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Review article

A glance over doxorubicin based-nanotherapeutics: From proof-of-concept studies to solutions in the market

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ABSTRACT

Cancer is one of the leading causes of death worldwide and, as such, efforts are being done to find new chemotherapeutic drugs or, alternatively, novel approaches for the delivery of old ones. In this scope, when used as vehicles for drugs, nanomaterials may potentially maximize the efficacy of the treatment and reduce its side effects, for example by a change in drug's pharmacokinetics, cell targeting and/or specific stimuli-responsiveness. This is the case of doxorubicin (DOX) that presents a broad spectrum of activity and is one of the most widely used chemotherapeutic drugs as first-line treatment. Indeed, DOX is a very interesting example of a drug for which several nanosized delivery systems have been developed over the years. While it is true that some of these systems are already in the market, it is also true that research on this subject remains very active and that there is a continuing search for new solutions. In this sense, this review takes the example of doxorubicin, not so much with the focus on the drug itself, but rather as a case study around which very diverse and imaginative nanotechnology approaches have emerged.

1. Introduction

Cancer is characterized by the uncontrolled growth and spread of abnormal cells, being a very important human health problem. In 2018 and according to estimates of the World Health Organization, there were about 18 million new cancer cases and 9.6 million deaths occurred as a consequence of cancer [1]. Although the genesis of cancer is related with an accumulation of mutations in critical genes that control cell behaviour, it is well known that factors like increased population ageing, environmental problems and adopted lifestyle, may strongly contribute for its incidence [2]. In this scope, it is of extreme importance to develop new strategies for cancer treatment that are more effective and prevent unnecessary deaths.

Nanomaterials, that may be defined as materials that have at least one dimension at the nanoscale [3], can provide solutions for the well-known problems associated with conventional anticancer therapy [4]. Particularly, they can be used as drug delivery vehicles and thus help to increase the effectiveness of medicines and simultaneously reduce their negative side effects. Indeed, nanomaterials are interesting tools for the delivery of therapeutic agents since they possess the right size to circulate inside the human body and to interact with biological targets, like macromolecules, cells and cell organelles [5,6]. In addition, depending on their size, shape/architecture, chemical composition and surface functionalization, nanomaterials may exhibit special chemical and physical properties that will impact their biological behaviour [7,8]. Regarding cancer treatment, their use as drug carriers may have a direct impact on the pharmacokinetics (PK) of the drug, including on the extent of drug's cell uptake, and as such on its efficacy [7,9]. In the case of solid tumours, it is also believed that nanomaterials may accumulate in the tumour site through the "Enhanced Permeation and Retention" (EPR) effect [9,10] which will be explained later in this review. Nanomaterials can also be designed to specifically target cancer cells (targeted nanomaterials) [11–19] or to release the drug only under the presence of specific environmental stimuli [20–29], thus helping to diminish unwanted side effects. Other advantages associated with the use of nanomaterials as drug delivery platforms include: the sustained delivery of the drug [17,30–33]; the delivery of more than one drug at the same place and at the same time (co-delivery of drugs) [15,23,25,28,34–40]; the possibility of transporting poorly soluble drugs that otherwise could not be administered by simple intravenous injection [41–43]; the protection of the drug from possible existent damaging environmental conditions [44–49]; the potential of bypassing

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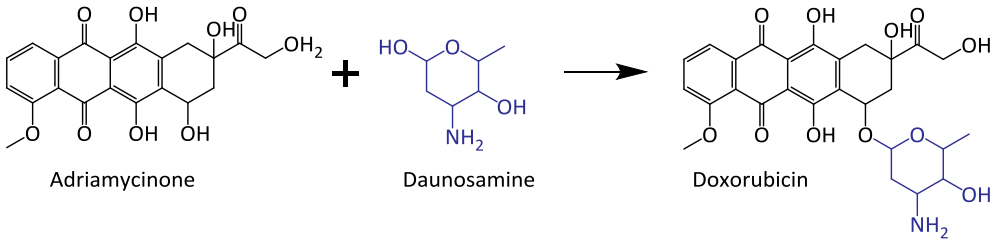

Fig. 1. Chemical structure of DOX. DOX molecule is based on a water-insoluble aglycone (adriamycinone, with lipophilic character) and a water-soluble amino-sugar moiety (daunosamine, with hydrophilic character); the adriamycinone consists in a tetracyclic ring with a quinine-hydroquinone group nearby; the amino-sugar moiety is linked to one of the rings through a glycosidic bond [63].

drug resistance mechanisms [50–52]; and the chance to associate drugs and contrast agents for medical imaging techniques in the same nanodevice thus allowing simultaneous therapy and diagnosis (theranostics) [53–57].

Doxorubicin (DOX, Fig. 1) is a non-selective anthracycline and is one of the most used chemotherapeutic drugs amongst those approved by Regulatory Agencies [58]. Indeed, DOX has shown a high activity against both solid and liquid tumours, including several types of lymphoma and leukaemia, as well as breast, bladder, stomach and ovary cancers [59]. Although DOX is routinely used as an anticancer drug, its exact mechanism of action is complex and still not completely clear. According to literature, DOX has multiple molecular targets but its cytotoxic effects are essentially based on two phenomena: (a) first, one should consider the intercalation of the planar adriamycinone moiety of DOX between adjacent DNA base pairs; this intercalation interferes with the action of the enzyme topoisomerase II (TOP2), preventing the DNA double helix from being resealed and, as a consequence, stopping DNA replication and RNA transcription [60,61]; and (b) second, by producing carbon-centred radicals and reactive oxygen species (ROS); these very reactive species disrupt the cell membrane, proteins and DNA [59,60,62]. In fact, most of the side effects of DOX are associated with ROS, as is the case of cardiotoxicity. Many body tissues possess enzymes responsible for the combat of free radicals that prevent or limit tissue damage. Since the cardiac tissue has a relatively low number of these enzymes, it will be more susceptible to these reactive species [60]. Beyond cardiotoxicity, a decrease of bone marrow activity (myelosuppression) is also often observed after cancer treatment with DOX [62]. For a more detailed knowledge about the discovery, physicochemical characteristics and biological action of this drug, we recommend the readers to consult the excellent reviews that may be found in the literature about these topics [63–67].

Nanotechnology approaches for DOX delivery started several decades ago in an attempt to reduce the severe side effects often observed after its use. Indeed, Doxil® was even the first nanotherapeutic to be approved for clinical use [68]. Since then, several other DOX-based nanotherapeutics were further introduced in the market or are under clinical trials. Also, ongoing laboratory research on this topic is still very active and promising new systems may soon go from the bench to the bedside.

This review aims at showing the potential of nanomaterials in cancer treatment, mainly as drug delivery vehicles, using doxorubicin-based nanosystems as a golden example. Since the effectiveness of a nanocarrier depends on its ability to deliver the drug in the therapeutic target, the biological barriers that may interfere in this process must be considered in its design. Also, the biological and physicochemical properties of the action's site should be taken into account when targeted and/or smart nanocarriers (sensitive to environmental conditions) are developed. Due to the importance of these aspects in the design of a nanocarrier, we start by briefly reviewing them. Then, an overview of the research that has been done around DOX-based nanotherapeutics is made, highlighting the general characteristics of the various systems under study and presenting representative examples in the form of tables. Finally, an important part of the review is dedicated to the DOX-based nanotherapeutics that have advanced from laboratory experiments to clinical studies and to those that are already available for clinical use.

2. Nanomaterial's design: physiological barriers, tumour targeting and stimuli-responsive properties

To be successful, nanomaterials should be designed to overcome several biological barriers that may appear along their pathway inside the body. Depending on the nanomaterial's method of administration and the localization of the cells/tissues to be treated, these may include the mononuclear phagocyte system, cellular barriers, stromal barriers and cell/organelle membranes [69,70]. Beyond surpassing these difficulties, targeted nanomaterials must find their molecular targets and interact with them in an effective manner, thus being even more challenging in terms of design. A brief description of the biological barriers that may be faced by DOX-based nanotherapeutics will be described in the next paragraphs bearing in mind that their administration in the body will be intravenously.

The mononuclear phagocyte system

The mononuclear phagocyte system (or reticuloendothelial system) makes part of our immune system and mainly consists of phagocytic cells, of which the most relevant are the macrophages [70,71]. Once inside the body, nanomaterials may suffer opsonisation (Fig. 2A) by interaction with opsonins in the blood and/or tissues, thus triggering an immune response, that is, resulting in phagocytosis and clearance from the body (or, in alternative, accumulation in organs such as the lymph nodes and the spleen). The surface charge of a nanomaterial can favour protein adsorption. It is reported that negatively charged nanomaterials are less prone to opsonisation and are consequently less recognized by the phagocytic cells, thus spending more time in blood circulation. On the contrary, other reports reveal that neutral nanomaterials, as well as positively charged ones, attract phagocytic cells attention [72–75]. In addition, a common strategy to diminish opsonisation is to cover the nanomaterial's surface with a hydrophilic polymer like polyethylene glycol (PEG). Beyond preventing phagocytosis, PEG also confers a higher biocompatibility to the nanocarrier and helps to improve its solubility in aqueous environment. Moreover, PEG prevents the *in vitro* aggregation of nanomaterials and increases the hydrodynamic diameter of very small nanomaterials, increasing their circulation half-time [76]. In fact, the impact of PEGylation over the overall performance of nanomaterials inside the human body is very important and that is the reason why many DOX-based nanotherapeutics include PEG in their design as will be highlighted in many examples along this review.

Cellular barriers and the EPR effect

DOX is usually administrated intravenously, being able to cross the vascular-endothelium cell lining and reach most of the body tissues. However, if the idea is to use nanomaterials to avoid side effects and target specific tissues/cells, then one should have in mind that they need to go through this cellular barrier. In fact, regarding solid tumours, this is facilitated due to a phenomenon already mentioned and known by the EPR effect (Fig. 2B). The EPR effect results from an angiogenesis process that is triggered by cancer cells. These new blood vessels within the tumour are immature and possess fenestrations that, depending on the tumour type, location and environment, can have a

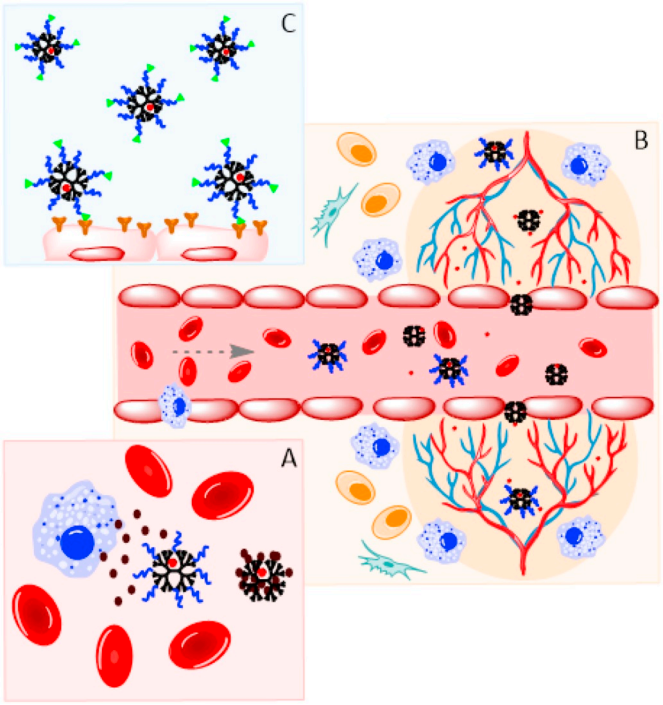

Fig. 2. A) Mononuclear phagocyte system recognition: opsonisation and phagocytosis, B) EPR effect: extravasation to tumour microenvironment through the leaky vessels and retention within the tumour tissue, and C) Active targeting: selective recognition of tumour cells through specific ligand-receptor interaction.

size ranging from 200 to 2000 nm [77,78]. The presence of these fenestrations associated with a lack of lymphatic drainage in the tumour site thus result in an enhanced permeability and retention effect [10]. Whereas small molecules can be re-absorbed by diffusion to the blood circulation, macromolecules or nanomaterials cannot due to their size and, for this reason, accumulate in the tumour site [79]. The EPR effect is, in fact, a passive form of targeting. This means that the targeting process depends on the characteristics of the biological system (the tumour and the new blood vessels, in this case) and not on especial properties of the nanomaterial.

Beyond extravasation due to the EPR effect, nanomaterials may be transported across the vascular-endothelium cell lining by transcytosis [80], a process that strongly depends on nanomaterial's physicochemical properties like composition, size, shape, flexibility and charge [10]. This mechanism may also be used to cross the blood-brain barrier (BBB) that is another important cellular barrier faced by a nanomaterial that is aimed at treating brain diseases. The BBB separates the circulating blood from the central nervous system and is highly selective [70]. DOX, for example, cannot cross the BBB which is a limitation for its use in the treatment of brain cancers [62]. In this case, the use of nanomaterials especially designed to serve as shuttles for DOX delivery into the brain would certainly extend the possibility of benefiting from the therapeutic properties of this drug. Several examples of DOX-based nanotherapeutics that were particularly designed to cross the BBB will appear along this review [49,81–85]. These nanomaterials were specifically designed to interact with cell surface receptors and, as such, follow an internalization mechanism known by receptor-mediated transcytosis [86,87].

Stromal barriers

After crossing the cellular barriers that separate the blood from the tissues, nanomaterials may further find stromal barriers, that is, they must be transported through the interstitial space around cells to reach their target. Stromal barriers in solid tumours can be even more difficult to be crossed as the extracellular matrix produced by cancer cells is distinct from the one existent in normal tissues, being stiffer and highly heterogeneous [88]. Also, the abnormal architecture of the blood vessels in tumours and the lack of lymphatic drainage leading to a lack of perfusion can result in an increase of the fluid pressure inside the tumour that, ultimately, will retard the movement of nanomaterials [69]. In part, this phenomenon counterbalances the EPR effect. In fact, the easiness with which the nanomaterial follows its path towards the target cells will depend on the characteristics of the biological tissue but also on their own properties, e.g., size, charge, and flexibility.

Cell/organelle membranes

DOX, like other drugs, can be internalized by cells through passive diffusion and accumulates intracellularly at high concentrations which is attributed to its lipophilic properties and easy DNA intercalation [65]. On the other side, the tumour microenvironment is often characterized by a privation of oxygen and low pH due to the change of cell metabolism towards fermentative processes [77,89]. This low pH can affect the cellular uptake of drugs that are weak bases, as is the case of DOX. The acid environment will retain the drug outside the cells in a great extent by a process called "ion-trapping". In this context, the use of nanomaterials can help to surpass this problem, that is, to increase the cellular uptake of DOX.

Notwithstanding, the cell membrane as well as organelle membranes constitute barriers for the nanomaterials themselves (loaded, or not, with a drug). Depending on the type of cells and on their own properties, nanomaterials can enter cells by phagocytosis (a process triggered by opsonisation) or by pinocytosis. The later can further be classified in four mechanisms: clathrin-mediated endocytosis, caveolin-mediated endocytosis, macropinocytosis and another class where all the other mechanisms different from the previous ones fall [90]. Then, once inside cells, nanomaterials should be able to release their therapeutic cargo near its molecular target. This means that, possibly, they will have to cross other biological membranes, such as the nuclear, lysosomal, or mitochondrial membranes. The design of a nanomaterial should, then, take all these issues into account which, by turn, are related with its specific application. In the case of DOX-based nanotherapeutics and since DOX itself is able of crossing biological membranes, the main barrier is indeed the cell membrane. So, one expects that after cell entry, the release of DOX in the cytosol will be enough to obtain a therapeutic effect. However, nanomaterials may be retained in the endolysosomal compartments and, even if DOX is there released, ion-trapping may occur inside these vesicles where an acid environment exists [32,91–93]. In this case, designing nanomaterials that are capable to act as "proton-sponges" (buffers) may help to disrupt the endolysosomal membrane so that the drug can easily reach the cytosol – this is known by the "proton-sponge effect". This proton adsorption/absorption achieved by the nanomaterial may conduct to additional pumping of protons by ATPase pumps present at the endolysosomal membrane which will be followed by an influx of chloride anions in order to maintain electric neutrality. The endolysosomal vesicles will then feel an increase in osmotic pressure and will be disrupted. Examples of this strategy are also present along this review [17,94–97].

Active targeting

Nanomaterials can actively be targeted towards biological entities. Active targeting, also known as ligand-mediated targeting, relies on the use of ligands (*e.g.* small molecules, carbohydrates, hormones, antibodies, peptides) with specific affinity for a molecular receptor that can, for example, be localized in the surface of the cells to be treated (Fig. 2C). This recognition between the ligand and the receptor may lead to a receptor-mediated mechanism for the cellular internalization of the nanomaterial, improving its efficacy as a delivery system [10]. In

Table 1
Examples of dendrimer-based nanomaterials for DOX delivery.

Dendrimer family	Nanocarrier description	Design strategy	Tumour model	Refs	Year
PAMAM	Partly PEGylated PAMAM-G4-cis-aconityl-DOX conjugates	- pH-responsive - DOX conjugation	Skin cancer (*in vitro* & *in vivo*)	Zhu *et al.* [72]	2010
	PEGylated PAMAM-G5 dendrimer modified with T7 peptide	- Targeted therapy - Co-delivery of drug and gene (DOX & pORF-hTRAIL plasmid) - DOX intercalation	Liver cancer cell line (*in vitro*)	Han *et al.* [137]	2011
	PAMAM-G4-oligodeoxynucleotides bioconjugates	- DOX intercalation onto hybridized oligonucleotides strands	Breast cancer (*in vivo*)	Lee *et al.* [138]	2011
	PAMAM-G4-PSMA-aptamer-oligonucleotide bioconjugates	- Targeted therapy - DOX intercalation onto hybridized oligonucleotides strands	Prostate cancer (*in vitro* & *in vivo*)	Lee *et al.* [11]	2011
	PEGylated PAMAM-G4 modified with wheat germ agglutinin and transferrin	- Targeted therapy - DOX encapsulation	Brain cancer (*in vitro* & *in vivo*)	He *et al.* [81]	2011
	PEGylated PAMAM-Gold nanorod conjugate	- Photothermal therapy - pH-responsive - DOX conjugation	Colon cancer (*in vitro* & *in vivo*)	Li *et al.* [21]	2014
	PEGylated PAMAM-G5 dendrimer modified with fluorescein isothiocyanate (FI) and RGD	- Targeted therapy - DOX encapsulation	Brain cancer (*in vitro*)	He *et al.* [139]	2015
	PEGylated hyperbranched PAMAM-cis-aconityl-DOX conjugates	- pH- and redox-responsive - DOX conjugation	Gastric cancer (*in vitro* & *in vivo*)	Nie *et al.* [24]	2016
	PAMAM-G4-SS-PEG conjugates	- pH- and redox-responsive - DOX encapsulation	Skin cancer (*in vitro* & *in vivo*)	Hu *et al.* [96]	2016
	PAMAM-G5 dendrimer modified with folic acid (FA) and borneol	- Targeted therapy - DOX encapsulation	Brain cancer (*in vitro* & *in vivo*)	Xu *et al.* [140]	2016
	PAMAM-G5 modified with cis-aconityl-PEG-NAcGal$_\beta$ and enzyme-sensitive linkages conjugates	- Targeted therapy - pH- and enzyme-responsive - DOX conjugation	Hepatic cancer (*in vitro* & *in vivo*)	Kuruvilla *et al.* [27]	2017
	Gold entrapped PAMAM-G5-FA-cis-aconytil-DOX conjugates	- Targeted therapy - pH-responsive - Imaging application (CT) - DOX conjugation	Brain cancer (*in vitro*)	Zhu *et al.* [57]	2018
	PAMAM-G4 dendrimer modified with cis-aconytil-DOX and trastuzumab conjugates	- Targeted therapy - pH-responsive - DOX conjugation	Breast cancer (*in vitro*)	Marcinkowska *et al.* [110]	2018
	PAMAM-G4 dendrimer modified with hyaluronic acid (HA), DOX and cisplatin	- Targeted therapy - Co-delivery (DOX and cisplatin) - DOX conjugation	Breast cancer (*in vitro* & *in vivo*)	Guo *et al.* [34]	2019
Polyglutamic	Poly(L-glutamic acid)-G3 dendrimer modified with biotin and DOX	- Targeted therapy - pH-responsive - DOX conjugation	Breast cancer (*in vitro* & *in vivo*)	Pu *et al.* [107]	2013
Phosphoramidate	Phosphoramidate dendrimers modified with zwitterionic groups	- Enzyme-responsive - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Zhang *et al.* [141]	2018
Polylysine	PEGylated poly-lysine-G5 4-(Hydrazinosulfonyl) benzoic acid (HSBA)-DOX dendrimer conjugate	- pH-responsive - DOX conjugation	Breast cancer (*in vitro* & *in vivo*)	Kaminskas *et al.* [106]	2011
	Poly-lysine-G6 dendrimers	- DOX encapsulation	Prostate cancer (*in vitro*) Lung cancer (*in vivo*)	Al-Jamal *et al.* [74]	2013
Polypropylene imine	PPI-G5-Dextran dendrimer conjugates	- DOX encapsulation	Lung cancer (*in vitro* & *in vivo*)	Agarwal *et al.* [44]	2009
	Acetylated PPI dendrimers	- DOX encapsulation	Breast cancer cell line (*in vitro*) Lung cancer cell line (*in vitro*)	Wang *et al.* [142]	2012
	PPI-G4-grafted nanocrystalline cellulose modified with FA	- Targeted therapy - DOX encapsulation		Golshan *et al.* [143]	2017

fact, the success of the process is dependent on several factors, the most important being the level of overexpression of the receptor in the target cells, and the affinity and selectivity of the ligand for the receptor [10,98]. In cancer therapy, active targeting has the important objective of overcoming drugs' side effects. When allied with the EPR effect (increased accumulation), active targeting approaches (increased specificity) can greatly improve the performance of nanomaterials as anticancer drug delivery vehicles [10]. The number of examples of targeted DOX-based nanotherapeutics is huge and some already moved forward towards advanced clinical trials [99–103].

Stimuli-responsiveness

Concerns about the side effects of anticancer drugs have led to the development of nanoparticles that release the drug only in the presence of specific environmental stimuli. These stimuli may be chemical or physical in nature and may correspond, for example, to a change in pH or temperature, or to the presence of reducing agents, specific enzymes or radiation [20,21,23–29,32,46,104,105]. Ideally, the stimulus should act only at the tumour site and may result in the release of an encapsulated drug or in chemical bonds' cleavage when the drug is covalently linked to the nanocarrier [21,24,27,57,94,106–110]. DOX-based nanotherapeutics with stimuli-responsiveness have been extensively investigated as will be shown in the next sections.

3. Proof-of-concept studies on DOX-based nanotherapeutics

The importance of DOX in the context of anticancer drugs justifies the large number of scientific studies that have been made in this area, as well as the variety of nanoscale systems that have been studied for its delivery in cancer cells. The following sections will review the research on DOX-based nanotherapeutics, highlighting the general characteristics, advantages and disadvantages of the different classes of nanomaterials. Representative examples of each class are shown in the form of tables with indication of the main concept behind their design.

3.1. Polymer-based nanocarriers

In nanomedicine, polymer-based systems are amongst the most successful nanocarriers due to their versatility. Their properties are easily tuned by playing with chemical composition, size and structure/architecture [111]. Polymers have proven that are capable to maintain a sustained drug release of encapsulated drugs, protecting them from the surrounding environment, and of targeting cancer tissues both in passive (through the EPR effect) and active forms. Importantly, they can provide shelter to hydrophobic drugs, improving their aqueous solubility [42]. Often, they are used in combination with other classes of nanomaterials to improve their properties, as is the case of PEG that, as mentioned before, among other objectives, is usually used to camouflage nanoscale systems and avoid opsonisation (stealth nanomaterials). Also, especially by varying the chemical composition, it is possible to tune polymer's toxicity and biodegradability, both relevant aspects for nanomaterials used in medicine. For instance, poly(lactic-co-glycolic acid) (PLGA) is one of the most known biodegradable and biocompatible polymers. When exposed to normal physiological conditions, PLGA is hydrolysed producing the original monomers (lactic acid and glycolic acid) that will be later metabolized through normal metabolic pathways. PLGA is considered safe and is approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA, in Europe) for human use [112]. Possibly, the main disadvantage associated with polymers, that may limit their use in nanomedicine, is the difficulty in preparing molecules with well-defined sizes (they usually present a high polydispersity) and to assure homogeneity among product batches. Even so, chemical synthesis methodologies are continuously evolving and allowing, more and more, a better control over this problem.

Dendrimers

Dendrimers constitute a special group of polymers as they possess a regular and well-defined architecture, narrow polydispersity (especially when comparing them with the classical polymers) and a high number of terminal groups (multivalency) which allows further modification [113]. Typically, the basic dendrimer structure consists of a core, branched shells (their number defines the dendrimer generation) and outer functional groups [114]. Dendrimers can carry drugs by electrostatic interaction, by chemical conjugation to their surface functional groups or by encapsulation inside their inner voids [113]. Not only due to their intrinsic chemical nature, but also through the modification of their peripheral groups, it is possible to control the drug release rate in dendrimers regardless of whether the drug has been encapsulated or conjugated [115].

Table 1 presents examples of nanotherapeutics based on dendrimers and evaluated for the cellular/tumour delivery of DOX. There, one can observe that dendrimers are ideal scaffolds for the simultaneous conjugation of different chemical entities, each one serving a distinct objective. In fact, due to dendrimer's multivalency, it is possible to congregate in the same structure ligands for targeting, PEG arms, and other nanomaterials for bioimaging purposes (imaging contrast agents) or additional therapy (like those used for cancer hyperthermia), as well as the drug itself. Indeed, dendrimers are being studied for the development of theranostic materials as they can act as vehicles for drug delivery and, also, have a role in the diagnosis of diseases, and especially of cancer. For instance, several studies showed that theranostic nanomaterials based on dendrimers are able to serve as contrast agents for Magnetic Resonance Imaging (MRI) or/and for Computed Tomography (CT) imaging and/or for Positron-Emission Tomography (PET) imaging [57,116–125]. Apart from the advantages, dendrimers also present weaknesses. In particular, those dendrimers that have terminal groups with a positive charge at physiological pH can present a high toxicity that grows with increasing generation [126]. This is the case of poly (amidoamine) (PAMAM) dendrimers which are the dendrimers most studied until now due to their early commercial availability [127]. In this regard, acetylation may be used to decrease dendrimers' surface charge and decrease their toxicity [128–134]. Also, several works reveal that dendrimers can have a great affinity for metal ions, lipids, proteins, salts and nucleic acids, leading to disruption of biological processes and, consequently, presenting a toxicity higher than desired [135]. Naturally, the possibility of constructing dendrimers with different chemical nature also opens new routes to surpass these difficulties, as is the case of biodegradable dendrimers which are expected to offer a better performance in terms of biocompatibility. On the other hand, cationic dendrimers have been described as acting like "proton-sponge materials" thus contributing for endolysome disruption and drug release into the cytosol [136].

Nanogels

Nanogels are hydrogel particles with nanoscale dimensions [144]. Research on hydrogels has become very popular since the 1960s with the interesting work of Wichterle and Lim [145]. Hydrogels are defined as three-dimensional (3D) networks made of cross-linked polymers that can absorb large amounts of water (or biological fluids) and swell still maintaining their 3D structure. Nanogels, like hydrogels, exhibit a high water content, soft consistency, flexibility and porosity [146,147]. Furthermore, they can result from physical or chemical crosslinking of natural and/or synthetic polymers, resulting in chemical stable systems, or eventually unstable by disintegration or dissolution [148]. The nanogel porosity can simply be regulated by adjusting the crosslinking density in the material which will, at the same time, control the material's affinity for water. This feature allows drug loading into the nanogel and further release with different diffusion rates depending on the drug molecule size [149]. The high biocompatibility degree and biodegradability of most hydrogels makes them especial candidates for introduction in the clinical scenario. In the meantime, many

Table 2
Examples of nanogels for DOX delivery.

Nanocarrier description	Design strategy	Tumour model	Refs	Year
Acetylated HA	- Targeted therapy - DOX encapsulation	HeLa cell line (*in vitro*)	Park *et al.* [156]	2010
Disulfide-core-crosslinked PEG-poly(amino acid)s star copolymers	- Redox-responsive - DOX encapsulation	HeLa cell line (*in vitro*)	Ding *et al.* [20]	2011
Poly(N-isopropylacrylamide-acrylic acid) nanogel	- Temperature- and pH-responsive - DOX conjugation	Liver cancer cell line (*in vitro*)	Xiong *et al.* [157]	2011
Alginate (AG) nanogel crosslinked with cystamine	- Redox-responsive - DOX encapsulation	Bone cancer cell line (*in vitro*)	Maciel *et al.* [158]	2013
AG/PAMAM-G5-FI nanogels	- Imaging therapy - DOX encapsulation	Bone cancer cell line and mouse fibroblasts (*in vitro*)	Gonçalves *et al.* [159]	2014
Poly(N-isopropylacrylamide-acrylic acid) nanogels crosslinked with N,N'-bis (acryloyl)cystamine or with N,N'-methylene bisacrylamide	- Temperature-, pH- and redox-responsive - DOX encapsulation	Bone cancer cell line (*in vitro*)	Zhan *et al.* [22]	2015
Dextrin nanogels crosslinked with formaldehyde or glyoxal	- pH-responsive - DOX encapsulation	Colon cancer (*in vitro* & *in vivo*)	Manchun *et al.* [160]	2015
FA-PEG-Poly(acrylic acid) nanogels crosslinked with N,N'-bis(acryloyl) cystamine	- Targeted therapy - Redox-responsive - Co-delivery (DOX and cisplatin)	Breast cancer (*in vitro* & *in vivo*)	Wu *et al.* [15]	2017
HA-7-N,N-diethylamino-4-hydroxymethylcoumarin nanogels	- Targeted therapy - NIR- and UV-responsive - DOX encapsulation	Breast cancer cell line (*in vitro*)	Hang *et al.* [161]	2017
Poly(acrylic acid-4-vinylphenylboronic acid) nanogels	- pH- and redox-responsive - Co-delivery (DOX and combretastatin-A4 phosphate) - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Yang *et al.* [28]	2018

nanotherapeutics based on hydrogels and used for the cellular/tumour delivery of DOX are being developed (Table 2). Smart hydrogels that respond to environmental changes (such as pH, redox conditions and temperature, among other stimuli) have been the focus of diverse works performed both *in vitro* and *in vivo* [150–155].

Polymeric micelles and polymersomes

Drugs can be covalently linked to polymers that will then act as carriers for their delivery inside the body. Usually, the conjugated drugs are hydrophobic, and the polymer provides to the system the required solubility in aqueous environment [162]. Often, due to their amphiphilic nature, when in aqueous solution, polymer-drug conjugates tend to organize and self-assemble as micelles [163,164]. Another possibility is to form micelles using amphiphilic polymers and encapsulate the hydrophobic drug in the core of the micelle only by physical means [165]. In both situations, micelles consist of a hydrophobic core containing the drug and an outer hydrophilic shell conferring solubility in water and preventing aggregation [104]. When present at low concentrations in water, the amphiphilic building blocks of the micelles exist as discrete entities. When concentration increases above the "critical micelle concentration" (CMC), they start to self-assemble into supramolecular structures (the micelles) to maintain the hydrophobic core protected from the polar surroundings. This turning concentration point is known to depend on several experimental conditions like the polymer's chemical composition, polymer's chain length, and temperature [105]. Indeed, an important advantage of polymeric micelles is their facility of production [166,167].

Amphiphilic block-copolymers can also be used to form polymersomes by self-assembly. Polymersomes consist in vesicles that can transport a cargo in their core or within their wall [168]. Usually, the membrane of these vesicles has a thickness between 5 and 50 nm which may be significant compared to the polymersome size and may impact the drug's release rate.

Table 3 shows several examples of polymeric micelles and polymersomes that can be found in the literature for the specific delivery of DOX. Only by the examples presented, it is already possible to see that these systems can be very diverse in terms of chemical composition and versatile in terms of the strategy followed for drug delivery.

3.2. Lipid-based nanocarriers

Since the 1960s, lipid-based nanomaterials have been deeply studied as potential systems for chemical and biomedical applications [183–185]. This kind of nanomaterials became popular due to their "natural" lipid composition and therefore low toxicity [186]. There are three major lipid-based nanomaterials: solid-lipid nanoparticles (SLNs), micelles and liposomes. SLNs are usually spherical and possess a solid lipid core matrix and an outer layer of a surfactant. Lipophilic drugs can be transported in the core. Lipid components of SLNs should be solid at both body and ambient temperature and can be prepared from triglycerides, complex glyceride mixtures or even waxes [187]. The other two systems are mainly prepared from naturally occurring and/or synthetic phospholipids by self-assembly. Micelles are considered the smallest and simplest self-assembled lipid structures formed by one layer of polar lipids in aqueous solutions, forming spheres. Liposomes also consist of spherical assemblies of phospholipids that, in this case, are organized in bilayers (sometimes multiple bilayers) with a diameter size typically in the 50-200 nm range [188]. In aqueous solutions, the formed micelles possess a hydrophobic core, whereas liposomes possess a hydrophilic core. Since liposomes have an aqueous core and a lipid bilayer, they can accommodate both hydrophilic and hydrophobic molecules [189]. Cholesterol is generally added to the formulations of liposomes to stabilize the lipid bilayers [190].

All lipid-based nanocarriers can transport lipophilic drugs and are able to protect them from severe environmental conditions. Additional advantages are the easiness of production, possibility of functionalization and control over the drug release process [115,191]. Over the last years, lipid-based nanocarriers have been used as delivery vehicles for a diversity of molecules, like chemotherapeutics, enzymes, peptides, nucleic acids, antigens, antifungals and imaging agents [191,192]. Table 4 presents recent examples of research studies on lipid-based systems for the release of DOX.

3.3. Metallic and metal oxide nanoparticles

Nanoparticles (NPs) made of metals and metallic oxides present special properties, such as electronic, magnetic and optical, that can be tuned by adjusting their size, shape and composition [204]. Numerous

Table 3
Examples of polymeric micelles and polymersomes for DOX delivery.

	Nanocarrier description	Design Strategy	Tumour model	Refs	Year
Polymeric micelles	PEG-poly(β-amino ester) block copolymer micelle	- pH-responsive - DOX encapsulation	Skin cancer (*in vitro* & *in vivo*)	Ko *et al.* [169]	2007
	PEGylated GPLGV- or GPLGVRG-peptide conjugates	- DOX conjugation and encapsulation	Lung cancer (*in vitro* &*in vivo*)	Lee *et al.* [170]	2007
	N-(2-hydroxypropyl) methacrylamide-based copolymer conjugates with different hydrophobic groups	- pH-responsive - DOX conjugation	Lymphoma (*in vitro* & *in vivo*)	Chytil *et al.* [171]	2008
	Chitosan oligosaccharide-stearic acid conjugates	- pH-responsive - DOX conjugation	Lung cancer (*in vitro* & *in vivo*)	Hu *et al.* [172]	2009
	PEG-poly(allyl glycidyl ether)-DOX block copolymers	- pH-responsive - DOX conjugation	Lymphoma (*in vitro* & *in vivo*)	Vetvicka *et al.* [173]	2009
	PEG-poly(ε-caprolactone) (PCL) polymeric micelles modified with RGD4C peptide	- Targeted therapy - pH-responsive - DOX conjugation	Breast cancer (*in vitro* & *in vivo*)	Xiong *et al.* [174]	2010
	Poly(ethylene oxide) (PEO)-poly[(R)-3-hydroxybutyrate] (PHB)-PEO micelles	- DOX encapsulation	Cervical cancer (*in vitro* & *in vivo*)	Kim *et al.* [45]	2010
	PEG-phosphatidylethanolamine (PE) micelles modified with monoclonal 2C5 antibody	- Targeted therapy - DOX encapsulation	Ovarian cancer cell line (*in vitro*)	Perche *et al.* [175]	2012
	FA-PEG-PLA, PEG-P(LA-*co*-2-mercaptoethanol) polymeric micelles	- Targeted- and imaging therapy - pH-responsive - DOX conjugation	Hepatic cancer (*in vitro* & *in vivo*)	Hu *et al.* [176]	2012
	PEG-PE polymeric micelles	- Co-delivery (DOX and vinorelbine) - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Qin *et al.* [36]	2013
	DOX-glucuronide prodrug linked to PEG-poly[N-(2-hydroxypropyl)-methacrylamide-lactate] copolymer micelle	- Targeted therapy - Enzyme-responsive - DOX conjugation	Lung cancer (*in vitro* & *in vivo*)	Ruiz-Hernández *et al.* [108]	2014
	PEG-PCL polymeric micelles modified with Rhenium-188	- Imaging therapy (SPECT/CT) - DOX encapsulation	Lung cancer (*in vitro* & *in vivo*)	Shih *et al.* [55]	2015
	PEG-SS-PCL polymeric micelles modified with cRGD	- Targeted therapy - Redox-responsive - DOX encapsulation	Brain cancer (*in vitro* & *in vivo*)	Zhu *et al.* [14]	2016
	Pluronic F127-Chitosan-cis-acontityl-DOX conjugates	- pH-responsive - Co-delivery (DOX and paclitaxel) - DOX conjugation	Pharmacokinetic studies in rats	Ma *et al.* [25]	2016
	HA-2-(octadecyloxy)-1,3-dioxane-5-amine conjugates	- Targeted therapy - pH-responsive - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Qiu *et al.* [177]	2017
	PEG-Polyurethane conjugates	- pH-responsive - DOX encapsulation	Macrophage cell line (*in vitro*)	Liao *et al.* [178]	2018
	PEG-Fmoc-glycyrrhetinic acid conjugates	- DOX encapsulation	Liver cancer (*in vitro* & *in vivo*)	Yang *et al.* [179]	2019

(*continued on next page*)

Table 3 (*continued*)

	Nanocarrier description	Design Strategy	Tumour model	Refs	Year
Polymersomes	Poly(γ-benzyl l-glutamate)-HA based polymersomes	- Targeted therapy - DOX encapsulation	Brain and breast cancer cell line (*in vitro*) Breast cancer (*in vivo*)	Upadhyay *et al.* [180]	2010
	PEG-PCL polymersomes modified with transferrin	- Targeted therapy - DOX encapsulation	Brain cancer (*in vitro* & *in vivo*)	Pang *et al.* [82]	2011
	PEG-(ethyl-p-aminobenzoate) phosphazenes) based polymersomes	- DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Xu *et al.* [48]	2014
	PEG-SS-PLA-SS-PLA-SS-PEG polymersomes modified with FA and trastuzumab monoclonal antibody	- Targeted therapy - Redox-responsive - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Lale *et al.* [181]	2015
	PEG-PLA based polymersomes	- Co-delivery (DOX and combretastatin-A4 phosphate - DOX encapsulation	Nasopharynx cancer (*in vitro* & *in vivo*)	Zhu *et al.* [37]	2015
	PEG-PLGA based polymersomes modified with FA	- Targeted therapy - Co-delivery (DOX and quantum dots) - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Alibolandi *et al.* [182]	2016
	PCL-PEG-PCL based polymersomes modified with FA	- Targeted therapy - Co-delivery (DOX and paclitaxel) - DOX encapsulation	Liver cancer (*in vitro* & *in vivo*)	Zhu *et al.* [40]	2017
	PEG-poly(trimethylene carbonate-dithiolane trimethylene carbonate) polymersomes modified with GE11 peptide	- Targeted therapy - DOX encapsulation	Liver cancer (*in vitro* & *in vivo*)	Fang *et al.* [16]	2017
	Poly(3-methyl-N-vinylcaprolactam)-poly(N-vinylpyrrolidone)	- pH-responsive - DOX encapsulation	Cardiotoxicity studies in rats (*in vivo*)	Kozlovskaya *et al.* [29]	2019

types of metallic NPs are under study for the purposes of therapy, medical imaging contrast enhancing or both (theranostic applications). Indeed, they can transport drugs adsorbed into their surface and, simultaneously, act as contrast agents in imaging techniques, like MRI (this is the case of iron oxide nanoparticles) or CT imaging (like gold nanoparticles). These NPs are also promising due to their robustness, stability, and resistance to enzymatic degradation [205]. Also, as is well-known, some metals possess antimicrobial and anti-inflammatory properties, for instance gold, silver and platinum [206]. Table 5 shows representative examples of the possible use of metallic nanoparticles for DOX delivery.

Iron oxide nanoparticles

Usually, it is not difficult to functionalize metallic NPs with different surface groups, keeping the inner properties for imaging applications. Amongst metallic NPs, those of iron oxide are quite well studied and explored due to their magnetic properties. Their size ranges from 5 to 50 nm and they can be easily synthesized, being possible to control their size, shape and solubility [207]. However, to achieve such stability, they need to be stabilized which is achieved by surface modification with different ligands, such as carboxylates, phosphates, and also with polymers, like PEG and polyvinyl alcohol (PVA) [208,209]. Interestingly, iron oxide NPs can be used as drug delivery systems, contrast agents in medical imaging and, in addition, by applying an adequate magnetic field, for the thermal ablation of cancer (cancer hyperthermia treatment). As naked iron oxide NPs are toxic, they are usually coated, for example with polysaccharides, PEG, and other inorganic materials. Also, in this case, the coating can confer stealth properties to the nanoparticles so that they can avoid recognition by the immune system and phagocytosis.

Gold nanoparticles

Gold nanoparticles (AuNPs) are unique amongst nanomaterials because of their inherent inert chemical properties, low toxicity, controllable size, shape and easy functionalization. The most usual method to synthesize AuNPs is through citrate reduction of chloroauric acid in water [210]. Their typical diameter is between 5-50 nm, being their colour shape/size-dependent. The colour shifts from red to blue which can be detected in the visible part of the electromagnetic spectra [211]. The role of AuNPs in the biomedical field include labelling (for example, they can be used as contrast agents in transmission electron microscopy), drug delivery (by adsorbing drugs at their surface), heating (like iron oxide NPs, they can be used for cancer hyperthermia treatment) and sensing (due to their optoelectronic properties). Furthermore, AuNPs can attenuate X-rays and, so, are being investigated to be used as contrast agents in CT imaging. Very important is also the fact that they are very easily functionalized at the surface through the reaction of gold with sulfhydryl (R–SH) groups present in organic or biological molecules.

Silver nanoparticles

Silver nanoparticles (AgNPs) are being used in our daily life in a wide range of fields, including food, healthcare, and industry [212]. AgNPs exhibit special features such as optical, thermal, electrical and biological properties. One important characteristic is their strong antimicrobial and antifungicidal activity. Due to this property, they have been employed in several materials for medical care, namely in silicon catheters, sterilizing filters, sutures and, also, as medicines for dermatitis. Recently, AgNPs have also been studied as anticancer agents themselves, beyond the possibility of being used as drug carriers. Like AuNPs, they may be applied as diagnostic or probing mediators [213,214].

3.4. Carbon-based nanomaterials

Beyond their technological applications, carbon-based nanomaterials are also being explored in the biomedical field [237,238].

Table 4
Examples of lipid-based nanomaterials with DOX*.

Lipid family	Nanocarrier description	Design Strategy	Tumour model	Refs	Year
Solid lipid NP	PEG-PE conjugates modified with transferrin	- Targeted therapy - Co-delivery (DOX and EGFP-encoding plasmid) - DOX encapsulation	Lung cancer (*in vitro* & *in vivo*)	Han *et al.* [193]	2014
	Glyceryl mono stearate and soya–lecithin based SLNs modified with Fluorescein isothiocyanate (FITC) and galactose	- Targeted therapy - DOX encapsulation	Lung cancer (*in vitro*) Pharmacokinetic and biodistribution studies in rats	Jain *et al.* [194]	2015
	DSPE-PEG, DPPC, cocoa butter and palmitic acid based SLNs modified with aprotinin and melanotransferrin antibody	- Targeted therapy - DOX encapsulation	Brain cancer (*in vitro*)	Kuo *et al.* [84]	2016
	Poloxamer 470 and precirol ATO 5 based SLNs	- DOX encapsulation	Melanoma cancer (*in vitro* & *in vivo*)	Tupal *et al.* [195]	2016
Micelle	Labrafac WL 1349® and Solutol HS 15® based micelles	- Co-delivery (DOX and docetaxel) - DOX encapsulation	N/A	Vrignaud *et al.* [35]	2011
	1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine based micelles	- DOX encapsulation and complexation	Blood cancer (*in vitro)*	Wang *et al.* [196]	2012
	PEG, PE, DC-Cholesterol and DOPE based micelles containing manganese NPs	- Imaging therapy (MRI) - Co-delivery (DOX and DNA) - DOX encapsulation	Kidney and lung cancer (*in vitro* & *in vivo*)	Howell *et al.* [54]	2013
	DSPE-PEG based micelles modified with CRGDK peptide	- Targeted therapy - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Wei *et al.* [47]	2013
	DSPE-PEG based micelles modified with GE11 peptide	- Targeted therapy - DOX encapsulation	Breast cancer (*in vitro*)	Fan *et al.* [13]	2016
Liposome	HSPC/Cholesterol and DSPE-PEG based liposomes modified with Fab'$_{222\text{-}1D8}$ antibody fragment	- Targeted therapy - DOX encapsulation	Fibrosarcoma (*in vitro* & *in vivo*)	Hatakeyama *et al.* [197]	2007
	DSPE-PEG and DPPC or HSPC based liposomes modified with HER2-antibody	- Targeted therapy - Light-responsive - Co-delivery (DOX and hollow gold nanospheres) - DOX encapsulation	Carcinoma and lung cancer (*in vitro* & *in vivo*)	Li *et al.* [198]	2015
	HSPC/Cholesterol based liposomes modified with sialic acid-octadecylamine	- Targeted therapy - Co-delivery (DOX and dexamethasone palmitate) - DOX encapsulation	Sarcoma cancer (*in vivo*)	Sun *et al.* [38]	2016
	DSPE-PEG based liposomes modified with GE11 peptide and cetuximab Fab' antibody fragment	- Targeted therapy - Temperature responsive - DOX encapsulation	Breast cancer cell line (*in vitro*)	Haeri *et al.* [199]	2016
	DPPC/DSPC/Cholesterol/DSPE-PEG based liposomes	- Imaging therapy (MRI) - Ultrasound responsive - Co-delivery (DOX and gadoteridol) - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Rizzitelli *et al.* [56]	2016
	DSPC/CH; DSPC/DOTAP/Cholesterol and DSPC/ PEG-DSPE/ DOTAP/Cholesterol based liposomes	- Co-delivery (DOX and 5-Flurouracil) - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Camacho *et al.* [39]	2016
	DSPE-PEG based liposomes modified with $H_7K(R_2)_2$ peptide	- Targeted therapy - pH responsive - DOX encapsulation	Brain cancer (*in vitro* & *in vivo*)	Zhao *et al.* [49]	2016
	DPPC/DSPC/$DPPG_2$ based liposomes	- Imaging therapy (MRI) - Temperature responsive - Co-delivery (DOX and gadoteridol) - DOX encapsulation	Sarcoma cancer (*in vitro* & *in vivo*)	Peller *et al.* [200]	2016
	PEG-DSPE/DPPC/Cholesterol based liposomes	- Co-delivery (DOX and curcumin) - DOX encapsulation	Colon cancer cell line (*in vitro*)	Sesarman *et al.* [201]	2017
	DOTAP/DSPE-PEG and POPC/Cholesterol/DSPE-PEG based liposomes	- Co-delivery (DOX-DNA aptamer complex and tobramycin - DOX-DNA aptamer complex encapsulation	HeLa cancer cell line (*in vitro*)	Plourde *et al.* [202]	2017
	Lecithin S100/DOTAP/Cholesterol based liposomes modified with selenium	- DOX encapsulation	Lung cancer (*in vitro* & *in vivo*)	Xie *et al.* [203]	2018
	DSPE-PEG based liposomes modified with D-mannose or L-fucose	- Targeted therapy - DOX encapsulation	Sarcoma cancer (*in vitro* & *in vivo*)	Li *et al.* [19]	2019

* DSPE: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine; HER2: Human epidermal growth factor receptor-2; HSPC: Hydrogenated soy phosphatidylcholine; DPPC: 1.2-Dipalmitoyl-sn-glycero-3-phosphocoline; DSPC: 1.2-Distearoyl-sn-glycero-3-phosphocoline; DC: 3ß-[N-(N′, N′-dimethylaminoethane)-carbamoyl]; DOPE: dioleoylphosphatidyl-ethanolamine; DOTAP: 1,2-dioleoyl-3- trimethylammonium-propane; $DPPG_2$: 1,2-dipalmitoyl-sn-glycero-3-phosphodiglycerol; POPC: 1-palmitoyl-2- oleoyl-sn-glycero-3-phosphocholine

Important examples are graphene, carbon nanotubes (CNTs) and the fullerene C_{60}. Graphene consists in a single layer sheet structure with a thickness of a carbon atom, CNTs possess cylindrical hollow structures with the walls also with a thickness of a carbon atom, and C_{60} is a spherical molecule having 60 carbon atoms and the shape of a soccer ball. These three kinds of nanomaterials can be easily functionalized to increase their solubility in water, to allow their use as drug carriers and to tune their interaction with biological targets [239]. Currently, other

Table 5
Examples of metallic-based NPs with DOX.

Metal family	Nanocarrier description	Design strategy	Tumour model	Refs	Year
Iron Oxide	Gelatin-coated iron oxide NPs	- DOX encapsulation	N/A	Gaihre *et al.* [215]	2009
	Poly-n-isopropylacrylamide-coated iron oxide NPs	- Temperature responsive - DOX encapsulation	Liver cancer (*in vitro* & *in vivo*)	Purushotham *et al.* [216]	2009
	Polyvinyl alcohol (PVA)-coated iron oxide NPs	- DOX encapsulation	N/A	Kayal *et al.* [217]	2010
	PEG-trimellitic anhydride chloride polymer modified with FA and iron oxide NPs	- Targeted therapy - Imaging therapy (MRI) - DOX encapsulation	Liver cancer (*in vitro* & *in vivo*)	Maeng *et al.* [218]	2010
	PEGylated iron-platinum/iron oxide core-shell NPs modified with FA	- Targeted therapy - Imaging therapy (MRI) - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Liu *et al.* [219]	2013
	PEG-poly(4-vinylbenzylphosphonate) polymer-coated iron oxide NPs	- DOX encapsulation	Colon cancer (*in vitro* & *in vivo*)	Hałupka-Bryl *et al.* [220]	2014
	PEGylated mesoporous silica-coated iron oxide NPs	- DOX encapsulation	N/A	Pourjavadi *et al.* [221]	2015
	HA coated-iron oxide NPs	- DOX encapsulation	Breast cancer cell line (*in vitro*)	Aval *et al.* [222]	2016
	PVA coated-iron oxide NPs	- DOX conjugation	N/A	Nadeem *et al.* [223]	2016
	Heparin-coated iron oxide NPs	- Imaging therapy (MRI) - DOX conjugation	Lung cancer (*in vitro* & *in vivo*)	Yang *et al.* [224]	2016
	Iron oxide NPs coated with PEG-polydopamine polymer crosslinked with N,N-Bis(acryloyl)	- Redox responsive - DOX encapsulation	N/A	Shang *et al.* [26]	2017
	Pluronic F127-coated iron oxide NPs	- DOX encapsulation	Brain cancer cell line (*in vitro*)	Mdlovu *et al.* [225]	2019
Gold	AuNPs stabilized with P(LA-DOX)-*b*-PEG-OH polymer modified with FA	- Targeted therapy - pH responsive - DOX conjugation	Breast cancer cell line (*in vitro*)	Prabaharam *et al.* [226]	2009
	Gold nanoclusters modified with FA	- Targeted and imaging therapy - Light responsive - Co-delivery (DOX and MPA) - DOX conjugation	Lung, liver, breast and colon cancer cells (*in vitro* & *in vivo*)	Chen *et al.* [227]	2012
	Multifunctional gold nanorods	- Targeted and imaging (PET) therapy - pH responsive - Co-delivery (DOX and ^{64}Cu chelator) - DOX conjugation	Brain cancer (*in vitro* & *in vivo*)	Xiao *et al.* [53]	2012
	DOX-CPLGLAGG peptide AuNPs conjugates	- Targeted therapy - Redox and enzyme responsive - DOX conjugation	Mouse head and neck carcinoma cell line (*in vitro* & *in vivo*)	Chen *et al.* [228]	2013
	PEG-AuNPs conjugates	- DOX conjugation	Breast cancer (*in vitro* & *in vivo*)	Sun *et al.* [229]	2014
	PLGA-core gold-shell conjugates modified with human serum albumin (HSA)-indocyanine green-FA	- Targeted therapy - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Topete *et al.* [230]	2014
	PEGylated magnetic AuNPs	- DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Elbialy *et al.* [231]	2015
	AuNPs crosslinked with PCL-Poly(2-(dimethylamino) ethylmethacrylate)-PEG	- DOX encapsulation	Mouse head and neck carcinoma cell line (*in vitro* & *in vivo*)	Jeon *et al.* [232]	2015
	PEGylated 3-[2-Pyridyldithio]propionyl hydrazide-AuNP conjugates	- pH responsive - DOX conjugation	Human head and neck squamous carcinoma cell line (*in vitro*)	Lee *et al.* [109]	2015
	AuNPs-DOX conjugate	- DOX conjugation	Skin cancer (*in vitro* & *in vivo*)	Zhang *et al.* [233]	2015
	Oligonucleotide-conjugated AuNPs	- Targeted therapy - DOX complexation	Colon cancer (*in vitro* & *in vivo*)	Lee *et al.* [234]	2017
	Polyvinylpyrrolidone-coated AuNPs	- DOX conjugation	Lung cancer (*in vitro*)	Ramalingam *et al.* [235]	2018
	PEGylated gold nanocages modified with biotin	- Targeted therapy - Light responsive - Co-delivery (DOX, quercetin and tetradecanol) - DOX encapsulation	Breast cancer cell line (*in vitro*)	Zhang *et al.* [18]	2018
	Oxidized HA-decorated dihydroxyphenyl/hydrazide bifunctionalized hydroxyethyl CHI-gold nanorods conjugates	- Targeted therapy - pH responsive - DOX encapsulation	Breast cancer cell line (*in vitro*)	Hou *et al.* [236]	2019
Silver	Alendronate-coated AgNPs	- pH responsive - Co-delivery (DOX and alendronate) - DOX conjugation	HeLa cancer cell line (*in vitro*)	Benyettou *et al.* [23]	2015

carbon nanomaterials are also being tested in the biomedical area, like carbon nanohorns, derived from CNTs but having a conical cap, and carbon dots which are fluorescent nanomaterials. Curiously, many of the mentioned carbon structures were tested for drug delivery applications and, particularly, for the delivery of DOX as can be seen in the examples listed in Table 6.

3.5. Clay-based nanomaterials

Clay-based nanomaterials are already being used for cosmetic and pharmaceutic applications and, so, researchers are now trying to take advantage of their properties for further application in tissue engineering, regenerative medicine, medical imaging and cancer

Table 6
Examples of carbon-based nanomaterials with DOX.

	Nanocarrier description	Design Strategy	Tumour model	Refs	Year
Nanohorns	PEGylated oxidized carbon single-walled nanohorns	- DOX conjugation	Lung cancer cell line (*in vitro*)	Murakami *et al.* [240]	2006
Carbon Nanotubes	Pluronics F127-coated multi-walled CNTs	- DOX encapsulation	Breast cancer cell line (*in vitro*)	Ali-Boucetta *et al.* [241]	2008
	AG and CHI-coated single-walled CNTs modified with FA	- Targeted therapy - pH responsive - DOX encapsulation	HeLa cancer cell line (*in vitro*)	Zhang *et al.* [242]	2009
	PEGylated single-walled CNTs	- DOX encapsulation	Lymphoma (*in vitro* & *in vivo*)	Liu *et al.* [243]	2009
	PEGylated single-walled CNTs	- pH responsive - DOX conjugation	Lung cancer cell line (*in vitro*)	Gu *et al.* [244]	2011
	Multi-walled CNTs modified with FA and iron NPs	- Targeted therapy - Light responsive - DOX encapsulation	HeLa cancer cell line (*in vitro*)	Li *et al.* [245]	2011
	PEGylated oxidized multi-walled CNTs modified with angiopep-2	- Targeted therapy - DOX encapsulation	Brain cancer (*in vitro* & *in vivo*)	Ren *et al.* [83]	2012
	PEGylated multi-walled CNTs modified with FA	- Targeted therapy - DOX encapsulation	HeLa cancer cell line (*in vitro*) Liver perfusion study (*in vivo*)	Dinan *et al.* [246]	2014
	Oxidized multi-walled CTNs modified with galactosylated CHI	- Targeted therapy - pH responsive - DOX encapsulation	Liver cancer (*in vitro* & *in vivo*)	Qi *et al.* [247]	2015
	Carbon dots functionalized with gold nanorods	- Imaging therapy - Temperature responsive - DOX conjugation	Vero cell line and breast cancer cell line (*in vitro*)	Pandey *et al.* [248]	2013
Fullerene	C_{60}	- DOX conjugation	Breast cancer cell line (*in vitro*)	Liu *et al.* [249]	2010
	C_{60}	- DOX conjugation	Chicken embryo (*in vitro* & *in vivo*)	Blazkova *et al.* [250]	2014
	C_{60}-Polyethylenimine (PEI) conjugates	- Photodynamic therapy - pH responsive - DOX conjugation	Skin cancer (*in vitro* and *in vivo*)	Shi *et al.* [251]	2014
	PEGylated C_{60}	- DOX conjugation	Breast cancer cell line (*in vitro*)	Magoulas *et al.* [252]	2015
Carbon dots	Carbon dots modified with FA and bovine serum albumin (BSA)	- Targeted therapy - DOX encapsulation	Vero cell line and HeLa cancer cell line (*in vitro*)	Mewada *et al.* [253]	2014
	Carbon dots modified with transferrin	- Targeted therapy - DOX conjugation	Brain cancer cell line (*in vitro*)	Li *et al.* [85]	2016
	Carbon dots modified with heparin	- pH responsive - Co-delivery (DOX and heparin) - DOX encapsulation	Breast and lung cancer cell line (*in vitro*) HeLa cancer cell line (*in vitro*)	Zhang *et al.* [254]	2017
	Polydopamine-coated carbon dots	- DOX encapsulation	HeLa cancer cell line (*in vitro*)	Sun *et al.* [255]	2017
	Carbon dots modified with PEI and HA	- Targeted therapy - DOX conjugation	Mouse fibroblast cell line (*in vitro*) HeLa cancer cell line (*in vitro*)	Gao *et al.* [256]	2017
	Carbon dots	- DOX conjugation	Breast cancer cell line (*in vitro*)	Kong *et al.* [257]	2018
	Magnetic hollow (Fe_3O_4) and porous carbon NPs modified with poly(γ-glutamic acid) and FA	- Targeted and imaging therapy - Temperature, redox and pH responsive - DOX encapsulation	HeLa cancer cell line (*in vitro*) Biodistribution studies (*in vivo*)	Wu *et al.* [258]	2018
	Carbon dots modified with catechol-borane moieties	- pH responsive - DOX encapsulation	HeLa cancer cell line (*in vitro*)	Wu *et al.* [259]	2019

treatment [260]. Especial attention is being given to the synthetic clay Laponite® ($Na^{+}_{0.7}[(Si_8Mg_{5.5}Li_{0.3})O_{20}(OH)_4]^{-0.7}$, LP) that can be produced with a controllable composition at a large scale and low cost. Although there are several different LP grades commercially available, for medical applications, the grades having high purity and low heavy metals content should be used [261]. LP is composed of nanoscale crystals with a disk shape (about 25 nm in diameter and 0.92 nm in thickness). In these disks, the faces are negatively charged whereas the

Table 7
Examples of clay-based nanomaterials with DOX.

Nanocarrier description	Design strategy	Tumour model	Refs	Year
LP nanodisks	- DOX encapsulation	Liver cancer (*in vivo*)	Li *et al.* [265]	2014
LP nanodisks modified with PEG-PLA polymer	- DOX encapsulation	Bone cancer (*in vitro*)	Wang *et al.* [92]	2014
Alginate-coated LP nanodisks	- DOX encapsulation	Bone cancer (*in vitro*)	Gonçalves *et al.* [32]	2014
PEGylated LP nanodisks modified with lactobionic acid	- Targeted therapy - DOX encapsulation	Liver cancer (*in vitro*)	Chen *et al.* [95]	2015
Poly(allylamine) hydrochloride (PAH)/ poly(sodiumstyrene sulfonate) (PSS)-coated LP nanohybrids	- DOX encapsulation	Breast cancer (*in vitro*)	Xiao *et al.* [105]	2016
LP nanodisks modified with PEG-PLA, PEI, AuNPs and HA	- Targeted therapy - DOX encapsulation	HeLa cancer cell line (*in vitro* & *in vivo*)	Zhuang *et al.* [17]	2017
LP nanodisks modified with HA	- Targeted therapy - DOX encapsulation	HeLa cancer cell line (*in vitro*)	Jiang *et al.* [97]	2019

Table 8
Examples of biological-based nanomaterials with DOX.

Biological family	Nanocarrier description	Design strategy	Tumour model	Refs	Year
Albumin	HSA NPs	- DOX encapsulation	Brain cancer cell line (*in vitro*)	Dreis *et al.* [267]	2007
	HSA NPs modified with DI17E6 monoclonal antibody	- Targeted therapy - DOX encapsulation	Skin cancer cell line (*in vitro*)	Wagner *et al.* [268]	2010
	PEGylated modified HSA NPs modified with RGD	- Targeted therapy - DOX encapsulation	Skin cancer cell line (*in vitro*)	Xu *et al.* [269]	2011
	HSA NPs modified with TRAIL ligand and transferrin	- Targeted therapy - DOX encapsulation	Pancreatic and breast cancer cell line (*in vitro*) Colon cancer (*in vitro* & *in vivo*)	Bae *et al.* [270]	2012
	HSA NPs modified with palmitoyl-poly-arginine peptides	- Targeted therapy - DOX conjugation	HeLa cancer cell line (*in vitro*)	Ichimizu *et al.* [271]	2018
	BSA NPs	- Co-delivery (DOX and cyclopamine) - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Lu *et al.* [52]	2019
	HSA NPs	- DOX encapsulation	Colon cancer (*in vitro* & *in vivo*)	Kimura *et al.* [272]	2019
Exosomes	DOX-loaded exosomes and exosome-mimetic nanovesicles obtained from macrophages and lung cell line	- DOX encapsulation	HUVEC cell line (*in vitro*) Colon cell line (*in vitro* & *in vivo*)	Jang *et al.* [273]	2013
	DOX-loaded exosomes obtained from immature dendritic cells labelled with iRGD-Lamp2b protein	- Targeted therapy - DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Tian *et al.* [12]	2014
	DOX-loaded tumour-cell-derived exosomes	- DOX encapsulation	Breast cancer (*in vitro* & *in vivo*)	Yang *et al.* [274]	2015
	Exosomes loaded with AuNPs-DOX conjugates	- pH responsive - DOX conjugation	Lung cancer cell line (*in vitro*)	Srivastava *et al.* [275]	2016
	DOX-loaded lung cell-derived nanovesicles	- DOX encapsulation	HEK293T cells and HeLa cancer cell line (*in vitro*)	Goh *et al.* [276]	2017

edges possess pH-dependent charge [262]. Like other clays, LP presents a high swelling capacity and tendency to adsorb many types of molecules at its surface. Furthermore, by reaction of the silanol groups present at its edges with alkoxysilanes with additional reactive groups, the clay can also be covalently linked to molecules of interest. Also, although this clay degrades when exposed to acidic environments, it

Table 9
DOX-based nanotherapeutics in the market and in clinical stages.

Nanocarrier platform	Generic name	Formulation type	Therapeutic indication	Phase status	Refs
Liposomes	Doxil®/Caelyx®	PEGylated liposomal DOX	Kaposi's sarcoma Breast cancer Ovarian cancer Multiple myeloma	Approved	Stewart *et al.* [284] Northfelt *et al.* [285] O'Brien *et al.* [286] Gordon *et al.* [287] Rifkin *et al.* [288]
	Myocet®	Liposomal DOX	Breast cancer	Approved	Batist *et al.* [289,290] Harris *et al.* [291] Chan *et al.* [292]
	ThermoDox®	Thermosensitive liposomal DOX	Non-resectable hepatocellular carcinoma	Phase III	NCT02112656 [293]
			Non-resectable hepatocellular carcinoma Breast cancer Solid liver tumours	Phase III	NCT00617981 [294]
			Refractory solid tumours	Phase I/II	NCT00826085 [295]
			Metastatic breast cancer	Phase I	NCT02181075 [296]
				Phase I	NCT02536183 [297]
				Phase I	NCT03749850 [298]
	Sarcodoxome™	Liposomal DOX containing lipochroman 6	Small cell lung cancer	Phase II	Lopez-Pousa *et al.* [299]
	2B3-101	Glutathione PEGylated liposomal DOX	Meningeal carcinomatosis Brain metastases	Phase II	NCT01818713 [99]
				Phase I	Kerklaan *et al.* [100]
	anti-EGFR ILs-DOX	EGFR targeted liposomal DOX	Solid tumours	Phase II	Mamot *et al.* [101]
	MM-302	HER2 targeted liposomal DOX	HER2 positive breast cancer	Phase II[a]	Miller *et al.* [102] ADCreview Website [300]
NPs	Livatag®	DOX-loaded poly(isohexyl-cyanoacrylate) NPs	Hepatocellular carcinoma	Phase III[b]	Onxeo company [301,302]
Polymer-drug conjugates	FCE28068/PK1	N-(2-Hydroxypropyl) methacrylamide-DOX copolymer	Breast cancer Non-small cell lung cancer Colorectal cancer	Phase II	Seymour *et al.* [303]
	FCE28069/PK2	N-(2-Hydroxypropyl) methacrylamide-DOX-Galactosamine	Primary/metastatic liver cancer	Phase II	Seymour *et al.* [103]
Polymeric micelles	SP1049C	DOX block copolymer micelle	Non-resectable stage IVb adenocarcinoma	Phase III	Valle *et al.* [304]
	NK911	mPEG-DOX-poly-aspartic acid conjugates	Solid tumours	Phase II	Matsumura *et al.* [305]
Bacteria-derived	EDV™ minicells	DOX-loaded EDV nanocells	Glioblastoma multiform	Phase I	Whittle *et al.* [306]

[a] MM-302 Phase II clinical trial was discontinued in March 2017.
[b] Livatag Phase III clinical trial was discontinued in September 2017.

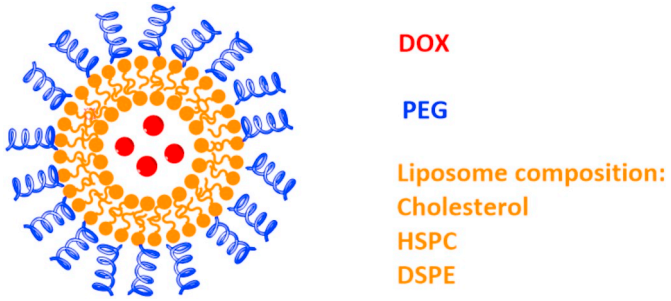

Fig. 3. Illustration of a PEGylated Doxil® liposome.

Fig. 4. Representation of the Myocet® liposome.

gives rise to non-toxic products such as aqueous silica, magnesium, sodium and lithium ions [263]. For these reasons, LP is now being evaluated as a nanocarrier for diverse biological and therapeutic molecules, including for the transport of DOX. Particularly interesting, is the behaviour of LP as a "proton-sponge material" thus helping to disrupt the endolysomal compartment inside cells [32].

The first work on the use of LP for DOX delivery, was authored by Wang and colleagues [264] that used naked LP for that purpose. As can be seen in Table 7, several other LP-based nanoscale systems (nanohybrid materials) were meanwhile assayed for DOX delivery too. All these platforms were developed having in view the improvement of the behaviour of LP as a drug delivery system, namely in what concerns the drug release profiles, stability in the physiological environment and targeting purposes.

3.6. Biological-based nanomaterials

Over the years, the long pursuit for non-toxic, non-immunogenic and biodegradable nanomaterials also led to the possibility of considering endogenous materials as vehicles for drugs since they already make part of the physiological system. In this sense, several works arose using proteins, cells and cellular vesicles from biological origin, *e.g.* platelets, macrophages, erythrocytes, exosomes and albumin (from these, exosomes and albumin can be classified as nanomaterials) [266].

Examples of works using biological-based nanomaterials as carriers for DOX can be seen in Table 8. These structures were considered as potential delivery systems for DOX mainly due to their inherent role in the transport of molecules inside the human body, also associated with their high *in vivo* biodistribution and long-life time. For instance, human serum albumin (HSA) is one of the most abundant proteins in the human plasma. This protein has an important role in the transport of many different molecules (like hormones, fatty acids, bilirubin) and metal ions. Moreover, exosomes are membrane vesicles which occur naturally during the excretion processes of cells, and that can be found in blood and other fluids of the body. These vesicles enclose a wide variety of proteins, RNA and lipids that are mediators in cell communication.

4. DOX-based nanotherapeutics in the clinical scenario

As will be detailed in the following sections, several DOX-based nanotherapeutics are under clinical trials or already in clinical use. In fact, from the discovery phase and before reaching the market, drugs (and nanodrugs too) must go through a selection process that starts with preclinical studies to obtain systematic data regarding drug's pharmacodynamics (PD, what the drug does to the body) and pharmacokinetics (PK, what the body does to the drug), and consequently the determination of the PK/PD profile of the drug. One of the main objectives of the preclinical phase is to provide knowledge concerning the safety of the drug and establish the safe dose for the first-in-man study. Then, experiments in humans start step-by-step from Phase I to Phase III clinical studies. For these, specific and defined protocols should be followed that clearly establish who is qualified to participate, number of persons, study duration, administration method, dosage and how data will be collected and analysed. Still, even after the drug reaching the market, it is important to gather information from the drug's performance while it is in active medical use [277,278].

Information regarding DOX-based nanotherapeutics that where already approved for clinical use or are under clinical studies is summarized in Table 9, such as their generic name, formulation type, therapeutic indications and clinical phase status. These nanotherapeutics cover different nanoplatform types, including liposomes, nanoparticles, polymer-drug conjugates, polymeric micelles, or even biological derivatives [9,70,279–283]. It must be mentioned that Table 9 does not include generic versions or very similar variants of the listed DOX-based nanotherapeutics that meanwhile appeared in the market.

4.1. Doxil®/Caelyx®

Liposomes were the first NPs to be successfully harnessed for drug delivery, and are experiencing an exponential evolution since almost 50 years ago [307]. Doxil® was the first nanotherapeutic approved by FDA in 1995 [308]. Doxil® was pioneer in the field of drug carriers in the US market and, in Europe, is commercialized under the name Caelyx® [68]. At the beginning, Doxil® was approved for the treatment of AIDS-related Kaposi's sarcoma [309] and, later on, for recurrent ovarian cancer (1998) [310], metastatic breast cancer (2003) [311] and multiple myeloma (2007) [312]. This system is based on a PEGylated liposome containing DOX in the internal cavity and has a mean diameter of about 80 to 90 nm [313]. Doxil® liposome is composed of three main lipid components: the hydrogenated soy phosphatidylcholine (HSPC); cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(methoxy-PEG) (DSPE-PEG) (Fig. 3). These lipids are considered safe once they do part of the diet and they are present on the cell membrane. The molar ratios (56: 38: 5) among them are responsible for maintaining the liposome structure [313,314]. The rigid bilayer at physiological temperature is achieved by the ratio between HSPC and cholesterol. DSPE is incorporated in the liposome bilayer and works as a docking point for PEG conjugation. Doxil® was conceived considering three main objectives: 1) to avoid the retention by the mononuclear phagocyte system and, as consequence, to prolong the circulation time; 2) to achieve a high and stable loading of DOX; and 3) to have the lipid bilayer in a "liquid order" phase. All of these requirements were achieved using: PEGylation to improve the biodistribution; the transmembrane ammonium-sulphate ($(NH_4)_2SO_4$) gradient driven force for DOX encapsulation; and the use of HSPC which exhibits a high phase transition temperature (melting temperature, Tm). Importantly, liposomal DOX displayed linear pharmacokinetics over the dose range of 10 to 20 mg/m^2 [313].

Interestingly, Barenholz and co-workers [313] developed a remote drug loading approach which was responsible for the high efficiency and stable drug loading. This method relies on a transmembrane gradient of $(NH_4)_2SO_4$, which involves a higher concentration of $(NH_4)_2SO_4$ inside the liposome when compared with the outside. This difference between the concentration in both compartments works as a driving force for the loading of DOX. With this loading technique, it was possible to reach a very high accumulation of DOX in the core (around 15,000 DOX molecules/liposome).

According to literature, more than ten Phase I/II clinical trials were performed in patients suffering from AIDS-related Kaposi's sarcoma. Overall, patients treated with Doxil® formulation presented improved responses when compared with conventional chemotherapy [315]. Passing to the Phase III clinical trials, two independent randomized studies were performed [284,285]. Stewart and colleagues [284] evaluated if Doxil® could be an effective approach when compared with the traditional bleomycin and vincristine treatment. They studied 241 patients in a randomized study where 20 mg/m^2 of Doxil® was administered against the combination of 15 IU/m^2 bleomycin with 1.4 mg/m^2 vincristine. In another study performed by Northfelt *et al.* [285], Doxil® efficacy was accessed *versus* the conventional treatment with DOX, bleomycin and vincristine. A total of 258 patients with AIDS-related Kaposi's sarcoma participated in the study. The treatment consisted in the administration of 20 mg/m^2 Doxil® versus the combination of 20 mg/m^2 DOX, 10 mg/m^2 bleomycin and 1 mg vincristine. In both studies, Doxil® improved the treatment, being more effective and less toxic than traditional chemotherapy. Regarding ovarian cancer treatment, Gordon and colleagues [287] performed a Phase III clinical trial with the purpose of evaluating the long-term survival. The study was performed with a total of 481 patients randomly distributed in two groups. In the first group, 50 mg/m^2 Doxil® was administered every 4th week; in the second group, 1.5 mg/m^2/day of topotecan was administered during 5 days, repeating the dose at every 3 weeks. This follow-up study demonstrated that treating patients suffering from recurrent and refractory ovarian cancer with Doxil® significantly improved the overall survival (OS) (from 70.1 weeks for topotecan to 108 weeks for Doxil® patients). Therefore, these results proved that Doxil® could be used as first-line treatment for this type of cancer. Also, O'Brien *et al.* [286] showed that Doxil® has higher efficacy and less cardiotoxicity than conventional DOX and that could be used as first-line treatment for metastatic breast cancer. In this Phase III clinical study, around 509 women received 50 mg/m^2 of Doxil® every 4 weeks or 60 mg/m^2 of DOX every 3 weeks. The results showed that Doxil® led to an overall reduction in cardiotoxicity and myelosuppression, having an efficacy equivalent to that of DOX. Rifkin and co-workers [288] conducted a Phase III clinical trial in patients newly diagnosed with active multiple myeloma. The patients (n = 192) were split and exposed to two different treatment methodologies. In the first method, the treatment consisted in the combination of 40 mg/m^2 Doxil® with 1.4 mg/m^2 vincristine plus the reduction in the oral dose of dexamethasone (40 mg) in the first 4 days. The second methodology was based on 0.4 mg/day vincristine, 9 mg/m^2/day DOX and, also, a reduction in the dexamethasone dose for 4 days. At the end, both approaches gave a similar response, less toxicity and improved OS when compared with conventional treatment with DOX.

Doxil® approval was the primary step to launch other nanomedicines. Following Doxil®, several other lipid-based systems were created, either based on stealth liposomes with a cocktail of loaded drugs or on liposomes with loaded drugs and targeted moieties conjugated at the surface.

4.2. *Myocet®*

Five years after Doxil® approval, Myocet®, a non-PEGylated liposomal DOX, was approved in Europe and in Canada [290]. Myocet® liposome presents a diameter size around 150-250 nm and is composed by cholesterol, egg phosphatidylcholine (PC) and, in the interior, a DOX citrate complex (Fig. 4) [316].

This formulation was approved as first-line treatment for metastatic breast cancer in combination with cyclophosphamide [292,317]. Bearing in mind the results from preclinical stage [318], a Phase I clinical trial [319] was conducted in 38 patients with refractory solid tumours. The study was made using two different approaches. The first consisted in intravenous *(i.v.)* administration of a dosage of 20 mg/m^2 escalating to 30, 45, 60, 75 and 90 mg/m^2 every 3 weeks. The second involved a consecutive administration for three days, starting with 20, then 25 and then 30 mg/m^2/day. The maximum-tolerated dose (MTD) was achieved by detection of leukopenia. In the first approach, the maximum dose was 90 mg/m^2 and for the second was 25 mg/m^2/day. In general, Myocet® was well tolerated and revealed fewer symptoms (nausea, vomiting and stomatitis) than free DOX. Cardiotoxicity was not detected in any of the patients. Phase III clinical trials were carried on by different research groups [289,291,292]. In the first study accomplished by Batist *et al.* [289], the purpose was to evaluate if the combination of Myocet® and cyclophosphamide could significantly reduce DOX cardiotoxicity and, at the same time, the improvement of the antitumor efficacy as first-line therapy for metastatic breast cancer. In this study, 297 patients received conventional DOX or Myocet® (60 mg/m^2, *i.v.)* and, additionally, a 600 mg/m^2 dose of cyclophosphamide every 3 weeks. Both groups revealed a similar response, but the Myocet® group demonstrated less cardiac toxicity. Another Phase III clinical trial developed by Harris *et al.* [291] consisted in the *i.v.* administration of 75 mg/m^2 Myocet® or DOX each 3rd week for the treatment of metastatic breast cancer. The obtained results revealed a comparable reaction for both groups, being more satisfactory the cardiotoxicity results of the Myocet® group. A few years later, one more Phase III clinical trial was carried on by Chan *et al.* [292]. In this study, they compared the combined effect of 75 mg/m^2 Myocet® and cyclophosphamide against 75 mg/m^2 epirubicin and cyclophosphamide (600 mg/m^2 for both approaches) as first-line treatment for metastatic

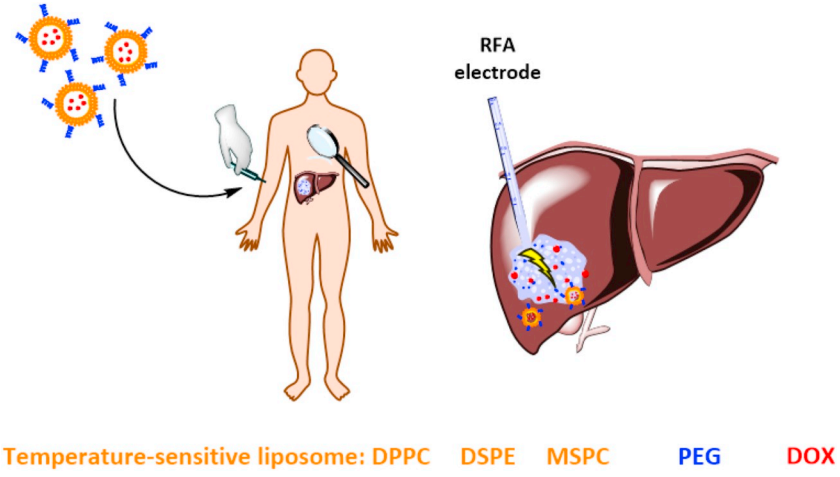

Fig. 5. Illustrative mechanism to trigger ThermoDox®.

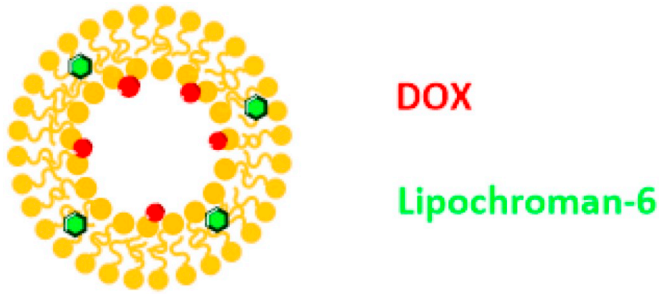

Fig. 6. Illustrative image of a liposomal formulation stabilized by lipochroman-6 for DOX delivery.

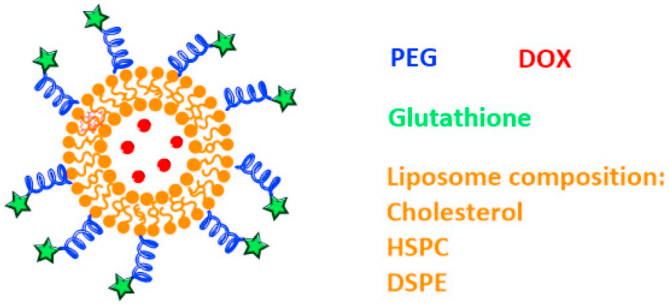

Fig. 7. Schematic structure of GSH-PEG liposomal DOX.

breast cancer. A total of 160 patients were randomized either to receive the first combined approach, either the second, every 3 weeks. At the end, the combination of Myocet® and cyclophosphamide revealed to be a more promising approach as first-line treatment since it gathers the dose-effect dependability of DOX with the safety of epirubicin. All the previous clinical trials demonstrated that Myocet® could be a good candidate for substitution of the traditional DOX.

4.3. ThermoDox®

ThermoDox® consists of thermosensitive liposomes with DOX that have a mean diameter size of 100 nm [320]. The liposomes are composed of 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC) and DSPE-PEG, at molar ratios of 86: 10: 4, respectively. These systems are described as low temperature-sensitive liposomes (LTSL) since, when exposed to a relatively high temperatures (~42°C), become leaky and release the encapsulated drug [321,322]. The Tm of phospholipids is very important. In LTSL systems, the transition temperature of lipids is usually around 40 to 45°C and it is for this reason that they are used [323,324]. Phospholipids can exist in a fluid state (when the temperature is higher than their Tm) or in a gel state (when the temperature is lower than their Tm). Temperature sensitive liposomes should exist in the gel state at body temperature to retain the drug while they are circulating in the bloodstream. If the temperature rises and reaches the Tm value, then the liposome changes to the fluid state and the drug is released.

ThermoDox® formulation was conceived for the treatment of primary liver cancer (hepatocellular carcinoma, HCC) and also for recurring chest wall breast cancer. Part of the typical treatment for these pathologies is based on the use of radiofrequency ablation combined with chemotherapy [321,325]. In this context, ThermoDox® liposomes are delivered by *i.v.* administration and, due to defective vasculature, they accumulate in the tumour site. Afterwards, a source of heat is applied and in response to that stimuli, the drug is released nearby and inside of the tumour tissue (Fig. 5). The key goal of ThermoDox® is to achieve the micro-metastases which are the main responsible for cancer recurrence.

In 2009, FDA gave the status of Orphan drug (a drug developed to treat a rare medical condition) to ThermoDox® for treatment of HCC. Several clinical studies with ThermoDox® are currently ongoing, although the results have not yet been disclosed. The Phase I/II DIGNITY study (NCT00826085) [295] involved ThermoDox® and microwave hyperthermia for the treatment of breast cancer recurrence at the chest wall. Another study which is ongoing is Phase III OPTIMA study (NCT02112656) [293] which is using ThermoDox® and radiofrequency ablation (RFA) for treatment of HCC. Still another study, the Phase I HEAT study (NCT00617981) [294], started with 24 patients suffering from HCC and metastatic liver tumours. The data from this study was not published but according to Poon and Borys [320], the MTD was achieved at 50 mg/m^2. Due to the outstanding Phase I results, this project jumped directly to Phase III. Phase III study has just been completed but the results are not yet known. The study was conducted in 701 patients aiming at treating non-resectable HCC using ThermoDox® and RFA. If ThermoDox® and RFA have synergistic effects in the treatment, then maybe this approach can be used as front-line treatment. ThermoDox® is also being tested using different approaches. For instance, the clinical trial Phase I TARDOX (NCT02181075) [296,326] consists in a proof-of-concept study where ThermoDox® is guided by focused ultrasound (FUS) to the target and later activated by mild hyperthermia. Another study is the Phase I trial of ThermoDox® and Magnetic Resonance-Guided High Intensity Focused Ultrasound (MR-HIFU) for the treatment of relapsed or refractory solid tumours in

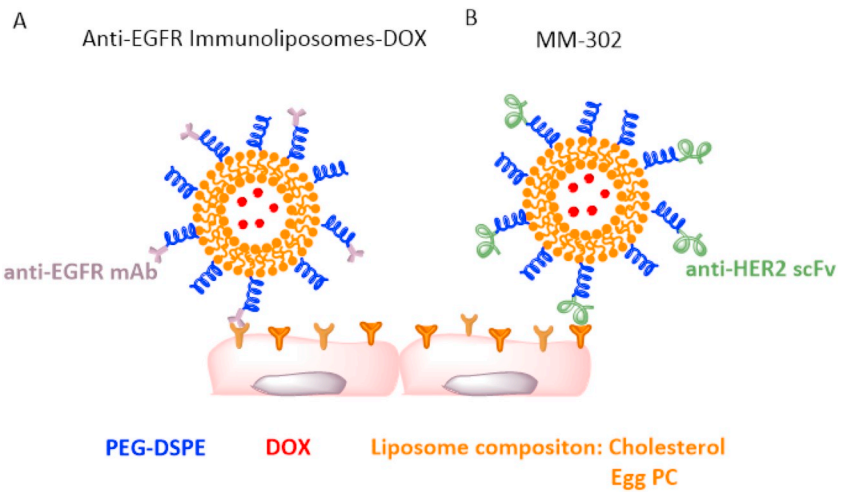

Fig. 8. A) Scheme showing the interaction of anti-EGFR ILs-DOX with cells; B) Illustration of the interaction of MM-302 with HER2 overexpressing cells.

children, adolescents and young adults (NCT02536183) [297]. This study started with 34 patients and is seeking for the MTD and the recommended Phase II dose of ThermoDox® to be administered in combination with MR-HIFU. The most recent study is the Phase I trial (NCT03749850) [298] of image-guided targeted DOX delivery with hyperthermia to optimize loco-regional control in breast cancer. In this study, clinicians are interested in the evaluation of the synergistic effect of ThermoDox® with local hyperthermia and cyclophosphamide in the treatment of primary breast tumour in patients with metastatic breast cancer.

4.4. Sarcodoxome™

A new liposomal formulation containing DOX, Sarcodoxome™, was developed for the treatment of soft tissue sarcoma (STS). These liposomes are not PEGylated and contain lipochroman-6 to improve their stability. In this system, DOX is loaded in the walls of the liposome (Fig. 6). In 2006, Sarcodoxome™ received the Orphan drug status by EMA and later the same status was approved by FDA (2007). Phase I/II clinical trials were launched in Spain [327]. A Phase II clinical trial [299] was performed in 37 patients with advanced or metastatic STS and with 65 years or older. In general, Sarcodoxome™ revealed a safe and acceptable toxicity profile, an MTD of 80 mg/m^2 and no cardiotoxicity associated. However, further studies are needed with younger patients.

4.5. 2B3-101

Brain tumours are considered devastating diseases, only starting to reveal symptoms already at a late stage. As mentioned before, the BBB is a protective shield of the central nervous system (CNS), being responsible for blocking the passage into the CNS of strange and potentially harmful molecules. As a consequence, treating brain malignancies is a very big challenge [87]. Aiming at overcoming this issue, the 2B3-101 system is being developed which consists of a PEGylated liposomal DOX formulation conjugated to glutathione (GSH) as targeting ligand. It has an average diameter of 95 nm (Fig. 7) [86,328]. The technology behind 2B3-101 (G-technology®) explores existing GSH-transport mechanisms across the BBB [86]. This system was designed for targeting glioma brain and metastases.

The performance (affinity and uptake) of 2B3-101 was evaluated *in vitro* using human brain capillary endothelial cells [328]. Results suggested that the uptake of 2B3-101 is time-, temperature- and concentration-dependent. Overall, the presence of GSH really improved the efficacy of the 2B3-101 system, increasing the efficacy of DOX delivery. Further PK and brain uptake studies were performed *in vivo* with concentrations comparable to those tested *in vitro*. Moreover, the efficacy of 2B3-101 was studied *in vivo* in mice using a brain tumour model of glioblastoma multiform (GBM). Basically, U87MG cells (human glioblastoma cell line) were injected directly into the brain of athymic FVB mice (mice lacking the thymus gland), originating a high vascularized brain tumour. In this study, the efficacy of free DOX, PEGylated liposomal DOX and 2B3-101 was compared. At the end, no neurological indicators were seen, and both systems were well tolerated. However, the presence of GSH in 2B3-101 resulted in a superior efficacy. The aim of this study was not to determine the toxicity and, thus, no MTD was determined [328]. However, these promising results were the initial impulse for the beginning of the clinical trials. A Phase I/IIa clinical trial [100] was performed in patients with solid tumours and brain metastases or recurrent malignant glioma. The patients received 40-70 mg/m^2 or 60 mg/m^2 dosages. In general, 2B3-101 was considered safe and it was well tolerated. In Phase IIa trial, the recommended doses were based on the tolerability of the previous results. The 2B3-101 system is also being studied for the treatment of meningeal carcinomatosis (NCT01818713) [99]. In the Phase II clinical trial, the aim is to evaluate the primary efficacy of 2B3-101 in patients suffering from leptomeningeal metastases of breast cancer. According to the clinical trials website, just a few patients received the treatment (n = 6). A single dose of 50 mg/m^2 was administered intravenously every 3 weeks. Up to date, no results were published.

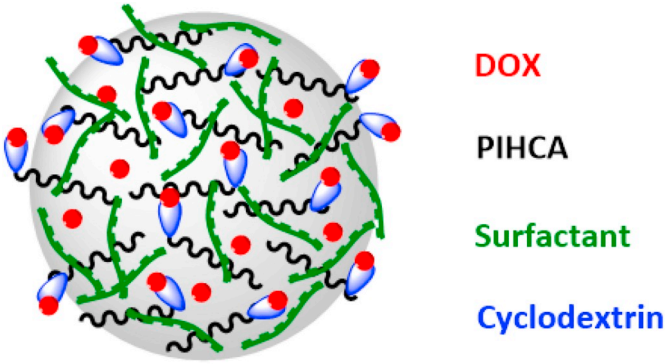

Fig. 9. Representative scheme of PIHCA NPs loaded with DOX.

4.6. Anti-EGFR immunoliposomes-DOX

The conjugation of monoclonal antibody fragments (mAb) to liposomes results in immunoliposomes (ILs). These anti-EGFR immunoliposomes-DOX, now in phase II clinical trial, are based on liposomes made of cholesterol and PC conjugated to a mAb against the epidermal growth factor receptor (EGFR) (Fig. 8 A) [329]. These liposomes present an average diameter of 100-120 nm [330]. They can target the EGFR overexpressing tumours and, at the same time, they can be used as drug carriers [331].

Mamot *et al.* studied the delivery of DOX from the anti-EGFR ILs-DOX system. According to these *in vitro* studies [331], a higher DOX internalization (about 29- fold more) was achieved in the presence of the EGFR ligand in EGFR-overexpressing cell lines (MDA-MB-468 human breast cancer and U87MG human GBM). In the *in vivo* studies [332], healthy rats revealed similar PK profiles between the liposomal DOX with and without EGFR ligand, suggesting that the mAb fragment was not crucial for biodistribution stability. The therapeutic efficacy of the anti-EGFR ILs-DOX was evaluated by using the cancer cell lines previously used, but this time as xenograft models. The results clearly showed that anti-EGFR ILs-DOX could significantly inhibit the tumour size and overcome the problem of multidrug resistance [333]. Considering the positive results, this anti-EGFR ILs-DOX system proceeded for Phase I clinical trial [101]. The main goal of this study was to determine the MTD in patients with EGFR-overexpressing advanced solid tumours. In this trial, 26 patients were treated with *i.v.* administration of anti-EGFR ILs-DOX. The concentration was scaled up (5-60 mg/m^2 DOX equivalents) over the six cycles. Interestingly, was the absence of cardiotoxicity, cumulative toxicity or alopecia. The suggested anti-EGFR ILs-DOX concentration for Phase II clinical trial was 50 mg/m^2, which corresponds to the MTD.

4.7. MM-302

A new drug delivery system, MM-302, was also developed that displays an average size of 75-110 nm and is a HER2-targeted antibody-liposomal DOX conjugate. HER2 is the human epidermal growth factor receptor-2 that may be overexpressed in breast cancer (Fig. 8 B) [334]. The targeting is accomplished by attaching a single-chain antibody fragment (scFv) of HER2 via a polyethylene glycol spacer (PEG-DSPE) to the DOX-loaded ILs surface [329].

In vitro studies revealed that the MM-302 liposomes were bind and internalized by HER2 overexpressing cells in a greater extent than the control [335,336]. According to the PK results in rats, there was no difference between the clearance rates of MM-302 and the control.

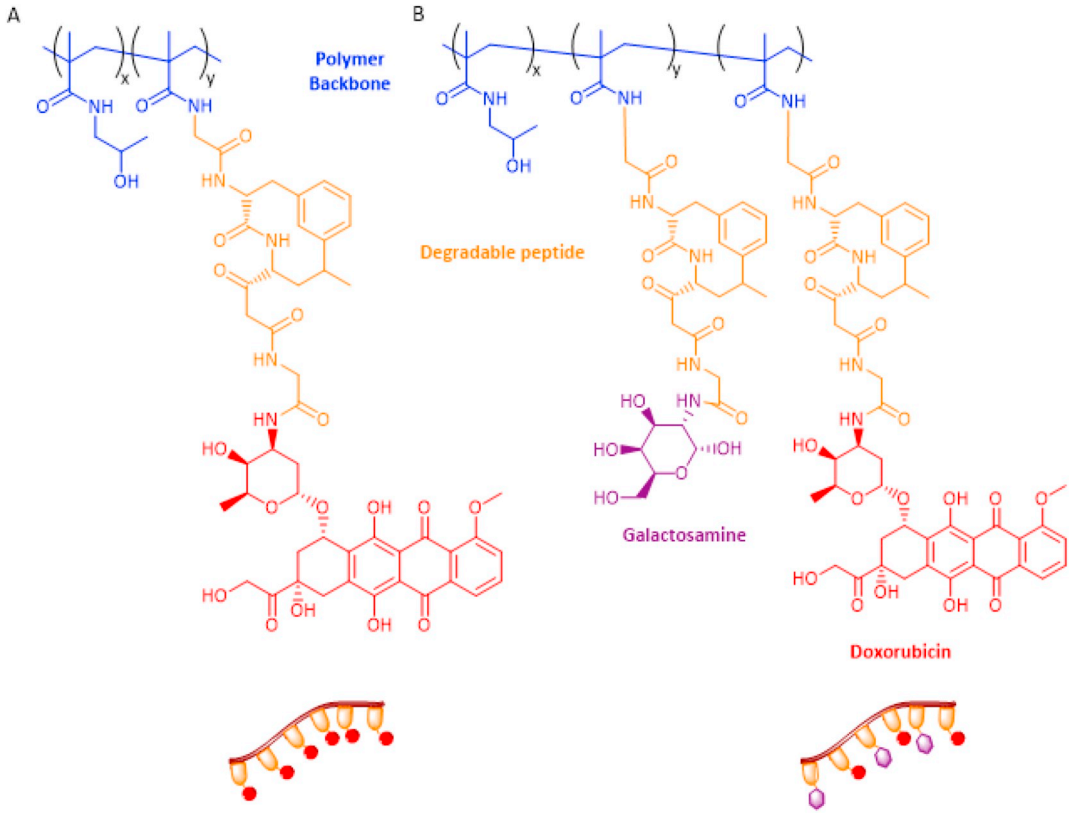

Fig. 10. A) HPMA copolymer–DOX (PK1) structure; B) HPMA copolymer–DOX structure containing galactosamine (PK2) to promote liver targeting.

Furthermore, the targeting capacity of MM-302 liposomes was extensively studied in four different human HER2-positive breast cancer xenograft models. These studies confirmed the selectivity for HER2 positive cells and, as a result, in some cases, a significant decrease in tumour growth [335,336]. According to Phase I trial results reported at the San Antonio Breast Cancer Symposium in 2012 [337], it was found that MTD was 40 mg/m^2. In this trial, 14 patients with positive HER2 advanced breast cancer received the MM-302. The administered dosage was 8, 16, 30 and 40 mg/m^2 every 4 weeks. In general, the results obtained suggested no cardiotoxicity associated up to the maximum dose administered.

Phase II clinical trial (HERMIONE study) consisted in the random administration of MM-302 plus trastuzumab in patients with locally advanced/metastatic HER2-positive breast cancer [102]. The main key points of this study were to assess the progression-free survival (PFS), the OS and also the safety, tolerability, quality of life and PK profile. For MM-302, the selected dose was 30 mg/m^2 every 3 weeks, and the combination with trastuzumab was also administered every 3 weeks. At the end, results demonstrated that the combination of both novel MM-302 and trastuzumab therapy could be well-tolerated and more effective. Unfortunately, after a recommendation of the independent monitoring panel "Data and Safety Monitoring Board" (DSMB), the HERMIONE clinical trial was stopped. The decision was taken considering that there were no improved safety signals after the treatment.

4.8. Livatag®

Livatag® is a NP formulation of DOX (Transdrug™ technology) that consists in DOX-loaded polyisohexylcyanoacrylate (PIHCA) NPs with 300 nm size (Fig. 9) [338]. The Transdrug™ technology relies on the use of NPs to overcome drug resistance, facilitating cell penetration and cell-drug contact.

Livatag® received the status of Orphan drug in Europe in 2004 and in the US in 2011. This NP formulation was developed with the aim to treat patients with primary liver cancer [339]. In Phase I clinical trial (ReLive study), Kattan and colleagues [338] have studied the effect of Livatag® in patients with refractory solid tumours. A total of 21 patients have received the formulation by *i.v.* administration with an initial dose of 15 mg/m^2 (30, 45, 60, 75 and 90 mg/m^2) every 4 weeks. The MTD revealed neutropenia at 90 mg/m^2. Consequently, for further Phase II trial, it was suggested a dose of 75 mg/m^2. According to the Onxeo website [340], the results from Phase II showed an increase in the survival time of patients with HCC. After that, a Phase III clinical trial was launched in 2012, in the US and Europe. This trial was designed to study the efficacy of Livatag® in 400 patients with HCC at advanced stage. At an early stage, Phase III results exhibited good results and tolerance. Unfortunately, in September 11, 2017, it was announced that the ReLive study did not met the principal purposes which were to improve the overall patient's survival when compared to the control group [301]. The final results from ReLive study were presented at the 11th Annual Conference of the International Liver Cancer Association in Seoul, South Korea (ILCA 2017) [302].

4.9. PK1

Currently, few anticancer-drug conjugates achieved the clinical phase. A few years back, Kopeček [341] and co-workers started the investigation on (N-(2-hydroxypropyl) methacrylamide) (HPMA) synthesis. Later on, fruit of collaborations with Duncan *et al.*, a patent application arise (1985) [342]. Until now, two types of HPMA copolymer conjugates were developed and reached the clinical trial stage. PK1 was the first to be designed and consists in a HPMA backbone in which DOX is conjugated through a peptide linker (Gly-Phe-Leu-Gly) (Fig. 10 A). This linker is stable at physiological pH but can be cleaved in the lysosomes by enzymes. PK1 presents a molecular weight (MW) ~ 30,000 g/mol and DOX content around 8.5 wt% [343]. At preclinical stage [344], this nanotherapeutic revealed to be promising when compared to the conventional drug. In a Phase I clinical trial [343], PK1

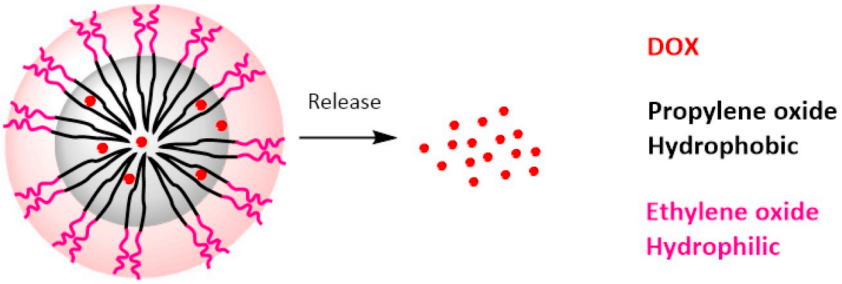

Fig. 11. Schematic representation of drug-loaded polymeric micelle.

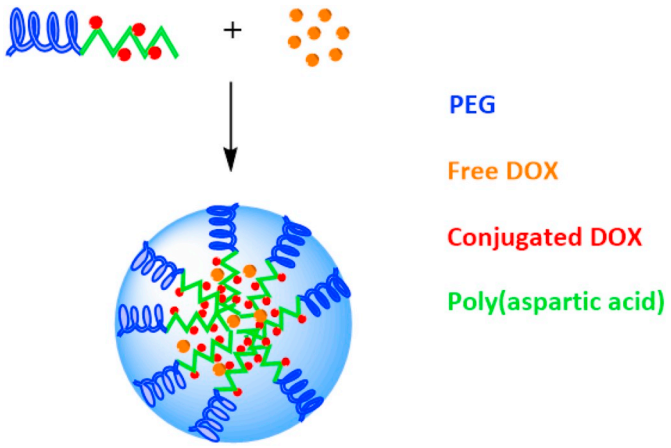

Fig. 12. Schematic representation showing the structure of NK911.

was administered to 36 patients with refractory or resistant cancer by *i.v.* administration with an interval of 3 weeks between cycles. The object of study was to determine the PK profile of PK1 and the toxicity associated with the determination of the MTD and dose-limiting toxicities (DLTs). At the beginning of the treatment, 20 mg/m^2 of PK1 were administered and increased until reaching 320 mg/m^2. At this step, few toxic effects were registered, namely mucositis and febrile neutropenia. Interestingly is that no cardiotoxicity was observed even at 1680 mg/m^2. Based on these results, the recommended dose to Phase II was 280 mg/m^2, to be implemented in patients with colorectal, non-small cell lung cancer (NSCLC) and breast cancer patients. In Phase II clinical trials [303], this recommendation was considered and 62 patients were divided into the three cancer types: breast (n = 17), NSCLC (n = 29) and colorectal (n = 16) cancer. The response was favourable in few cases (3 for breast and 3 for NSCLC) and no response for colorectal patients. In contrast, these studies demonstrated that the administration of high doses of PK1 (> 20 g/m^2) did not triggered any toxicity related to the polymer or even immunogenicity.

4.10. PK2

Bearing in mind the PK1 system and the positive results achieved, PK2 was developed, being structurally similar to PK1 but with an additional galactosamine residue (Fig. 10 B). This residue introduction is supposed to facilitate and improve the efficacy of the system by targeting the hepatocyte asialoglycoprotein receptors for hepatic cancer treatment. The MW of PK2 is about 25,000 g/mol with a DOX content of ~7.5% and 1.5-2.5 mol% of galactosamine content [103]. PK2 is the first drug conjugate which was designed for active targeting. In preclinical studies with mice, reduced cardiotoxicity was observed when using PK2 [345]. In a Phase I study [103], the PK profile, toxicity and the targeting specificity were evaluated in 31 patients with primary or metastatic liver cancer. PK2 was administered by *i.v.* with an initial concentration of 20 mg/m^2 (DOX equivalents) every 3 weeks. Consequently, with the escalation of the concentration (160 mg/m^2, further MTD), some side effects started to appear, such as severe fatigue, neutropenia and mucositis. Moreover, after 24 h injection, the biodistribution revealed that approximately 16.9% of the PK2 drug was targeting the liver, while the untargeted control did not. For further Phase II trials, a 120 mg/m^2 dosage was recommended to be administered every 3 weeks.

4.11. SP1049C

SP1049C consists in micelles resulting from the combination of two different Pluronic® copolymers, *i.e.*, Pluronic® L61 and Pluronic® F127 [346]. Basically, pluronics consist in ternary copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO). Each of these

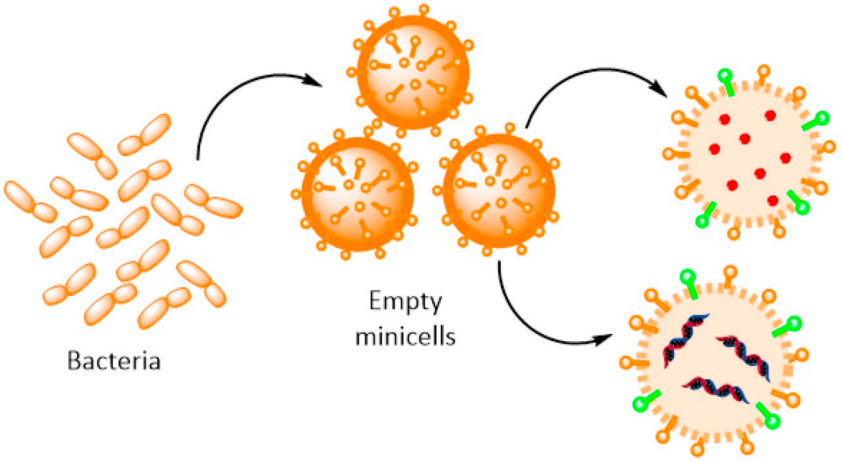

Fig. 13. Scheme showing bispecific antibody-targeted, drug/siRNA-packaged minicells.

segments is responsible for one part of the micelle formation. The PPO segment is hydrophobic and will assemble forming the hydrophobic core, whereas the PEO segment is hydrophilic and will be responsible for the corona formation (Fig. 11) [347]. The ratio Pluronic® L61:Pluronic® F127 used to obtain the desired polymeric micelle was 1:8 (w/w). Afterwards, DOX was loaded into the hydrophobic core by noncovalent interactions achieving a diameter of 22-27 nm [346].

In vitro assays demonstrated that SP1049C had an improved efficacy when compared to free DOX [348]. Furthermore, the preclinical *in vivo* studies showed that the antitumor efficacy improved with SP1049C usage [346,349]. SP1049C had antitumor potential especially for treating adenocarcinoma in the oesophagus and gastroesophageal junction. In Phase I clinical trials [350], the goal was to assess all the PK and toxicity profiles, specifically, the DLTs and MTD. The study started with 28 patients with refractory tumours and a 5 mg/m^2 (DOX equivalents) dose every 3 weeks till reaching the 6th cycle. When the maximum dose was administered (90 mg/m^2), some toxic effects were observed, such as myelosuppression. Considering these results, a Phase II clinical trial was proposed but with a DLT around 70 mg/m^2. The Phase II clinical trial [304] included 21 patients with adenocarcinoma in the oesophagus and gastroesophageal junction. In this study, a 75 mg/m^2 (DOX equivalents) dose was injected every 3 weeks. Despite neutropenia manifestation, this Phase II revealed that SP1049C was really effective as monotherapy for the previously mentioned types of cancer. A Phase III clinical trial is currently under way for metastatic adenocarcinoma of the oesophagus, gastroesophageal junction and stomach. In the meantime, FDA approved SP1049C as an orphan drug for gastric cancer.

4.12. NK911

The NK911 is also a polymeric micellar formulation of DOX. This system is made of a copolymer of PEG (MW ~ 5,000 g/mol) and polyaspartic acid (ASP) (Fig. 12). To achieve a higher hydrophobicity, DOX was partially conjugated in the side chains of ASP (~45%). Therefore, when the copolymer is dissolved in water, it assembles as a micelle with a high hydrophobic inner core. The hydrophobicity of the core provides additional accommodation to encapsulate free DOX. As a result, the DOX which will be responsible for the antitumor activity is the loaded one, since the conjugated one does not reveal any activity. This lack of response is probably due to the stable coupling of DOX to the backbone of the polymer. NK911 exhibits a small size, nearby 40 nm in diameter, which is within the NPs size for passive targeting by the EPR effect [351].

This NK911 system successfully accumulated in solid tumours in mice and was thus considered for Phase I clinical trial. In this study, a total of 23 patients with metastatic or recurrent solid tumours were followed. The aim was to analyse the PK profile of NK911 nanotherapeutics through the MTD and the DLTs. The treatment consisted in *i.v.* administration of the NK911 formulation, starting with 6 mg/m^2 DOX equivalent every 3 weeks. The haematological side effect most common was neutropenia when the doses were increased till 50 to 67 mg/m^2 DOX equivalents. Other associated effects were mild alopecia, anorexia and stomatitis. In general, NK911 was well tolerated and presented a good safety profile. A Phase II clinical trial was proposed with a recommended dosage of 50 mg/m^2 every 3 weeks, however, it is uncertain if the clinical trials proceeded [305].

4.13. Bacterial-derived EDV™ minicells

MacDiarmid and colleagues [352] accomplished a novel technology based on a bacterial-derived nanoplatform (EDV™ minicells) for drug/gene encapsulation with specific targeting ability (Fig. 13). These systems are obtained through a genetically *min*CDE-chromosomal deletion mutant from: *Salmonella enterica serovar Typhimurium* (S. Typhimurium); *Escherichia coli*; *Shigella flexneri*; *Pseudomonas aeruginosa* (Gram-negative) and *Listeria monocytes* (Gram-positive) strains. Essentially, bacterial minicells are anucleate NPs that present a uniform diameter (~400 nm), acquired by the inactivation of the genes that control normal bacterial cell division, therefore depressing the polar sites of cell fission. They are produced with high yields from both Gram-positive and Gram-negative bacteria. After the production and purification process, the bacterial minicells can be lyophilized and stored for about 4 months. They can be used as vectors for a wide range of chemotherapeutics with different charge, structure, solubility and hydrophobicity. The encapsulation process occurs by unilateral diffusion and shows to be dependent on concentration and time of incubation with the drug.

The targetability of these systems is accomplished by using bispecific antibodies, in which one arm will recognize the surface lipopolysaccharide, and the other will recognize a cell-surface receptor specific for the targeted cell, such as EGFR [353]. A single minicell can accommodate approximately 1 million molecules of DOX [352]. Once in the tumour microenvironment, the endocytosis process is triggered by the binding of the targeted-minicell to the specific antibody receptor present on the tumour cell surface. According to the *in vitro* studies (MDA-MB-468 breast, SKOV-3 ovarian, A549 lung, and HL-60 promyelocytic leukaemia cancer cell lines), minicells are internalized and degraded by the endosomes/lysosomes and, as a result, the cargo is released into the cytosol [352,354]. *In vivo* studies were performed with targeted DOX-loaded bacterial minicells to evaluate the antitumor potential. These experiments resulted in a huge inhibition and regression of the tumour growth either for mice with cancer xenografts (breast, lung, ovarian and breast) and for dogs with Non-Hodgkin's lymphoma (NHL) [352]. Besides cancer models, healthy pigs were also used to evaluate the safety of the *i.v.* administration of bacterial minicells. Despite the five consecutives *i.v.* administrations, pigs tolerate well and did not reveal side effects for all haematological indices, serum chemistries, growth and food intake. The same was verified for the NHL dogs. Furthermore, there is the need to highlight that more exhaustive toxicology and stability studies are necessary for using this bacterial minicell in humans. The previous *in vitro* and *in vivo* results were responsible for the achievement of the "first-in-man" clinical trial. This study was based on EDV with the anti-human EGFR Erbitux and paclitaxel ($^{Erbitux}EDV_{Paclitaxel}$) [355]. Another Phase I clinical trial which is currently under progress is the CerebralEDV study (NCT02766699) [306]. The purpose of this research is to study the safety and tolerability of the EDV minicell (EGFR(V)-EDV-DOX ($^{V}EDV_{DOX}$)) packaged with DOX and coupled to panitumumab/Vectibix (V) to target the EGFR protein on the tumour cell membrane. The choice of the EGFR as target moiety and the Vectibix as the antibody was based on literature, where EGFR seems to be important for GBM [356,357]. In this study, the patients with recurrent or progressive GBM randomly received one of two $^{V}EDV_{DOX}$ doses (5x10^9 or 8x10^9) by *i.v.* administration, once a week, for a period of 8 weeks. In general, $^{V}EDV_{DOX}$ was well tolerated and no severe side effects were reported, being the most common, fever, nausea and chills. However, MTD was not achieved. In summary, this Phase I trial revealed that $^{V}EDV_{DOX}$ can be administered to the patients with no severe risks [306]. Nevertheless, further research is needed to validate the safety of this novel technology. Meanwhile, in 2017, FDA approved the $^{EGFR}EDV_{DOX}$ minicells as an orphan drug status for the treatment of GBM.

5. Conclusions and future perspectives

Nanotechnology, being a multi and interdisciplinary field, offers new opportunities for patient treatment. In the context of cancer, the introduction of nanomaterials as nanocarriers for conventional drugs is extending the possibility of their use, by improving their efficacy and safety. This is the case of DOX, an anthracycline widely applied in cancer treatment which has been associated to the occurrence of severe side effects. Although there is a long road to pursue until a

nanotherapeutic reaches the market, a few DOX-based nanotherapeutics are now in the clinical scenario and others are currently under different phases of clinical trials. While liposomes are clearly ahead in the field of DOX-based nanotherapeutics, other nanoscale formulations are also now showing their applicability and specific advantages, such as nanoparticles, polymer-drug conjugates, micelles and nanocapsules from biological origin. Interestingly, one can notice that these DOX-nanotherapeutics are evolving, not only exploring the EPR effect to accumulate and exert their action in the tumour site, but they are getting smarter over time and equipped with new tools that allow them to overcome physiological barriers, respond to environmental stimuli and reach specific cells/molecular targets.

Meanwhile, research on the area of DOX-based nanotherapeutics is still very active and results are exciting. Given the number of publications that can be found in the literature, of which only representative examples are presented in this review, new and better solutions for the delivery of doxorubicin in cancer cells may be expected in the future, which will also possible be extended for the delivery of other drugs. Hopefully, in a medium/long-term, the future of cancer therapy will rely on personalized nanomedicine approaches, custom-made for each patient, and capable of treating not only primary tumours but also their metastases.

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