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Synergistic Nanomedicine: Passive, Active, and Ultrasound-Triggered Drug Delivery in Cancer Treatment

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Nanocarriers are heavily researched as drug delivery vehicles capable of sequestering antineoplastic agents and then releasing their contents at the desired location. The feasibility of using such carriers stems from their ability to produce a multimodel delivery system whereby passive, ligand and triggered targeting can be applied in the fight against cancer. Passive targeting capitalizes on the leaky nature of tumor tissue which allows for the extravasation of particles with a size smaller than 0.5 μm into the tumors. Ligand targeting utilizes the concept of receptor-mediated endocytosis and involves the conjugation of ligands onto the surface of nanoparticles, while triggered targeting involves the use of external and internal stimuli to release the carriers contents upon reaching the diseased location. In this review, micelles and liposomes have been considered due to the promising results they have shown *in vivo* and *in vitro* and their potential for advancements into clinical trials. Thus, this review focuses on the most recent advancements in the field of micellar and liposomal drug delivery and considers the synergistic effect of passive- and ligand-targeting strategies, and the use of ultrasound in triggering drug release at the tumor site.

Keywords: Liposomes, Micelles, Ligand Targeting, Triggers, Ultrasound.

CONTENTS

1. INTRODUCTION

Advancements in molecular drug design have contributed to more efficient administration of chemotherapy in our fight against cancer. Nevertheless, since the strength of the chemotherapeutic drugs not only destroys tumor cells at the required location, but also spreads out to other areas in the body and destroys healthy cells, patients experience a range of side effects that may be lethal.[1,2] Additionally, this lack of specificity leads to a decrease in the efficacy of the utilized drugs.[3] As a result, the challenge in curing cancer arises from developing an approach that can carry the drug, increase its availability, and administer it only to the specific tumor site, hence preventing its cytoxicity to healthy cells.[4] To solve this predicament, drug delivery research focuses on the discovery of compounds specific to the tumor, as well as ways to package the drug into a nanocarrier, and only make it available at the tumor site at the required time.[1]

With the advancements in drug delivery during the 1980s and 1990s, directing anticancer drugs to the tumor site by only relying on physiological properties, i.e., passive targeting, was, and still is, a breakthrough in the field.[1] Passive targeting relies on the enhanced permeability and retention (EPR) effect in tumor tissues (Section 3.1). The EPR effect results in the preferential permeation and accumulation of nanocarriers, including liposomes and micelles as well as other macromolecules, at the disease site where

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they can accumulate for an extended period of time, allowing for the slow release of their contents. Liposomes and polymeric micelles, due to their unique attributes, are characteristic examples of biocompatible macromolecular nanocarriers that are heavily researched in drug delivery. While the EPR effect is a huge advantage of these drug delivery vehicles, delayed drug pharmacokinetics and restricted drug biodistribution are major obstacles faced when merely relying on passive targeting.[3,5,6] As a result, effective nanocarriers must additionally possess the ability to remain in the blood for long periods and to specifically target the disease sites via active- or ligand-targeting (Section 3.3).[3,5,6]

Both internal and external stimuli can be employed to trigger the release of the drug from the nanocarriers once they have made their way to the tumor site. This review focuses on the application of acoustic waves as an external stimulus to trigger the drug release from nanocarriers, namely micelles and liposomes (Section 4).

Synergy involves the effect by which nanoparticles improve their toxicity in a specific tumor tissue by a combination of:

(i) passive targeting (optimized EPR effect),
(ii) active targeting (addition of specific ligands to carrier) and
(iii) a stimulus (or stimuli), such as US, to trigger the release.

The research in this area has been promising. For example, the work of Yuh et al.[7] showed that there was an increase of 124% in the concentration of doxorubicin (Dox) in mice tumor tissue when they received a liposome-encapsulated Dox injection into their tails' veins and were exposed to high intensity US. Promising results were also reported by Pitt et al.,[8] using a combination of Dox encapsulated in stealth liposomes and low-frequency 20-kHz US.

2. NANOCARRIERS USED FOR DRUG DELIVERY

2.1. Types of Nanocarriers

The advancement of nanotechnology in delivering biologically active compounds has provided several accepted drug carrier nanosystems.[3,5,6,9–16] A wide range of nanocarrier designs for the treatment of various diseases, with a size range of 10–800 nm, is available.[13] These include nanocrystals, nanosuspensions, nanotubes, nanowires, micelles, liposomes, ceramic nanoparticles, solid lipid nanoparticles, dendrimers, and hydrogel nanoparticles among others. For each design, it is essential that vital factors be taken into account to make the nanocarrier most suitable to its application. Here, we will discuss the two most common types of nanocarriers researched in drug delivery systems (DDS): micelles and liposomes.

2.2. Micelles

2.2.1. Polymeric Micelles

Micelles are self-assembling colloidal nanocarriers. They are made of "amphiphiles," or surface active molecules, that consist of a hydrophilic tail and a hydrophobic head (Fig. 1).[17–19] Once placed in water, these molecules spontaneously assemble to form a spherical monolayer consisting of an external hydrophilic shell (or corona) and a hydrophobic core. Micelles have been known to sequester only hydrophobic drugs,[20] but recently a report by Huang and co-workers[21] described core-inversible micelles that can sequester hydrophilic drugs in their core. These micelles, made of poly(ethylene glycol) (PEG) and a dendritic octamer of cholic acid (named $PEG^{5k}CA_8$), self-assemble normally in aqueous solution, but assemble in a reverse way in certain a polar solvents, which gives them a hydrophilic core capable of sequestering hydrophilic drugs. The hydrophobic core (shown in blue in Fig. 1) is made of PPO polymer and encapsulates the hydrophobic drug. The hydrophilic polymer PEO (shown in red in Fig. 1) is oriented towards the aqueous surrounding.

The hydrophobic core of regular micelles is maintained by van der Waals interactions, while the hydrophilic shell is joined to its aqueous surrounding by hydrogen bonds.[22] The critical micelle concentration (CMC) and critical micelle temperature (CMT) are the given concentration and temperature, respectively, at which micelles spontaneously assemble.[23] The CMC of micelles is very important in defining its stability, i.e., a lower CMC value means the micelle will be stable at relatively low concentration. In general, surfactants (amphiphiles of low-molecular weight) have a higher CMC value (10^{-4} to 10^{-3}) than amphiphilic block copolymers.[22] Polymeric micelles are made of amphiphilic block copolymers, with a CMC range of 10^{-6} to 10^{-7} M,[22] and will be the focus of this section.

Polymeric micelles are the most commonly used micelles for drug delivery, and are promising nanocarriers for chemotherapy.[20, 24–29] They consist of two or more block polymers and their size ranges from 10 to 200 nm.[20] Their low CMC value makes them very stable at low concentration, which is important for prolonged blood circulation and increased EPR effect. Hydrophobic drugs can be loaded into the core of the polymeric micelles, while their shell provides colloidal stability *in vitro* and *in vivo*.[30]

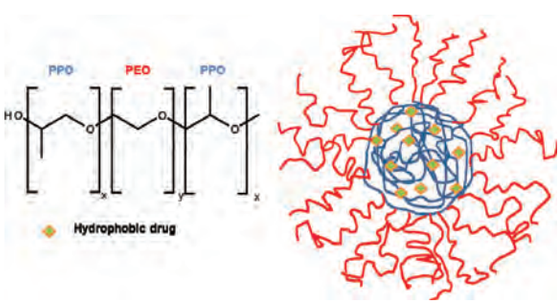

Figure 1. Schematic of a polymeric micelle.

Additionally, several polymers used in forming micelles must be above their CMC and these concentrations are sometimes relatively high and thus the body may not tolerate. For example, the critical micellar concentration of Pluronic P-105 is approximately 1 wt.% at room temperature.[31] For this reason, stabilizers such as *N*,*N*-diethylacrylamide, NIPAAm, pENHL are often used to stabilize the core of these polymeric micelles.[32, 33]

The hydrophilic block of polymeric micelles is usually made up of PEG or poly(ethylene oxide) (PEO), a low molecular weight polymer, which is biocompatible, has low cytotoxicity, and provides steric protection against uptake by the mononuclear phagocyte system (MPS)[20] (Section 3.2). Therefore, the variation in the different types and functions of polymeric micelles arises from the composition of their hydrophobic block, adapted for sequestering a variety of drug molecules. Some frequently used hydrophobic blocks include: poly(propylene oxide) (PPO)[34] and aliphatic polyesters polymers such as poly(*D*,*L*-lactide) (PLA) and poly(ε-caprolactone) (PCL).[35] A good hydrophobic block should have high drug loading capacity and compatibility with the encapsulated drug.[22]

The polymer block arrangement usually consists of di-block copolymers (A–B) and tri-block copolymers (ABA or ABC).[19, 22] The PEG^{5K}-CA_8 polymer used in the delivery of Paclitaxel is a good example of a di-block copolymer,[21, 36] while Pluronic® polymers (also known as Poloxamer) are commonly used as tri-block copolymers. They consist of hydrophilic PEO and hydrophobic PPO, with a PEO-PPO-PEO block arrangement.[37]

Pluronic®-based polymeric micelles (especially Pluronic® P105 micelles), which consist of 37 PEO monomers and 56 PPO monomers, have been widely researched as DDS.[38–45] The PPO monomers form the hydrophobic core of the micelle, where the drug is loaded, while the PEO monomers form a shell or corona, which is responsible for the stabilization of the micelle at the solvent-core interface.[46] The PEO chains extending into the aqueous solution are very important since they allow the saturation of the micellar surface with water molecules, and this stabilizes their structure.[47] Additionally, these chains decrease the process of opsonization, which is the adsorption of proteins on the surface of the micelles. This is beneficial because opsonization allows the reticulo-endothelial system (RES) to recognize and eliminate nanocarriers from the blood stream, and should therefore be reduced.[37, 48–51] Hence, Pluronic®-based micelles have good stealth properties,[28] and have been widely studied for application in chemotherapy.[19, 20, 22, 24–27, 29, 52]

Polymeric micelles have several advantages over other nanocarriers. Micellar drug carriers are usually smaller in size than liposomal nanocarriers and this could potentially

lead to an advantageous increase in the EPR effect in drug delivery by ensuring deep tumor penetration.[22, 53] On the other hand, their size is usually large enough to allow them to escape renal excretion.[37] Additionally, these micelles have a higher structural stability than other micelles, and dissociate slowly at levels below their CMC.[48] Furthermore, it is possible to modify polymeric micelles to be stable in biological fluids, hence increasing their blood circulation time (Section 3.2).[54] Micelles are easy to prepare, and the chemotherapeutic agents can be easily loaded into their cores by the simple act of mixing.[49] Furthermore, micelles can be modified for targeted drug delivery, hence increasing their accumulation at the diseased site (Section 3.3).[55, 56]

From a pharmaceutical point of view, polymeric micelles also have several advantages such as their high carrying capacity, long shelf-life when lyophilized, the fact that they can be sterilized by microfiltration, and the low viscosity of their respective micellar solutions.[57] The incorporation of drugs into these nanocarriers increases the drug solubility and stability, thus enhancing its pharmacokinetics and biodistribution.[58] Micelle-encapsulated drugs have shown reduced cytotoxicity and have enhanced drug efficacy when compared to free drug formulations, as shown in *in vitro* studies.[20, 22, 55] Another important advantage of Pluronic® micelles is that they are able to sensitize multidrug resistance (MDR) cell lines at low concentrations, by potentiating the effects of the chemotherapeutic drug they encapsulate and decreasing the ATP levels in the cells.[59]

Additionally, polymeric micelles can be custom-made to respond to a certain stimulus including changes in pH, temperature, US, and others.[46] Some studies have successfully used Pluronic® P105 micelles, which are sensitive to ultrasound (US), as nanocarriers to administer Dox to tumors developed in mice models.[37, 48, 55, 56] By including thermosensitive and pH-sensitive polymers in the micellar formulation, it is possible to produce pH- and temperature-sensitive micelles, respectively, which also have relevant applications as DDS.[22]

2.2.2. Micelle Synthesis

With the increased research in the use of micelles as nanocarriers, several different methods have been proposed for the synthesis of drug-loaded micelles. These include common methods such as direct dissolution, dialysis and solvent evaporation, as well as more recent methods such as freeze-drying[25] and near-critical micellization (NCM).[60]

The method used for the preparation of poloxamer-based micelles, such as Pluronic®, which are moderately hydrophobic, is by direct dissolution: both block copolymer and drug are dissolved in an aqueous solution and the micelle formation is spontaneous, depending on the charge ratio of both compounds.[25]

Most hydrophobic polymers which are not easily soluble in water must be dissolved, along with the drug, in a common organic solvent.[28] In the dialysis method, the polymer-drug dissolved in the organic solvent is placed in a dialysis bag, and this is placed in water, causing the micelles to form as the solvent is exchanged with water by dialysis. Afterwards, freeze-drying is used to remove the water. Meanwhile, micellization using solvent evaporation involves using rotary evaporation or spray drying to remove the organic solvent. The formation of a polymeric film increases the polymer-drug interaction, and the rehydration of the film with an aqueous solvent leads to the formation of the micelles. The two methods produce different sizes of micelles and thus have different applications in drug delivery.[25, 28] A more efficient method known as near-critical fluid micellization (NCM) has been developed. NCM has higher and more efficient drug loading capacity than the former methods discussed.[60] Therefore, different researchers adopt different techniques, depending on the desired micelle properties.

2.2.3. Clinical Uses

Polymeric micelles as drug-encapsulating nanocarriers have been widely researched in *in vitro* and *in vivo* studies,[55] but only a few micellar formulations have been approved for clinical use, although several others are undergoing clinical trials.[22]

Genexol-PM® is a methoxy-PEG-poly(*D*,*L*-lactide) polymeric micellar formulation encapsulating Paclitaxel, constituting particles of 20–50 nm.[61, 62] This formulation encloses the paclitaxel in the hydrophobic core, while the aqueous solubility is ensured by the PEG-hydrophilic shell.[62] Genexol-PM® has been approved for the therapy of breast cancer in Europe and South Korea,[63] and is undergoing phase III and IV clinical trials in the USA.[61, 62] It has also been undergoing clinical trials in patients with non-small-cell lung cancer,[64] pancreatic cancer,[65] and others (overview of clinical trials available via www.clinicaltrials.org).

Paclical, another Paclitaxel micellar formulation is undergoing Phase III clinical trials for treatment of ovarian cancer.[63] Also undergoing phase III clinical trials is NK105 (https://clinicaltrials.gov/ct2/show/NCT01644890), another micellar formulation of Paclitaxel, with polymeric micelles composed of PEG and modified polyaspartate.[66] This DDS is being evaluated for the treatment of metastatic or recurrent breast cancer. In all cases, the micellar formulations have been showing promising results when compared to the administration of free Paclitaxel, which has several serious side effects.[66]

2.3. Liposomes

2.3.1. Definition

The structural form and features of liposomes, formerly called banghasomes, were first described by Alec D. Bangham in 1965.[67] Only a few years afterwards did Weismann name these phospholipid spherules

"liposomes,"[68] derived from the greek *lipos* (fat) and *soma* (body).[69] Their discovery led to extensive research in their use as drug delivery vehicles.[70–72]

The basic component of any liposome is a natural or synthetic amphiphilic lipid molecule—a phospholipid—with a hydrophilic head and a hydrophobic tail. A combination of the lipid formulation results in a spherical-shaped vesicle, characterized by a unique self-forming bilayered membrane structure (Fig. 2).[73] This membrane has a structure similar to the cell membrane: the hydrophobic tails of the lipids come together to form its hydrophobic region, thus distinguishing its aqueous internal compartment (hydrophilic region) from the bulk aqueous phase. This unique property allows liposomes to readily encapsulate hydrophilic molecules in their aqueous core, while hydrophobic ones can be encapsulated within the phospholipid bilayer.

2.3.2. Classification of Liposomes

Liposomes can be classified according to their size and number of bilayers as shown in Table I. The liposomes used as nanocarriers *in vivo* should be smaller than 500 nm to effectively circulate through the blood system. It was observed that an enhanced EPR effect can be obtained when the liposome size ranges from 63 to 388 nm in diameter.[53]

Simple liposomes demonstrated a disadvantage of relatively low blood circulation times, and were easily recognized and cleared by the RES.[72] While different modifications to the conventional liposomes have been made to enhance the EPR effect and targeting ability of liposomes, which are major goals in chemotherapy, the most significant was covalently attaching PEG to the surface of liposomes, thus stabilizing them and helping them evade the MPS. PEGylated (or *stealth*) liposomes are commonly used in DDS, and will be further discussed in Section 3.2.

Liposomes can also be classified according to the targeting moiety conjugated to their surface when used for active targeting (Section 3.3.). For example, proteoliposomes bind a protein or peptide that interacts with certain receptors over expressed on the surface of cancer cells. Immunoliposomes have their surface modified with an antibody or antibody fragment. Furthermore, immunoliposomes modified with PEG are also more likely to escape the RES and reduce toxicity to other healthy cells, and could therefore enhance the chemotherapeutic effectiveness of the anti-neoplastic agents.[74]

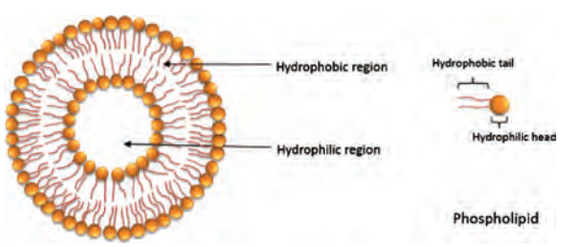

Figure 2. Structure of a conventional liposome.

Table I. Classification of liposomes according to number of bilayers and size.

Liposome	Number of bilayers	Diameter range (nm)
Small unilamellar vesicle (SUV)	1	20–100
Large unilamellar vesicle (LUV)	1	>100
Giant unilamellar vesicle (GUV)	1	>1000
Oligolamellar vesicle	~5	100–1000
Multilamellar vesicle	5–25	>1000

Similarly, it is possible to classify liposomes based on the stimuli they are sensitive to. Liposomes can be modified to increase their responsiveness to US and subsequent release of their drug content. Echogenic liposomes encapsulate gas in their inner compartment, thus rendering them sensitive to ultrasonic waves.[75] Meanwhile, eLiposomes contain nanoemulsions along with the drug molecules. eLiposomes enhance triggered drug delivery by a quick change in phase from liquid to gas when US is applied (via acoustic droplet vaporization).[76] In both cases US is used to facilitate drug release.[72] This type of liposomes is discussed in further detail in Section 4.2.4. pH-Sensitive liposomes have also gained the attention of researchers due to their fusogenic properties.[74] These liposomes are sensitive to pH changes, and will release the encapsulated drug at the set pH trigger point, usually in acidic conditions.[74,77] Additionally, it is possible to prepare liposomes that are light-, microwave-, and temperature-sensitive. Light-sensitive liposomes are modified by using special lipid molecules that respond to a "defined mechanism of photo-activation."[78] Likewise, temperature-sensitive liposomes (TSL) allow for TSL-based delivery by responding to rising environmental temperature triggers (e.g., hyperthermia applicators like ultrasound, microwave, radiofrequency ablation etc.).[79]

2.3.3. Advantages and Disadvantages of Liposomes in Drug Delivery

The use of liposomal nanocarriers for drug delivery has created much interest for researchers due to their biological and physicochemical properties.[71] Additionally, liposomes have the unique ability to deliver both hydrophobic and hydrophilic drugs, in contrast to micelles, which have been used to encapsulate hydrophobic drugs.[80] Furthermore, liposomes are known to have relatively high drug loading efficacy, up to 0.25 mg drug/mg lipid.[81]

2.3.4. Liposomes Synthesis

The membrane bilayer of liposomes is made of natural and/or synthetic phospholipids, such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). Moreover, it is important to consider the properties

(charge, molecular weight, stability, critical temperature) of the phospholipids to be used for synthesis.[82] Cholesterol is another important component of the liposomes, which increases their stability in the blood stream, and decreases the permeability of the membrane to foreign substances.[82]

Numerous techniques have been described for the synthesis of liposomes. However, the most commonly used synthesis technique, described by most researchers, is the hydration film method that was first used by Bangham.[71] In this method, the phospholipids, along with other membrane components (e.g., PEG, and cholesterol), are dissolved in an organic solvent such as chloroform, sometimes along with an adjuvant (e.g., methanol or ethanol) in a determined ratio. The organic solvent is then dried under reduced pressure, using a rotatory evaporator, until a dry thin film of lipid forms. Afterwards, the water-soluble form of the drug to be encapsulated is added to rehydrate the lipid film, followed by sonication at low shear rates (e.g., using a sonication bath), which leads to the formation of MLV liposomes. To obtain unilamellar liposomes, the resulting heterogeneous MLV liposomes are extruded several times using an extruder of polycarbonate membrane of a certain diameter.[71]

Loading the drug into the liposome can be done remotely using different techniques. Citric gradient and ammonium sulfate gradient used for drug loading in Myocet® and Doxil® (liposomal formulations approved for clinical use), respectively, are common examples.[83] A pH-gradient is also a commonly used technique.[83, 84]

2.3.5. Clinical Uses

Since liposomes gained interest for use as nanocarriers, many liposomal drugs have been approved for clinical use. The first liposomal formulation that was approved for clinical use in Europe (and later, in 1997, in the USA) was AmBisome®,[62] amphotericin B encapsulated in 100 nm liposomes, which is used for the treatment of serious fungal and protozoal infections.[72, 80, 85] In 1995, Doxil®/Caelyx®, which consists of PEG-stabilized liposome-encapsulated Dox, was approved by the Food and Drug Administration (FDA)-USA for the treatment of Kaposi's sarcoma found in AIDS victims.[72, 80, 85] Afterwards other liposomal formulations were approved, such as DaunoXome® (liposome-encapsulated daunorubicin, used for the treatment of Kaposi's sarcoma, FDA-approved in 1996), Myocet® (non-PEGylated liposome-encapsulated Dox, used for the treatment of breast cancer, approved in 2000 in Europe and Canada, and undergoing clinical trials in the USA), and DepoCyt® (liposome-encapsulated cytosine arabinoside, used for the treatment of lymphoma complications, FDA-approved in 1999).[63]

Several other liposomal formulations are currently undergoing clinical trials in the USA; for example: Onco-TCS (liposomal cytarabine) for the treatment of non-Hodgkin lymphoma (phase I/II trial), SPI-77 (stealth liposomal cisplatin) for the treatment of head and neck, and lung cancers (phase III trials), and Lipoplatin (liposomal cisplatin) for the treatment of pancreatic, head and neck, and breast cancers (phase III trials).[63]

2.4. Factors Determining the Selection of the Nanoparticles to Use

Advantages and disadvantages of using micelles and liposomes as drug carrier systems have been outlined in the previous sections. In general, three main factors govern the choice of which type of nanoparticle to use: size, *in vitro* and *in vivo* stability, and surface charge.

The tumor fenestration (openings) present in the vessel walls of tumor tissues, that result from rapid tumor angiogenesis, vary in size and structure depending on the tumor type, stage and location.[19, 86, 87] Accordingly, the selection of the size of the nanocarriers, which directly affects their performance, will depend on the microenvironment of the tumor.[88, 89] Thus, the size and shape of the nanocarriers are important factors to consider when optimizing their biodistribution and pharmacokinetics.[70, 81, 86, 90] In general, nanocarriers should be smaller than the size of the tumor fenestrations[91] in order to allow for deeper penetration into the tumor parenchyma.[86] Usually, the size and geometrical structure of the nanoparticles can be determined by dynamic light scattering (DLS).[92] It is important to note here that micelles are better than liposomes at evading breakdown in the kidneys, due to their smaller size.[62]

The stability of the nanocarrier should be equally considered when deciding on a good choice of the DDS. In order to reach and advance in the clinical trial stages, liposomes and micelles should demonstrate good efficacy and stability, both *in vivo* and *in vitro*.[56, 70, 93] *In vivo*, nanocarriers interact with different blood constituents including proteins and cells, which can cause high dilution in the blood. Chen et al. showed that polymeric micelles of poly(ethylene glycol)-block-poly(*D*,*L*-lactide) (PEG-*b*-PDLLA) were unstable in blood vessels of mice, mainly due to the presence of alpha- and beta-globulins.[94] Furthermore, the presence of enzymes, which may not be accounted for in *in vitro* studies, could affect the functionality or stability of nanocarriers. For instance, the stability of PEG-*b*-poly(3-hydroxybutyrate)-*b*-PEG (PEG-*b*-PHB-PEG) was shown to vary with concentration of PHB depolymerase, an enzyme that degrades the micelle.[93]

Lastly, since the plasma membranes of cells are negatively charged, the surface charge of nanocarriers must be considered. Cells easily take in positively charged (cationic) nanocarriers by adsorption-mediated endocytosis in comparison to negatively charged and neutral nanocarriers.[70, 95, 96] However, cationic liposomes may not be very effective *in vivo*; therefore, some researchers have resorted to a "charge-reversal technique"

which imparts positive charge to liposomes prior to endocytosis.[96]

3. DELIVERY OF NANOCARRIERS TO TUMOR TISSUES

3.1. The EPR Effect

To increase the efficiency of drugs and reduce their toxicity to healthy tissue, drugs should target specific tumor sites. This is achieved by first understanding the anatomical and physiological differences between normal and cancerous tissues. As explained below, these vital differences work together to primarily lead to the permeability and retention features that are specific to tumor tissues and organs.[1, 97–101] First, EPR allows large molecules (i.e., macromolecules, liposomes and micelles) to permeate to the diseased site via the tumor's hyperpermeable vasculature.[101] EPR also allows the prolonged retention of the large molecules at the cancer site due to its deficient lymphatic system.[102] Additionally, the generation of factors that enhance the permeability, including vascular endothelial growth factor (VEGF), prostaglandins, nitric oxide, matrix metalloproteinases and other enzymes, may enhance the EPR effect.[103]

3.1.1. Permeability

Compared to normal tissue, the ability of molecules to permeate into the tumor site results from differences in the progression of angiogenesis, the process by which endothelial cells and soft tissue create new blood vessels, between healthy and cancerous tissue.[104] By allowing tumor cells to regress from the tumor's primary site into the blood stream circulation, angiogenesis is a critical process for tumor development of secondary malignant growths (e.g., metastasis).[104] A pre-existing membrane degrades, leaving a gap to be filled by the endothelial cells and soft tissue that respond to certain molecular signals. The entire process is controlled by the presence or absence of these molecules. The rate at which angiogenesis occurs in tumor cells is considerably faster than that of normal adult cells, with the exception of vessel rate formation during pregnancy and wound healing. As a result, leaky tumor vasculature is formed, as shown in Figure 3. Furthermore, endothelial cells of tumor tissues are not wrapped with contractile cells, known as pericytes, to maintain the blood vessels, as is the case with normal tissue.[97, 99, 105] Thus, the borders of tumor vessels lack a smooth muscle layer, which is essential in controlling the blood flow pressure and direction, leading to an elevated blood pressure in these diseased tissue.[99] Compared to the linear blood vessels of normal tissues, the absence of these smooth muscle tissues results in a non-aligned endothelial vascular lining structure in tumor blood vessels.[98] Consequently, tumor tissues contain hyper-permeable vessels (sometimes referred to as *leaky* vasculature), which create openings in the walls of the tissue structure.[97, 105–107]

Due to the openings between the endothelial cells, as well as the vascular properties, tumor tissues allow nanocarriers to be effortlessly administered into the interstitial fluid to accumulate at the tumor site thus facilitating selective delivery.

3.1.2. Retention of Nanocarriers

Opportunely, the presence of the drug carrier in the tumor vicinity implies its accumulation in the tumor. This is due to the fact that, in contrast to normal tissues, tumor tissues lack a functional lymphatic drainage system, which removes foreign particles (i.e., drug carrier) from the interstitial fluid bathing around the cells.[108, 109] As a result, the nanocarriers are not efficiently cleared from the blood stream, and the long circulation time establishes a high nanocarrier concentration at the tumor surrounding area, allowed by the leaky vasculature, as discussed above. Subsequently, through the prolonged retention, a unique characteristic of tumor tissue, the drug's cytotoxicity at the tumor site increases.[110, 111]

With the exception of hypovascular tumors, including pancreatic and prostate cancers, the EPR effect has been observed in almost all types of cancers of the human body.[101] A polymer conjugate of poly(styrene-co-maleic acid) and neocarzinostatin (SMANCS) used as a prototype of a macromolecular drug was the onset of the EPR effect formulation. In 1986, Matsumura et al. observed that SMANCS preferentially accumulates in tumors, and hypothesized that this was due to the tumor hypervasculature. This study confirmed the applicability of the EPR effect.[103] Further evidence of the EPR effect observed by Maeda et al. was provided by a traditional tumor imaging technique that uses radioactive gallium (^{65}Ga) and scintigraphy. After the radioactive gallium is injected into the body, it binds to the plasma protein transferrin, which is entrapped in the tumor due to the EPR effect. Two to three day afterwards, the scintigraphy is performed and the ^{65}Ga can still be observed in the tumor tissue, but not in normal tissues, where the lymphatic system cleared it.[10] The group of Maeda and co-workers has extensively reviewed the EPR effect.[10, 100–102, 107, 109]

Although the discovery of the EPR effect was a major breakthrough in cancer treatment, it must be mentioned here that several clinical studies provided evidence that encapsulated drugs are only marginally more efficient than the free drug, a problem recently reviewed by Nichols and Bae,[1] and previously posed by Bae.[106] Apparently, the EPR effect observed in *in vivo* studies is more pronounced than that observed in human cancers in clinical trials. The authors suggest that this may be due to irregular vascularity, which leads to poor blood flow inside tumors, as well as the high tumor interstitial fluid pressure. The efficiency of the EPR effect thus depends on the tumor type, and should be considered on a case-by-case basis.

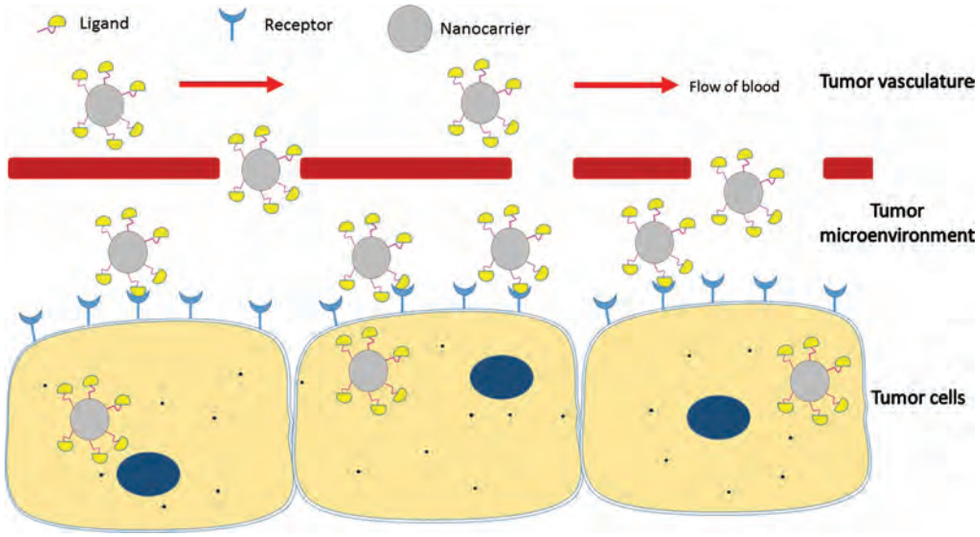

Figure 3. Active targeting: effect of receptor mediated endocytosis.

3.2. Solving Longevity Issues: Stabilization of Micelles and Liposomes

Nanocarriers face several barriers that prevent their specific targeting to the required site. Predicting and preventing such barriers associated with the biodistribution and pharmacokinetics of nanocarriers during their early design stage can help achieve better targeting.[112] The longevity of a nanocarrier system is essential. While most of the drugs have a plasma half-life of 20 minutes in human and mice, it takes around 6 hours of circulation for any drug to accumulate via the EPR effect.[102]

3.2.1. Decreasing Opsonization by Using PEG

Researchers studied the improvement of the EPR effect by using different strategies. One of the most important strategies is to decrease the occurrence of opsonization, a major obstacle in controlled drug delivery. Opsonization is a complex process that disturbs the pharmacokinetics of nanocarrier systems. As soon as nanocarriers are administered in the bloodstream, blood serum components, known as opsonin proteins, bind to the surface of the nanocarrier based on its surface properties and size, as explained by Lundqvist et al.[113] and Aggarwal et al.[108] The nanocarrier then becomes enclosed with opsonin proteins and is thus more prone to detection by phagocytes. As a result, the nanocarrier is taken up and removed from the blood circulation system by the body's RES via phagocytosis.[108, 114, 115] Consequently, opsonization leads to a rapid destabilization of the nanocarrier in the blood stream, thereby rendering it ineffective.[112, 114]

A simple way to avoid opsonization, thus increasing the carrier's accumulation at the cancer site via the EPR effect, is to conjugate groups to their surface that enhance their circulation time in the blood, creating what are called *stealth* nanocarriers.[116] The most utilized of these groups are PEO and PEG chains. The modified nanocarriers have a more stable structure and will not rupture when subject to physiologically relevant shear stresses. The attachment of PEG to the surface of the nanocarrier creates an outer hydrophilic protective layer, which has the ability to repel opsonin proteins. As a result, the interaction between the PEG-modified nanocarrier systems and blood components is decreased, preventing the recognition and consequent uptake of nanocarriers by phagocytes.[5, 115, 117, 118] Additionally, PEG-modified systems have a prolonged circulation, increasing the number of times they pass through the tumor vasculature, which favors their accumulation at the tumor site by the EPR effect.[3, 98, 110, 117, 119] For instance, in a study done on 17 cancer patients, Harrington et al.[120] observed the pharmacokinetics and biodistribution of ^{111}In-DPTA (diethylenetriaminepentaacetic acid anhydride)-radiolabeled PEGylated liposomes. The liposomes were injected into the patients' bodies and scintigraphy was used for whole body scans, which showed a clear gathering of the liposomes at the tumor sites for an extended period. Likewise, in an *in vivo* study using a colloidal gold nanocarrier, encapsulating tumor necrosis factor targeted to a solid tumor growing in mice, the results showed a significant decrease in the uptake by the RES, particularly in the liver and spleen, upon modification with PEG.[121]

Nevertheless, a PEG layer on the outer surface of nanocarriers only allows for non-specific interactions between the tumor tissue and the nanocarrier system.[115] It is essential to optimize the amount of PEG-lipid to be incorporated into the nanocarrier formulation, so there is enough to provide a defense system against opsonization, but not so much that will prevent the interaction between the nanocarrier and the cancer cells, essential for its cellular uptake and internalization.[106, 115] This concept

is known as the "PEG dilemma," and it should be critically considered in order to optimize the EPR effect.[70] It also needs to be taken into account that, 2 to 4 days after the administration of the PEGylated nanoparticles, namely liposomes, the production of anti-PEG antibodies has been observed, which leads to the accelerated blood clearance of PEG-liposomes administered at repeat doses.[122–124] Other modifications that increase the stability and longevity of micelles and liposomes will now be discussed.

3.2.2. Increasing the Stability and Longevity of Polymeric Micelles

The use of polymeric micelles is limited by their recognition by the immune system and also due to stability issues. When these micelles are injected into the blood stream, if they are diluted below their CMC, they will dissolve and release the drug prematurely.[49] In order to make them stable enough to reach the target site, the concentration of the polymer would have to be above the CMC, and such concentrations may not be tolerated by the human body.[56] This disadvantage can be overcome by using "stealth micelles," which have their surface modified, usually by an exterior layer of PEO, which prevents the micelles' fast clearance.

For instance, in *in vitro* studies conducted by Opanasopit et al.[125] and Liu et al.,[126] human albumin or serum attached to micellar-encapsulated drugs was shown to reduce their stability. In addition, PEG blocks with sizes greater than 15 kDa were claimed to reduce stability.[19]

To solve this challenge, researchers introduced cross-linked micelles which improved the stability and drug delivery efficiency over that of non-cross-linked micelles.[30, 36, 96, 127, 128] Pruitt et al.[129] synthesized P105 stabilized micelles, NanoDeliv™, by polymerizing an interpenetrating network of temperature-sensitive *N*,*N*-diethylacrylamide inside the hydrophobic core of the micelles. This network was found to expand at room temperature, which allows the accumulation of the drug in the hydrophobic core, and contracts at temperatures above 31 °C, which allows the drug to be entrapped at physiological temperatures. Unlike the regular P105, the NanoDeliv™ are stable upon injection and the network has a half-life of about 17 h.[38]

Yang et al.[130] stabilized Pluronic® L121 (PEO5–PPO68–PEO5) micelles by forming crosslinks in their outer shells. They first converted the terminal hydroxyl groups of their micelle to aldehydes. Then, via Schiff bases, they bridged the terminal aldehydes. The size (100 nm) and shape (spherical) of their micelles remained the same after cross-linking; however, the stability was significantly increased.

Other approaches to increasing stability of micelles involve optimizing the hydrophilic-hydrophobic block mass ratio.[96] More information on cross-linked polymeric micelles can be found in a recent report by van Nostrum.[30]

3.2.3. Increasing the Stability and Longevity of Liposomes

The advancements in strategies to modify conventional liposomes and yield more efficient drug delivery carriers have led to significant strides, making the future of drug delivery very appealing.[80] However, some challenges still exist when using hydrophobic drugs in liposomes. Unlike polymeric micelles, liposomes can lose their targeting potential at high hydrophobic drug loads. This could be because in liposomes, contrary to micelles, the hydrophobic drug is loaded in the very thin lipid bilayer.[131] Cholesterol is usually introduced in order to improve the stability of liposomes.[132] Furthermore, the hydrophobic nature of amphiphilic PEG was claimed to be a cause of destabilization of liposomes. Hence, Cao et al. proposed experimenting with liposomes in which "super-hydrophilic" zwitterionic poly(carboxybetaine) (PCB) replaced PEG. Doxorubicin in poly(zwitterionic) liposomes without cholesterol showed better blood-circulating property and hydrophilic drug retention than Doxil®, which contains 39% cholesterol.[132]

3.3. Active Targeting

3.3.1. Fundamentals of Active Targeting

In spite of the advantages of passive targeting and its distinct localization effect, DDS may still lack enough efficacy due to insufficient uptake[91, 133–135] and the delayed drug pharmacokinetics previously discussed. Hence, the need for ligand–receptor mediated targeting arises.

One of the main differences between tumor cell biology and normal cell biology is that tumor cells frequently overexpress certain receptors on their membranes. This creates a higher affinity of tumor cells towards the molecules that specifically bind these receptors. Active targeting involves attaching these molecules to the surface of the nanocarrier as a targeting ligand, and uses the increased affinity towards higher internalization/uptake, thus increasing efficacy and specificity through receptor-mediated endocytosis.[91, 105, 115, 133–137] As shown in Figure 3, after the nanocarrier reaches the tumor microenvironment via the EPR effect, the targeting ligand is recognized by its corresponding receptor, eventually achieving a higher drug concentration in tumor cells.

The targeting ligands can be antibodies,[136, 138, 139] peptides,[140, 141] nucleic acids,[142–144] or other small molecules.[145, 146] The choice of the ligand to be used depends on the characteristics of the receptor that is overexpressed by the tumor cells to be targeted, the degree of overexpression, whether the ligand is internalized or not, the affinity of the ligand, and on the nanocarrier itself.[136] The success of the targeted DDS depends on several factors, including the characteristics of the DDS, the intracellular barriers, the bioavailability of the carried drug, the biodistribution and pharmacokinetics.[147] In this section, we will start with the targeting strategies currently being investigated.

3.3.2. Targeting Strategies

The existence of different types of malignancies and the variety of tumor cell characteristics accentuate the need for different active targeting strategies. However, the different strategies have one characteristic in common: the ability to target a receptor that is distinctly overexpressed in tumor cells, and which is easily accessible.[136] The most common targeting strategies are: targeting tumor's angiogenesis, targeting tumor's uncontrolled cell division, and targeting tumor specific receptors.[137] This diversity opens the door for a future of personalized chemotherapy where the active targeting system is specifically tailored to result in the maximum specific damage for a patient's tumor.

3.3.3. Targeting Angiogenesis

The angiogenesis process is controlled by the presence or absence of several angiogenic factors, the most important being the vascular endothelial growth factor (VEGF), extracellular matrix (ECM) ligands and basic fibroblast growth factors.[104] The use of these molecules by cells is regulated through specific receptors including the vascular endothelial growth factor receptor (VEGFR),[148] integrins,[149, 150] and vascular cell adhesion molecules (VCAM).[151–153]

In the case of solid tumors, significantly faster cell growth causes a higher demand for nutrients and therefore, higher demand for new vascular systems. In many cases, the extra demand is met through secretion of angiogenesis-associated molecules by tumor associated macrophages (TAMs).[139] As a result, receptors associated with angiogenesis were found to be overexpressed on most solid tumors.[137, 148, 154, 155]

The VEGFR is one of the most important receptors in targeting neo-angiogenesis due to its proven overexpression in the majority of solid tumors and its significance in tumor signaling cascades.[137, 148] Backer et al. designed an active targeting system consisting of a boronated dendrimer with a VEGF, a protein ligand, on its surface. IR imaging showed that the VEGF boronated dendrimer, unlike the nontargeted dendrimer, killed a significantly higher amount of tumor cells with negligible effect on normal cells.[156]

The integrins family of integral protein surface receptors represents another very promising target in tumor cells as these proteins play a key role in any tumor progression.[143, 149] Furthermore, integrins bind to a whole range of ECM proteins, including fibrinogen, vitronectin, collagen and fibronectin, which creates more targeting ligand options.[157] To target $\alpha_5\beta_1$ in colon cancer, Garg and co-workers[158] designed a targeted liposome with a synthesized peptide on its surface. The peptide, synthesized to specifically mimic fibronectin, is a combination of two common integrin specific peptide sequences, RGDSP and PHSRN, which is recognized by $\alpha_5\beta_1$, an integrin receptor. Flow cytometry results showed that accumulation of the peptide-coated liposomes was significantly higher in colon cancer cells compared to the accumulation of non-targeted PEGylated liposomes.

Vascular cell adhesion molecules, on the other hand, are targets that can provide a much higher specificity to tumor cells, when compared to the majority of other receptors. This is due to the very low expression of the receptor on normal cells. These transmembrane glycoproteins bind to ECM proteins and are very important for metastasis.[151, 152] Gosk et al.[153] studied the effect of attaching an anti VCAM-1 monoclonal antibody to the surface of liposomes, on their accumulation in tumor endothelium cells both *in vitro* and *in vivo*. In both cases, more accumulation of immunoliposomes was observed in tumor cells, than of the PEGylated liposomes control. Moreover, unlike the control, internalization of the drug was observed when using the immunoliposomes: drug was found inside the tumor cells instead of on the membrane surface.

3.3.4. Targeting Uncontrolled Cell Division

Tumor cells are generally defined by their uncontrolled cell division. This is a result of mutations in tumor suppressor genes, e.g., p53.[153] Instead of regulating the cell division pathways, the mutated genes cause continuous cell division. Therefore, a good targeting strategy would be to use the overexpression of tumor's distinct cell division receptors to target cancer. Main cell division targets are Human Epidermal Receptors (HER),[159] transferrin receptors[150, 160] and folate receptors.[134, 161]

The continuous cell division process causes tumor cells to display overdependence on growth factors and therefore overexpress the surface receptors that mediate cell division pathways.[137] The major family of receptors that exhibit this function is that of HERs,[159] which have been widely researched as cancer therapeutic targets and their overexpression has been observed in major tumors including gastric cancer,[162] prostate cancer,[163] ovarian cancer, and breast cancer.[164] Sandoval et al.[165] studied the effect of attaching a recombinant murine epidermal growth factor (EGF) protein ligand to the surface of a stearoyl gemcitabine nanoparticle (GemC18-NPs), a nucleic acid based nanocarrier, on the targeting of both human breast sarcoma cells *in vitro* and tumors in mice *in vivo*. *In vitro* results showed a statistically significant higher cytotoxicity in cells treated with EGF-GemC18-NPs compared to the non-targeted GemC18-NPs. More interestingly, *in vivo* results showed 2 times more drug accumulation in the tumor treated with the targeted nanocarrier compared to the untargeted one, causing a significantly lower tumor diameter in mice treated with the targeted one. Moreover, after adding the ligand, the survival time of the mice improved from 69 to 160 days.

Another receptor that is distinctly overexpressed on numerous malignant tumors is the transferrin receptor. Transferrin is an iron transport protein that is essential

when cells undergo division, hence the transferrin receptor is overexpressed on tumor cells.[150, 160, 166] Wang et al. designed a polymer-based nanocarrier conjugated to Herceptin, a transferrin monoclonal antibody, to target 6 different tumor cell lines. Targeted nanocarriers resulted in up to 80% drug accumulation in tumor cell, while less than 10% drug accumulation was achieved by untargeted nanocarriers.[115]

Finally, the folate receptor, a transmembrane glycoprotein that binds to folic acid, is also a very widely researched targeting ligand. The regulation of folic acid has been linked to tumor cell division through its abundance during metastasis and tumor recurrence, and its complete absence in the majority of normal cells.[161] This, along with folic acid's availability and relatively small size, makes the folate receptor one of the most used targets for cancer therapeutics.[167–169] To study these advantages, Cao and co-workers[170] designed amphiphilic biodegradable dendrimer-like star polymers (DLSPs) and conjugated them with folic acid. After running flow cytometry analysis, a higher amount of drug accumulation inside and on the surface of human epidermal carcinoma cells was evident compared to the non-targeted control.

3.3.5. Targeting Specific Malignancies

The previously mentioned strategies all depend on general biological phenomena that apply to all malignancies, but researchers also focus their strategies on specific tumors rather than general phenomena.[137] This is because the overexpression of receptors, and in some cases just their presence, depends on the type of malignancy. Table II highlights common specific malignancy targets and their ligands.

4. TRIGGERING THE DRUG DELIVERY

4.1. Internal and External Triggers

Nanosystems for drug delivery may be designed to either respond to external stimuli and release their contents, or to make use of changes in their environment in order to release the drug. External triggers include ultrasound, light, temperature increase, and magnetic or electric fields.[171] Ultrasound as a trigger for drug release from nanocarriers will be discussed in detail in the next section.

Light is used as an external stimulus with photo-triggerable liposomes. These liposomes are made of modified lipids, called photoactivable lipids, that have an added light-sensitive chemical group, while still maintaining the properties that are essential for the formation and drug-loading of liposomes.[172] Temperature increase is another external trigger that may be used for drug release; the use of local hyperthermia with Dox-TSL (temperature-sensitive liposomes loaded with Dox) was studied and the results indicated an increase in the uptake of Dox by the tumor and increased tumor disappearance.[173] Magnetically responsive nanocarriers, which include a paramagnetic material such as iron oxide, have been synthesized to be sensitive to a magnetic field, which is used as an external trigger for the release of the drug.[171] Weak electric fields (with a strength of approximately 1V) can be used to trigger drug release from nanocarriers that have been modified with conductive materials; on the other hand, strong electric fields may be used to permeabilize cell membranes and increase their uptake of drugs.[171]

Internal triggers make use of pH conditions in the endosome of a cell, overexpression of certain enzymes in the target site, or the electron affinity and reduction potentials of liposomes.[174] Drug release from PHSM/f (pH-sensitive micelles with folate) was studied at different pH values and it was shown that 82 wt% of Dox was released at pH 5.0 as opposed to 32 wt% only at pH 7.0.[174] Disulphide bonds can be used to make DDS more redox-sensitive; these bonds are subjected to reduction by the tripeptide glutathione (GSH), which triggers drug release due to its different concentrations in tumor tissues versus healthy cells. Different types of micelles have been synthesized following this principle, and other DDS such as liposomes have been created using different conjugates, materials, and nanogels to increase redox-sensitivity.[171] Secretory phospholipase A_2 ($sPLA_2$) is an enzyme that is overexpressed in tumor tissues and therefore, by creating PEGylated liposomes using phospholipids that allow for $sPLA_2$ hydrolysis, enzyme overexpression may be used as an internal trigger for drug release from nanocarriers.[175]

Table II. Targeting specific malignancies.

Malignancy	Ligand	Target	Source
Breast cancer	Anti-HER-2 monoclonal antibody	HER-2	205
	Folic-acid	Folate receptor	206
	Estrone-3-sulphate	Organic anion transporting peptides (OATPs)	207
Colorectal cancer	Guanylyl cyclase C (GCC)	Peptide enterotoxin ST	208
	Cetuximab	EGFR	209
Lung cancer	NeutrAvidin	EGFR	210
	siRNA and RGD	$\alpha v\beta 3$ integrin	211
Prostate cancer	A10 PSMA aptamer	prostate specific membrane antigen (PSMA)	142, 212
Melanoma	Peptide PHSCNK	$\alpha 5\beta 1$ integrin	140
	Peptide RGD	$\alpha v\beta 3$ integrin	213

4.2. Ultrasound

In the process of drug delivery to tumor cells, whether by passive or active targeting, ultrasound (US) may be used once the drug carrier reaches the target site in order to trigger the release of the drug.

Ultrasound waves transmitted from a transducer are sinusoidal in nature, alternating between high and low pressures.[81, 176] These waves move through a medium, transferring energy from one element to the next in a manner referred to as propagation; however, some of the energy is lost in a process called dispersion. The combination of both events results in attenuation, which can be summarized as the collective process of absorption and scattering of energy waves.[176, 177] Two important parameters of US waves are the frequency and the power density; frequency of an US wave refers to the number of sinusoidal cycles occurring per second (Hz), whereas the power density is defined as the power per cross-sectional area (W/cm^2).

In order to achieve targeted delivery and prevent drug accumulation in non-target tissues, the drug carrier needs to travel in the blood stream until it reaches the target, then extravasate in the space between cells and penetrate the tumor.[178] Using US for triggered drug delivery allows for the time- and location-specific release of anti-cancer drugs at the desired target cells, thus decreasing the known side effects resulting from chemotherapy spreading to several parts of the body.

The effects of US in drug delivery may be either thermal or mechanical (Fig. 4). Thermal effects occur due to the absorption of thermal energy by the tissues or cells, which results in an increase in temperature. When hyperthermia occurs, this may result in heating of the drug and carrier, or heating and damaging of the tumor tissues in the absence of chemotherapy.[178] On the other hand, the more prominent effects, which are observed and exploited for triggered drug delivery, are the mechanical effects, which occur due to oscillating bubbles, cavitation, wave pressure, and acoustic streaming.[178, 179]

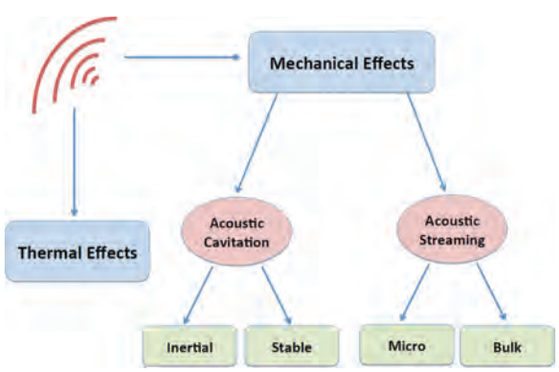

Figure 4. Effects of ultrasound.

4.2.1. Mechanical Effects of US

There are several non-thermal effects when applying US to target sites during drug delivery, and they result from the mechanical nature of the wave propagation through a medium, as well as the pressure variations. Acoustic cavitation is one such effect that occurs due to the oscillation, growth, and collapse of gas bubbles in the medium due to the varying pressure caused by the US wave. This effect depends on the intensity parameter of the wave and requires a certain threshold after which it can occur.[177, 180] Cavitation may be divided into two categories, namely inertial and stable, as shown in Figure 4. As the name indicates, stable cavitation involves constant oscillation of the gas bubble and occurs when the pressure amplitude is low.[177, 180, 181] On the other hand, inertial cavitation involves unstable oscillation of the bubble, after which it grows rapidly in size and implodes violently, resulting in a series of events.[176, 180, 181] Inertial and stable cavitation are not independent events that occur separately in different situations. However, the former effect occurs at low amplitudes and linear oscillations, whereby the bubble grows in size until it reaches the resonance size; this is followed by the latter effect, which occurs as the oscillations become non-linear at high pressures, and the bubble grows rapidly and collapses.[81, 177]

The collapse of the bubble due to inertial cavitation results in a series of events as mentioned earlier. Shock waves result from the implosion of the gas bubble and that leads to shear stresses that can damage surrounding cells. Moreover, when inertial cavitation occurs near a solid surface, a sonic jet of liquid is produced and it pierces the surface of the nearby vesicle (e.g., a micelle, liposome or another drug carrier).[176, 177, 180, 182]

Mechanical US waves moving through the medium result in acoustic streaming, which is the directional current of flowing fluid. This could be bulk-streaming or micro-streaming (Fig. 4), where the former refers to unidirectional flow and the latter refers to swirls of flow. Microstreaming is found to be more powerful and facilitates improved drug delivery.[180]

In a study by Zhao et al., the effect of combining US with Dox liposomes on tumor growth inhibition was studied; it was found that acoustic cavitation enhanced the inhibition effect even at low concentrations of Dox, and that tumors were more susceptible to the drug even after US exposure.[183] A review on the use of US as a trigger for the release of drugs from micelles and liposomes have been recently published, and summarizes the most important *in vitro* and *in vivo* studies in this area.[55]

4.2.2. Mechanisms of Triggered Drug Delivery

With the properties and effects of US in mind, the proposed mechanisms by which it affects drug delivery will now be discussed. As the encapsulated drug travels through the bloodstream towards the target cells and tissues, and is exposed to US, the release is achieved by

mechanisms that can be explained in terms of the carrier, surrounding tissues, the drug itself, or a combination of these factors.

4.2.2.1. Cell Membrane Permeabilization. The aforementioned mechanical effects caused by the use of US play an important role in the mechanism of cell membrane permeabilization. As the bubbles collapse and shear stresses or sonic jets of liquid are produced, the surrounding cell membranes are affected. The holes that are produced in the membranes enhance the permeability of these cells, which in turn facilitates the successful delivery of the drug to the target tissues.[81, 177] Nomikou and McHale conducted studies, which involved the use of pulsed US, and the results indicated that cell membrane permeabilization was enhanced in the use of camptothecin with *in vitro* targets.[184] Several other studies that support this mechanism have been recently described.[55]

4.2.2.2. Enhanced Drug Distribution in Target Tissues. In addition to increasing the permeability of cells, US induces another mechanism that contributes to effective drug delivery to tumors. By studying tissues that are not well vascularized, it was observed that improved drug delivery of chemotherapeutic drugs was due to the enhanced distribution of the drug throughout tumor tissues after applying US.[184] This effect may be explained by the enhanced transport effect, which is attributed to the motion of surrounding fluid. The transport of drugs into the cells occurs naturally by the mechanism of simple diffusion; therefore, even when the cell membrane permeability is increased, there will still be limitations to the extent of drug distribution. However, the current effects and bubble oscillations that are created due to the use of US enhance the motion of fluid in the vicinity of the drug and the target tissues.[177] This in turn leads to an improvement in the dispersion of the chemotherapeutic drug throughout the tumor cells and tissues.

4.2.2.3. Disruption of the Drug Carrier. The mechanism by which US affects the drug carrier is greatly relevant to the site-specificity of triggered drug delivery. The mechanical effects created by the pressure waves that result from insonation lead to disruption of the drug carrier.[184] Oscillating bubbles and cavitation create shear stresses that may open up the drug carrier and lead to the release of the encapsulated drug; this mechanism is particularly useful in triggering the delivery of the drug at the required target site and avoiding side effects of toxicity elsewhere in the body.[177, 184] A very important example is the use of polymeric micelles as nanocarriers in conjunction with pulsed US as a trigger. When US is applied in a pulsated method, there is an *ON* period and an *OFF* period; since polymeric micelles are capable of self-assembly, as mentioned previously, any amount of drug that has not entered the tumor during the *ON* period, can be re-encapsulated in the carrier during the *OFF* period and circulate in the blood stream once again.[185] This was shown in experiments conducted by Diaz de la Rosa et al.; the drug, Dox, was only released from the core of the P105 micelles when exposed to the US in the environment of the tumor, and then the remaining Dox was re-encapsulated in the absence of US. This helps in reducing the interaction of the drug with non-tumor tissues and successfully enhancing chemotherapeutic drug delivery while reducing unwanted side effects.[185]

4.2.3. Effects of Frequency and Intensity on Triggered Drug Delivery

When US is being used to trigger drug release from nanocarriers, the frequencies usually range from 20 kHz to 16 MHz; however, different frequencies result in different efficiency of drug release.[180] In experiments by Diaz de la Rosa et al., it was reported that Dox was released from the core of micelles at frequencies of 20, 45, 70, and 90 kHz, whereas no release was observed at 476 kHz.[185] This can possibly be explained by the effect of frequency on the cavitation effect which facilitates the release of drugs from carriers. Ueda et al. have studied the cavitation effect at 41, 158, and 455 kHz, and obtained results that confirm the increase of cavitation at lower frequencies.[186] To further confirm this effect, a study was conducted by Cohen-Levi to study the release of Dox from liposomes at 20 kHz, 1 MHz, and 3 MHz. At the low frequency of 20 kHz, up to 85% of Dox was released from the liposomes in saline, and up to 61% in human plasma. However, the level and speed of release was observed to be much lower (~5%) at the higher frequency of 1 MHz, and even lower at 3 MHz.[187]

In studies conducted by Husseini et al. to study the effect of intensity, or power density, on drug release from micelles, it was observed that higher power densities resulted in higher release at all frequencies studied. The effect of intensity on drug release is related to the frequency as well; if high frequencies of US are used, higher levels of power density are required in order to obtain significant amounts of release.[188] More recently, the same group has found similar Dox release from targeted micelles (using folic acid as a targeting moiety).[167, 168]

4.2.4. Enhancements: Microbubbles and eLiposomes

The US-triggered drug delivery from nanocarriers can be enhanced by designing modified drug carriers. Two important examples that increase the echogenicity of drug carriers are microbubbles and eLiposomes.

4.2.4.1. Microbubbles. Microbubbles are US contrast agents that are micro-sized, gas-filled bubbles surrounded by a stabilizing shell and used to enhance the aforementioned mechanisms involved in US triggered drug delivery.[189, 190] The reason for their effectiveness arises from the discrepancy in densities between the gas inside the microbubbles and the blood in the human body.[191]

This leads to a difference in acoustic impedance, the propagation of sound waves through a medium, and hence, oscillations occur when the microbubbles are exposed to US waves.[179, 191] These oscillations may eventually lead to some of the mechanical effects explained earlier such as microstreaming, cavitation, collapse of the bubbles, and the production of sonic liquid jets.[179, 189, 190] Such interactions between the contrast agents and the applied US enhance the drug delivery process by permeabilization, increased drug uptake, and improved diffusion into tumor tissues.[179, 190, 192–194]

Microbubbles are either encapsulated in drug carriers or used as drug carriers themselves,[184] and they provide encouraging opportunities for use in drug delivery in combination with clinical/low frequencies of US by facilitating cavitation at lower pressures.[195] This was confirmed by Unger et al. in an experiment where significantly more fluorescent material was delivered to tissues when US and microbubbles were used.[196] Moreover, by using model fluorescent materials to study intracellular delivery, it was demonstrated that an increased effect occurs when using insonated microbubbles, as opposed to using either of them separately.[197] Escoffre et al. demonstrated the importance of using microbubbles in drug delivery by preparing Dox liposome-loaded microbubbles and studying the effect of combining US with these agents. The results showed that the uptake by glioblastoma U-87 MG cells was enhanced greatly in comparison to Dox liposome-loaded microbubbles alone.[190]

4.2.4.2. eLiposomes. eLiposomes are liposomes with an added element that increases the drug carrier's echogenity and allows for enhanced and site-specific drug release. These drug carriers contain liquid emulsion droplets and require low intensities of US in order to release their drug content at the tumor site.[198] The liquid emulsions are made from perfluorocarbons (PFCs), which are liquids that have high vapor pressures at biological temperatures[199] and are non-toxic to the human body.[200] The phenomenon that forms the basis for the development of eLiposomes is called acoustic droplet vaporization, during which a liquid droplet expands greatly as it vaporizes when exposed to an US wave;[201, 202] this occurs during the low-pressure phases of the wave, when the pressure is lower than the vapor pressure of the liquid.[198, 200] This expansion leads to the rupture of the liposome and effective release of the encapsulated drug at the target site; moreover, the small size of the eLiposome allows its accumulation (by endocytosis), and delivery of its contents, inside tumor cells.[198, 203] In a study conducted by Lin et al., 20-kHz US, with a power density of 1 W/cm^2 was used to trigger the release of Dox from both LDox (liposome-encapsulated Dox) and eLipoDox (Dox encapsulated in eLiposomes). The results showed that for the same period of insonation, more release occurred from the eLipoDox than the LDox (80% vs. 50%).[204]

5. CONCLUSION

In this review, we have explored the use of drug nanocarriers (targeted and non-targeted) in conjunction with internal and external stimuli, with a focus on ultrasound. Passive targeting of nanocarriers, particularly micelles or liposomes, is the starting point of site-specific drug delivery, because of the EPR effect. Utilizing the second component of the DDS, active targeting, allows for the addition of various ligands to the nanocarrier surface to increase drug internalization through specific receptor-ligand binding. The final key element of the DDS is to trigger the maximum release of the drug from the activated nanocarriers. Despite the existence of extensive research that studies the effect of these individual components, the true potential of the chemotherapeutic drug delivery lies in a synergistic DDS that combines passive, active, and triggered targeting. As our fight against cancer continues, we have been lucky with several discoveries that have a potential to achieve site-specific delivery, which would reduce the side effects of conventional chemotherapy. This would, no doubt, improve the quality of life of cancer patients worldwide.

ABBREVIATIONS

CMC: Critical micelle concentration;
CMT: Critical micelle temperature;
DDS: Drug delivery system;
DLSP: Dendrimer-like star polymer;
DOPE: 1:2-dioleoyl-sn-glycero-3-phosphoethanolamine;
Dox: Doxorubicin;
DPPC: 1:2-dipalmitoyl-sn-glycero-3-phosphocholine;
ECM: Extracellular matrix;
EGF: Epidermal growth factor;
EPR: Enhanced permeability and retention;
HER: Human epidermal receptors;
MLV: Multilamellar vesicles;
MPS: Mononuclear phagocyte system;
NCM: Near-critical fluid micellization;
PCB: Poly(carboxybetaine);
PCL: Poly(ε-caprolactone);
PEG: Poly(ethylene glycol);
PEG-*b*-PDLLA: Poly(ethylene glycol)-block-poly(*D*:*L*-lactide);
PEG-*b*-PHB-PEG: PEG-b-poly(3-hydroxybutyrate)-b-PEG;
PLA: Poly(*D*:*L*-lactide);
PEO: Poly(ethylene oxide);
PPO: Poly(propylene oxide);
RES: Reticulo-endothelial system;
SMANCS: Polymer conjugate of poly(styrene-co-maleic acid) and neocarzinostatin;

TAM: Tumor-associated macrophages;
US: Ultrasound;
VCAM: Vascular cell adhesion molecules;
VEGF (R): Vascular endothelial growth factor (R).

References and Notes

1. J. W. Nichols and Y. H. Bae, *J. Control Release* 190, 451 (**2014**).
2. K. Strebhardt and A. Ullrich, *Nat. Rev. Cancer* 8, 473 (**2008**).
3. S. Ganta, H. Devalapally, A. Shahiwala, and M. Amiji, *J. Control Release* 126, 187 (**2008**).
4. W. G. Pitt and G. A. Husseini, *Adv. Drug. Deliv. Rev.* 60, 1095 (**2008**).
5. V. P. Torchilin, *Adv. Drug. Deliv. Rev.* 58, 1532 (**2006**).
6. V. P. Torchilin, *AAPS J.* 9, E128 (**2007**).
7. E. L. Yuh, S. G. Shulman, S. A. Mehta, J. Xie, L. Chen, V. Frenkel, M. D. Bednarski, and K. C. Li, *Radiology* 234, 431 (**2005**).
8. W. G. Pitt, G. A. Husseini, B. L. Roeder, D. J. Dickinson, D. R. Warden, J. M. Hartley, and P. W. Jones, *J. Nanosci. Nanotechnol.* 11, 1866 (**2011**).
9. P. Couvreur and C. Vauthier, *Pharm. Res.* 23, 1417 (**2006**).
10. H. Maeda, G. Y. Bharate, and J. Daruwalla, *Eur. J. Pharm. Biopharm.* 71, 409 (**2009**).
11. V. Torchilin, *Eur. J. Pharm. Biopharm.* 71, 431 (**2009**).
12. V. P. Torchilin, *Pharm. Res.* 24, 2333 (**2007**).
13. I. F. Uchegbu and A. Siew, *J. Pharm. Sci.* 102, 305 (**2013**).
14. K. E. Uhrich, S. M. Cannizzaro, R. S. Langer, and K. M. Shakesheff, *Chem. Rev.* 99, 3181 (**1999**).
15. S. Singh, *J. Nanosci. Nanotechnol.* 10, 7906 (**2010**).
16. R. Singh and H. S. Nalwa, *J. Biomed. Nanotechnol.* 7, 489 (**2011**).
17. P. S. Goyal and V. K. Aswal, *Curr. Sci.* 80 (**2001**).
18. E. J. R. Sudhölter, G. B. van de Langkruis, and J. B. F. N. Engberts, *Recl. Trav. Chim. Pay-B* 99, 73 (**1980**).
19. V. P. Torchilin, *Pharm. Res.* 24, 1 (**2007**).
20. C. Tan, Y. Wang, and W. Fan, *Pharm.* 5, 201 (**2013**).
21. W. Huang, C. Shi, Y. Shao, K. S. Lam, and J. Luo, *Chem. Commun. (Camb)* 49, 6674 (**2013**).
22. C. Oerlemans, W. Bult, M. Bos, G. Storm, J. F. Nijsen, and W. E. Hennink, *Pharm. Res.* 27, 2569 (**2010**).
23. P. Alexandridis and T. A. Hatton, *Colloid. Surface. A* 96, 1 (**1995**).
24. H. M. Aliabadi and A. Lavasanifar, *Expert. Opin. Drug. Deliv.* 3, 139 (**2006**).
25. G. Gaucher, M. H. Dufresne, V. P. Sant, N. Kang, D. Maysinger and J. C. Leroux, *J. Control. Release* 109, 169 (**2005**).
26. U. Kedar, P. Phutane, S. Shidhaye, and V. Kadam, *Nanomedicine* 6, 714 (**2010**).
27. A. Mahmud, X. B. Xiong, H. M. Aliabadi, and A. Lavasanifar, *J. Drug. Target* 15, 553 (**2007**).
28. S. C. Owen, D. P. Y. Chan, and M. S. Shoichet, *Nano Today* 7, 53 (**2012**).
29. W. Xu, P. Ling, and T. Zhang, *J. Drug. Deliv.* 2013, 340315 (**2013**).
30. C. F. van Nostrum, *Soft. Matter* 7, 3246 (**2011**).
31. G. A. Husseini, R. I. El-Fayoumi, K. L. O'Neill, N. Y. Rapoport, and W. G. Pitt, *Cancer Lett.* 154, 211 (**2000**).
32. J. D. Pruitt, W. G. Pitt, and N. Rapoport, Stabilization of pluronic P-105 micelles with an interpenetrating network of *N*,*N*-diethylacrylamide, *Sixth World Biomaterials Congress*, Society for Biomaterials, Hawaii (**2000**), Vol. 1, p. 201.
33. G. A. Husseini, W. G. Pitt, D. A. Christensen, and D. J. Dickinson, *J. Control Release* 138, 45 (**2009**).
34. D. A. Chiappetta and A. Sosnik, *Eur. J. Pharm. Biopharm.* 66, 303 (**2007**).
35. R. Gref, Y. Minamitake, M. T. Peracchia, V. Trubetskoy, V. Torchilin, and R. Langer, *Science* 263, 1600 (**1994**).
36. Y. Li, K. Xiao, J. Luo, W. Xiao, J. S. Lee, A. M. Gonik, J. Kato, T. A. Dong, and K. S. Lam, *Biomaterials* 32, 6633 (**2011**).
37. G. A. Husseini and W. G. Pitt, *Adv. Drug Deliv. Rev.* 60, 1137 (**2008**).
38. G. A. Husseini, D. A. Christensen, N. Y. Rapoport, and W. G. Pitt, *J. Control Release* 83, 303 (**2002**).
39. G. A. Husseini, N. Y. Rapoport, D. A. Christensen, P. J. D., and W. G. Pitt, *Colloids Surf. B* 24, 253 (**2002**).
40. A. V. Kabanov and V. Y. Alakhov, *Crit. Rev. Ther. Drug Carrier Syst.* 19, 1 (**2002**).
41. A. V. Kabanov, E. V. Batrakova, and V. Y. Alakhov, *Adv. Drug Deliv. Rev.* 54, 759 (**2002**).
42. A. V. Kabanov, E. V. Batrakova, N. S. Melik-Nubarov, N. A. Fedoseev, T. Y. Dorodnich, V. Y. Alakhov, I. R. Nazarova, and V. A. Kabanov, *J. Control Release* 22, 141 (**1992**).
43. M. D. Muniruzzaman, A. Marin, Y. Luo, G. D. Prestwich, W. G. Pitt, G. A. Husseini, and N. Rapoport, *Colloids Surf. B* 25, 233 (**2002**).
44. N. Munshi, N. Rapoport, and W. G. Pitt, *Cancer Lett.* 118, 13 (**1997**).
45. N. Y. Rapoport, J. N. Herron, W. G. Pitt, and L. Pitina, *J. Control Release* 58, 153 (**1999**).
46. Z. L. Tyrrell, Y. Shen, and M. Radosz, *Prog. Polym. Sci.* 35, 1128 (**2010**).
47. C. Allen, D. Maysinger, and A. Eisenberg, *Colloids Surf. B* 16, 3 (**1999**).
48. G. A. Husseini and W. G. Pitt, *J. Nanosci. Nanotechnol.* 8, 2205 (**2008**).
49. W. G. Pitt, G. A. Husseini, and B. J. Staples, *Expert. Opin. Drug Deliv.* 1, 37 (**2004**).
50. B. J. Staples, W. G. Pitt, B. L. Roeder, G. A. Husseini, D. Rajeev, and G. B. Schaalje, *J. Pharm. Sci.* 99, 3122 (**2010**).
51. B. J. Staples, B. L. Roeder, G. A. Husseini, O. Badamjav, G. B. Schaalje, and W. G. Pitt, *Cancer Chemoth. Pharm.* 64, 593 (**2009**).
52. N. Rapoport, W. G. Pitt, H. Sun, and J. L. Nelson, *J. Control Release* 91, 85 (**2003**).
53. C. L. Waite and C. M. Roth, *Crit. Rev. Biomed. Eng.* 40, 21 (**2012**).
54. G. S. Kwon, M. Naito, K. Kataoka, M. Yokoyama, Y. Sakurai, and T. Okano, *Colloids Surf. B* 2, 429 (**1994**).
55. S. E. Ahmed, A. M. Martins, and G. A. Husseini, *J. Drug. Target.* 23, 16 (**2015**).
56. G. A. Husseini and W. G. Pitt, *J. Pharm. Sci.* 98, 795 (**2009**).
57. K. Kataoka, G. S. Kwon, M. Yokoyama, T. Okano, and Y. Sakurai, *J. Control Release* 24, 119 (**1993**).
58. E. V. Batrakova and A. V. Kabanov, *J. Control Release* 130, 98 (**2008**).
59. E. V. Batrakova, S. Li, W. F. Elmquist, D. W. Miller, V. Y. Alakhov, and A. V. Kabanov, *Br. J. Cancer* 85, 1987 (**2001**).
60. Z. L. Tyrrell, Y. Shen, and M. Radosz, *J. Phys. Chem. C* 115, 11951 (**2012**).
61. G. Ledet and T. Mandal, *US Pharm.* 37, 7 (**2012**).
62. M. Peplow, *Pharm. J.* 292, 467 (**2014**).
63. G. Pillai, *SOJ Pharm. Sci.* 1, 1 (**2014**).
64. D. W. Kim, S. Y. Kim, H. K. Kim, S. W. Kim, S. W. Shin, J. S. Kim, K. Park, M. Y. Lee, and D. S. Heo, *Ann. Oncol.* 18, 2009 (**2007**).
65. M. W. Saif, N. A. Podoltsev, M. S. Rubin, J. A. Figueroa, M. Y. Lee, J. Kwon, J. Yu, and R. O. Kerr, *Cancer Invest.* 28, 186 (**2008**).
66. T. Hamaguchi, Y. Matsumura, M. Suzuki, K. Shimizu, R. Goda, I. Nakamura, I. Nakatomi, M. Yokoyama, K. Kataoka, and T. Kakizoe, *Br. J. Cancer* 92, 1240 (**2005**).
67. D. W. Deamer, *FASEB J.* 24, 1308 (**2010**).
68. G. Sessa and G. Weissmann, *J. Lipid. Res.* 9, 310 (**1968**).
69. J. S. Dua, A. C. Rana, and A. K. Bhandari, *Int. J. Pharm. Stud. Res.* 3, 14 (**2012**).
70. F. Perche and V. P. Torchilin, *J. Drug Deliv.* 2013, 32 (**2013**).

71. A. Laouini, C. Jaafar-Maalej, I. Limayem-Blouza, S. Sfar, C. Charcosset, and H. Fessi, *J. Coll. Sci. Biot.* 1, 147 (**2012**).
72. T. M. Allen and P. R. Cullis, *Adv. Drug Deliv. Rev.* 65, 36 (**2013**).
73. S. Huang, *Adv. Drug Deliv. Rev.* 60, 1167 (**2008**).
74. H. Karanth and R. S. Murthy, *J. Pharm. Pharmacol.* 59, 469 (**2007**).
75. S. L. Huang, D. D. McPherson, and R. C. Macdonald, *Ultrasound Med. Biol.* 34, 1272 (**2008**).
76. M. Javadi, W. G. Pitt, D. M. Belnap, N. H. Tsosie, and J. M. Hartley, *Langmuir* 28, 14720 (**2012**).
77. J. Liu, Y. Huang, A. Kumar, A. Tan, S. Jin, A. Mozhi, and X.-J. Liang, *Biot. Adv.* 32, 693 (**2014**).
78. A. Yavlovich, B. Smith, K. Gupta, R. Blumenthal, and A. Puri, *Mol. Membr. Biol.* 27, 364 (**2010**).
79. A. Gasselhuber, M. R. Dreher, F. Rattay, B. J. Wood, and D. Haemmerich, *PLoS ONE* 7 (**2012**).
80. L. Zhang, F. X. Gu, J. M. Chan, A. Z. Wang, R. S. Langer, and O. C. Farokhzad, *Clin. Pharm. Ther.* 83, 761 (**2008**).
81. K. W. Ferrara, *Adv. Drug Deliv. Rev.* 60, 1097 (**2008**).
82. A. Samad, Y. Sultana, and M. Aqil, *Curr. Drug. Deliv.* 4, 297 (**2007**).
83. A. Fritze, F. Hens, A. Kimpfler, R. Schubert, and R. Peschka-Suss, *Biochim. Biophys. Acta* 1758, 1633 (**2006**).
84. P. R. Harrigan, K. F. Wong, T. E. Redelmeier, J. J. Wheeler, and P. R. Cullis, *Biochim. Biophys. Acta* 1149, 329 (**1993**).
85. S. L. Huang, *Adv. Drug Deliv. Rev.* 60, 1167 (**2008**).
86. H. B. Frieboes, M. Wu, J. Lowengrub, P. Decuzzi, and V. Cristini, *PLoS One* 8, e56876 (**2013**).
87. S. K. Hobbs, W. L. Monsky, F. Yuan, W. G. Roberts, L. Griffith, V. P. Torchilin, and R. K. Jain, *Proc. Natl. Acad. Sci. USA* 95, 4607 (**1998**).
88. F. Yuan, M. Dellian, D. Fukumura, M. Leunig, D. A. Berk, V. P. Torchilin, and R. K. Jain, *Cancer Res.* 55, 3752 (**1995**).
89. F. Yuan, M. Leunig, S. K. Huang, D. A. Berk, D. Papahadjopoulos, and R. K. Jain, *Cancer Res.* 54, 3352 (**1994**).
90. Y. Zhang, H. F. Chan, and K. W. Leong, *Adv. Drug Deliv. Rev.* 65, 104 (**2013**).
91. N. Bertrand, J. Wu, X. Xu, N. Kamaly, and O. C. Farokhzad, *Adv. Drug Deliv. Rev.* 66, 2 (**2014**).
92. B. Chu, Soft Matter Characterization, edited by R. Borsali and R. Pecora, Elsevier (**2008**), p. 335.
93. S. Kim, Y. Shi, J. Y. Kim, K. Park, and J. X. Cheng, *Expert. Opin. Drug. Deliv.* 7, 49 (**2010**).
94. H. Chen, S. Kim, W. He, H. Wang, P. S. Low, K. Park, and J. X. Cheng, *Langmuir* 24, 5213 (**2008**).
95. A. Panariti, G. Miserocchi, and I. Rivolta, *Nanotechnol. Sci. Appl.* 5, 87 (**2012**).
96. Q. Sun, M. Radosz, and Y. Shen, *J. Control. Release* 164, 156 (**2012**).
97. J. Fang, H. Nakamura, and H. Maeda, *Adv. Drug Deliv. Rev.* 63, 136 (**2011**).
98. K. Greish, *Drug Discovery Today: Technol.* 9, e161 (**2012**).
99. A. K. Iyer, G. Khaled, J. Fang, and H. Maeda, *Drug Discov. Today* 11, 812 (**2006**).
100. H. Maeda, *Bioconjug. Chem.* 21, 797 (**2010**).
101. H. Maeda, J. Wu, T. Sawa, Y. Matsumura, and K. Hori, *J. Control Release* 65, 271 (**2000**).
102. H. Maeda, *Adv. Enzyme Regul.* 41, 189 (**2001**).
103. Y. Matsumura and H. Maeda, *Cancer Res.* 46, 6387 (**1986**).
104. B. R. Zetter, *Annu. Rev. Med.* 49, 407 (**1998**).
105. F. Danhier, O. Feron, and V. Preat, *J. Control Release* 148, 135 (**2010**).
106. Y. H. Bae, *J. Control Release* 133, 2 (**2009**).
107. H. Maeda, *Adv. Drug. Deliv. Rev.* 46, 169 (**2001**).
108. P. Aggarwal, J. B. Hall, C. B. McLeland, M. A. Dobrovolskaia, and S. E. McNeil, *Adv. Drug. Deliv. Rev.* 61, 428 (**2009**).
109. H. Maeda, T. Sawa, and T. Konno, *J. Control. Release* 74, 47 (**2001**).
110. K. Greish, *J. Drug Target* 15, 457 (**2007**).
111. C. Heneweer, J. P. Holland, V. Divilov, S. Carlin, and J. S. Lewis, *J. Nucl. Med.* 52, 625 (**2011**).
112. N. Bertrand and J. C. Leroux, *J. Control Release* 161, 152 (**2012**).
113. M. Lundqvist, J. Stigler, G. Elia, I. Lynch, T. Cedervall, and, K. A. Dawson, *Proc. Natl. Acad. Sci.* 105, 14265 (**2008**).
114. D. E. Owens, 3rd and N. A. Peppas, *Int. J. Pharm.* 307, 93 (**2006**).
115. M. Wang and M. Thanou, *Pharmacol. Res.* 62, 90 (**2010**).
116. D. D. Lasic and D. Needham, *Chem. Rev.* 95, 2601 (**1995**).
117. F. M. Veronese and G. Pasut, *Drug Discov. Today* 10, 1451 (**2005**).
118. K. Zhang, X. Tang, J. Zhang, W. Lu, X. Lin, Y. Zhang, B. Tian, H. Yang, and H. He, *J. Control Release* 183, 77 (**2014**).
119. K. R. Vega-Villa, J. K. Takemoto, J. A. Yanez, C. M. Remsberg, M. L. Forrest, and N. M. Davies, *Adv. Drug Deliv. Rev.* 60, 929 (**2008**).
120. K. J. Harrington, S. Mohammadtaghi, P. S. Uster, D. Glass, A. M. Peters, R. G. Vile, and J. S. Stewart, *Clin. Cancer Res.* 7, 243 (**2001**).
121. G. F. Paciotti, L. Myer, D. Weinreich, D. Goia, N. Pavel, R. E. McLaughlin, and L. Tamarkin, *Drug Deliv.* 11, 169 (**2004**).
122. T. Ishida, K. Atobe, X. Wang, and H. Kiwada, *J. Control. Release* 115, 251 (**2006**).
123. A. Judge, K. McClintock, J. R. Phelps, and I. MacLachlan, *Mol. Ther.* 13, 328 (**2006**).
124. X. Wang, T. Ishida, and H. Kiwada, *J. Control Release* 119, 236 (**2007**).
125. P. Opanasopit, M. Yokoyama, M. Watanabe, K. Kawano, Y. Maitani, and T. Okano, *J. Control Release* 104, 313 (**2005**).
126. J. Liu, F. Zeng, and C. Allen, *J. Control Release* 103, 481 (**2005**).
127. S. Y. Lee, S. Kim, J. Y. Tyler, K. Park, and J. X. Cheng, *Biomaterials* 34, 552 (**2013**).
128. M. Talelli, M. Iman, A. K. Varkouhi, C. J. Rijcken, R. M. Schiffelers, T. Etrych, K. Ulbrich, C. F. van Nostrum, T. Lammers, G. Storm, and W. E. Hennink, *Biomaterials* 31, 7797 (**2010**).
129. J. D. Pruitt, G. Husseini, N. Rapoport, and W. G. Pitt, *Macromolecules* 33, 9306 (**2000**).
130. T. F. Yang, C. N. Chen, M. C. Chen, C. H. Lai, H. F. Liang, and H. W. Sung, *Biomaterials* 28, 725 (**2007**).
131. T. Inoue, Y. Yamashita, M. Nishihara, S. Sugiyama, Y. Sonoda, T. Kumabe, M. Yokoyama, and T. Tominaga, *Neuro-Oncol.* 11, 151 (**2009**).
132. Z. Cao, L. Zhang, and S. Jiang, *Langmuir* 28, 11625 (**2012**).
133. D. Peer, J. M. Karp, S. Hong, O. C. Farokhzad, R. Margalit, and R. Langer, *Nat. Nanotechnol.* 2, 751 (**2007**).
134. B. Yu, H. C. Tai, W. Xue, L. J. Lee, and R. J. Lee, *Mol. Membr. Biol.* 27, 286 (**2010**).
135. Y. Zhong, F. Meng, C. Deng, and Z. Zhong, *Biomacromolecules* 15, 1955 (**2014**).
136. T. M. Allen, *Nat. Rev. Cancer* 2, 750 (**2002**).
137. J. D. Byrne, T. Betancourt, and L. Brannon-Peppas, *Adv. Drug Deliv. Rev.* 60, 1615 (**2008**).
138. F. Fay and C. J. Scott, *Immunotherapy* 3, 381 (**2011**).
139. P. Kocbek, N. Obermajer, M. Cegnar, J. Kos, and J. Kristl, *J. Control Release* 120, 18 (**2007**).
140. W. Dai, T. Yang, Y. Wang, X. Wang, J. Wang, X. Zhang, and Q. Zhang, *Nanomedicine* 8, 1152 (**2012**).
141. X. Jiang, X. Sha, H. Xin, L. Chen, X. Gao, X. Wang, K. Law, J. Gu, Y. Chen, Y. Jiang, X. Ren, Q. Ren, and X. Fang, *Biomaterials* 32, 9457 (**2011**).
142. O. C. Farokhzad, J. Cheng, B. A. Teply, I. Sherifi, S. Jon, P. W. Kantoff, J. P. Richie, and R. Langer, *Proc. Natl. Acad. Sci.* 103, 6315 (**2006**).
143. O. C. Farokhzad, S. Jon, A. Khademhosseini, T. N. Tran, D. A. Lavan, and R. Langer, *Cancer Res.* 64, 7668 (**2004**).

144. J. Mi, Y. Liu, Z. N. Rabbani, Z. Yang, J. H. Urban, B. A. Sullenger, and B. M. Clary, *Nat. Chem. Biol.* 6, 22 **(2010)**.
145. S. Marrache and S. Dhar, *Proc. Natl. Acad. Sci.* 109, 16288 **(2012)**.
146. W. L. Ye, J. B. Du, B. L. Zhang, R. Na, Y. F. Song, Q. B. Mei, M. G. Zhao, and S. Y. Zhou, *PLoS One* 9, e97358 **(2014)**.
147. S. E. Leucuta, *J. Drug Target* 22, 95 **(2014)**.
148. K. H. Plate, G. Breier, H. A. Weich, and W. Risau, *Nature* 359, 845 **(1992)**.
149. J. S. Desgrosellier and D. A. Cheresh, *Nat. Rev. Cancer* 10, 9 **(2010)**.
150. A. Calzolari, V. Finisguerra, I. Oliviero, S. Deaglio, G. Mariani, F. Malavasi, and U. Testa, *Blood Cell. Mol. Dis.* 42, 5 **(2009)**.
151. G. J. Byrne, A. Ghellal, J. Iddon, A. D. Blann, V. Venizelos, S. Kumar, A. Howell, and N. J. Bundred, *J. Natl. Cancer Inst.* 92, 1329 **(2000)**.
152. N. Makrilia, A. Kollias, L. Manolopoulos, and K. Syrigos, *Cancer Invest.* 27, 1023 **(2009)**.
153. S. Gosk, T. Moos, C. Gottstein, and G. Bendas, *Biochim. Biophys. Acta* 1778, 854 **(2008)**.
154. S. Kim, K. Bell, S. A. Mousa, and J. A. Varner, *Am. J. Pathol.* 156, 1345 **(2000)**.
155. G. Zhao and B. L. Rodriguez, *Int. J. Nanomedicine* 8, 61 **(2013)**.
156. M. V. Backer, T. I. Gaynudhinov, V. Patel, A. K. Bandyopadhyaya, B. T. Thirumamagal, W. Tjarks, R. F. Barth, K. Claffey, and J. M. Backer, *Mol. Cancer Ther.* 4, 1423 **(2005)**.
157. U. Marelli, F. Rechenmache, T. Sobahi, C. Mas-Moruno, and H. Kessler, *Front. Oncol.* 3, 1 **(2013)**.
158. A. Garg, A. W. Tisdale, E. Haidari, and E. Kokkoli, *Int. J. Pharm.* 366, 201 **(2009)**.
159. J. J. Laskin and A. B. Sandler, *Cancer Treat. Rev.* 30, 1 **(2004)**.
160. J. Wang, S. Tian, R. A. Petros, M. E. Napier, and J. M. Desimone, *J. Am. Chem. Soc.* 132, 11306 **(2010)**.
161. J. Sudimack and R. J. Lee, *Adv. Drug Deliv. Rev.* 41, 147 **(2000)**.
162. J. T. Jorgensen, *World J. Gastroenterol.* 20, 4526 **(2014)**.
163. M. Siampanopoulou, G. Galaktidou, N. Dimasis, and A. Gotzamani-Psarrakou, *Hippokratia* 17, 108 **(2013)**.
164. D. J. Slamon, W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J. Levin, S. G. Stuart, J. Udove, and A. Ullrich, *Science* 244, 707 **(1989)**.
165. M. A. Sandoval, B. R. Sloat, P. D. Lansakara, A. Kumar, B. L. Rodriguez, K. Kiguchi, J. Digiovanni, and Z. Cui, *J. Control. Release* 157, 287 **(2012)**.
166. J. Yue, S. Liu, R. Wang, X. Hu, Z. Xie, Y. Huang, and X. Jing, *Mol. Pharmaceut.* 9, 1919 **(2012)**.
167. G. A. Husseini, L. Kherbeck, W. G. Pitt, J. A. Hubbell, D. A. Christensen, and D. Velluto, *J. Nanosci. Nanotechnol.* 15, 2099 **(2015)**.
168. G. A. Husseini, D. Velluto, L. Kherbeck, W. G. Pitt, J. A. Hubbell, and D. A. Christensen, *Colloids Surfaces B* 101, 153 **(2013)**.
169. G. A. Husseini, S. Kanan, and M. Al-Sayah, *J. Nanosci. Nanotechnol.* accepted **(2014)**.
170. W. Cao, J. Zhou, Y. Wang, and L. Zhu, *Biomacromolecules* 11, 3680 **(2010)**.
171. S. Mura, J. Nicolas, and P. Couvreur, *Nat. Mater.* 12, 991 **(2013)**.
172. A. Puri, *Pharmaceutics* 6, 1 **(2014)**.
173. A. M. Ponce, Z. Vujaskovic, F. Yuan, D. Needham, and M. W. Dewhirst, *Int. J. Hyperthermia* 22, 205 **(2006)**.
174. E. S. Lee, Z. Gao, and Y. H. Bae, *J. Control Release* 132, 164 **(2008)**.
175. T. L. Andresen, D. H. Thompson, and T. Kaasgaard, *Mol. Membr. Biol.* 27, 353 **(2010)**.
176. A. Schroeder, J. Kost, and Y. Barenholz, *Chem. Phys. Lipids* 162, 1 **(2009)**.
177. G. A. Husseini, W. G. Pitt, and A. M. Martins, *Colloids Surfaces B* 123C, 364 **(2014)**.
178. K. Kwon, S. C. Lee, H. Bumsoo, and P. Kinam, *J. Control Release* 164, 108 **(2012)**.
179. Y. Y. Liu, H. Miyoshi, and M. Nakamura, *J. Control Release* 114, 89 **(2006)**.
180. A. Azagury, L. Khoury, G. Enden, and J. Kost, *Adv. Drug Deliv. Rev.* 72, 127 **(2014)**.
181. J. Wu and W. L. Nyborg, *Adv. Drug Deliv. Rev.* 60, 1103 **(2008)**.
182. T. J. Evjen, S. Hupfeld, S. Barnert, S. Fossheim, R. Schubert, and M. Brandl, *J. Pharm. Biomed. Anal.* 78–79, 118 **(2013)**.
183. Y.-Z. Zhao, D.-D. Dai, C.-T. Lu, H.-F. Lv, Y. Zhang, X. Li, W.-F. Li, Y. Wu, L. Jiang, X.-K. Li, P.-T. Huang, L.-J. Chen, and M. Lin, *Drug Dev. Ind. Pharm.* 38, 1090 **(2012)**.
184. N. Nomikou and A. P. McHale, *Cancer Lett.* 296, 133 **(2010)**.
185. M. A. Diaz de la Rosa, G. A. Husseini, and W. G. Pitt, *Ultrasonics* 53, 377 **(2013)**.
186. H. Ueda, M. Mutoh, T. Seki, D. Kobayashi, and Y. Morimoto, *Biol. Pharm. Bull.* 32, 916 **(2009)**.
187. D. Cohen-Levi, Ultrasound for targeted delivery of cytotoxic drugs from liposomes, Faculty of Engineering Sciences, Ben Gurion University **(2000)**.
188. G. A. Husseini, G. D. Myrup, W. G. Pitt, D. A. Christensen, and N. Y. Rapoport, *J. Control Release* 69, 43 **(2000)**.
189. M. C. Cochran, J. Eisenbrey, R. O. Ouma, M. Soulen, and M. A. Wheatley, *Int. J. Pharm.* 414, 161 **(2011)**.
190. J. M. Escoffre, C. Mannaris, B. Geers, A. Novell, I. Lentacker, M. Averkiou, and A. Bouakaz, *IEEE Trans. Ultrason. Ferroelectr. Freq. Control* 60, 78 **(2013)**.
191. B. Geers, I. Lentacker, N. N. Sanders, J. Demeester, S. Meairs, and S. C. De Smedt, *J. Control Release* 152, 249 **(2011)**.
192. A. van Wamel, K. Kooiman, M. Harteveld, M. Emmer, F. J. Ten Cate, M. Versluis, and N. de Jong, *J. Control Release* 112, 149 **(2006)**.
193. A. L. Klibanov, T. I. Shevchenko, B. I. Raju, R. Seip, and C. T. Chin, *J. Control. Release* 148, 13 **(2010)**.
194. V. Sboros, *Adv. Drug Deliv. Rev.* 60, 1117 **(2008)**.
195. S. Wrenn, S. Dicker, E. Small, and M. Michal, Controlling cavitation for controlled release, *IEEE Ultrasonics Symposium*, IEEE, Rome **(2009)**, p. 104.
196. E. C. Unger, T. O. Matsunaga, T. McCreery, P. Schumann, R. Sweitzer, and R. Quigley, *Eur. J. Radiol.* 42, 160 **(2002)**.
197. S. Hernot and A. L. Klibanov, *Adv. Drug Deliv. Rev.* 60, 1153 **(2008)**.
198. J. R. Lattin, D. M. Belnap, and W. G. Pitt, *Colloids Surfaces B* 89, 93 **(2012)**.
199. G. A. Husseini, W. G. Pitt, and J. Marjan, *Technol. Cancer Res. Treat* **(2014)**, in press.
200. M. Javadi, W. G. Pitt, C. M. Tracy, J. R. Barrow, B. M. Willardson, J. M. Hartley, and N. H. Tsosie, *J. Control Release* 167, 92 **(2013)**.
201. R. Singh, G. A. Husseini, and W. G. Pitt, *Ultrason. Sonochem.* 19, 1120 **(2012)**.
202. W. G. Pitt, R. N. Singh, K. X. Perez, G. A. Husseini, and D. R. Jack, *Ultrason. Sonochem.* **(2013)**.
203. J. R. Lattin, W. G. Pitt, D. M. Belnap, and G. A. Husseini, *Ultrasound Med. Biol.* 38, 2163 **(2012)**.
204. C. Y. Lin, M. Javadi, D. M. Belnap, J. R. Barrow, and W. G. Pitt, *Nanomedicine* 10, 67 **(2014)**.
205. S. R. Johnston, *Lancet. Oncol.* 14, 1145 **(2013)**.
206. V. Saxena, Y. Naguib, and M. D. Hussain, *Colloids Surfaces B* 94, 274 **(2012)**.
207. N. Banerjee, H. Fonge, A. Mikhail, R. M. Reilly, R. Bendayan, and C. Allen, *PLoS ONE* 8, e64069 **(2013)**.
208. P. Fortina, L. J. Kricka, D. J. Graves, J. Park, T. Hyslop, F. Tam, N. Halas, S. Surrey, and S. A. Waldman, *Trends Biotechnol.* 25, 145 **(2007)**.
209. Y. S. Cho, T. J. Yoon, E. S. Jang, K. Soo Hong, S. Young Lee, O. Ran Kim, C. Park, Y. J. Kim, G. C. Yi, and K. Chang, *Cancer Lett.* 299, 63 **(2010)**.

210. C. L. Tseng, T. W. Wang, G. C. Dong, S. Yueh-Hsiu Wu, T. H. Young, M. J. Shieh, P. J. Lou, and F. H. Lin, *Biomaterials* 28, 3996 (**2007**).

211. J. Conde, F. Tian, Y. Hernandez, C. Bao, D. Cui, K. P. Janssen, M. R. Ibarra, P. V. Baptista, T. Stoeger, and J. M. de la Fuente, *Biomaterials* 34, 7744 (**2013**).

212. S. Dhar, N. Kolishetti, S. J. Lippard, and O. C. Farokhzad, *Proc. Natl. Acad. Sci.* 108, 1850 (**2011**).

213. M. Benezra, O. Penate-Medina, P. B. Zanzonico, D. Schaer, H. Ow, A. Burns, E. DeStanchina, V. Longo, E. Herz, S. Iyer, J. Wolchok, S. M. Larson, U. Wiesner, and M. S. Bradbury, *J. Clin. Invest.* 121, 2768 (**2011**).

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