# Ultrasonic Drug Delivery Using Micelles and Liposomes

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Abstract

The encapsulation of drugs in nanocarriers revolutionized research in drug delivery, especially in cancer chemotherapeutics. Several nanosystems have been developed including liposomes, polymeric micelles, dendrimers, solid lipid nanoparticles, and others.

The surface of nanocarriers can be modified to alter their characteristics and improve their efficiency as drug delivery systems. The addition of polyethylene

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glycol chains, for example, increases the blood circulation time of nanocapsules and, in some cases, improves their stability.

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Once the structure of nanocarriers is optimized, the next logical step is to explore the feasibility of using one or several trigger mechanisms to release their therapeutic contents at the required time and space. Abundant literature is available on both internal and external trigger mechanisms in cancer drug delivery. Internal mechanisms include changes in pH, enzyme concentration, and temperature, while external mechanisms include light, magnetic/electromagnetic waves, and acoustic power.

This review focuses on the utility of ultrasound and polymeric micelles in cancer drug delivery. The idea is to control the release of chemotherapeutics from micelles to cancerous cells by focusing the ultrasound waves on the diseased tissue while sparing other healthy cells in the body. Thus, the side effects of conventional chemotherapy can be minimized.

Keywords

Drug delivery systems (DDS) • Pluronic® • Polymeric micelles • Triggered release • Ultrasound (US)

Abbreviations

| | |
|---|---|
| AC | Alternating current |
| CMC | Critical micellar concentration |
| CW | Continuous wave US |
| DDS | Drug delivery system(s) |
| Dox | Doxorubicin |
| EPR | Enhanced permeability and retention |
| FA | Folic acid |
| FA-CLC/SPIO | Mixed micelles of folic acid-conjugated carboxymethyl lauryl chitosan and superparamagnetic iron oxide |
| HEMA | Hydroxyethyl methacrylate |
| ICAM-1 | Intercellular adhesion molecule |
| IPN | Interpenetrating network |
| LFA-1 | Leukocyte function-associated antigen |
| mAb | Monoclonal antibody |
| MDR | Multidrug resistance |
| MI | Mechanical index |
| PEG | Polyethylene glycol |
| PEG-b-PBLA | Polyethylene glycol-co-poly(beta-benzyl-L-aspartate) |
| PEG-PCL | Polyethylene glycol)-b-poly(caprolactone) |

| | |
|---|---|
| PEG-PE | Polyethylene glycol-phosphatidylethanolamine |
| PEG-PLLA | Polyethylene glycol-b-poly(L-lactide) |
| PEO | Poly(ethylene oxide) |
| PEO-b-PTHPMA | Copolymer of poly(ethylene oxide) and poly (2-tetrahydropyranyl methacrylate) |
| $PFC_5$ | Perfluoropentane |
| PLA | Poly(lactic acid) |
| PLA-b-PEG | Copolymer of poly(lactic acid) and poly(ethylene) glycol |
| PPO | Poly(propylene oxide) |
| PTHPMA | Poly(2-tetrahydropyranyl methacrylate) |
| RES | Reticuloendothelial system |
| SLN | Solid lipid nanoparticles |
| US | Ultrasound |

## Introduction

Cancer is a leading cause of death in the western world [1]. Chemotherapy, a term first coined by the German physician and scientist Paul Ehrlich more than 100 years ago [2], is widely used as a cancer treatment, alongside surgery and radiotherapy. Several chemical compounds have been and are currently being researched as possible chemotherapeutic agents, but, in general, although efficient, they are not selective, killing both cancer cells and healthy cells. This leads to the development of serious side effects in patients undergoing treatment, such as hair loss, weight loss, nausea, fatigue, and decreased immunity [1]. Hence, there has been intense research in the area of drug delivery systems (DDS), engineered systems for the controlled spatial and/or temporal release of therapeutic drugs.

Nanoparticles are an essential part of a DDS. They are particles made of a defined stable material, which must be compatible with the human body, and their role is to carry the chemotherapeutic agent until this reaches the tumor site and then being able to release it. This way, there is a lower interaction of the drug with healthy cells, and also there is a decrease in the dose needed for the treatment than when using the free drug, which is economically desirable. There are several types of nanocarriers, namely, liposomes, micelles, dendrimers, nanotubes, solid lipid nanoparticles, nanoshells, quantum dots, and others [3].

Multifunctional nanoparticles can be engineered to target a certain site, usually based on specific characteristics of the cancer cells and, once there, an internal or external stimulus can be applied to trigger the release of the drug. Examples of internal stimuli are a change in pH, temperature, or enzyme activity, while external triggers include light, magnetic fields, heat, and ultrasound (US).

Ultrasound has been widely used in medicine for several decades due to its noninvasive nature. Its application in diagnostic imaging is well known [4]. Only recently, however, US has been tested as a trigger in DDS.

This chapter describes the recent advances on the use of polymeric micelles to encapsulate chemotherapeutic agents and the use of US as a trigger to promote the leakage of the drug from these nanocarriers.

## Drug Delivery Systems

### Diversity of Nanocarriers

There are several types of nanocarriers (Fig. 1 and Table 1). This paper focuses on polymeric micelles, but a brief description of other nanocarriers will be presented below.

One of the most widely researched nanoparticles, already in use in the clinical setting, are liposomes [5, 6]. Liposomes were discovered in the 1960s by Alec Bangham when conducting experiments with phospholipids. Liposomes are spherical structures with a size in the range of 20 nm to 1 μm comprised of a phospholipid bilayer membrane, similar to the cell membrane, surrounding an internal aqueous core. Due to the zwitterionic nature of the phospholipids, liposomes can be used to carry both hydrophilic and hydrophobic molecules, the first in their aqueous core, the latter in their hydrophobic membrane.

Micelles are smaller than liposomes with sizes ranging between 10 and 100 nm in diameter [7], with polymeric micelles having an average diameter of 20 nm [8]. Micelles are colloidal dispersions, consisting of amphiphilic surfactant molecules that aggregate spontaneously when in water, forming a spherical structure with a hydrophobic core that can encapsulate hydrophobic drugs [9]. Polymeric micelles will be further discussed in section "Triggered Release."

Dendrimers are small (1–20 nm diameter) synthetic, branched macromolecules, with a central core (ethylenediamine or ammonia) surrounded by multiple layers with active terminal surface groups [10]. The multiple hydrophobic or hydrophilic cavities that are formed in the interior of the dendrimer tridimensional structure are well suited for the encapsulation of molecules.

Solid lipid nanoparticles (SLN) are colloidal dispersions composed of solid lipids, such as waxes, fatty acids, and glycerides, stabilized by surfactants. In SLN, the drug can be entrapped inside the lipid matrix, encapsulated, or adsorbed to the surface of the particles [11].

Polymer-drug conjugates are drug delivery nanosystems composed of several drug molecules covalently attached to a polymer. Polymers used in this type of DDS include PEG, PGA, HPMA, and the drugs attached include Dox, camptothecin, docetaxel, and paclitaxel [12].

Other nanoparticles include quantum dots, niosomes, and carbon nanotubes [3, 13].

With such a diversity of nanocarriers, a pertinent question is: which one of these have been approved for clinical use? Recent review papers described which DDS are currently used in the clinical setting, as well as the ones undergoing clinical trials [14–17] (Table 2). In 2013, it was reported that 4,520 nanomedicines were undergoing clinical trials for cancer treatment [3].

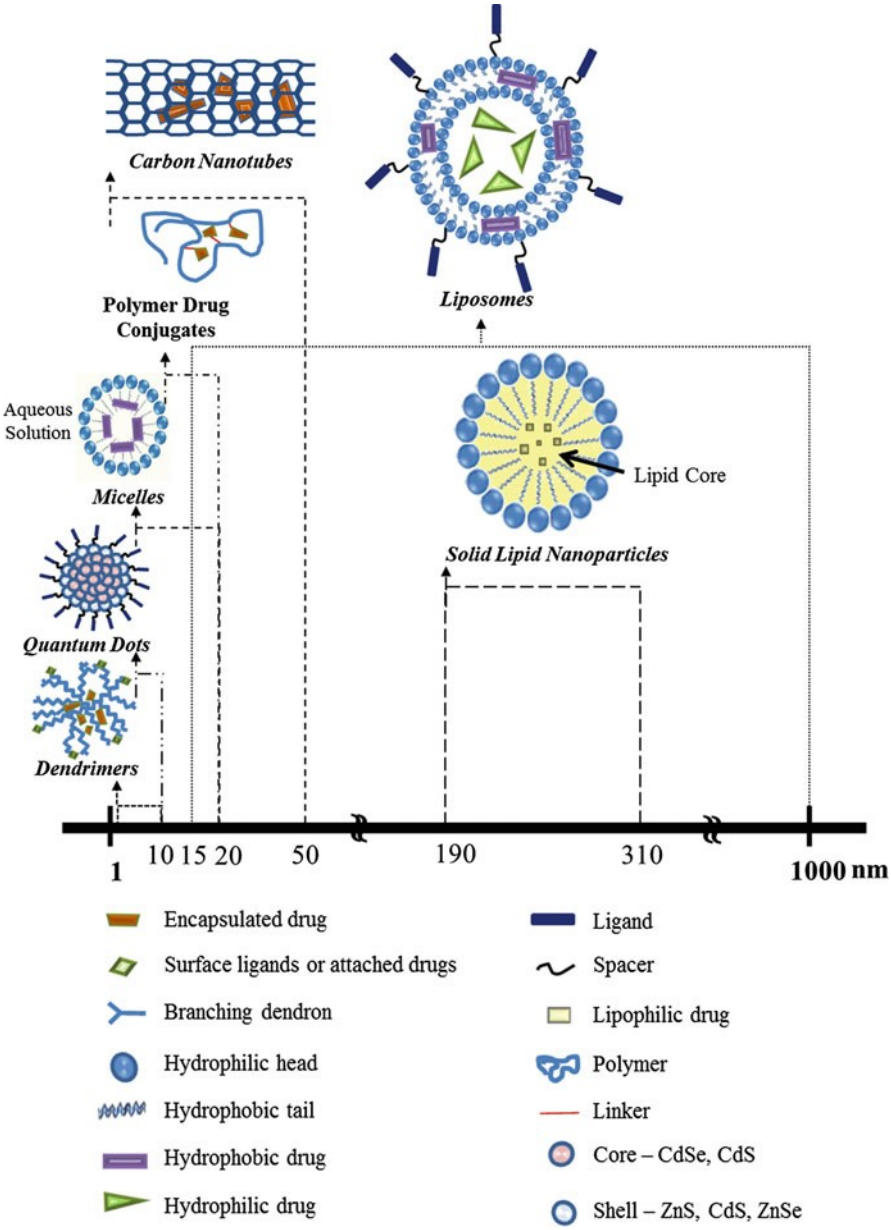



Fig. 1 Most used nanoparticles and their size ranges Table 1 Types of nanocarriers used in drug delivery systems [14, 17]

| Nanocarrier | Types | Composition | Examples |
|---|---|---|---|
| Drug conjugates | Polymer-drug conjugate | Linear polymers, drug | PGA, HPMA |
| | Antibody-drug conjugate | Targeted antibodies, drug | Gemtuzumab, brentuximab |

| | | | |
|---|---|---|---|
| | Polymer-protein conjugate | Polymer, anticancer protein | SMANCS |
| Lipid-based | Liposome | Phospholipids, cholesterol, others | DPPC, DOPE, DSPC, DPPE, DSPG |
| | Solid lipid nanoparticle | Lipids with low melting points | |
| Polymerbased | Dendrimers | Hyperbranched synthetic polymers | PAMAM |
| | Polymeric micelles | Amphiphilic block copolymers | Pluronic, PAA, PLA, PEG |
| | Polymeric nanoparticle | Glycan, cyclodextrin, Albumin | |
| | Protein nanoparticle | | |
| Inorganic | Silica nanoparticle | Mesoporous silica | MCM-41, SBA-15 |
| | Metal nanoparticle | Gold, iron oxide | |
| Viral-based | | Self-assembled protein cages | CPMV |

CPMV cowpea mosaic virus, DPPC 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DOPE 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, DSPC 1,2-distearoyl-sn-glycero-3-phosphatidylcholine, DSPE 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol)2000, DSPG 1,2-distearoyl-sn-glycero-3-phosphatidylglycerol, HPMA N-(2-hydroxypropyl)methacrylamide, PAA poly-(L-aspartate), PAMAM poly(amidoamine), PEG polyethylene glycol, PGA poly-(L-glutamate), PLA poly-(L-lactide), SMANCS styrene maleic anhydride neocarzinostatin

## The EPR Effect

Chemotherapeutic drugs preferentially accumulate at the tumor location due to several physiological characteristics of tumors, which makes them distinguishable from normal tissues, such as pH, capillary structure, enzyme concentration, and others. The preferential extravasation and accumulation of macromolecules and nanoparticles at the tumor site is called the enhanced permeability and retention (EPR) effect, a phenomenon which was originally described by the group of Maeda in 1986 [18] and which has been extensively reviewed by this research group [19–21]. The EPR effect has been widely described in all types of cancer, excluding the hypovascular ones, such as prostate and pancreatic cancers [21]. However, a recent review of clinical trials showed that the efficiency of the encapsulated drug is only marginally higher than that of the free drug, a result which contrasts with that obtained in in vivo studies using animal models [22]. Apparently, the EPR effect depends on the tumor type, and in some tumors with irregular vascularity, it may be prevented due to low blood flow and high tumor interstitial fluid pressure. A very important factor for the EPR effect is the tumor's hyper-permeable or leaky vasculature [20]. Due to a very fast angiogenesis, the capillary vessels of the tumors show a deficient structure; hence, it is easier for macromolecules and nanocarriers to extravasate into the tumor site than into a healthy tissue. Additionally, tumors

have deficient lymphatic drainage systems, which allows for the extended retention of large molecules [20]. The EPR effect may also be enhanced due to the generation of factors that increase the permeability at the tumor site, such as prostaglandins, enzymes such as matrix metalloproteinases, vascular endothelial growth factor, nitric oxide, and others [18].

The so-called first-generation nanomedicine drugs are based on the EPR effect, also termed passive targeting. In contrast, active targeting involves the modification of the nanocarrier surface with a ligand, which selectively binds a receptor overexpressed in the membrane of the cancer cells. This will be further discussed in the following section.

## Passive Versus Active Targeting

There are two broad types of targeting techniques, namely, passive and active [3, 5]. In passive targeting, the nanocarriers preferentially accumulate at the tumor site due to the EPR effect. In active targeting, besides the aid of the EPR effect, the nanocarrier has a feature that allows it to specifically target and interact with the cancer cells. Usually, the nanocarrier possesses a targeting moiety, such as a ligand, which is conjugated on its surface and is free to interact with a cell surface receptor overexpressed on the cancer cells [23]. The type of chemical modification of the nanocarrier surface depends on the type of receptor present on the surface of the tumor cells. Hence, active targeting can be generally defined as the use of targeting moieties or ligands to enhance the delivery of nanoparticles to the target tumor site, thus significantly increasing the therapeutic effects while decreasing the undesired side effects [23].

Several targeting moieties have been used in studies of targeting strategies, such as peptides, oligosaccharides, antibodies, aptamers, and low molecular weight molecules, the most widely used being folic acid [24]. The choice of the targeting moiety is also crucial since it affects the circulation time of the modified nanocarrier, its extravasation at the cancer site, its affinity for the cell receptor or antigen, and its cellular uptake [25]. Some examples will be discussed next.

Peptides are commonly used as targeting moieties in DDS. These small chains of amino acids are usually derived from sequences of proteins that bind to receptors in the cell membrane, such as intercellular adhesion molecule (ICAM-1), bombesin, leukocyte function-associated antigen (LFA-1), and others. Peptides have been investigated as targeting ligands in multifunctional nanoparticles used in triggered drug delivery for cancer therapy and imaging strategies [26].

Antibodies are widely used as targeting moieties due to their variety and specificity for receptors on the surface of cancer cells. Both human and nonhuman antibodies have been used in DDS research, but since the nonhuman may induce an immunogenic response, new methods are being investigated for the development of chimeric, fragmented, and humanized antibodies [27]. Monoclonal antibodies (mAb) are identical (clones) and monospecific and can be used in targeted cancer therapy, after the identification of which antigens are expressed on the surface of

tumor cells [28]. There are several reports describing the use of monoclonal antibodies for targeted drug delivery [29–31].

Small molecular weight molecules have also been described as modifiers of nanocarriers used for targeted drug delivery. Carbohydrates such as galactose and mannose have been described [32], but folate is the most important one in this category since its receptor is overexpressed in many cancer cells due to the increased biosynthesis of nucleotide bases in these fast-dividing cells [33]. Folic acid, vitamin $B_9$, easily recognizes the folate receptor on the surface of several cancer cells in ovarian, brain, kidney, breast, lung, and other types of cancer [32, 34]. The inclusion of this molecule in DDS has been used in imaging [35] and therapeutic processes [32].

Aptamers, small molecules of single-stranded nucleic acids that can specifically bind proteins and peptides, have also been researched as targeting moieties in DDS [32, 36]. Their high specificity is due to the fact that they can fold into unique threedimensional structures and target proteins including transcription factors and cell membrane receptors. They offer some advantages over other ligands, e.g., their high stability and low immunogenicity [36].

Other ligands have been described and were reviewed elsewhere [29–31].

In both passive and active targeting, it is essential that the nanocarrier remains in circulation long enough, which allows its accumulation at the desired location before release occurs.

## Triggered Release

The aim of a good DDS is to deliver the encapsulated drug to the tumor site and release it there, thus avoiding healthy tissues and minimizing the side effects of the drug. The release of the drug from nanoparticles that reached the tumor site either by passive or active targeting can be controlled by triggers or stimuli, a process known as triggered release [37]. In this case, the nanocarriers have to be responsive to an external stimulus or to sense changes in the environment (internal stimulus) and release the drug load [38]. External triggers include hyperthermia, magnetic and electric fields, US and light, while changes in the pH of the environment, enzyme concentration, and/or redox potential are examples of internal triggers [6, 37–39].

The choice of the trigger depends on the type of nanocarrier used, the encapsulated drug, and the tumor environment. For example, it was observed that drug release from pH-sensitive micelles targeted with folic acid (named PHSM/f) was much higher at acidic pH (5.0) than at neutral pH [40]. Also, micelles can be synthesized with disulfide bonds that are sensitive to reduction by the antioxidant intracellular tripeptide glutathione, which concentrations are significantly different in healthy and tumor cells [38]. Although these are examples of internal triggers, micelles can also be designed to be sensitive to external ones, such as hyperthermia, US, magnetic and electrical fields, light, and others.

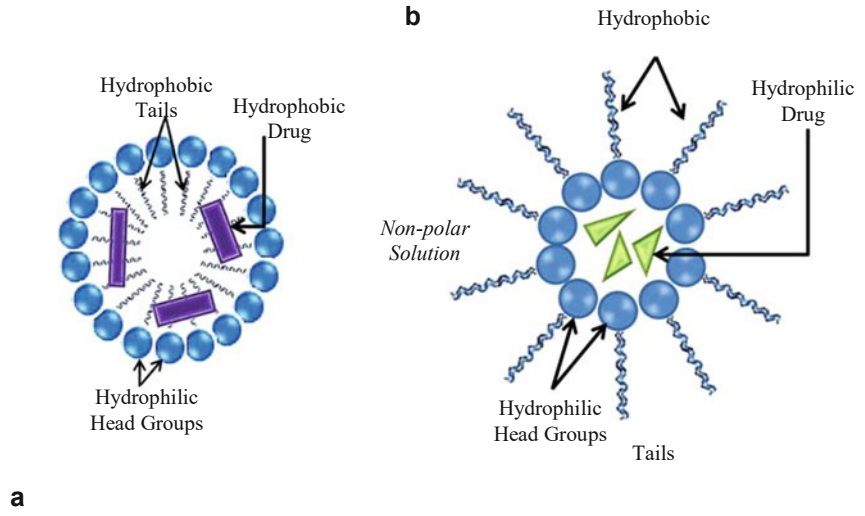
Ultrasound, the focus of this chapter, is considered one of the best trigger mechanisms in triggered drug delivery [6, 37], and it will be discussed in section "Ultrasound-Triggered Drug Release from Micelles."

## Micelles

### Polymeric Micelles

Micelles are colloidal nanocarriers made of amphiphilic molecules that spontaneously self-assemble and form a spherical monolayer structure of 10–100 nm in diameter when dissolved in water [7]. Amphiphiles are surface-active molecules that consist of a hydrophilic tail and a hydrophobic head [31]. Hence, the structure of a micelle consists of an external hydrophilic corona and a hydrophobic core. Micelles can transport hydrophobic drugs and imaging agents in their hydrophobic cores [9], and recently, the preparation of core-inversible micelles that can sequester hydrophilic molecules in a hydrophilic core was also described [41] (Fig. 2). Additionally, polymeric micelles can transport drugs attached to the hydrophilic polymer of their corona [5]. When discussing micelles, a very important parameter to take into consideration is the critical micellar concentration (CMC), the concentration above which micelles assemble and form in water [29].

Micelles are not used widely in clinics yet (Table 2) but have several advantages over other nanocarriers: (i) they are biocompatible; (ii) they are easy to prepare and load with the drug; (iii) their small size allows their deep penetration into tissues



*Solution*

Fig. 2 Schematic of the structure of polymeric micelles. (a) Normal micelles in a polar solvent; (b) reverse micelles in a nonpolar solvent

Table 2 Different types of drug delivery systems that have been clinically approved [14, 17]

| Drug delivery system | Nanoparticle/ polymer/protein/ antibody | Drug/protein | Cancer indication | Commercial name (approval date) |
|---|---|---|---|---|
| Antibodydrug conjugate | Brentuximab vedotin | MMAE | Non-Hodgkin lymphoma | Adcetris® (2011) |
| | Trastuzumab emtansine | DM1 | Breast cancer | Kadcyla® (2013) |
| Liposomes | PEGylated liposomes | Doxorubicin | Kaposi's sarcoma, ovarian, breast | Doxil®/Caelyx® (1995) |
| | | Doxorubicin | Kaposi's sarcoma, ovarian, breast | Lipo-Dox® (1998, Taiwan) |
| | Non-PEGylated liposomes | Cytosine arabinoside | Neoplastic meningitis | DepoCyt® (1999) |
| | | Daunorubicin | Kaposi's sarcoma | DaunoXome® (1996) |
| | | Doxorubicin | Breast | Myocet® (2000, Europe) |
| Polymerprotein conjugate | PEG | L-asparaginase | Leukemia | Oncaspar® (2006) |
| | Styrene maleic anhydride | Neocarzinostatin | Liver, renal | Zinostatin stimalamer (1994, Japan) |
| Polymeric micelles | PEG-PLA | Paclitaxel | Breast, lung, ovarian | Genexol-PM® (2007, South Korea) |
| Proteindrug conjugate | Albumin | Paclitaxel | Breast, pancreatic, nonsmall-cell lung | Abraxane® (2005) |

DM1 maytansine derivative, MMAE Monomethyl auristatin E, PEG polyethylene glycol, PLA poly-(L-lactide)

and organs and increases the blood circulation time, since they escape renal excretion; (iv) the release of drugs from their core can be controlled; (v) they are stable in biological fluids; and (vi) they can be successfully used for drug solubilization [5, 8, 37, 42].

Polymeric micelles are formed from polymers with hydrophilic and hydrophobic monomers. Poly(ethylene oxide) (PEO) is widely used as the hydrophilic building block, while the hydrophobic monomers may be poly(propylene oxide) (PPO), poly (lactic acid) (PLA), or others [8, 9, 43]. The hydrophobic drug accumulates in the hydrophobic core of the micelle, while the hydrophilic PEO chains extend into the aqueous environments, stabilizing the micelles [37]. It has been observed that the pharmacokinetics and biodistribution of pharmaceuticals are enhanced when these are incorporated into polymeric micelles when compared to the free formulation [44].

Polymeric micelles of Pluronic® copolymer are widely used in DDS, especially in US-induced drug release. They are composed of triblock copolymers of PPO and PEO and have a hydrodynamic radius ranging between 5 and 20 nm at 37 C, which allows their extravasation into the tumor [9, 37]. Besides the previously mentioned advantages of micelles in general, Pluronic® has several others [5, 9, 37]: (i) the ability to sensitize multidrug-resistant (MDR) cancer cells when used at low concentrations [45]; (ii) its high loading capacity; (iii) its increased shelf life when lyophilized; (iv) the low viscosity of its micellar solutions; (v) the fact that it can be sterilized by microfiltration; (vi) the ability to make it sensitive to a certain release trigger, as previously mentioned in section "Triggered Release"; (vii) its low in vivo toxicity [45]; and (viii) its enhanced structural stability and lower CMC when compared to micelles composed of low molecular weight surfactants. The physical and biological properties of Pluronic® compounds have been reviewed by Batrakova and coworkers [44, 45].

The most used type of Pluronic® micelles in research is Pluronic® P105 [8, 9, 29], composed of PEO and PPO blocks with the formula $PEO_{37}$-$PPO_{56}$-$PEO_{57}$ [43]. P105 may exist as unimers, loose aggregates or dense micelles, depending on the solution concentration. These micelles have a CMC of ~1 wt.% at room temperature [46], but dense micelles that can encapsulate hydrophobic drugs form at concentrations 4 wt.% and above [47].

Polymeric micelles have several advantages as DDS, but their use in vivo is still challengingduetotheirrecognitionandeliminationbytheimmunesystemandalsodue to stability problems when the micellar solution is diluted in the bloodstream [5, 37].

While circulating in the bloodstream, micelles adsorb proteins onto their surface and they are subsequently recognized by the reticuloendothelial system (RES) and cleared by phagocytosis, endocytosis, and/or other biological mechanisms. This can be prevented by covering the surface of the micelles with PEO that inhibits adsorption and opsonization [9]. Indeed, the hydrophilic PEO chains extend into the aqueous environment and associate with water molecules, leading to a steric repulsion of proteins, preventing their adsorption and further recognition of these stealth micelles by the RES [9]. This increases the circulation time of the micelles and enhances their accumulation at the tumor site by passive or active targeting [48].

Pluronic® micelles have the advantage of having PEO as an integral part of their structure, forming their coronas. The PEO corona, however, may pose problems for the micelles to interact with and enter the cells, where they would release their drug load. The use of sheddable polymers that allow the unmasking of the particles upon arrival at the target site has been studied. Further details on sheddable polymers and shedding techniques can be found on a review by Romberg et al. [49].

Another problem that arises when using polymeric micelles in vivo is their micellar stability. When injected into the bloodstream, the micellar solution may be diluted below the CMC, leading to their quick dissolution and the early release of the encapsulated drug [50]. One way to avoid this is to use higher concentrations of the polymer, but these may be toxic for the human body [43]. Research into this subject led to the design and synthesis of stabilized, cross-linked micellar formulations [51–55]. One of such formulations, named NanoDeliv™, was synthesized from P105 by creating an interpenetrating network (IPN) of the temperatureresponsive N,N-diethylacrylamide polymer inside the hydrophobic core of the micelles [51]. NanoDeliv™ micelles are more stable upon dilution [51] and have a half-life of approximately 17 h [56]. The IPN expands at room temperature, allowing the accumulation of the drug in the core of the micelles but contracts when the temperature is higher than 31 C, thus trapping the drug. Hence, when the micellar solution is injected into the body at 37 C, it is diluted, but the IPN prevents its dissolution and keeps the drug entrapped in the structure [51]. Although larger than the non-stabilized P105, the NanoDeliv™ are still small enough (~60 nm) to extravasate at cancer capillaries [8]. The group of Yang and coworkers [53] created a different type of stabilized micelles by the formation of cross-links in their outer shells. The size of these micelles was still small enough (100 nm), but they were significantly more stable than the non-stabilized ones.

Another approach to increase the micellar stability involves the optimization of the mass ratio of the hydrophilic and hydrophobic blocks that constitute the micelles [52]. Zeng and Pitt [54, 55] synthesized micelles with time-controlled degradation using PEO, N-isopropyl acrylamide (NIPAAm), and the polylactate ester of hydroxyethyl methacrylate (HEMA-lactate). The systemic circulation lifetime of this formulation could be controlled by changing the ratio of the copolymer concentration, and this DDS was shown to release the encapsulated drugs upon ultrasonication at 70 kHz.

## Micellar Modifications for Active Targeting

The differences between passive and active targeting were mentioned in section "Passive Versus Active Targeting," and here, we shall discuss active targeting as a way to enhance the efficiency of polymeric micelles as DDS.

When using micelles as a DDS, several factors can be studied and modified to enhance their biodistribution and uptake, and these include the micellar composition, the drug encapsulated, and the tumor location [42]. The drug carrier, in this case, the micelle, is one of the most important components of the DDS, which

can be modified to improve drug delivery. When used for passive targeting, which is based on the nanoparticle size, the formulation parameters are carefully selected in order to obtain micelles with an enhanced circulation half-life (previously mentioned) and that can easily extravasate at the tumor site. These stealth micelles are water soluble, have higher molecular weights than regular micelles, and higher structural stability [42]. The solubility of the drugs inside the micellar core, which also depends on the charge of the hydrophilic copolymers, can be enhanced by the addition of anioinic, nonionic, or zwitterionic surfactants [39].

In addition to passive targeting, polymeric micelles can be modified with ligand moieties, which can be attached to the hydrophobic blocks that form the micellar corona, to enhance active targeting [57].

One option to targeted delivery using micelles is to design immunomicelles, which consist of micelles with an antibody or antibody fragment (e.g., the Fab fragment) as a targeting moiety [28]. One of the first studies on the use of antibody-targeted micelles was done by Kabanov et al. [58], who reported the modification of Pluronic® P85 micelles with brain-specific polyclonal antibodies, as a way to enhance the delivery of the drug haloperidol to mice brain. Monoclonal antibodies (mAb), derived from the IgG isotype antibody, have also been widely used for targeted cancer therapy [28]. Micelle-mAb conjugates are diblock copolymeric micelles with antibodies or antibody fragments (Fab, antigen-binding fragment) chemically attached to their surface. The first micelle-mAb to be synthesized used polyethylene glycol-phosphatidylethanolamine (PEG-PE) conjugated to the 2C5 mAb [28]. This antibody is reactive toward a wide variety of cancer cells, unlike the majority of anticancer mAb. The results showed that the antibodies conjugated to the micelles retained their activity and specificity, binding to several different cancer cells in vitro. In vivo experiments revealed an increased accumulation of these immunomicelles at the tumor site when compared to non-targeted micelles.

Polymeric micelles conjugated with folic acid have also been researched as targeted nanocarriers. Husseini et al. [59] synthesized Dox-encapsulating Pluronic® P105 micelles with a folate moiety and studied the Dox release triggered by 70-kHz LFUS at several power densities.

Presently, micelle-based DDS consisting of multifunctional nanocarriers are the most promising for anticancer therapies [29]. Drug delivery systems based on these multifunctional nanocarriers combine targeted (sections "Passive Versus Active Targeting" and "Micellar Modifications for Active Targeting") and targeted and triggered delivery (section "Triggered Release"). The combination of ligand targeting of cancer cells with the use of an internal or external stimulus to trigger the drug release from the nanocarrier ensures the highest specificity toward cancer cells with increased cytotoxicity and antitumor activity [60]. The use of stimuli-sensitive micelles in combination with a trigger for the drug release, in particular US, has been extensively studied by Husseini and coworkers [8, 9, 37, 43] and will be discussed in the following sections.

## Ultrasound-Triggered Drug Release from Micelles

When using a DDS in anticancer therapy, it is necessary to optimize the rate of drug release from the nanocarrier in order to obtain the maximum efficiency and decrease the deleterious side effects that chemotherapy has on healthy cells. This is easily understandable: if the drug is released early, it may affect healthy tissues while preventing the drug to reach the intended tumor target, while if the release is too slow, the drug will not reach the necessary concentration to exert its therapeutic effect. The use of carefully designed nanocarriers sensitive to a certain stimulus or stimuli allows the time- and space-controlled release of the drug from the nanocarrier, the previously mentioned triggered release process. Several external triggers can be used for this purpose (see section "Triggered Release"), but here, we will focus on the use of US to trigger the release of the drug encapsulated in polymeric micelles.

### Brief Introduction to the Physics of Ultrasound

Ultrasound has been widely studied as a trigger mechanism in DDS, due to the fact that it is safe, it is already being used for medical purposes, and it has a low cost. Ultrasound in medicine is mainly known as an imaging (diagnosis) technique without adverse side effects, although it may also be used for therapeutic purposes, e.g., physical therapy [61]. The physics of US has been recently reviewed [6]; here, a brief description will be presented.

#### What Is Ultrasound?

An ultrasound wave is a pressure wave (sound wave) that propagates through a medium and has frequencies of 20 kHz or higher, above the normal human hearing range (40 Hz to 20 kHz) [50, 62]. Just like any sound wave, US waves are sinusoidal and propagate by means of energy transfer between the molecules that constitute the medium, since they consist of cycles of alternating pressures: high pressure – compression – and low pressure – refraction [62].

Acoustic waves possess all the properties of a wave, i.e., attenuation (caused by dispersion due to energy losses while the wave propagates), reflection, refraction, amplification, absorption, and scattering [6]. Ultrasound waves, however, also have the capability of propagation on the surface of matter without traveling through it [63, 64].

Ultrasound waves propagate in a medium, namely, the fluid medium, by a series of compression and rarefaction states, and this propagation depends on the density of the material. Since it is a process where energy is transferred from molecules to molecules, the propagation is faster in solid medium than in liquid medium and is slower in gases [61]. In this process, the particles do not move, they just oscillate in place, while the energy is transferred, thus propagating the pressure wave. The physical nature of the ultrasonic waves explains how they can interact with cells and tissues, being able to shear open cells and nanoparticles. Usually, however, these physical forces are not able to cause these effects by themselves but only in the

presence of gas bubbles [65, 66], as will be discussed in section "Mechanisms of US-Induced Micellar Drug Release and Cellular Uptake."

### What Are the Parameters of Ultrasound?

Since acoustic waves are sinusoidal waves, they have the same important parameters that define any sinusoid. The high-pressure phases coincide with the upper peaks of the sinusoid, while the lower pressures coincide with the lower peaks. It is then possible to calculate wavelength (λ), frequency (1/T), amplitude of the wave, and speed (λ/T) of the wave [4].

The frequency, defined as the number of sinusoidal cycles per second (Hertz, Hz), is one of the most important parameters that defines the application of the US [67]. Low-frequency US (LFUS) refers to frequencies lower than 1 MHz, mediumfrequency US usually ranges from 1 to 5 MHz, while high-frequency (HFUS) is greater than 5 MHz [43]. Higher frequencies (1 MHz) have lower penetration depth and lower wavelengths, and they are used in medical diagnostic imaging [4].

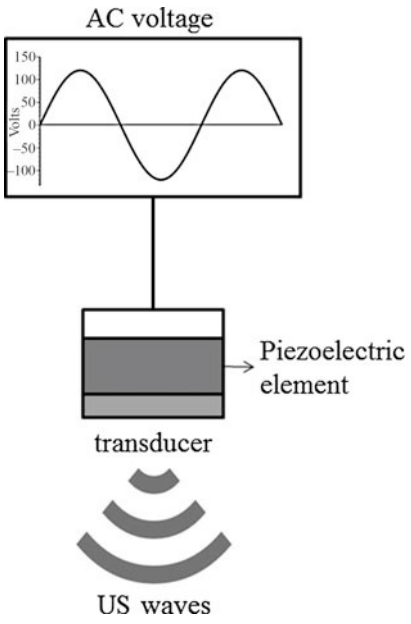



Fig. 3 Generation of ultrasound waves from an alternating current (AC) source

The power density, commonly referred to as US intensity, is defined as the power carried per cross-sectional area of the US beam (W/cm$^2$) [50]. High intensities cause hyperthermia and are used for tissue ablation as an example. On the other hand, low intensities do not cause hyperthermia and are used for imaging purposes [4, 61].

The mode of operation is also a very important US parameter: in continuous mode (continuous wave, CW), the generated US wave is applied continuously for a determined period of time, while in pulsed mode, the wave is generated in a cycle of on and off periods that usually last a few seconds [68].

Although frequency, power density, and mode of operation are the main controllable parameters of US with a crucial importance in triggered drug delivery

[69], it is also very important to consider attenuation that occurs while the wave passes through a medium. Attenuation is the intensity loss of the traveling wave, which occurs due to absorption and reflection phenomena. Attenuation depends on the frequency of US, decreasing as frequency decreases; hence, LFUS can penetrate tissues more deeply than HFUS [70]. Additionally, attenuation also depends on the medium through which the wave propagates: attenuation is very low in water and ultrasonic gel, but it is high in muscle and bone tissue [6]. This parameter is very important to consider when choosing the frequency of US to be used in different medical applications.

### How Is Ultrasound Generated?

Ultrasound waves can be generated by an oscillating piezoelectric crystal, a transducer of the alternating current (AC) produced by an actuator [67] (Fig. 3). The transducer basically translates the applied voltage waveform into linear motion of the transducer's face, producing the pressure waves that are transmitted into fluid or tissue through a medium or gel contacting the transducer.

## Mechanisms of US-Induced Micellar Drug Release and Cellular Uptake

### Interaction of US with Biological Systems

The interaction of US with biological systems is classified as thermal effects or nonthermal effects. Thermal effects are associated with the absorption of acoustic energy by body tissues and fluids, leading to hyperthermia [65]. Hyperthermia is very important in anticancer therapy, being used by itself, to heat the tumor, or as an adjuvant in DDS, to heat the tissues and/or as a trigger for drug release from temperature-sensitive nanocarriers [6, 50].

Nonthermal effects, which are usually exploited for triggered drug delivery, usually refer to cavitation, the formation,and oscillation ofgas bubbles in theacoustic field in response to the oscillating pressure referred to previously [37]. Cavitation depends on the parameters of the US wave and only occurs after a certain threshold is achieved when the resonant frequency of the oscillating bubbles approaches the frequency of the ultrasonic field [37]. At low-pressure US amplitudes, stable (or non-inertial) cavitation occurs, during which the gas bubbles oscillate, slightly expanding and contracting [37, 71]. During stable cavitation, the size of the bubbles increasesandmicrostreaming–circulatingfluidflow aroundthebubbles– alsooccurs [72, 73]. This phenomenon has been described as enhancing drug delivery [74] and, if the pressureishighenough, itmayshearopencellsand nanoparticles(Fig. 4)[72, 75]. When the size of the bubbles approaches their resonant size and/or when the acoustic pressure increases, the oscillations become unstable and eventually lead to the collapse of the bubbles, generating extremely high pressures and temperatures and free radicals, a process known as collapse (or inertial) cavitation [37, 71, 74, 76, 77].

Themechanicalindex(MI)isanotherfrequentlyusedparameterrelatedtoultrasound, and it is a measure of the probability of collapse cavitation occurring [78].

When the bubbles collapse near a solid surface, a directional sonic jet of liquid is produced [37, 76]. These events, if occurring near cells, will damage or even destroy them. Following the collapse, new, smaller bubbles form, and these may serve as cavitation nuclei, reinitiating the process [76]. In summary, both inertial and collapse cavitation may shear open both nanoparticles, such as micelles and liposomes, and cells, thus enhancing the drug delivery process by allowing the drug release and possibly allowing the direct entry of the drug into the cell cytosol [66, 75]. Several US-related parameters must be carefully controlled for cavitation and drug release to occur, such as the frequency, power density, duration of sonication, and position of the transducer [5].

## Mechanisms of US-Triggered Drug Release

The knowledge of the properties of US, as well as the ways by which it interacts with matter, leads to the proposal of several mechanisms for US-triggered drug

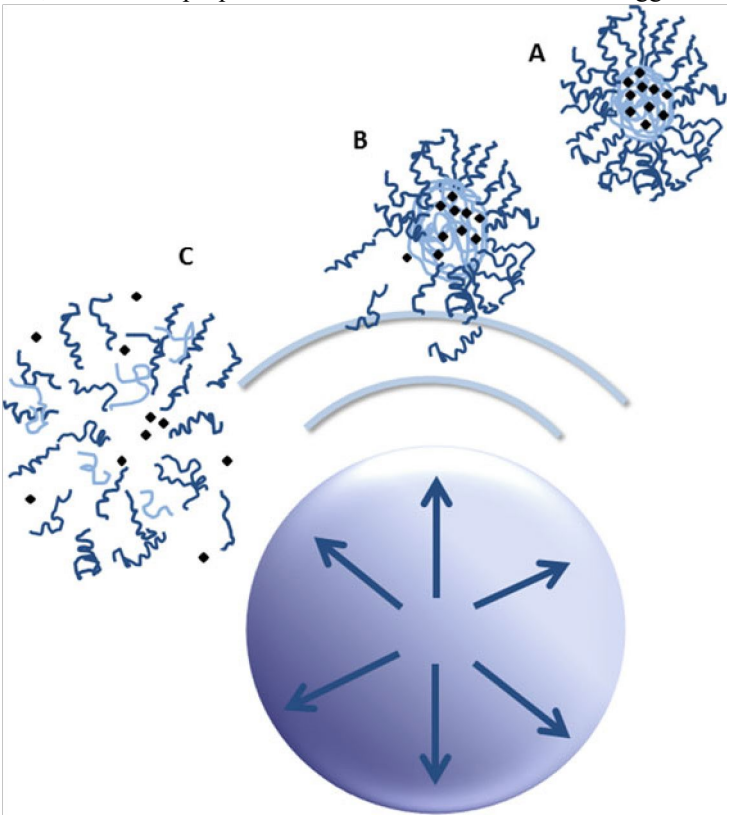



Fig. 4 Drug release from polymeric micelles triggered by ultrasound. (a) Intact micelle; (b) start of structural collapse of the micelle and initial release of the encapsulated agent; (c) complete collapse of the micelle and agent release due to the ultrasonic shockwave

delivery. The objective is to find a combination of acoustic parameters leading to a cavitational level and all associated effects that allow the delivery of the drug without killing the cells [50].

Several mechanisms have been proposed for US-triggered drug delivery, including drug release and cell drug uptake. These mechanisms are still being researched but seem to include [37, 50]: (i) disruption of the drug nanocarriers; (ii) enhanced drug transport and distribution in target tissues; (iii) enhancement of endocytosis and pinocytosis events, which increase drug uptake by the cells; and (iv) permeabilization of the cell membrane, which facilitates the transport of free and micelle-encapsulated drugs. Each of these mechanisms will be further discussed below.

### Disruption of the Drug Nanocarriers

For the drug to be released from the nanocarriers, they have to be disrupted. The mechanical effects caused by US lead to the disruption of the drug carriers by shear stresses and/or extreme stresses [79]. In the first case, when the shear stress caused by acoustic pressure and velocity gradients exceeds the cohesive forces of the nanocarrier, this will rupture and release the encapsulated drugs. On the other hand, the shock waves, microjets, extreme pressures, and temperatures and generation of free radicals caused by collapse cavitation all lead to the rupture of the drug carrier [37].

The disruption of the drug carrier triggered by US should occur at the tumor site, thus decreasing the deleterious side effects of chemotherapy. When the nanocarriers are polymeric micelles, this is particularly emphasized when pulsed US is used as a trigger. Polymeric micelles are capable of self-assembly: when the US is on, the drug is released from the micelles, but during the off US period, the drug that did not enter the cells can be re-encapsulated in the micelles that reform and circulate in the bloodstream again [80].

How does US increase the uptake of drugs – free or encapsulated – by the cell? Do these enter the cell by simple diffusion via endocytotic events, or does US induce transient pores in the cell membrane thus allowing the entrance? All mechanisms have been proposed, and while the first has been dismissed as having a major contribution, there are studies that support the other two.

### Enhanced Drug Transport and Distribution in Target Tissues

This mechanism relies on the oscillatory movement of the fluid medium upon exposure to US, which increases the micro-convection phenomenon, thus enhancing the transport of molecules by simple diffusion [81]. This mechanism, which may occur even in the absence of cavitation, was suggested after the observation of drug distribution in poorly vascularized tumor tissues after exposure to US [79]. Diffusion, as the main mechanism of cellular drug uptake after US-triggered release from polymeric micelles, has been proposed but was dismissed by several experiments by the group of Pitt et al. [82–84].

When cavitation occurs, the drug transport is obviously enhanced by the convection currents generated from stable oscillating bubbles. The motion of the

fluid near the drug (free or encapsulated) and the target tissues leads to the dispersion of the drug throughout the tissues. Additionally, when in the proximity of these bubbles, bodies that are denser than the fluid medium, such as drug carriers, are pushed toward the bubbles and eventually are sheared open and release their contents [72, 73].

#### Upregulation of Endocytosis/Pinocytosis

This mechanism suggests that the application of US upregulates the endocytosis and/or pinocytosis of the micelles encapsulating the drug by the tumor cells. Several in vitro cellular studies provided evidence for this mechanism, while others support the sonoporation mechanism discussed below (see section "Relevant In Vitro and In Vivo Studies" – in vitro cellular studies). In any case, this hypothesis concerns nonspecific endocytosis, since it is unlikely that cells possess receptors for polymeric micelles, rejecting the possibility of receptor-mediated endocytosis [37].

#### Cell Membrane Permeabilization

This mechanism suggests that the mechanical effects caused by the interaction of US with the cells cause the transient permeabilization of the cell membrane, facilitating the delivery of the drug to the target tissues [77]. Without bubble cavitation, hyperthermia is the major US effect, and this has little effect on cells [65]. When cavitation occurs, cells and drug-encapsulating nanocarriers are subjected to shock waves, microjets, and microstreaming, and this leads to the rupture of vesicles and the formation of pores in the cell membrane, enhancing the cell permeability [37, 77]. Blood vessel poration and rupture has also been noted [37]. Several studies support this mechanism, and these will be further discussed in section "Relevant In Vitro and In Vivo Studies."

## Relevant In Vitro and In Vivo Studies

Most of the research done on ultrasound DDS using polymeric micelles as nanocarriers uses Pluronic®-based micelles, especially P105 [5, 37]. Other micellar formulations have also been used, and these are also briefly described in this section.

#### In Vitro Micellar Drug Release

The first evidence that US can release drugs from polymeric micelles came from in vitro studies. Most studies were performed using micelles encapsulating a fluorescent drug, such as the model drug calcein or the anticancer drug doxorubicin.

The group of Pitt and coworkers [85] designed an ultrasonic exposure chamber (Fig. 5) to measure the real-time fluorescence decrease due to drug release from polymeric micelles, especially Pluronic® P105, triggered by US. In this type of studies, the fluorescence inside the hydrophobic core of the micelle is higher, and it decreases when the fluorescent probe is released from the micelle and diluted in the medium.

In vitro studies investigated the effect of frequency on the efficiency of release. Ultrasound used for drug release usually ranges from 20 kHz to 16 MHz [74]. Using

the ultrasonic chamber shown in Fig. 5, Husseini et al. [85] studied the effect of using 20–90 kHz pulsed US on the release of Dox and ruboxyl from polymeric micelles. The results showed that the release decreased with increasing frequencies, even when the power density increased, and this suggested an important role for cavitation in the process. Further studies by the same group showed a correlation between drug release and the appearance of subharmonic emissions and broadband noise, suggesting that collapse cavitation was involved [86].

Diaz de la Rosa and coworkers [87] also studied the effect of frequency on drug release from polymeric micelles and showed release at 70 kHz but no release at 476 kHz, even if inertial cavitation occurred at all frequencies. To explain this difference, the authors performed dynamic modeling studies of the bubble oscillation at 70 kHz and 500 kHz [80, 88] at different mechanical indices, a parameter that measures the probability of occurrence of collapse cavitation [89]. Their results

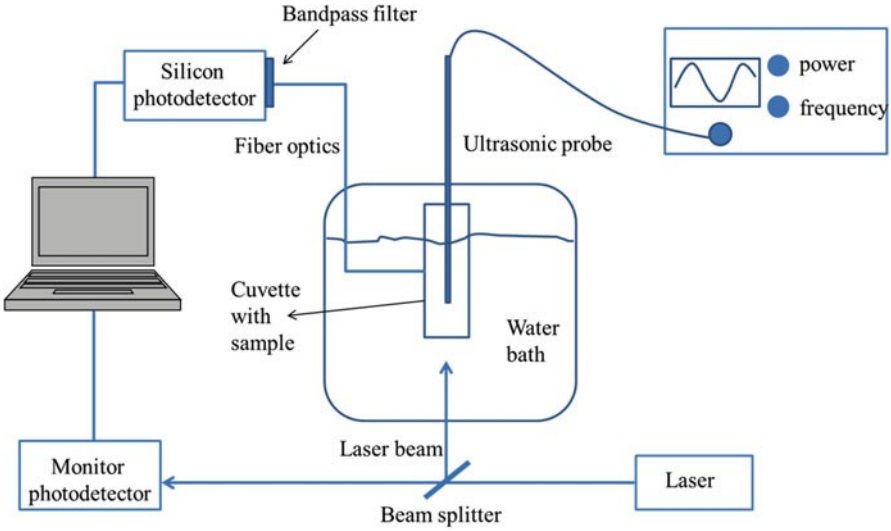



Fig. 5 Custom ultrasonic chamber designed by Husseini et al. [84]. The chamber detects realtime fluorescence, allowing the measurement of US-triggered drug release from polymeric micelles

showed different behaviors of the bubbles under the 70-kHz and 476-kHz ultrasonic fields, with two different routes to chaos, which explained the experimental results [80]. Further modeling studies by the same group showed that the drug release observed experimentally at 70-kHz US is due to an intermittent route to chaos, which does not occur at 476 kHz [80, 88].

Husseini et al. [85] studied the effect of changing power densities at a constant frequency on drug release from Pluronic® P105 micelles. They observed that as the power density increased, so did the release. At 70-kHz, the power density threshold required for drug release was on the range of 0.35–0.41 W/cm$^2$ [88]. When higher frequencies were applied, higher power densities had to be applied to obtain significant amounts of release [85].

Another factor that was studied was the use of pulsed US versus continuous wave (CW) US. In the same study mentioned previously, Husseini et al. [85] observed that when using pulsed US, the drugs were released while the US was on, but they were re-encapsulated in the micelles during the off period between short pulses. This provides a great advantage of this micellar system, since in vivo, when the micelles and the drug leave the diseased area, the drug will be re-encapsulated, thus decreasing the side effects due to the interaction of the drug with healthy cells. The kinetics of release and encapsulation was studied by the same group [90], and the results showed that a 20-kHz US pulse of 0.2 s was necessary to observe release, while the re-encapsulation required an off phase of 0.1 s. Maximum release occurred after 0.6 s insonation, and re-encapsulation occurred immediately after the pulse stopped and was also completed 0.6 s after the beginning of the off period. The data obtained was described by simple physical models, and the best fit was obtained with a zero-order release and first-order re-encapsulation simple kinetics. New results were obtained and modeled in subsequent studies, using artificial neural network (ANN) [91] and chemical kinetic mechanistic models [92]. These studies investigated the drug release and re-encapsulation as functions of frequency, power density, micelle concentration, and temperature. Stevenson-Abouelnasr and coworkers [92] proposed four mechanisms for the release and re-encapsulation of Dox from P105 micelles using 20-kHz US with a power density of 0.058 W/cm$^2$: (i) micelle destruction and Dox release, (ii) cavitating nuclei destruction, (iii) micelles reassembly, and (iv) Dox re-encapsulation. While the first mechanism is due to cavitation, the second one is a slow partial recovery phase, when the re-encapsulation of a small amount of drug occurs. The last two mechanisms are independent of US. The modeling and sensitivity analysis of Dox release kinetics from P105 using an ANN model [91] showed that the drug release was inversely proportional to the US frequency and directly proportional to power density. The power density threshold to release at 20-kHz US was much lower (0.015 W/cm$^2$ at MI 0.15) than that at 70-kHz US (0.38 W/cm$^2$ at MI 0.40), emphasizing the role of inertial cavitation in the process. The same modeling strategy was further used to optimize the US parameters – US frequency, power density, pulse length, sonication duration – to achieve an optimal drug release at the tumor site via a model-predictive controller (MPC) [93, 94]. The parameters of the controller can then be adjusted to reach good reference signal tracking and sustain constant drug release.

Several studies were also performed to unravel the effect of micelle stabilization on the drug release rate. The release rate of Dox from stabilized and non-stabilized Pluronic® P105 micelles, triggered by 70-kHz US, was studied in vitro [95]. It was observed that Dox release from non-stabilized micelles (10 % release) was higher than from NanoDeliv™ stabilized ones (3 % release). Although 3 % is a low value, theoretically, the entire micellar drug load could be released if pulsed US was applied for a period long enough, in the presence of cells that compete for Dox. Another study by the same group [96] showed that the release rate of Dox from non-stabilized micelles is also significantly higher than from NanoDeliv™. Additionally, a study of the degradation kinetics of NanoDeliv™ micelles exposed to 70-kHz and 476-kHz US (both at MI 0.9), showed that, although US perturbs the IPN of the

stabilized micelles, the degradation time is long when compared to the drug release rate, and no significant difference in the degradation rates could be observed after 2 h of insonation at both frequencies.

Temperature was studied as another factor that influences US-triggered drug release from polymeric micelles. The kinetic model developed by StevensonAbouelnasr et al. [92] was also used to study the kinetics of US-induced release and re-encapsulation of Pluronic® P105 micelles at different power densities and temperatures (namely, 25 C, 37 C and 56 C) [97]. A negative correlation was reported between 70-kHz US power intensity and residual activation energy of the micelle destruction, and it was observed that an increase in temperature increased the rate of micelle destruction (a function accurately represented using the Arrhenius equation) and decreased the rate of micelle reassembly. The ANN model developed by Husseini et al. [91] also predicted that Dox release is not dependent on temperature, suggesting that the major mechanism of release is not mainly due to the thermal effects of US. The same group [96] further studied the effect of temperature on Dox release and re-encapsulation from stabilized (NanoDeliv™) and unstabilized P105 micelles. Temperature did not have any effect on Dox release and re-encapsulation time constants for the unstabilized micelles. For the same temperature, the observed release was higher for the unstabilized micelles than for the NanoDeliv™, emphasizing the importance of the IPN in preserving the integrity of the micellar structure subjected to an external stimulus. On the contrary, no significant differences could be observed for the re-encapsulation rate constants of stabilized and unstabilized micelles.

The concentration of Pluronic® also influences the drug release from these polymeric micelles. The first evidence was published by Husseini et al. [85], who observed that the drug release was higher for lower concentrations of Pluronic® P105, possibly due to the higher local drug concentration in the hydrophobic core of the micelles, when the number of micelles was low. This study also provided evidence that the release was lower when the drug was deeply inserted into the micelle core. The ANN model developed by Husseini et al. [91] of the steady-state acoustic release of Dox from P105 micelles further indicated that higher release was obtained at lower copolymer concentrations.

The previously described studies used non-targeted Pluronic® P105 micelles, but targeted ones have also been studied. The first system combining polymeric micelles, targeting, and triggered release using US was described by Husseini and coworkers [59]. The polymeric micelles were synthesized with a folate moiety, and the release of encapsulated Dox was studied using 70-kHz US as a trigger. It was observed that Dox was released above a power density threshold of 0.55 W/cm$^2$, which again suggested the critical role of cavitation in the process. Above this threshold, the amount of drug release increased with increasing power densities but reached a maximum of 14 % release at 5.4 W/cm$^2$. A subsequent study by the same group [98] compared the kinetics of Dox release from folated and non-folated micelles exposed to 70-kHz US at different power densities. The results showed a higher percentage of release from folated micelles. Additionally, a mathematical model with a zero-order release and first-order re-encapsulation rate was used to fit

the data and the existence of a power density threshold emphasized the importance of inertial cavitation for the drug release.

Although Pluronic® P105 has been the most researched type of polymeric micelles in acoustically-triggered drug delivery, several different formulations have been studied, including different types of Pluronic® and mixed micelle formulations. Ugarenko et al. [99], for example, synthesized DSPE-$PEG_{2000}$ (1,2-diasteroyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000])-Pluronic® mixed micelles and studied the release of Dox and formaldehyde-releasing prodrugs, triggered by 20-kHz US at 100 W/$cm^2$. Upon micelle formation, it was observed that 60 % of Dox was encapsulated but no formaldehyde-releasing products. However, these were administered separately to the Dox-loaded micelles. Upon exposure to US, 7–10 % of Dox was released from the micelles. This system was considered promising in cancer treatment, since it can potentially form cytotoxic DNA adducts in cancer cells.

Zhang and coworkers [100] designed and synthesized micelles of the block copolymer poly(lactic acid)-b-poly(ethylene) glycol (PLA-b-PEG), encapsulated Nile Red, and studied the release triggered by HIFU. They suggested an irreversible mechanism elicited by transient cavitation. The same group synthesized micelles of poly(ethylene oxide) and poly(2-tetrahydropyranyl methacrylate) (PEO-bPTHPMA) [101] and also studied the effect of HIFU in this formulation. The observed disruption was due to the US-induced hydrolysis of 2-tetrahydropyranyl groups at room temperature.

### In Vitro Cellular Studies

This section describes the main in vitro studies performed on polymeric micelles and US as a DDS. The uptake of Dox and other fluorescence molecules by cancer cells in vitro can be monitored by direct or indirect methods [8, 9, 37, 43, 102–104]. The direct methods measure the fluorescence of the cells by flow cytometry and/or fluorescence microscopy and also allow the study of the intracellular distribution of the drug. Indirect methods measure the depletion of the drug from the medium by using a spectrofluorometer.

In vitro cell studies provided evidence that US induces cavitation-related processes which mediate a synergistic effect between US exposure, pharmacological activity of the encapsulated drug, and polymeric micelles. The exposure to US releases the drug encapsulated in the nanocarriers and simultaneously enhances the intracellular uptake of micellar-encapsulated drugs, but different studies suggested different mechanisms of uptake, either the endocytosis of the carrier or the sonoporation of the cell membrane.

The first in vitro cellular study was performed by Munshi et al. [105] using Pluronic® P105-encapsulating Dox and 80-kHz US to study delivery of the agent to HL-60 human leukemia cells. The synergistic effect between US and encapsulated Dox was observed since the Dox $IC_{50}$ was lowered from 2.35 to 0.19 mg/ml. Another study, using the same cell line and 70-kHz US [106] also showed a synergism between the Dox, polymeric micelles, and US. In the absence of US, the encapsulation protected the cells from the bioeffects of Dox, while the application

of US triggered the drug release and/or uptake by the cells, causing DNA damage, as determined by the comet assay. Similar assays performed with NanoDeliv™ stabilized micelles [107] showed that these were able to protect cells from much higher Dox concentrations when compared to unstabilized micelles. Exposure to 70-kHz US led to a synergistic effect, similar to that observed on the previous study [106].

Several other in vitro studies on the drug release from Pluronic® micelles and cellular uptake induced by US were performed by the Pitt and Rapoport groups, using either LFUS or HFUS [82, 102–104]. Several US parameters were investigated, such as the power density, pulsed vs. CW US, insonation duration, and interpulse intervals. Marin et al. [102] studied the mechanism of US-induced drug delivery to HL-60 cells in vitro. Using 20-kHz LFUS and Dox encapsulated in Pluronic® P105 micelles, they observed an increase in cellular drug uptake. They suggested that LFUS caused acoustic cavitation that induced both drug release from Pluronic® micelles and enhanced uptake of the micellar-encapsulated drugs. Additionally, the authors observed that, when using US, the same level of drug uptake could be attained when having a much lower extracellular concentration of drug.

The same group studied the effect of using CW or pulsed 20-kHz US on Dox uptake by the same cell line [103]. Drug uptake was observed in both cases, and the authors further studied the effect of the duration of the pulse (on period) and interpulse (off period) intervals. Dox uptake increased with increasing pulse duration from 0.1 to 2 s using the same total insonation time, and no significant effect of the interpulse interval could be observed, suggesting that the cells are very effective in competing with the drug re-encapsulation in micelles. The authors suggested two independents mechanisms that seem to control the acoustic-controlled drug uptake by the cells: (i) US-induced Dox release from micelles, with the consequent increase of the free drug in the medium; (ii) US-caused perturbation of the cell membrane with the consequent increase of the intracellular uptake of the micellar formulation.

In a study using different cell lines [104], the same group compared the drug release and cellular uptake when the cells were exposed to LFUS (20–100 kHz) or HFUS (1 MHz). They observed that the onset of acoustic cavitation at higher frequencies required much higher power densities than at low frequencies.

Rapoport and coworkers [84] studied the effect of copolymer concentration on the uptake of fluorescently labeled Pluronic® P105 micelles by ovarian carcinoma cell lines (A2780 drug sensitive and A2780/ADR MDR). Their data supported the internalization of drugs via fluid-phase endocytosis, followed by a nuclear accumulation enhanced by the use of the polymeric micelles and further increased by the application of 20-kHz US. The data also showed that the membranes of the endosomes and lysosomes of A2780/ADR MDR cells were more susceptible to the action of polymeric surfactants than those of drug-sensitive A2780 cells.

Pitt and coworkers [106–108] studied the differences between exposing HL-60 cells to free Dox, Dox encapsulated in Pluronic® P105, and Dox encapsulated in NanoDeliv™, with and without US. It was observed that, in the absence of US, cells

exposed to free Dox were killed faster than those exposed to encapsulated Dox [106]. When exposed to 70- kHz US, however, the scenario was opposite: cells exposed to the encapsulated drug were killed at a faster rate than those exposed to the free drug. Hence, in the absence of US, the micelles protect the cells from the effects of the drug, and when US is used, the released Dox kills cells faster than the free drug. These studies also used the comet assay to monitor DNA damage caused by the treatment [106, 108]. They documented the correlation between cell death and DNA damage, thus indicating that apoptosis was the main mechanism of cell death caused by these insonation levels, not necrosis which irreversibly damages the cell membrane.

Howard and coworkers [109] used a different polymeric formulation – micelles of methyl-capped poly(ethylene oxide)-co-poly-(L-lactide) encapsulating the anticancer drug paclitaxel – and also demonstrated the synergy between this system and US (1 MHz, power density 1.7 W/cm$^2$) used on a drug-resistant breast cancer cell line (MCF7/ADmt). In the absence of US, the micelles protected the cells from the toxic effects of the drug, but, upon exposure to the ultrasonic field, there was a dramatic increase in the accumulation of the micellar formulation inside the cells.

Similarly, Ugarenko et al. [99] demonstrated the previously described synergism, using Pluronic® P105 micelles stabilized with disteroyl-phosphoethanolamine-PEG200, encapsulating Dox, and 20-kHz US. They used this system in MDA-MB-231 breast cancer cells in vitro, and showed that, in the absence of US, the micellar system protected the cells from Dox, while upon application of US, 10 % of the drug was released and the cellular uptake was significantly increased. However, when US was applied for more than 5 s, the cells died, which emphasizes the importance of a careful control of all US parameters, when doing drug delivery research.

The groups of Pitt and Rapoport carried several studies [82–84] that demonstrated the uptake of micelles into cells, thus dismissing the proposed mechanism that US triggers the drug delivery from micelles outside the cells, followed by diffusion (see section "Mechanisms of US-Induced Micellar Drug Release and Cellular Uptake"). They designed Dox-encapsulating Pluronic® P105 micelles with the end hydroxyl groups labeled with a fluorescent probe possessing a different fluorescence than that of Dox. Studies of confocal microscopy and flow cytometry showed that, upon insonation, the labeled P105 micelles entered HL-60 cells and were distributed between the membrane, the cytosol, and other vesicles.

The same group further investigated whether the mechanism of cellular drug uptake triggered by US involved endocytosis/pinocytosis [82, 83]. A preliminary study [83], performed in the absence of US, used fluorescently labeled Pluronic® P105, and concluded that the aggregation state of the copolymer influenced the uptake by HL-60 cells, with unimers entering the cell by diffusion, while uptake of micelles occurred via fluid-phase endocytosis. A subsequent study [82] used Pluronic® P105 micelles labeled with a pH-sensitive fluorescent probe, which has higher fluorescence in acidic conditions, i.e., endosomes. Flow cytometry studies showed that, upon insonation with 70-kHz US, there was an increase in the fluorescent inside HL-60 and HeLa cells but no increase was observed inside

endosomes and lysosomes. The researchers suggested that sonoporation was the main mechanism of US-enhanced drug uptake.

Sonoporation has been supported by several other studies, including the ones by Tachibana et al. [110–114], which provided direct evidence of this mechanism by using, for example, scanning electron microscopy. One of their early studies [111] used merocyanine 540 as a tracer and exposed HL-60 cells to 255-kHz US for 30 s. Results obtained by scanning electron microscopy showed the formation of pores in the cell membrane, which resulted in cell death. Another study [112], using the same cell line and the cytotoxic drug cytosine arabinoside, showed increased cell death upon exposure to 48-kHz US. Scanning electron microscopy showed some disruption of the cell membranes as well as a decreased number of microvilli, and it was hypothesized that this increased drug uptake. Saito and coworkers [115] showed that sonoporation was implicated in the injury of corneal endothelium cells by US. They observed that some cells died due to necrosis, while others survived and recovered, with the membranes recovering integrity several minutes after the exposure. The experiments of Prentice et al. [116] also supported the sonoporation mechanism. They exposed MCF-7 breast cancer cells to high power densities of 1-MHz US and used atomic force microscopy to observe holes in the cell membranes. Stringham and coworkers [117] used a rat colon cancer cell line (DHD/K12 TRb) and the model drug calcein to unravel the relationship between cavitation and sonoporation. The cells were exposed to 476-kHz US at different power densities, and it was observed that, in these conditions, calcein entered and accumulated intracellularly, unlike in the absence of US. To test the hypothesis that inertial cavitation was directly related to the drug uptake, further experiments were performed at a pressure of 3 atm. At this higher pressure, it was observed that the accumulation of calcein inside the cells decreased. Since cavitation decreases as pressure increases at a constant US power density [118], these results proved the direct relation hypothesized by the authors. A similar result was obtained when using 1-MHz HFUS in an in vitro study with bovine endothelial cells [119]. Studies by Schlicher et al. [120], using flow cytometry coupled to electron and fluorescence microscopy, concluded similarly: the accumulation of calcein in prostate cancer cells was caused by the reversible increase in membrane permeability induced by acoustic cavitation caused by 24-kHz US. A paper by Zhou et al. [121] went further and reported the size of the pores produced in *Xenopus laevis* oocytes exposed to 1.075-MHz US.

Several studies, however, supported the endocytosis hypothesis of cellular drug uptake enhanced by US. Muniruzzaman et al. [83] investigated the effect of the copolymer aggregation state – micelles or unimers of Pluronic® P105 – on the intracellular uptake by HL-60 cells, in the absence of US. Their results suggested that below the CMC, the unimers enter the cell via simple diffusion, while micelles enter via fluid-phase endocytosis. A later study by the same group [84] used 20-kHz US and reported that sonication enhanced the rate of endocytosis of micelles by several types of human cell lines. In the same study, the intracellular distribution of Dox was studied by confocal microscopy, and its accumulation was observed in the nucleus. Sheikov et al. [122] provided evidence that US-enhanced pinocytosis in the

endothelial cells that line brain arterioles and capillaries. The enhancement of endocytosis by US was also reported in human fibroblasts with no detectable cellular membrane injury [123].

As mentioned before, the mechanism of drug uptake is still being researched. A study by Meijering et al. [124], published in 2009, suggested that both mechanisms, endocytosis and sonoporation, contribute for drug cellular uptake induced by US. They studied microbubble-targeted delivery of therapeutic compounds to primary endothelial cells, using pulsed 1-MHz US, observing that both endocytosis and transient pore formation were involved in drug uptake, and that the contribution of endocytosis was dependent on the molecular size of the molecules to be delivered. Additionally, it has been suggested that different cells may respond differently to US and that maybe a general mechanism cannot be derived [5]. In any case, the in vitro cellular studies provided evidence of the synergism between chemotherapy and US exposure, with US both enhancing the release of the drugs from the micelles and the uptake of the drug by the cells by creating transient pores in the cell membrane and/or increasing endocytosis.

### In Vivo Research Using Animal Models

Several in vivo studies have been conducted since 2002 in order to test controlled delivery systems composed of polymeric micelles sequestering anticancer drugs and triggered using US in either rat or mouse models. It should be noted that, even in the absence of micellar systems, there is a decrease in tumor growth upon exposure to free drug and US, which confirms the synergistic effect between the pharmacological activity of the chemotherapeutic drug and US [37].

The first in vivo studies were performed by Nelson and coworkers [125, 126], who treated a group of 42 BDIX rats injected with a DHD/K12/TRb colorectal tumor cell line in both of their hind legs, with different concentrations of free Dox or Dox encapsulated in stabilized NanoDeliv™ micelles. The tumor in one leg was sonicated using 20- or 70-kHz US at different power intensities, duty cycles, and US application regimens (once or twice a week), while the other leg was left without sonication. An observable reduction of the tumor size was reported when the combined system was applied (micellar-encapsulated Dox and US), compared to the noninsonated, micellar-loaded Dox formulation and the free drug control. According to the authors, this could be due to the increased drug uptake by cancer cells when sonicated, or that the US assists the extravasation of the drug-loaded carriers into the tumor tissues.

Another in vivo experiment conducted by the same group [46] used immunecompromised athymic nu/nu mice model bearing ovarian carcinoma tumors and studied the effect of Pluronic® P105 and 1-MHz HFUS. The mice were treated with either micellar-encapsulated Dox or free Dox, and one group was insonated, while the control group was not exposed to US. It was observed that the intracellular encapsulated drug uptake by tumor cells was higher than the uptake by other sites or organs. The uptake by tumors was even more enhanced when localized sonication was applied, which resulted in an increase in mice survival rates when compared to noninsonated mice treated with a similar concentration of micellar Dox. More

importantly,Dox did not accumulatein theheart,anorgan which is severely affected by the cytotoxicity of this drug [127]. The advantage of using HFUS is due to the fact that it can be more precisely focused then LFUS and causes less sonolysis [85, 104].

Fluorouracil (5-FU) encapsulated in stabilized P105 micelles in conjunction with 20-kHz US was tested as a DDS in a BALB/c nude mice model inoculated with the WiDr human colon cancer cell line [128]. The group treated with US showed a significant reduction in tumor volumes, when compared to noninsonated groups, emphasizing the synergy resulting from the use of the combined delivery system, which became more evident for lower 5-FU concentrations.

Howard and coworkers [109] performed in vitro cellular studies using methylcapped poly(ethylene oxide)-co-poly-(l-lactide)-tocopherol micelles encapsulating Paclitaxel further tested this formulation in vivo, in conjunction with 1-MHz US. The results showed that this DDS was effective in the complete tumor regression in nu/nu mice inoculated with an MCF-7/ADM drug-resistant breast cancer cell line.

Gao and coworkers [129] studied the effect of 1- and 3-MHz US on the biodistribution of fluorescently labeled unstabilized and PEG-diacylphospholipidstabilized Pluronic® P105 micelles in ovarian cancer-bearing nu/nu mice. The results showed that US enhanced the accumulation of the micelles in the tumors and that the degree of targeting depended on the local tumor sonication. A later study by the same group [130] used Dox encapsulated in Pluronic® P105 and mixed Pluronic® P105, PEG2000-diacylphospholipid and PEG-co-poly(beta-benzyl-Laspartate) (PEG-b-PBLA) to treat the same mice cancer model and observed that 30 s of 1-MHz US increased the intracellular Dox uptake by eightfold. In a later study [131], nu/nu mice implanted with breast (MDA-MB-231) or ovarian cancer (A2780) cells were treated with Dox encapsulated in micelles of copolymers PEG-b-poly(L-lactide) (PEG-PLLA) or PEG-b-poly(caprolactone) (PEG-PCL) and loaded with perfluoropentane ($PFC_5$) nanoemulsions. The delivery system accumulated selectively in the tumor sites due to the EPR effect. This was followed by either 1- or 3- MHz US applied locally in order to release and increase the intracellular uptake of the encapsulated drug. The researchers suggested that this selective release of drug in tumor sites occurred mainly due to the collapse of the highly echogenic microbubbles developing from the nanodroplets that grew in size as a result sonication.

A group of researchers from Brigham Young University [132–134] conducted several in vivo experiments using BDIX rats bearing bilateral leg DHD/K12/TRb colorectal epithelial tumors. For their studies, they used US at 20- and 476-kHz at different pulse intensities as a trigger to release Dox from NanoDeliv™ micelles. The aims of the research were: (i) to study the pharmacokinetics of the drug, (ii) to quantitatively analyze the temporal Dox concentration profiles in cancerous and healthy rat tissues, and (iii) to study the effect of using different US frequencies (at the same MIs and temporal average intensities) on the development of cancer cells and drug delivery. Results showed that an initial, although not significant, accumulation of the drug in the blood-perfused organs, such as the liver and heart,

took place. However, this accumulation decreased with time, when the drug started to preferentially accumulate in tumor tissues, with faster clearance rates from the healthy tissues achieved in the insonated groups when compared to the noninsonated groups. Consequently, this caused the tumors in the groups exposed to 20- and 476-kHz US to grow significantly slower than in the controls [134]. At 476-kHz, however, even if there was a tumor regression in treated groups, the differences in Dox concentration in cancerous cells were not significantly different between both groups, 6 h after the injection of the micellar system [132]. This result supported the main role of cell membrane permeabilization as the mechanism of drug uptake by cells, since it could not be explained by simple Dox release [132, 134, 135]. Additionally, these results were not obtained when the animals were exposed to US alone or to empty drug carriers, emphasizing the synergism between all these factors, just like in in vitro cellular studies.

A mouse model of breast cancer (spontaneous breast adenocarcinoma xenografted in female BALB/c mice) was used to study the dual application of 28-kHz and 3-MHz US when using a stabilized Pluronic® P105 micellar Dox system [136]. Dox was administered either free or encapsulated in the micelles, and some mice were exposed to US while a control group only received Dox in free form. As reported by the previous groups, it was observed that the US and polymeric micelle system were significantly more effective in facilitating drug accumulation in tumor cells when compared to either the free Dox or micellar noninsonated formulations. On the contrary, the concentration of Dox in non-tumor tissues was lower when micellar drug was used, compared to free drug. According to the authors, this was due to the role that US plays in cavitation and sonoporation.

Another set of experiments were conducted using docetaxel (DTX)-loaded P105/F127 mixed micelles in the treatment of male Sprague-Dawley rats and BALB/c nude mice models bearing Taxol-resistant human lung adenocarcinoma tumors (A549/Taxol) [137]. No US treatment was used in this work. The results obtained when the hybrid micellar formulation was used were compared to the results obtained from negative controls that were not treated at all and/or controls that received only the poorly soluble DTX and/or its commercially soluble form, Taxotere®. It was observed that the Taxol elimination half-life was extended when the micellar drug system was used. Moreover, the sizes of tumors injected with the DTX-hybrid micelles were significantly smaller than the sizes of the negative controls and the groups treated with Taxotere®. These promising results were probably due to the enhanced drug uptake by the tumor caused by the EPR effect, which indicates that such a system may be used in future clinical trials to overcome MDR in lung cancer.

Recently, the same group of researchers [138] developed a novel DDS composed of mixed micelles of folic acid-conjugated carboxymethyl lauryl chitosan (FA-CLC) and superparamagnetic iron oxide (SPIO) (FA-CLC/SPIO), sequestering camptothecin, and triggered using both magnetic and ultrasonic (1 MHz) fields. The system was tested against MDA-MB-231 (FA-positive) breast cancer cells implanted in 68-week-old female nude mice, and fluorescence and magnetic resonance imaging were used to confirm the active drug targeting of the system in

vivo. It was observed that the therapeutic efficacy of this system was considerably enhanced when compared to other systems that used either the free drug or camptothecin-loaded FA-CLC/SPIO micelles triggered passively or actively (whether by US alone or the magnetic field alone). Nevertheless, it was recommended that extra caution should be taken in future experiments in order to prevent any possible unwanted accumulation of iron-containing vehicles in the liver when the cancer is located near this organ.

### Clinical Trials and Uses

From the previous sections, it is clear that several micellar formulations have been and are being studied as possible chemotherapies, with or without the concomitant use of US. However, so far, there are no FDA-approved micellar systems for the treatment of cancer [5, 139]. Some micellar formulations have been approved for use in other countries and several others are undergoing clinical trials around the world, as recently reviewed by Wicki et al. [17].

Genexol-PM® is a polymeric micelle composed of methoxy-PEG-poly(D,Llactide) and encapsulating the chemotherapeutic drug Paclitaxel, which has been approved for the therapy of breast cancer in Europe and South Korea, and is undergoing clinical trials in the USA for the treatment of breast, small-cell lung, and pancreatic cancers [15, 16, 140]. This formulation is a regular micelle encapsulating the drug in its hydrophobic core, stabilized and soluble in water due to the PEG hydrophilic corona [15]. Paclital and NK105 are other Paclitaxel micellar formulations that are undergoing clinical trials for the treatment of ovarian and metastatic or recurrent breast cancer, respectively [16, 141]. Nanoxel, a micellar formulation of Paclitaxel, has also been approved for the treatment of breast cancer in India [17].

The combination of micelle-encapsulated drugs and US did not reach the clinical trial stage yet.

## Conclusion and Future Directions

The high toxicity of potent chemotherapeutic drugs limits the therapeutic window in which they can be applied. This window can be expanded by controlling the drug delivery in both space and time such that non-targeted tissues are not adversely affected. This review chapter focuses on using US to control the release of antineoplastic agents from nanocarriers spacially and temporally. These nanovehicles include polymeric micelles and liposomes. The potential benefits of such controlled chemotherapy compels a thorough investigation of the role of US and the mechanisms by which US accomplishes drug release and/or enhances drug potency which is the focus of our drug delivery group.

As is widely known, the current practice in chemotherapy requires the use of high dosages of antineoplastic agents to increase its effectiveness on tumors which also

results in detrimental side effects on healthy cells. These side effects significantly decrease the quality of life of the patient and result in several life-threatening conditions. Therefore, researchers have directed ample time to improve the practice of chemotherapy in an attempt to increase the effectiveness of the drug, which results in decreasing the need for high doses and in turn decreasing the side effects.

In addition to advancements in cancer drug delivery, other promising areas have evolved including, but not limited to, vaccines and gene delivery. The first area entails the discovery of the virus causing different cancers and vaccinating toddlers against each virus. On the other hand, gene delivery involves transfecting cancer cells with the intention of controlling or eliminating the DNA mutations. Gene delivery is divided into two main areas: viral and non-viral. Viral gene delivery involves the use of a virus to transfect the DNA of diseased cell. Its main drawback is low specificity since the virus could transfect healthy cells in the process. Non-viral techniques include US, and they suffer from low transfection rates.

This chapter focused on drug delivery in cancer treatment with the utility of US. As mentioned above, this research area involves the sequestration of the drug inside nanocarriers designed to target the tumor cells specifically while sparing the healthy cells. Once at the tumor site, focused waves of US are used on the tumor to break open the carriers, releasing the drug into the cancer cells. The novelty of this line of research is the fact that it is the first combination of the two technologies, (i) nanocarriers or nanocapsules and (ii) US waves, to generate a new drug delivery methodology for cancer treatment.

When deciding on a drug delivery vehicle, several principles are examined to improve their performance including passive, ligand, and triggered targeting. Passive targeting is the main reason behind the success of liposomal Dox-Doxil (which achieved FDA approval in 1996). Passive diffusion takes place because of the leaky defective vasculature of cancerous tissues compared to health tissue. The extent to which passive diffusion improves drug accumulation at the tumor while reducing the systemic concentration is still being researched for a variety of chemotherapeutic agents and with different formulations of liposomes to achieve more efficient cancer treatments. Active targeting (or more correctly, ligand targeting) involves the decoration of targeting moieties unto the surface of drug delivery vehicles in the hope that receptor-mediated endocytosis will improve the antineoplastic accumulation at the tumor site via the key-and-lock mechanism. Naturally, the main obstacle faced by scientists in this area is to insure that the stability, drug efficiency, and other characteristics will not be affected by conjugating these molecules to these nanostructures. Some targeted nanocarriers have shown promising results in vitro, but the same improvement was not observed when tested in vivo. There is no doubt that ligand targeting will continue to be researched heavily to reach the optimal conditions of loading efficiency, moiety surface concentration, type of cancers that can be targeted, etc.

External and internal triggers constitute the third type of drug delivery targeting. External triggers including US, magnetic, electrical fields, and light have been reported widely in cancer treatment literature. Similarly, internal triggers (e.g., temperature variations, pH, and enzymes) have shown promise both in vivo and in

vitro. This review has focused on the use of US as a trigger mechanism for several reasons. First, US waves can easily be focused on the tumor noninvasively. Additionally, the physics of US is very well understood and documented. US has also been used to induce hyperthermia (by increasing the temperature of the tissue to above 42 C) which would be an added advantage to the use of this technique. More importantly, there is a well-documented synergism between the action of chemotherapeutic agents and US, thus rendering acoustic waves more attractive for this area of research.

In conclusion, we reiterate the importance of finding a multimodal drug delivery system that employs all three targeting techniques into one system that can be classified as a "magic bullet" in the fight against one of the most prevalent killers of the twenty-first century.

## References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) CA Cancer J Clin 61:69
2. DeVita VT Jr, Chu E (2008) Cancer Res 68:8643
3. Uchegbu IF, Siew A (2013) J Pharm Sci 102:305
4. Aldrich JE (2007) Crit Care Med 35:S131
5. Ahmed SE, Martins AM, Husseini GA (2015) J Drug Target 23:16
6. Moussa HG, Martins AM, Husseini GA (2015) Curr Cancer Drug Targets 15:282
7. Sutton D, Nasongkla N, Blanco E, Gao J (2007) Pharm Res 24:1029
8. Husseini GA, Pitt WG (2008) J Nanosci Nanotechnol 8:2205
9. Husseini GA, Pitt WG (2008) Adv Drug Deliv Rev 60:1137
10. Paleos CM, Tsiourvas D, Sideratou Z, Tziveleka L-A (2010) Expert Opin Drug Deliv 7:1387
11. Souto EB, Fangueiro J, M€uller RH (2013) In: Uchegbu IF, Scha¨tzlein AG, Cheng WP, Lalatsa A (eds) Fundamentals of pharmaceutical nanosciences. Springer, New York
12. Fante C, Greco F (2013) In: Uchegbu IF, Scha¨tzlein AG, Cheng WP, Lalatsa A (eds) Fundamentals of pharmaceutical nanosciences. Springer, New York
13. Uchegbu IF (2013) In: Uchegbu IF, Scha¨tzlein AG, Cheng WP, Lalatsa A (eds) Fundamentals of pharmaceutical nanoscience. Springer, New York, p 9
14. Cho K, Wang X, Nie S, Chen ZG, Shin DM (2008) Clin Cancer Res 14:1310
15. Peplow M (2014) Pharm J 292:467
16. Pillai G (2014) SOJ Pharm Pharm Sci 1:1
17. Wicki A, Witzigmann D, Balasubramanian V, Huwyler J (2015) J Control Release 200:138
18. Matsumura Y, Maeda H (1986) Cancer Res 46:6387
19. Maeda H (2001) Adv Enzyme Regul 41:189
20. Maeda H, Nakamura H, Fang J (2013) Adv Drug Deliv Rev 65:71
21. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K (2000) J Control Release 65:271
22. Nichols JW, Bae YH (2014) J Control Release 190:451
23. Allen TM (2002) Nat Rev Cancer 2:750
24. Torchilin VP (2007) AAPS J 9:E128
25. Byrne JD, Betancourt T, Brannon-Peppas L (2008) Adv Drug Deliv Rev 60:1615
26. Yu B, Tai HC, Xue W, Lee LJ, Lee RJ (2010) Mol Membr Biol 27:286
27. Gu FX, Karnik R, Wang AZ, Alexis F, Levy-Nissenbaum E, Hong S, Langer RS, FarokhzadOC (2007) Nano Today 2:14
28. Torchilin VP, Lukyanov AN, Gao Z, Papahadjopoulos-Sternberg B (2003) Proc Natl AcadSci U S A 100:6039
29. Oerlemans C, Bult W, Bos M, Storm G, Nijsen JF, Hennink WE (2010) Pharm Res 27:2569
30. Torchilin VP (2009) Eur J Pharm Biopharm 71:431
31. Torchilin VP (2007) Pharm Res 24:1

32. Yu MK, Park J, Jon S (2012) Theranostics 2:3
33. Zhao X, Li H, Lee RJ (2008) Expert Opin Drug Deliv 5:309
34. Shen Z, Li Y, Kohama K, Oneill B, Bi J (2011) Pharmacol Res 63:51
35. Ai J, Xu Y, Li D, Liu Z, Wang E (2012) Talanta 101:32
36. Shigdar S, Qiao L, Zhou SF, Xiang D, Wang T, Li Y, Lim LY, Kong L, Li L, Duan W (2013) Cancer Lett 330:84
37. Husseini GA, Pitt WG, Martins AM (2014) Colloids Surf B 123C:364
38. Mura S, Nicolas J, Couvreur P (2013) Nat Mater 12:991
39. Pitt WG, Husseini GA, Kherbeck LN (2013) In: Alvarez-Lorenzo C, Concheiro A (eds) Smart materials for drug delivery, vol 1. RSC Publishing, Cambridge, UK, p 148
40. Lee ES, Gao Z, Bae YH (2008) J Control Release 132:164
41. Huang W, Shi C, Shao Y, Lam KS, Luo J (2013) Chem Commun (Camb) 49:6674
42. Siepmann J, Siegel R (2012) Fundamentals and applications of controlled release drugdelivery. Springer, New York
43. Husseini GA, Pitt WG (2009) J Pharm Sci 98:795
44. Batrakova EV, Kabanov AV (2008) J Control Release 130:98
45. Batrakova E, Li S, Li S, Venne A, Alakhov V, Kabanov A (1999) Pharm Res 16:1373
46. Rapoport NY, Christensen DA, Fain HD, Barrows L, Gao Z (2004) Ultrasonics 42:943
47. Rapoport N, Caldwell K (1994) Colloids Surf B 3:217
48. Kabanov AV, Batrakova EV, Melik-Nubarov NS, Fedoseev NA, Dorodnich TY, Alakhov VY,Nazarova IR, Kabanov VA (1992) J Control Release 22:141
49. Romberg B, Hennink WE, Storm G (2008) Pharm Res 25:55
50. Pitt WG, Husseini GA, Staples BJ (2004) Expert Opin Drug Deliv 1:37
51. Pruitt JD, Husseini G, Rapoport N, Pitt WG (2000) Macromolecules 33:9306
52. Sun Q, Radosz M, Shen Y (2012) J Control Release 164:156
53. Yang TF, Chen CN, Chen MC, Lai CH, Liang HF, Sung HW (2007) Biomaterials 28:725
54. Zeng Y, Pitt WG (2005) J Biomat Sci Polym 16:371
55. Zeng Y, Pitt WG (2006) J Biomater Sci Polym 17:591
56. Husseini GA, Christensen DA, Rapoport NY, Pitt WG (2002) J Control Release 83:303
57. Basile L, Pignatello R, Passirani C (2012) Curr Drug Deliv 9:255
58. Kabanov AV, Chekhonin VP, Alakhov V, Batrakova EV, Lebedev AS, Melik-Nubarov NS,Arzhakov SA, Levashov AV, Morozov GV, Severin ES et al (1989) FEBS Lett 258:343
59. Husseini GA, Velluto D, Kherbeck L, Pitt WG, Hubbell JA, Christensen DA (2013) ColloidsSurf B 101:153
60. Rapoport N (2007) Prog Polym Sci 32:962
61. Hendee WR, Ritenour ER (2003) Medical imaging physics, 4th edn. Wiley, New York
62. Shriki J (2014) Crit Care Clin 30:1
63. Coltrera MD (2010) Otolaryngol Clin North Am 43:1149
64. Edwards H (2010) Ultrasound 18:100
65. Nyborg WL (2001) Ultrasound Med Biol 27:301
66. Rooney JA (1988) Ultrasound: its chemical, physical, and biological effects. VCHPublishers, New York, p 65
67. Hall DO, Selfridge AR (1995) US Patent 5,460,595A
68. Buldakov MA, Hassan MA, Zhao QL, Feril LB Jr, Kudo N, Kondo T, Litvyakov NV,Bolshakov MA, Rostov VV, Cherdyntseva NV, Riesz P (2009) Ultrason Sonochem 16:392
69. Rapoport N (2012) Int J Hyperthermia 28:374
70. Goss SA, Johnston RL, Dunn F (1978) J Acoust Soc Am 64:423
71. Wu J, Nyborg WL (2008) Adv Drug Deliv Rev 60:1103
72. Marmottant P, Hilgenfeldt S (2003) Nature 423:153
73. Nyborg WL (1982) Br J Cancer Suppl 5:156
74. Azagury A, Khoury L, Enden G, Kost J (2014) Adv Drug Deliv Rev 72:127

75. Rooney JA (1970) Science 169:869
76. Brennen CE (1995) Cavitation and bubble dynamics. Oxford University Press, New York, p 282
77. Ferrara KW (2008) Adv Drug Deliv Rev 60:1097
78. Church CC, Yang X (2005) J Acoust Soc Am 117:2530
79. Nomikou N, McHale AP (2010) Cancer Lett 296:133
80. Diaz de la Rosa MA, Husseini GA, Pitt WG (2013) Ultrasonics 53:377
81. Kaviany M (1986) Int J Heat Mass Transfer 29:2002
82. Husseini GA, Runyan CM, Pitt WG (2002) BMC Cancer 2:20
83. Muniruzzaman MD, Marin A, Luo Y, Prestwich GD, Pitt WG, Husseini GA, Rapoport N(2002) Colloids Surf B 25:233
84. Rapoport N, Marin A, Luo Y, Prestwich GD, Muniruzzaman MD (2002) J Pharm Sci 91:157
85. Husseini GA, Myrup GD, Pitt WG, Christensen DA, Rapoport NY (2000) J Control Release69:43
86. Husseini GA, Diaz de la Rosa MA, Richardson ES, Christensen DA, Pitt WG (2005) J ControlRelease 107:253
87. Diaz de la Rosa MA (2007) High-frequency ultrasound drug delivery and cavitation, M.Sc. Thesis. Brigham Young University, Provo, UT
88. Diaz de la Rosa MA, Husseini GA, Pitt WG (2013) Ultrasonics 53:97
89. Parlitz U, Englisch V, Scheffczyk C, Lauterborn W (1990) J Acoust Soc Am 88:1061
90. Husseini GA, Rapoport NY, Christensen DA, Pruitt JD, Pitt WG (2002) Colloids Surf B24:253
91. Husseini GA, Abdel-Jabbar NM, Mjalli FS, Pitt WG (2007) Technol Cancer Res Treat 6:49
92. Stevenson-Abouelnasr D, Husseini GA, Pitt WG (2007) Colloids Surf B 55:59
93. Husseini GA, Abdel-Jabbar NM, Mjalli FS, Pitt WG, Al-Mousa A (2011) J Franklin Inst348:1276
94. Husseini GA, Mjalli FS, Pitt WG, Abdel-Jabbar N (2009) Technol Cancer Res Treat 8:479
95. Husseini GA, Diaz de la Rosa MA, Gabuji T, Zeng Y, Christensen DA, Pitt WG (2007) J NanosciNanotechnol 7:1028
96. Husseini GA, Diaz de la Rosa MA, AlAqqad EO, Al Mamary S, Kadimati Y, Al Baik A, PittWG (2011) J Franklin Inst 348:125
97. Husseini GA, Stevenson-Abouelnasr D, Pitt WG, Assaleh KT, Farahat LO, Fahadi J (2010) Colloids Surf A 359:18
98. Husseini GA, Kherbeck L, Pitt WG, Hubbell JA, Christensen DA, Velluto D (2015) J NanosciNanotechnol 15:2099
99. Ugarenko M, Chan CK, Nudelman A, Rephaeli A, Cutts SM, Phillips DR (2009) Oncol Res17:283
100. Zhang H, Xia H, Wang J, Li Y (2009) J Control Release 139:31
101. Wang J, Pelletier M, Zhang H, Xia H, Zhao Y (2009) Langmuir 25:13201
102. Marin A, Muniruzzaman M, Rapoport N (2001) J Control Release 75:69
103. Marin A, Muniruzzaman M, Rapoport N (2001) J Control Release 71:239
104. Marin A, Sun H, Husseini GA, Pitt WG, Christensen DA, Rapoport NY (2002) J ControlRelease 84:39
105. Munshi N, Rapoport N, Pitt WG (1997) Cancer Lett 118:13
106. Husseini GA, El-Fayoumi RI, O'Neill KL, Rapoport NY, Pitt WG (2000) Cancer Lett154:211
107. Pruitt JD, Pitt WG (2002) Drug Deliv 9:253
108. Husseini GA, O'Neill KL, Pitt WG (2005) Technol Cancer Res Treat 4:707
109. Howard B, Gao A, Lee S-W, Seo M-H, Rapoport N (2006) Am J Drug Deliv 4:97
110. Sivakumar M, Tachibana K, Pandit AB, Yasui K, Tuziuti T, Towata A, Iida Y (2005) CellMol Biol 51(Suppl):OL767
111. Tachibana K, Uchida T, Ogawa K, Yamashita N, Tamura K (1999) Lancet 353:1409

112. Tachibana K, Uchida T, Tamura K, Eguchi H, Yamashita N, Ogawa K (2000) Cancer Lett149:189
113. Taniyama Y, Tachibana K, Hiraoka K, Aoki M, Yamamoto S, Matsumoto K, Nakamura T,Ogihara T, Kaneda Y, Morishita R (2002) Gene Ther 9:372
114. Taniyama Y, Tachibana K, Hiraoka K, Namba T, Yamasaki K, Hashiya N, Aoki M, Ogihara T, Yasufumi K, Morishita R (2002) Circulation 105:1233
115. Saito K, Miyake K, McNeil PL, Kato K, Yago K, Sugai N (1999) Exp Eye Res 68:431
116. Prentice P, Cuschierp A, Dholakia K, Prausnitz M, Campbell P (2005) Nat Phys 1:107
117. Stringham SB, Viskovska MA, Richardson ES, Ohmine S, Husseini GA, Murray BK, PittWG (2009) Ultrasound Med Biol 35:409
118. Bailey MR, Couret LN, Sapozhnikov OA, Khokhlova VA, ter Haar G, Vaezy S, Shi X,Martin R, Crum LA (2001) Ultrasound Med Biol 27:695
119. van Wamel A, Kooiman K, Harteveld M, Emmer M, ten Cate FJ, Versluis M, de Jong N(2006) J Control Release 112:149
120. Schlicher RK, Hutcheson JD, Radhakrishna H, Apkarian RP, Prausnitz MR (2010) Ultrasound Med Biol 36:677
121. Zhou Y, Kumon RE, Cui J, Deng CX (2009) Ultrasound Med Biol 35:1756
122. Sheikov N, McDannold N, Jolesz F, Zhang YZ, Tam K, Hynynen K (2006) Ultrasound MedBiol 32:1399
123. Hauser J, Ellisman M, Steinau HU, Stefan E, Dudda M, Hauser M (2009) Ultrasound MedBiol 35:2084
124. Meijering BD, Juffermans LJ, van Wamel A, Henning RH, Zuhorn IS, Emmer M, VersteilenAM, Paulus WJ, van Gilst WH, Kooiman K, de Jong N, Musters RJ, Deelman LE, Kamp O (2009) Circ Res 104:679
125. Nelson JL, Roeder BL, Carmen JC, Roloff F, Pitt WG (2002) Cancer Res 62:7280
126. Rapoport N, Pitt WG, Sun H, Nelson JL (2003) J Control Release 91:85
127. Chlebowski RT (1979) West J Med 131:364
128. Myhr G, Moan J (2006) Cancer Lett 232:206
129. Gao Z, Fain HD, Rapoport N (2004) Mol Pharm 1:317
130. Gao ZG, Fain HD, Rapoport N (2005) J Control Release 102:203
131. Gao Z, Kennedy AM, Christensen DA, Rapoport NY (2008) Ultrasonics 48:260
132. Staples BJ (2007) MSc Thesis, Brigham Young University, Provo, Utah, USA
133. Staples BJ, Pitt WG, Roeder BL, Husseini GA, Rajeev D, Schaalje GB (2010) J Pharm Sci99:3122
134. Staples BJ, Roeder BL, Husseini GA, Badamjav O, Schaalje GB, Pitt WG (2009) CancerChemother Pharmacol 64:593
135. Staples BJ, Roeder BL, Pitt WG (2006) In: Annual meeting of the society for biomaterials.Pittsburgh
136. Hasanzadeh H, Mokhtari-Dizaji M, Bathaie SZ, Hassan ZM (2011) Ultrason Sonochem18:1165
137. Chen L, Sha X, Jiang X, Chen Y, Ren Q, Fang X (2013) Int J Nanomedicine 8:73
138. Chen H, Chen M, Tung F, Liu T (2015) J Med Chem 58:3704
139. Elkhodiry MA, Momah CC, Suwaidi SR, Gadalla D, Martins AM, Vitor RF, Husseini GA(2015) J Nanosci Nanotechnol 15:1–19
140. Ledet G, Mandal T (2012) US Pharm 37:7
141. Hamaguchi T, Matsumura Y, Suzuki M, Shimizu K, Goda R, Nakamura I, Nakatomi I, Yokoyama M, Kataoka K, Kakizoe T (2005) Br J Cancer 92:1240