conjugated with MAb) can be prepared by either attachement of the MAb directly to the liposome phospholipid headgroup or to PEG endings [31, 32]. One particular target of immunoliposomes is the human epidermal growth factor receptor-2 (HER-2). Several investigations have shown that DOX-loaded anti-HER-2 immunoliposomes are able to display increased therapeutic efficacy towards different breast cancer xenograft models when compared to naked PEGylated liposomes [26, 27]. PE38KDEL-loaded anti-HER2 PEGylated liposomes (PE-HER-liposomes) have exhibited superior antitumor activity and less non-specific toxicity than free PE38KDEL (a 38 kDa mutant form of *Pseudomonas* exotoxin A). These liposomes were constructed with Fab′ of recombinant humanized anti-HER2 MAb (anti-HER2 Fab′) covalently linked to PEGylated liposomes containing PE38KDEL (PE-liposomes). Incorporation of pyridylthiopropionoylamino-PEG-distearoylphosphatidylethanolamine (PDP-PEG-DSPE)

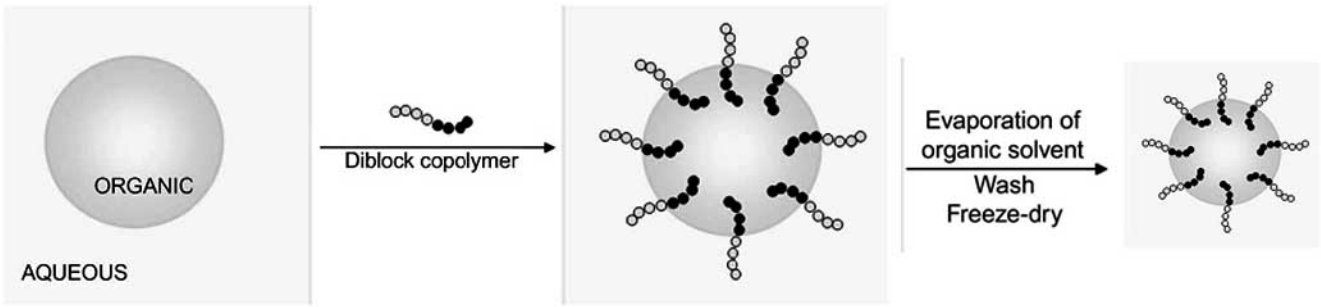

Fig. (2). Behavior of an amphiphilic diblock copolymer in an oil/water biphasic system. This characteristic disposition is the base of an easy single-step surface functionalizing technique for preparing long-circulating polymeric NPs. Reprinted with permission from Ref. [24]. Copyright Elsevier (2009).

into PEGylated liposomes followed by mild thiolysis of the PDP groups resulted in the formation of reactive thiol groups at the periphery of the liposomes. Efficient attachment of maleimide-derivatized anti-HER2 Fab′ took place under mild conditions. Flow cytometry and confocal microscopy showed that PE-HER-liposomes developed receptor-specific binding and internalization for HER2-overexpressing SK-BR3 cells. Remarkably, PE-HER-liposomes were more cytotoxic than non-functionalized liposomes in HER2-overexpressing breast cancer cells [33]. PEGylated immunoliposomes have also been conjugated to rat 8D3 MAb to the mouse transferrin receptor (in order to cross the blood brain barrier) and to 83-14 MAb to the human insulin receptor (to target the implanted U87 human glioma cells within the brain parenchyma). These immunoliposomes were loaded with a DNA expression plasmid that encode for a short hairpin RNA (shRNA) fragment designed to silence the expression of the oncogenic gene EGFR (human epidermal growth factor receptor). Results revealed that this gene therapy strategy resulted in an increase of ≈ 90 % in the survival time of mice with advanced intracranial brain cancer [34].

Polymeric nanoplatforms have also been surface decorated with MAb for drug active targeting to cancer. Poly(D,L-lactide-co-glycolide) (PLGA) NPs surface functionalized with cytokeratin (a specific MAb to invasive breast epithelial cells, that additionally prevents the generation of plasmin, a central extracellular protease involved in malignant progression) and loaded with cystatin (a potent protease inhibitor that can neutralize the excessive proteolytic activity associated with the invasive and metastatic potential of breast tumor cells) have been designed as a very promising approach to improve the efficacy of therapy in patients with breast tumors, compared to the application of individual protease inhibitors [35]. Paclitaxel-loaded PLGA/montmorillonite NPs were conjugated with the HER-2 Trastuzumab antibody for targeted breast cancer chemotherapy. It was observed in Caco-2 colon adenocarcinoma cells and in SK-BR-3 breast cancer cells that this surface functionalized composite polymer achieved significantly higher cellular uptake than the pure colloid. Moreover, *in vitro* cytotoxicity on SK-BR-3 cells showed that the anticancer action of the drug formulated in the surface-decorated NPs was 12.7-fold higher than that of the bare polymer, and 13.1-fold higher than the free drug (Taxol®) [36].

3.1.2. Peptide-Mediated Targeting

The existence of the RGD sequence (arginine-glycine-aspartic acid) in peptides and peptidomimetics, an important recognition system for cell adhesion, gives them the possibility of binding to various integrins overexpressed on endothelial cells in tumor neovasculature [37]. Hence, the conjugation of anticancer drug carriers with these peptides can increase their targetability to the tumor vasculature, inducing its destruction [38]. In addition, surface functionalization with adequate peptides will also allow selective targeting to malignant cells that overexpress the corresponding integrin and, subsequently, the anticancer efficacy of the drug will be significantly enhanced. Inulin multi-methacrylate (IMMA) NPs (≈ 30 nm) loaded with DOX and surface functionalized with a cyclic peptide containing the RGD sequence cyclo-(Arg-Gly-Asp-D-Phe-Cys) have enhanced the antitumor efficacy of this drug after i.v. injection [39]. Paclitaxel-loaded albumin NPs were surface functionalized with the peptides CREKA and LyP-1 for active tumor targeting. It was demonstrated that after i.v. injection into mice bearing MDA-MB-435 human cancer xenografts, the colloid selectively accumulated in tumor blood vessels, forming aggregates that contained red blood cells and fibrin. This drug carrier produced a statistically highly significant inhibition of tumor growth compared to the untargeted carrier [40]. 5-FU-loaded PEGylated liposomes with PR_b as the functionalizing moiety (a peptide sequence that mimics the cell adhesion domain of fibronectin) were able to target colon cancer cells that express the integrin $\alpha_5\beta_1$. These surface functionalized PEGylated liposomes were internalized through $\alpha_5\beta_1$-mediated endocytosis, and exerted higher cytotoxicity on CT-26 *wt* cells than non-surface decorated 5-FU-loaded PEGylated liposomes [41]. A novel liposomal system specifically directed by the peptide ligand PH1 to Tie2 expressing cancer cells has been recently developed. The PH1 peptide was selected by phage display library screening combined with surface plasmon resonance binding assays. It was covalently conjugated to the distal end of PEG2000-DSPE lipid and loaded onto liposome membranes as the targeting ligand. These PH1-PEG-liposomes containing cisplatin were showed to binded tightly to Tie2 positive cells, mediated active endocytosis of the drug, and resulted in much higher cell specific cytoxicities than non-targeted PEGylated liposomes [42].

New delivery approaches have been investigated in order to overcome the problems related to the systemic delivery of small interfering RNA (siRNA) in gene therapy: low penetration ability through the cellular plasma membrane, and limited stability in blood. siRNA NPs were formulated with poly(propyleneimine) (PPI) dendrimers and stabilized with dithiol containing cross-linker molecules and a PEG coating. A synthetic analog of the luteinizing hormone-releasing hormone (LHRH) peptide was conjugated to the distal end of PEG, in order to direct the siRNA NPs specifically to the cancer cells. *In vivo* results demonstrated that this layer-by-layer modification and targeting approach confers to the siRNA NPs stability in plasma and intracellular bioavailability, provides for their specific uptake by tumor cells, and assures the accumulation of siRNA in the cytoplasm of malignant cells and an efficient gene silencing. In addition, biodistribution data confirmed the high specificity of this targeting delivery approach [43]. A non-viral nanovector has also been developed for gene therapy by PEGylation of DNA-complexing polyethylenimine (PEI) in NPs functionalized with an Alexa Fluor 647 near infrared fluorophore, and the chlorotoxin (CTX) peptide which binds specifically to many cancers (Fig. **3**). Surface engineering of PEI NPs minimized the potential toxicity of PEI (a gene carrier that induces gene transfection with high efficiency) to healthy cells. Compared to conventional PEI NPs, this nanovector demonstrated high levels of targeting specificity and gene transfection efficiency with both C6 glioma and DAOY medulloblastoma tumor cells. It is expected that by using CTX as the targeting ligand, the

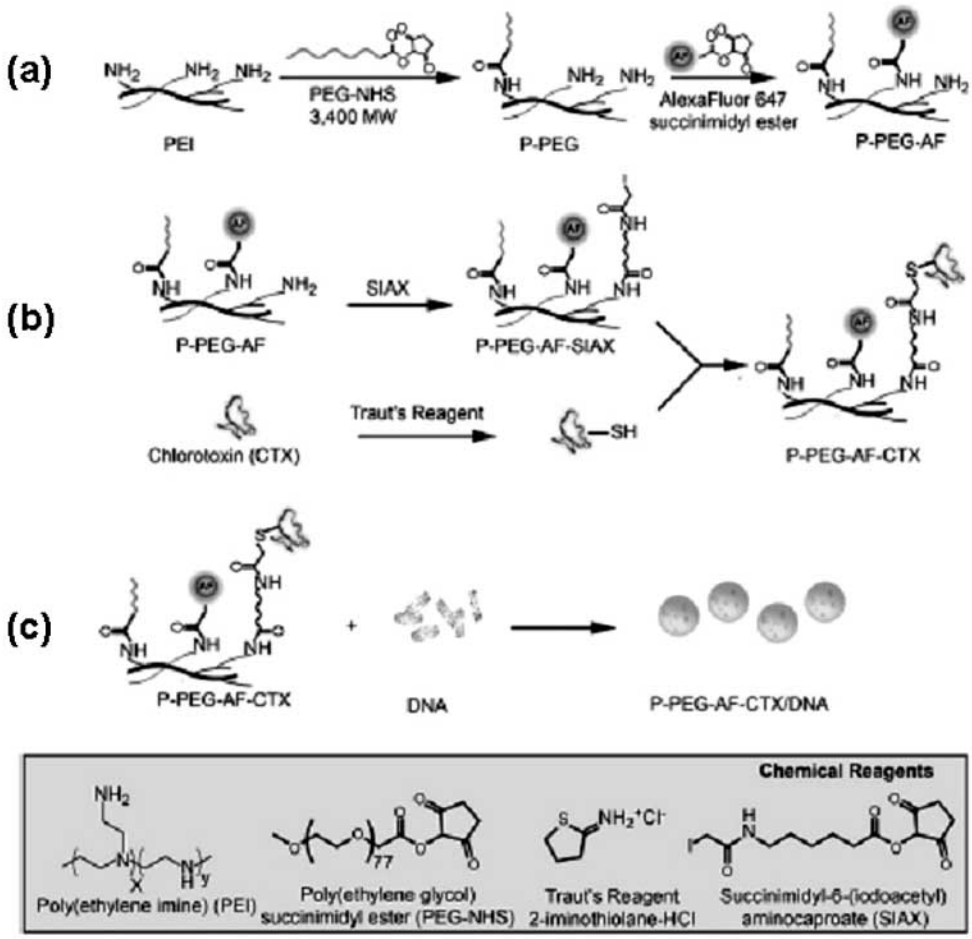

Fig. (3). Formulation of PEGylated DNA-complexing polyethylenimine (PEI) NPs surface functionalized with an Alexa Fluor 647 near infrared fluorophore (AF) and the chlorotoxin (CTX) peptide. Nanovector preparation scheme. (a) PEGylation of PEI polymer and modification with AFs. (b) P-PEG-AF modification with succinimidyl-6-(iodoacetyl) aminocaproate (SIAX), modification of chlorotoxin (CTX) with Traut's reagent to produce free thiols on peptide and subsequent reaction of the thiol modified CTX peptide with reactive iodoacetate group of P-PEG-AF-SIAX yielding P-PEG-AF-CTX. (c) The polymeric construct complexed with DNA to generate the targeting nanovector (P-PEG-AF-CTX/DNA). Reprinted with permission from Ref. [44]. Copyright Elsevier (2009).

nanovector may serve as a widely applicable gene delivery system for several cancers [44].

Very recently, it has been investigated the formulation of 17-allylamino-17-demethoxy geldanamycin (17-AAG) in sterically stabilized (PEGylated) phospholipid nanomicelles. 17-AAG is an inhibitor of the heat shock protein 90 (Hsp90) function that has been proposed as a chemotherapy agents in breast cancer. Unfortunately, its null hydrosolubility and its very high hepatotoxicity limit its use. The vehiculization of this novel molecule into PEGylated phospholipid nanomicelles (16 ± 1 nm, and drug content ≈ 97 %) further surface functionalizated by grafting the vasoactive intestinal peptide (VIP) as an active targeting moiety, proved to be more cytotoxic to MCF-7 human breast cancer cells than 17-AAG loaded into non-targeted nanomicelles ($p < 0.05$) [45].

3.1.3. Integrin-Mediated Targeting

Integrins are small peptides expressed in the tumor neo-vasculature. They can be used in active targeting strategies aimed at directing chemotherapy agents to cancer cells. As an example, H2009.1 is a peptide with high affinity for the cell receptor integrin $\alpha v\beta 6$ that has been successfully incorporated to DOX-loaded polyglutamic acid conjugates [46]. Flow cytometric analysis and fluorescent microscopy proved that the conjugates were selectively internalized into $\alpha v\beta 6$ positive cells. This cellular uptake was supposed to be mediated by H2009.1, as no internalization of DOX-loaded polyglutamic acid was observed when it was conjugated to a control peptide.

Integrin targeting has also been proposed for gene delivery to cancer. DNA-encapsulated cationic polymerized liposomes were prepared bearing *avb*3 ligand targeting integrins of M21-melanoma xenograft tumors. It was demonstrated that gene expression was selectively enhanced in the tumor and that the delivery of a mutant Raf gene blocked the endothelial cell signalling and angiogenesis, causing sustained tumor regression after just one injection [28].

3.1.4. Aptamer-Mediated Targeting

Aptamers are nucleic acid ligands (DNA or RNA oligonucleotides) capable of selectively bind to target antigens. These biomolecules are characterized by an ease chemical synthesis and a small size, which make them

attractive for targeting diseases, or as therapeutics. In cancer, they can be formulated directly for tumor treatment [47], but they can also be coupled to drug delivery systems to enhance the targeting of cancer cells [48]. For instance, docetaxel-loaded PEGylated PLGA NPs were surface-decorated with A10 2'-fluoropyrimidine RNA aptamers that recognize the extracellular domain of the prostate-specific membrane antigen (PSMA), a surface antigen expressed on prostate cancer cells. It was demonstrated that the docetaxel-encapsulated NP-aptamer bioconjugates very efficiently bind to the PSMA protein expressed onto the surface of the cancer cells, being subsequently uptaken. Significantly higher *in vitro* cellular toxicity in LNCaP prostate epithelial cells was described, compared to non-targeted NPs that lack the PSMA aptamer ($p < 0.0004$). After a single intratumoral (i.t.) injection of these nanosystems, complete tumor reduction was observed in five of seven LNCaP xenograft nude mice (initial tumor volume ≈ 300 mm^3), and all animals were alive at the end of the study (109 days). In contrast, mice in the docetaxel-loaded NP group, and in the docetaxel group presented a survivability of only 57 % and 14 %, respectively [49].

3.1.5. Folate Receptor Targeting

Folate receptors are frequently overexpressed by cancer cells as a consequence of enhanced folate requirements for DNA synthesis. The interaction of a folate moiety with the folate receptor on tumor cells leads to an endocytic transport which results in cytosolic accumulation [29]. Folate-decorated NPs have been proposed not only for drug delivery to cancer, but also for cancer phototherapy, and for the preparation of quantum dots formulations (as luminescence probes) for targeted and sustained imaging in cancer diagnosis at its early stage [50-52]. Folate-coated liposomes can enhance the accumulation of chemotherapy agents in several types of tumor cells, increasing their cytotoxicity [53-55], but they can also bypass MDR of tumor cells [56]. For example, the vehiculization of DOX in folate-coated liposomes enhanced the *in vitro* drug uptake by KB (human epidermal carcinoma), and HeLa (cervical cancer) cells, which vastly overexpress folate receptors [57]. Polymeric NPs have been also surface-decorated with folate and derivatives in order to induce selective drug targeting [58, 59]. Anticancer prodrugs have been also loaded to drug carriers surface functionalized with folate to increase their cell uptake and cytotoxicity. For instance, folate-functionalized SLNs were formulated for the selective delivery of a paclitaxel prodrug (paclitaxel-2′-carbonyl-cholesterol). This formulation enhanced the inhibition of tumor growth in tumor-bearing mice, compared to non-targeted SLNs, and paclitaxel in Cremophor® EL [60].

PEGylated PEI NPs surface functionalized with folic acid (FA) (FA-PEG-PEI) and loaded with cytosine deaminase/5-fluorocytosine (CD/5-FC) or TNF-related apoptosis-inducing ligand (TRAIL) genes (FA-PEG-PEI/pCD/5-FC or FA-PEG-PEI/pTRAIL, respectively) have shown very interesting results when they were co-administered to folate receptor expressed C6 glioma cells and Wistar rats. This combination generated an additive cytotoxic effect in C6 glioma cells, this indicating that such treatment schedule using both enzyme/prodrug therapy and TRAIL immunotherapy could be a very promising approach to the treatment of gliomas [61]. Folate grafted to PEGylated poly(alkylcyanoacrylate) NPs showed a 10-fold higher apparent affinity for the folate-binding protein (FBP, a glycosylphosphatidylinositol anchored cell surface folate receptor) than free folate NPs. This conjugated nanoplatform not only selectively target tumor cells, but also improves drug internalization within them [62]. Amphiphilic block copolymer NPs (< 80 nm) prepared from methoxy-PEG and PCL can successfully enhance paclitaxel delivery to tumor cells [63]. Paclitaxel-loaded PLA-PEG NPs surface functionalized with biotin and FA have also shown very interesting results in a mouse xenograft tumor model (NCR-NU mice bearing MCF-7 xenografts). Compared to unfunctionalized PLA-PEG NPs, NP accumulation in tumors was significantly increased, resulting in a greatly improved efficacy (Fig. **4**) [24].

3.1.6. Transferrin Receptor Targeting

Transferrin receptors are overexpressed on the surface of a wide range of malignant cells. Because of the possibility of receptor saturation by endogenous plasma transferrin, alternative routes of administration (e.g., intra-site administration) [64] or specific MAb to this receptor (e.g., OX26 and TfRscFv) [65] have been proposed to achieve effective tumor targeting. Transferrin has shown very promising activity in overcoming tumor resistance to drugs caused by MDR proteins and P-glycoprotein (P-*gp*) [66]. Despite tranferrin can be directly conjugated to drugs, assuring a greater targeting [67], the conjugation of this biomolecule to drug delivery systems allows optimizing their pharmacokinetics, extending the exposure of cancer cells to chemotherapy, thanks to a selective accumulation into the tumor and to a sustained drug release from the nanocarrier [68]. *In vitro* studies have shown that transferrin-conjugated PLGA NPs loaded with paclitaxel can undergo higher uptake (3-fold) by human prostate cancer cells (PC3) than the unconjugated ones. Interestingly, a single i.t. injection of transferrin-conjugated NPs (paclitaxel: 24 mg/Kg) in a mouse model (PC3 cells injected subcutaneously) resulted in complete tumor regression. This paclitaxel delivery system also developed a greater efficacy in MCF-7 breast cancer cells due to an increased intracellular retention [68, 69]. Active targeting of brain malignancies could be also achieved by conjugation of nanoplatforms with transferrin, as this molecule could facilitate the transcytosis of the drug-loaded colloids across the blood brain barrier [70]. Recently, novel drug delivery systems have been formulated for combined cancer photothermal therapy and cancer cell imaging [71].

3.2. Stimuli-Sensitive Carriers

Stimuli-sensitive, stimuli-responsive, environmental-sensitive, smart or intelligent carriers are essentially polymeric materials wisely engineered to experience rapid changes in their structure and physical properties (disruption/aggregation, swelling/deswelling, etc.) under exposure to small modifications in the environment. These changes are reversible and, therefore, the polymer is in principle capable of returning to its initial state as soon as the trigger is removed. Stimuli may occur internally (e.g., a change in pH in certain tissues or diseased states, a change in temperature

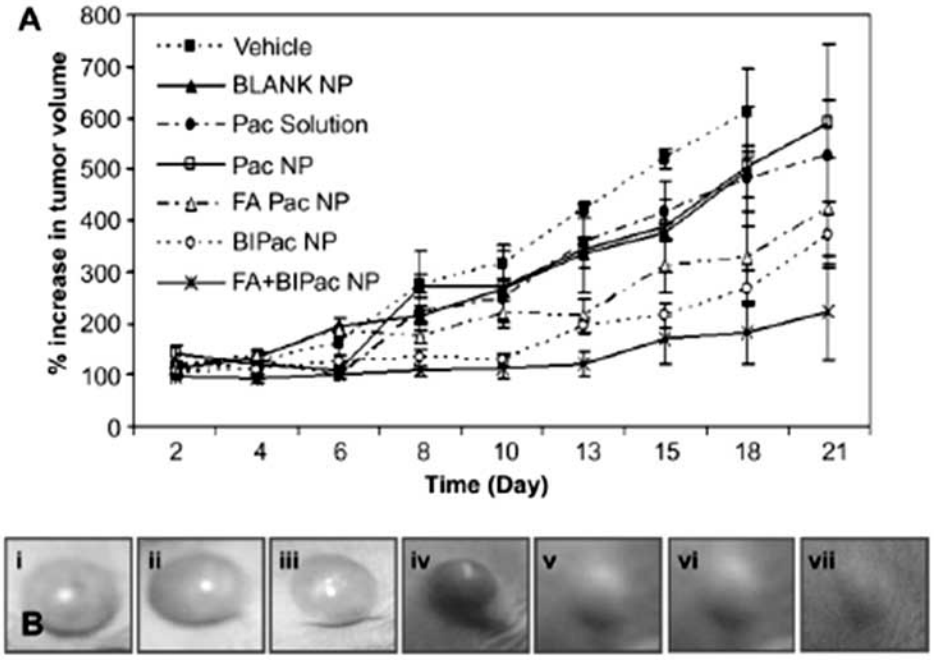

Fig. (4). Incorporation of PEG-folic acid (FA) and/or PEG-biotin (BI) onto the surface of paclitaxel-loaded nanoparticles (Pac NP) results in enhanced anticancer effectiveness. NCR-NU mice bearing MCF-7 xenografts were injected with Pac (20 mg/Kg) in solution or encapsulated into these nanoparticles (≈ 3 mg/animal) with or without surface functionalization. (A) Growth in tumor volume was determined over a period of 21 days. Data as mean ± S.D. (n = 6). (B) Representative photographs of tumor-bearing mice that received: *i*) vehicle, *ii*) blank NP, *iii*) Pac solution, *iv*) Pac NP, *v*) FA Pac NP, *vi*) BI Pac NP, and *vii*) FA + BI Pac NP. Reprinted with permission from Ref. [24]. Copyright Elsevier (2009).

or the presence of specific enzymes or antigens). Externally controlled systems rely on outwardly applied stimuli (magnetic or electric fields, light, ultrasound, etc.) that are produced with the help of different stimuli-generating devices, which ultimately results in pulsed drug delivery. This special property is widely used to trigger drug release at any given target site, but can be also utilized to concentrate the drug at the target region before allowing its release (e.g., magnetically responsive carriers). Nonetheless, temporal modulation and site-specific drug targeting is supposed to occur, and the biodistribution of the drug is minimized (and hence, the undesired side effects), meanwhile the therapeutic efficacy is enhanced [16, 72, 73].

3.2.1. Acid-Triggered Release

Local pH changes in response to specific substrates can be generated and exploited for modulating drug release from nanocarriers. In tumor interstitial fluids, as one move away from tumor vessels, the spatial and temporal heterogeneities in blood flow lead to a compromised metabolic micro-environment that determines a slightly significant decrease in pH down to ≈ 6.6. This is the consequence of a higher aerobic and anaerobic glycolisis. Thus, the acid pH that the drug carrier will face inside the tumor region differs from the physiological pH (≈ 7.4). This fact is used to control drug release into the tumor interstitium. Furthermore, the lysosomes can also be targeted: after cellular uptake, the drug conjugate will be disrupted in the lysosome under the influence of the acid pH (pH ≈ 4.5 - 5.0), and hydrolytic enzymes (e.g., cathepsin B) may also contribute to drug release [2, 16, 72, 74].

pH-sensitive drug carriers are stable in physiological pHs but, under exposure to acidic environments, they degrade releasing the entrapped drug [75-77]. All pH responsive polymers contain pendant acidic (e.g., carboxylic and sulfonic acids) or basic (e.g., ammonium salts) groups that are capable of either accepting or releasing protons in response to environmental changes in pH, thus leading to conformational modifications in their solubility or in their swelling behavior. The most commonly studied ionic polymers for pH-responsive drug release include poly(methacrylic acid), poly(diethylaminoethyl methacrylate), poly(acrylamide), poly(acrylic acid) and poly(dimethylaminoethyl methacrylate) [16, 72, 78, 79].

Poly(vinylpyrrolidone-*co*-dimethylmaleic anhydride) (PVD) is a pH-sensitive polymer that can be radically synthesized with vinylpyrrolidone and 2,3-dimethylmaleic anhydride, a pH-reversible amino-protecting reagent. PVD can release fully active drugs in response to slightly acidic pHs. Adriamycin-loaded PVD has shown superior antitumor activity against Sarcoma 180-bearing mice and less toxic side effects than free adriamycin [80]. DOX-encapsulated pH-sensitive micelles composed of poly(L-histidine)-*b*-PEG and PLA-*b*-PEG-folate have demonstrated a great antitumor efficacy in MDR ovarian A2780/DOX(R) xenografted nude mice. These drug-loaded micelles inhibit very efficiently the growth of MDR ovarian tumors in mice, with minimum weight loss (toxicity). This anticancer activity was based on folate receptor-mediated endocytosis and subsequent lysosomal disruption [81].

pH-sensitive copolymers have been also studied for drug delivery to tumors. A copolymer of poly(*N*-isopropylacryl-

amide) and chitosan loaded with paclitaxel exhibited pH-sensitive responses to tumor pH. It was observed that the cumulative drug release rate was significantly enhanced below pH 6.8, and decreased rapidly above pH 6.9. A fluorescence microscopic study confirmed that drug release was drastically promoted in tumor surroundings, while exerting less effect in normal conditions. S-180-bearing KM mice treated with these NPs showed a limited decrease in body weight and a significant tumor regression, with complete regression in > 50 % of the mice. The life span of tumor-bearing mice was significantly prolongued when they were treated with these NPs [82]. A copolymer of *N*-(2-hydroxypropyl)methacrylamide and 6-methacrylamidohexanohydrazide bearing hydrazide groups randomly distributed along the polymer chain, was conjugated with DOX (through its C13 keto group) or with keto esters [two derivatives of the anti-inflammatory drug dexamethasone: 4-oxopentanoate and 4-(2-oxopropyl)benzoate esters]. These drugs were covalently attached to the polymer backbone *via* hydrolytically labile pH-sensitive hydrazone bonds. It was determined that polymer-drug conjugates incubated in buffers modeling intracellular environment released the drugs at a rate significantly higher compared to conditions mimicking the blood stream [83].

Finally, several investigations have proved that pH-sensitive liposomes are more efficient in delivering chemotherapy agents to tumors than conventional and long-circulating liposomes, due to their fusogenic properties [7, 84, 85]. Long-circulating pH-sensitive liposomes (PSLs) surface functionalized with the epidermal growth factor receptor (EGFR) antibody and loaded with gemcitabine have shown a very interesting antitumor activity in A549 tumor-bearing BALB/c-nu/nu mouse tumor model. PSL (mean diamenter ≈ 150 nm) were synthesized using small unilamellar vesicles of dioleylphosphatidylethanolamine and cholesterylhemisuccinate (6:4 molar ratio) by the reverse-phase evaporation vesicle (REV) method [86]. Recently, a polymeric pH-sensitive liposomal nanosystem was formulated to release its content inside endosomes, through a polymer structural change following receptor-mediated internalization. Specifically, pH-sensitive immunoliposomes were obtained by including a terminally alkylated copolymer of *N*-isopropylacrylamide in the liposome bilayer and by surface coupling the anti-CD33 MAb to target leukemic cells. This novel system was very efficiently internalized by various CD33+ leukemic cell lines, while limited interaction was found for liposomes decorated with an isotype-matched control antibody. Thus, this pH-sensitive liposomal formulation showed greatly interesting possibilities in the treatment of acute myeloid leukemia [87].

3.2.2. Thermo-Sensitive Drug Delivery

Temperature-sensitive or thermo-responsive drug carriers are the most widely studied type of stimuli-responsive colloids. They are frequently made of hydrogels or polymers that present several hydrophobic groups in their structure (e.g., methyl, ethyl and propyl groups). A unique property of temperature-responsive polymers is the presence of a critical solution temperature, which is the temperature at which the physical state of the system is changed according to their composition. Generally, under heating (high temperatures) the solubility of these materials increases and drug release occurs. Poly(*N*-isopropylacrylamide) is the most widely studied and used synthetic temperature-responsive polymer in thermo-sensitive drug delivery, because its phase transition occurs at approximately body temperature but can be easily adjusted to ≈ 42 ºC by the incorporation of a hydrophilic co-monomer such as *N*,*N*-dimethylacrylamide. We can also cite other widely investigated temperature-responsive polymers, such as poly(*N*-(l)-1-hydroxymethyl-propylmethacrylamide), poly(2-carboxyisopropylacrylamide), poly(*N*-acryloyl-*N*′-alkylpiperazine), or poly(*N*,*N*′-diethylacrylamide) [16, 72].

In cancer therapy, hyperthermia is an interesting technique that allows increasing the tumor permeability and enhances the tumor uptake of several biomolecules and colloids [88, 89]. Hyperthermia itself has been shown to be cytotoxic [90]. The main limitation in the use of hyperthermia is that the tumor location must be known and accessible [91]. The technique involves locally heating the tumor region, inducing an increase in the microvascular pore size and in the tumoral blood flow. Consequently, the extravasation of the drug delivery system into the tumor region will be significantly increased. A maximum effect is observed when heating at 42 ºC, as the tumor vascular pore size is increased from ≈ 7 – 20 nm to > 400 nm. An optimal enhancement of the extravasation can be easily achieved after a careful selection of the temperature and time of heating, which could be of extreme interest in thermo-sensitive drug carriers [92]. This approach is not only used to increase tumor permeability, but also to trigger drug release exclusively into the targeted site. As previously commented, hyperthermia treatment of cancer is usually performed at 42 ºC; thus, thermo-responsive drug carriers should have their critical solution temperature above that of healthy body (37 ºC). Several liposomal formulations have been designed with a narrow temperature phase transition region, with lipid membrane heterogeneity, and leaky interfacial membrane regions, resulting in selective and controlled drug release after adequate heating [93, 94]. Sterically stabilized thermo-sensitive liposomes have been also formulated to increase tumor accumulation (due to the stealth protection), and to release > 60 % of their contents when heated at 42 ºC for 30 min [89]. Thermo-sensitive magnetoliposomes (TMs) loaded with methotrexate (MTX) have been formulated with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and cholesterol by the REV method. It was observed that > 80 % of the MTX loaded was released from TMs within 30 min when the environmental temperature increased from 37 ºC to 41 ºC, while 60 % of the drug remained inside the TMs for up to 24 h at 37 ºC. Interestingly, TMs significantly increased the accumulation of MTX in the skeletal muscular tissue of mice when exposed to an external constant magnetic field and heated to 41 ºC, compared to the absence of the magnetic field exposure and heating [95]. Furthermore, anchoring temperature-sensitive polymers to liposomes has been found to facilitate the desestabilization of the latter [96]. For this application, poly(*N*-isopropylacrylamide) is the most extensively used thermo-sensitive polymer [96-98]. For instance, thermo-sensitive magnetic nanocarriers made of a Fe_3O_4 nanocore surface coated with dextran-*g*-poly(*N*-isopropylacrylamide-*co*-*N*,*N*-dimethylacrylamide) have been

developed for DOX delivery to tumors. This multifunctional delivery system assures a controlled DOX release in response to changes in temperature, a consequence of the collapse of the polymer and cleavage of the acid-labile hydrazone linkage [99]. Poly(*N*-isopropylacrylamide) magnetic gels were also proposed for combined hyperthermia and drug release applications [100].

Special attention has been recently given to the use of superparamagnetic iron oxides for temperature-sensitive drug release, and hyperthermia treatment of cancer [101-103]. Magnetic field hyperthermia is based on the selective delivery and accumulation of magnetic NPs into cancer, and the local heating of these magnetic colloids with an externally applied alternating magnetic field (AMF). When the magnetic NPs are subjected to an AMF of high frequency (≈ 1 MHz), heat is generated as a consequence of magnetic hysteresis loss [104-107]. This temperature increment will then facilitate drug desorption from the non-magnetic matrix where the drug is embedded [108]. Interestingly, under exposure to an AMF, these nanomaterials will be converted in heaters, and can be used for heat induction of the targeted tissue up to ≈ 41 – 45 ºC. Tumor cells heated at this temperature will be irrevocably and specifically damaged, and will die [106, 109-111].

3.2.3. Magnetic Drug Targeting

Due to their magnetic-field responsiveness, magnetic colloids are capable of carrying chemotherapy agents specifically to the targeted place. A magnetic field will drive the magnetic system to the targeted tumor, keeping it there for a given period of time until the drug is fully released [112]. Magnetic carriers could, in principle, be only made of a magnetic material such as iron oxides (mainly, magnetite or maghemite). However, magnetic inorganic NPs are characterized by very limited drug delivery properties: low drug loading capacity and difficult control of the drug release [4, 5, 113]. Unlike them, biodegradable polymers and liposomes can release drugs at a rate defined by their biodegradation or, alternatively, by a physical stimuli. Hence, the majority of the research efforts are focussed on the development of nanoplatforms composed of a magnetic core and a biodegradable polymer or liposome shell (a matrix in which the magnetic nucleus is embedded). These NPs will take advantage of the properties of its two components. The biodegradable shell [principally, liposomes, chitosan, PLGA, PCL or poly(alkylcyanoacrylates), to cite just a few] will play the role of improving the biodegradability and biocompatibility of the system, and transporting the drug to the tumor tissue [5, 114]. Meanwhile, the magnetic core will induce the accumulation of the drug carrier into the intended location, keeping it there, by means of a magnetic field. Since the magnetic gradient decreases with the distance to the target, the main limitation of this drug delivery strategy relates to the strength of the external magnetic field that is applied to control the residence time of a magnetic colloid at the target region, or to trigger drug release. In order to elude this limitation, internal magnets (implants) can be located inside or in the vicinity of the target by using minimally invasive surgery. The use of magnetic implants in combination with an externally applied magnetic field will optimize the delivery of magnetic particles to the tumor site [115-117]. With the aim of enhancing the $t_{1/2}$ of magnetic drug nanocarriers and to specifically direct these systems to the tumor site, the association with ligand-mediated targeting strategies has been extensively investigated [118]. Another area of intensive investigation has been the targeting of magnetic colloids to receptors overexpressed on tumor neovasculature [119-121].

3.2.4. Light-Triggered Drug Release

Light responsive liposomes, polymers and hydrogels can be made UV or visible light sensitive. Visible light-responsive systems are more beneficial in drug delivery because they are safe, inexpensive, readily available, clean and easily manipulated. As an example, bis(4-dimethylamino) phenylmethyl leucocyanide, a leuco derivative molecule, was loaded into a polymeric network to synthesize a UV light-responsive hydrogel. On the other hand, visible light-responsive hydrogels were prepared by introducing a light-sensitive chromophore (e.g., trisodium salt of copper chlorophyllin) into poly(*N*-isopropylacrylamide) hydrogels. This technique is highly advantageous over others, since the stimulus of light can be imposed instantaneously, and can be delivered in specific amounts with high accuracy [16, 72, 122]. Despite the significant possibilities of this strategy, more investigations are needed to prove the viability of obtaining *in vivo* a tumor targeted drug release. As an example, plasmalogen photooxidation is an interesting approach that relies on an increase in membrane permeability upon photooxidative cleavage of plasmenyl-choline to single-chain surfactants [123].

3.2.5. Ultrasound-Mediated Drug Delivery

This approach to cancer treatment is based on the exposition of tumor regions to ultrasounds, leading to localize and complete release of the drug from the delivery system [124]. Ultrasound-mediated drug delivery is a non-invasive strategy, capable of penetrating deep into the body, and hence drug delivery can be focused and controlled through a number of parameters including frequency, power density, duty cycles and time of application. The mechanism of action of this technique is related to enhanced permeability of blood capillaries, the generation of thermal energy, and perturbation of cell membranes under the influence of micro-convection or inertia cavitation [72].

Generally, passive targeting of the tumor takes place through the EPR effect, followed by the application of ultrasound to ensure the cellular uptake by the alteration of the cell membrane permeability, and to induce drug release as a consequence of the nanocarrier disruption [125]. For example, this technique was successfully applied in the treatment of highly resistant human colon KM20 tumor-bearing mice with 5-FU-loaded polystyrene NPs [126].

Low frequency ultrasound (LFUS) has been used to trigger drug release from nano-sterically stabilized liposomes (nSSL), without affecting the chemical integrity and the biological potency of chemotherapy molecules. nSSL loaded with cisplatin have shown a very significant antitumor activity after intraperitoneally (i.p.) injection to mice bearing

well-developed J6456 murine lymphoma tumors in their peritoneal cavity, or C26 tumors in the footpad, and external application of LFUS to the target site. Briefly, 1 h after i.p. injection, a rubber cylinder was sealed over the abdominal tumor of the anesthetized mice, and filled with water. The LFUS probe was then immersed in the water-filled cylinder. Alternatively, in order to treat C26 tumors in the footpad, 24 h after drug injection (in order to enable liposome accumulation at the target site), the foot with the tumor of LFUS-treated (and i.p. anesthetized) mice was immersed in a water bath (24 °C) and the LFUS probe was placed into the bath. In both treatment schedules, LFUS irradiation (frequency: 20 kHz) was conducted at an intensity of 5.9 W/cm^2 for 60 – 120 s at a continuous mode, depending on the characteristics of the skin. Interestingly, ≈ 70 % of the amount of cisplatin loaded into nSSL was selectively released into tumors exposed to LFUS, compared to < 3 % in those not exposed to LFUS. The group of mice treated with cisplatin-loaded nSSL in combination with LFUS had the best therapeutic results, compared to other groups (i.e., free cisplatin with or without LFUS, or cisplatin-loaded nSSL without LFUS, or LFUS alone, or control): tumors stopped proliferating and then regressed over time (Fig. **5**). It was also highlighted that LFUS are hardly focussed, and dissipate rather near the body's surface. Therefore, it can be accepted that LFUS are principally appropriate for superficial tumors (e.g., skin, and some head and neck, and gynecological cancers). In the case of deeper tumors, high intensity ultrasounds could be more suitable [127].

3.2.6. Enzyme-Triggered Drug Release

Enzymes that are naturally expressed in tumors can be also used for inducing the release of anticancer agents from a drug carrier. This strategy is based on drug delivery systems that are susceptible to a specific enzyme overexpressed by the tumor. Under the influence of this enzyme, the nanocarrier is disrupted, leading to the release of the drug [128]. Enzyme-responsive polymers form the basis for hydrogels that are susceptible only to specific enzymes. These enzymes have been used very successfully as signals for the site-specific delivery of several drugs to specific organs. This strategy has shown very interesting results in colonic drug delivery [72]. Cancer cells express and release unique enzymes such as matrix metalloproteinases, which are implicated in their movement and survival mechanisms. An albumin-bound form of DOX incorporating a matrix metalloproteinase-2 specific octapeptide sequence between the drug and the carrier was efficiently and specifically cleaved *in vitro* by matrix metalloproteinase-2 [7].

Liposomes are frequently engineered to be disrupted by enzymes. Particularly, they are formulated to be biodegraded by secretory phospholipase A_2, a lipid hydrolyzing enzyme that is significantly up-regulated in the extracellular microenvironment of tumors [129, 130]. Thus, when liposomes extravasate in the tumor interstitium, this enzyme will act as an active trigger, resulting in drug release in close vicinity to the target site. Other widely used enzymes in enzyme-induced drug delivery are alkaline phosphatase

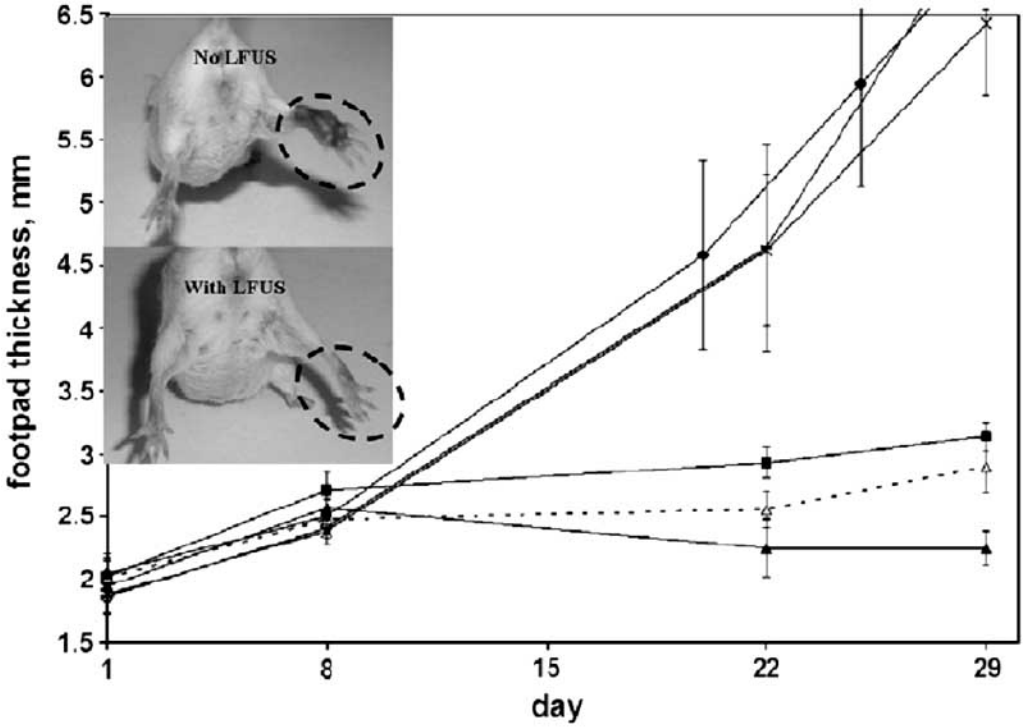

Fig. (5). Antitumor activity (in terms of footpad thickness) of different treatment schedules on C26 tumors in the footpad of BALB/c mice: (*i*) control, no drug no LFUS (upper image in graph) (♦); (*ii*) control, saline (placebo) plus LFUS (5.9 W/cm^2, 60 s, -); (*iii*) cisplatin-loaded nSSL without LFUS (×); (*iv*) cisplatin-loaded nSSL plus LFUS (upper image in graph) (▲); (*v*) free (non-liposomal) cisplatin plus LFUS (–Δ–); (*vi*) free cisplatin without LFUS (■). Insert: footpad of mice treated with cisplatin-loaded nSSL, without (top) or with (bottom) LFUS. At day 29, using Student's t-test, a statistically significant difference ($p < 0.05$) was demonstrated between animals in group (*iv*) and group (*v*), and ($p < 0.006$) between group (*iv*) and all other groups. Data points indicate mean footpad thickness of six mice, in two experiments, ± SD. Reprinted with permission from Ref. [127]. Copyright Elsevier (2009).

[131], transglutaminase [132] or phosphatidylinositol-specific phospholipase C [133].

4. STRATEGIES FOR OVERCOMING MULTI-DRUG RESISTANCE (MDR)

Drug resistance is one of the major obstacles limiting the therapeutic efficacy of anticancer agents. MDR contributes to the persistence of cancer despite of the high doses of chemotherapy agents that are used, and the combined chemotherapy. To achieve an efficient delivery of chemotherapy agents to MDR cancer cells some challenges must be overcome: *i*) cellular based drug resistance mechanisms; *ii*) non-cellular based drug resistance mechanisms; and *iii*) biodistribution, biotransformation and clearance of antitumor drugs. Non-cellular drug resistance can be associated to poorly vascularized tumors that reduce drug access. Low microvascular pressure and high interstitial pressure can also avoid drug diffusion across the cell membrane. Moreover, the acid conditions inside cancer can protect the tumor cells from basic drugs that could be ionized preventing their extravasation. Cell-based drug resistance mechanisms can be summarized as: blockage of apoptosis (decreased ceramide), increased drug efflux (e.g., up-regulated P-*gp*), decreased drug influx, and DNA repair activation and detoxification (due to the activity of specific enzymatic systems, such as, topoisomerase). The MDR phenotype is usually the synergistic result of a combination of different MDR mechanisms [10, 134, 135].

It has been suggested that the entrapment of chemotherapy agents within NPs reduces the incidence of MDR: the drug is not recognized by the cell surface, and it is only released when the system is internalized into the cell. Multi-functional nanocarriers are of great interest in drug delivery because of their special ability to enhance drug delivery in refractory tumors, overcoming MDR by simultaneous delivery of agents that regulate intracellular pH, resistance modulators (e.g., with P-*gp* substrates), agents that lower the apoptotic threshold (e.g., ceramide), or in combination with energy delivery (e.g., sound, heat or light). However, these nanoplatforms should address some parameters to effectively reverse MDR: *i*) they must be able to bypass rapid MPS clearance, with PEGylation allowing long-circulation and higher uptake efficiency; *ii*) they must be loaded with high concentrations of combined chemotherapy agents that can divert MDR and elicit a antitumor effect; and *iii*) they should allow tumor-specific targeting, and facilitate the uptake through surface modification (e.g., EGFR1 peptides) [135].

Pluronic block copolymers have been highlighted as potential drug carriers, as they can cause drastic sensitization of MDR tumors to anticancer agents. The biological activity of these amphiphilic block copolymers is based on their ability to incorporate into membranes followed by subsequent translocation into the cells and alteration of various cellular functions, such as mitochondrial respiration, ATP synthesis, drug efflux transport, apoptotic signal transduction, and gene expression (Fig. **6**) [136]. Due to this multiple action, pluronics can also cause enhance drug transport across the blood brain and intestinal barriers, and transcriptional activation of gene expression. As an example, DOX incorporated in mixed micelles of pluronic block copolymers, has shown a very interesting activity as monotherapy in patients with advanced esophageal carcinoma. The formulation contains pluronics with the unique ability to chemosensitize MDR tumors by inhibiting the P-*gp* drug efflux system, and enhancing the pro-apoptotic signaling in cancer cells [137].

A common strategy to overcome MDR in cancer is the administration of an antitumor drug along with a drug efflux modulation. The use of transferrin-decorated liposomes loaded with both DOX and verapamil (a P-*gp* inhibitor) has been suggested as a highly promising approach. This nanosystem enhances the amount of DOX retained in the MDR cancer cells, significantly increasing the effectiveness of this agent [138]. In another study, PEGylated PLGA NPs surface functionalized with biotin (for active tumor targeting) and loaded with paclitaxel and tariquidar (a third

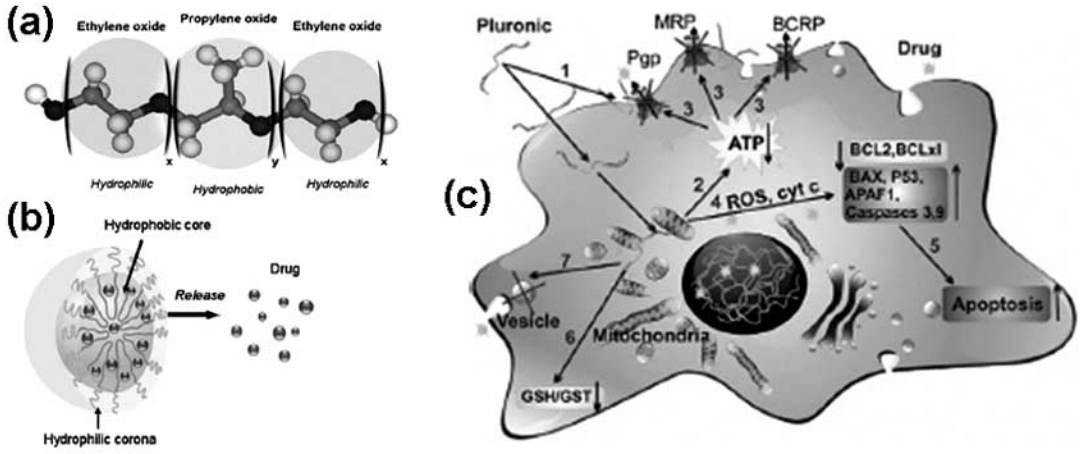

Fig. (6). Chemical structure of a pluronic block copolymer (a), micelle loaded with a solubilized drug (b), and (c) multiple activity of pluronic block copolymers in MDR cells: *1*) incorporation of pluronic molecules into membranes, and decrease of the membrane microviscosity; *2*) induction of ATP depletion; *3*) inhibition of drug efflux transporters; *4*) release of cytochrome C from mitochondria, and increase in reactive oxygen species (ROS) levels in cytoplasm; *5*) increase of pro-apoptotic signalling and decrease of anti-apoptotic defense in MDR cells; *6*) inhibition of the glutathione/glutathione S-transferase detoxification system; and *7*) abolishment of drug sequestration within cytoplasmic vesicles. Reprinted with permission from Ref. [136]. Copyright Elsevier (2009).

generation P-*gp* modulator) were formulated to very efficiently increase the accumulation of paclitaxel in MDR tumor cells, significantly enhancing its antitumor efficacy (Fig. **7**) [139]. Another very useful possibility for obstructing the drug efflux is the use of MDR1 targeted antisense oligonucleotides (ASO) [140].

Immunoliposomes has been also developed to effectively by-pass drug transporters located at the plasma membrane [141]. The administration of immunoliposomes directed against P-*gp* enhanced the cytotoxicity in P-*gp* expressing tumor cell lines [142, 143]. Vincristine-loaded liposomes conjugated to MRK-16 (a MAb against P-*gp*) have induced an enhancement of the drug cytotoxicity against resistant human myelogenous leukemia cell lines, compared to conventional vincristine-loaded liposomes. This enhanced efficacy was attributed to the inhibition of P-*gp* mediated efflux of vincristine by MRK-16 [143]. Paclitaxel-loaded PEGylated PLA NPs have been surface functionalized with transferrin to target glioma cells (BT4C). *In vitro* results demonstrated an enhancement of the antitumor activity of paclitaxel, when compared to the commercial drug formulation Taxol® and non-targeted NPs [144]. DOX-encapsulated pH-sensitive micelles composed of poly(L-histidine)-*b*-PEG and PLA-*b*-PEG-folate showed significantly superior efficacy in MDR ovarian A2780/DOX(R) xenografted nude mice, compared to free DOX and DOX-loaded pH-insensitive micelles composed of PLA-*b*-PEG/PLA-*b*-PEG-folate. It was observed an efficient inhibition of the growth of the MDR ovarian tumors in mice, with minimum weight loss (toxicity). This nanosystem proved its capacity to undergo folate receptor-mediated endocytosis and endosomal disruption, releasing the drug inside the cancer cell [85].

The simultaneous combination of antitumor drug delivery and the modulation of the apoptotic threshold has also offered very interesting results. PEGylated PCL NPs have been formulated for the co-administration of paclitaxel and ceramide to a MDR human ovarian cancer cell line. It was proved that this formulation very importantly enhances the cytotoxicity of paclitaxel in MDR cells [145]. Another interesting strategy to overcome MDR deals with the simultaneous use of drug delivery and intracellular pH modulation. The acidic pH associated with MDR cells can be exploited in two different ways: design of pH-sensitive nanocarriers for controlled drug release and/or alteration of the intracellular pH. As an example, DOX-loaded pH-sensitive polymeric micelles were prepared with two block copolymers poly(L-histidine)-β-PEG-folate and PLA-β-PEG-folate to achieve folate-receptor targeting and dissolution below pH 6.8. Interestingly, these micelles developed higher cytotoxicity in MDR breast cancer cells at pH 6.8 (cell viability: 20 %) in comparison to free DOX (cell viability: 85 %) [146].

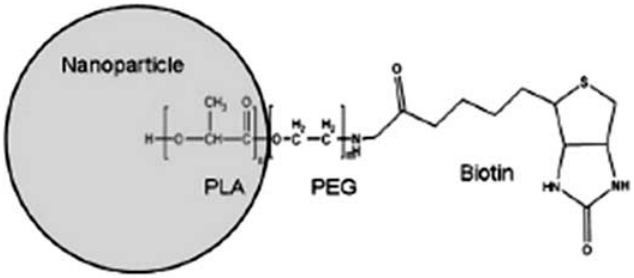

Fig. (7). Chemical structure of PLGA nanoparticles surface functionalized with biotin. The nanoparticles consist of a PLGA matrix, with the PLA chain of the PLA-PEG block copolymer anchored into the PLGA matrix. PEG chains with the terminal biotin group are present on the nanoparticle surface. Paclitaxel and tariquidar are dispersed inside the PLGA matrix. Reprinted with permission from Ref. [139]. Copyright Elsevier (2009).

5. NANOTOXICITY IN DRUG DELIVERY TO CANCER

Biocompatibility is a key role parameter that must be assured in the formulation of any given colloid for drug delivery. It is determined by both the toxicity of the material, and the interaction of its biodegradation products with the immune system [147]. Biocompatibility is exclusively associated to nanosystems with an adequate physiochemistry (chemical composition, geometry and structure, surface chemistry, and solubility), that must be also cleared out of the body in the shortest period of time. It has been determined that the toxicity and, hence, the biocompatibility of drug nanocarriers also relies on the dose of exposure (mass administered), delivered dose (mass per cell or cm^3), cellular dose (internalized mass), method of administration, biodegradability, pharmacokinetics, and biodistribution [113, 148-150].

The geometry (particularly, the surface area) and the chemical composition are very important aspects determining the cytotoxicity of the drug delivery system. In order to keep it to a very minimum, NP formulations must be hydrophilic, with a pH ≈ 7.4, and will not accumulate in the body [151, 152]. For engineering purposes, it must be also taken into account that the biodegradation products of the nanomaterials can also contribute to the final toxicity, primarily by stimulating cells to release inflammatory mediators [153, 154]. For instance, it has been studied that magnetic nanocomposites consisting of an inorganic core and an organic shell do not increase significantly the individual toxicity of the components. Clinical trials have confimed the low toxicity of these formulations [155-159].

Recently, the key role of protein-NP interactions in nanotoxicity has been given very important attention. The interactions of drug delivery colloids with biological components (e.g., proteins and cells) could lead to unique biodistribution, clearance, immune response, and metabolism. At the present moment, it is clear that further extensive investigations are needed to clarify the relationship between the physicochemistry of the nanocarrier and its *in vivo* behavior. This would allow assessing toxic response, and determining predictive models for toxicity evaluations [147-150].

6. CONCLUSION

Significant progress has been made in the development of new drug delivery approaches to cancer. These strategies have contributed very importantly to the overall enhancement in the efficacy of the treatment of numerous cancers. However, to date, very few formulations [mainly

liposomal (i.e., Myocet™, Doxil® or Caelyx®), but also polymeric preparations (Genexol-PM™)] have been approved by the FDA for this purpose. The immediate future and possibilities of nanotechnology in the battle against cancer will depend on improving the knowledge of tumor biology, as well as on advances in the nanoengineering of colloidal drug carriers. Hence, more research efforts are needed to clearly determine the viability, biological fate, nanotoxicity, and efficacy of these new antitumor drug nanoplatforms that, from a preclinical point of view, are really promising.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

17-AAG	=	17-allylamino-17-demethoxy geldanamycin
5-FU	=	5-fluorouracil
AF	=	Alexa Fluor 647 near infrared fluorophore
AMF	=	Alternating magnetic field
ASGP	=	Asialoglycoprotein
ASO	=	Antisense oligonucleotide
AUC	=	Area under the time-concentration curve
BI	=	Biotin
CTX	=	Chlorotoxin
DOX	=	Doxorubicin
EGFR	=	Human epidermal growth factor receptor
EPR effect	=	Enhanced permeability and retention effect
FA	=	Folic acid
FDA	=	Food and Drug Administration
FBP	=	Folate-binding protein
HER-2	=	Human epidermal growth factor receptor-2
i.p.	=	Intraperitoneal
i.t.	=	Intratumoral
i.v.	=	Intravenous
IMMA	=	Inulin multi-methacrylate
LFUS	=	Low frequency ultrasound
MAb	=	Monoclonal antibody
MDR	=	Multi-drug resistance
MPS	=	Mononuclear phagocyte system
MTX	=	Methotrexate
NPs	=	Nanoparticles
nSSL	=	Nano-sterically stabilized liposome
P-*gp*	=	P-glycoprotein
PCL	=	Poly(ε-caprolactone)
PDP-PEG-DSPE	=	Pyridylthiopropionoylamino-PEG-distearoylphosphatidylethanolamine
PE-HER-liposomes	=	PE38KDEL-loaded anti-HER2 PEGylated liposomes
PEG	=	Poly(ethylene glycol)
PEI	=	Poly(ethyleneimine)
PEO	=	Poly(ethylene oxide)
PLA	=	Poly(D,L-lactide)
PLGA	=	Poly(D,L-lactide-co-glycolide)
PPI	=	Poly(propyleneimine)
PSLs	=	pH-sensitive liposomes
PSMA	=	Prostate-specific membrane antigen
PVD	=	Poly(vinylpyrrolidone-*co*-dimethylmaleic anhydride)
RES	=	Reticuloendothelial system
REV method	=	Reverse-phase evaporation vesicle method
RGD sequence	=	Arginine-glycine-aspartic acid sequence
shRNA	=	Short hairpin RNA
SIAX	=	Succinimidyl-6-(iodoacetyl) aminocaproate
SLNs	=	Solid lipid nanoparticles
ROS	=	Reactive oxygen species
$t_{1/2}$	=	Half-life
TMs	=	Magnetoliposomes

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