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Drug Delivery Systems Based on Polymeric Micelles and Ultrasound: A Review

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Abstract: ***Background***: Anti-neoplastic drugs used for cancer treatment often produce damage to healthy cells, leading to severe side effects in patients undergoing chemotherapy. The encapsulation of these agents in nanoparticles reduces the adverse side effects of conventional chemotherapy on healthy tissues. Such nanoparticles, considered as drug delivery vehicles, are diverse and include micelles, liposomes, dendrimers, nanocapsules, nanospheres and others. Polymeric micelles have been widely researched as nanocarriers for hydrophobic drugs. They can be designed to have increased stability and blood circulation time, as well as binding specificity to certain receptors overexpressed on the surface of cancer cells. Once these drug-encapsulating nanoparticles reach the tumor site, an external stimulus, such as ultrasound, can be used to spatially and temporally trigger drug release. ***Methods***: This review paper focuses on the recent advances of cancer drug delivery systems employing polymeric micelles and ultrasound. An extensive literature review was performed mainly using PubMed. The introduction explains how nanocarriers are related to chemotherapy and the several modalities of use for this application. Afterwards, the review focuses on polymeric micelles used for drug delivery, their advantages and disadvantages. Subsequently, the physics of ultrasound is briefly reviewed, as well as the way it interacts with polymeric micelles to trigger the delivery of the drug transported by these nanocarriers. The following section focuses on targeting, discussing the several ways by which the nanoparticles can be directed to the target cells, and there deliver their cargo. Finally, a selection of relevant *in vitro* and *in vivo* studies, as well as clinical trials, is presented and discussed. ***Conclusion***: Although there are still several research studies to be performed before the combination of micelles and ultrasound can enter clinical trials, the future of controlled delivery using this drug delivery system is promising as a way to reduce the mortality and morbidity of cancer and the noxious side effects of conventional chemotherapy.

Keywords: Cancer, chemotherapy, drug release, polymeric micelles, triggered release, ultrasound.

1. INTRODUCTION

In the human body, cells are continuously regenerated in a cycle where older cells die in accordance with genetic programming that defines their life cycle. In some cases mutations occur in genes related to regulation, cell division and death, which may lead to abnormal or uncontrolled cell growth, thus producing tumors in tissues and organs [1, 2]. For example, one of the main players in the cell cycle regulation is the gene p53, and it has been observed that mutations in this gene lead to p53 proteins that acquire oncogenic properties. The mutant proteins participate in processes that promote metastasis – spreading of abnormal cells, proliferation and cell survival, which are characteristics of malignant tumors, i.e., cancers [3].

Cancer is the leading cause of death in Europe and North America, while being the second leading cause of death in the less developed countries, especially in Africa [4, 5]. The treatment of cancer often involves subjecting the patient to a chemical treatment (chemotherapy), which usually requires repeated doses of drugs. Although chemotherapy may be effective in cancer treatment, it also has unwanted effects on healthy cells and organs [6]. Hence, scientists have been searching for better drug delivery methods in order to decrease these side effects, without compromising the efficacy of the chemotherapeutic drug in killing cancer cells. Such drug delivery systems (DDS) include the use of nanoparticles that sequester the drug inside their structure and release it at the tumor site using internal and/or external stimulus. This helps in decreasing the damage caused to healthy cells because it localizes the effect of chemotherapy on the tumor cells by controlling the time and space in which the drugs are released [7-10].

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1381-6128/16 $58.00+.00 © 2016 Bentham Science Publishers

There are many types of nanosized drug carriers that can be used in chemotherapy, including micelles, liposomes, nanospheres, nanocapsules and dendrimers (Fig. **1**). Following liposomes, micelles are the second most commonly used nanoparticles in drug delivery [11], and are the focus of this review. Controlled drug delivery via liposomes has been recently reviewed by our group, particularly the combination of these lipid bilayer-based nanocarriers and the use of ultrasound (US) as a triggering mechanism [12, 13]. Drugs can also be delivered from solid or solid-shell particles, such as nanospheres or nanocapsules, made of a polymeric shell or matrix within which the drug is encapsulated. Dendrimers are highly branched molecules, which form cavities where the drug can be sequestered. The drug can also be conjugated to the exterior surface of the dendrimer [14].

The present review is concerned with drug delivery from micelles. Micelles are colloidal dispersions of self-assembled amphiphilic molecules, i.e., molecules with two distinct regions with opposite affinities towards a solvent, e.g. hydrophilic and hydrophobic [15]. The sizes of these assemblies are determined by their composition and associated packing geometry, and usually range from 5 to 100 nm in diameter [15]. Polymeric micelles are made up of polymers that impart specific characteristics. Block copolymers of amphiphilic composition self-assemble into core-shell structures with the hydrophilic blocks forming the external layer. The type of drugs that can be encapsulated inside these carriers depends on the micelle geometry and the hydrophobic character of the drugs that partition to the core [16].

Modifications on drug carrier systems can improve the results of the drug treatment. The modifications can be done to the carrier itself, for example by adding targeting moieties to improve their capability to bind to tumor cells. This is called *active* or *ligand targeting*, and one example is the folate moiety, which binds the folate receptor overexpressed on several types of cancer cells. Additionally, external factors including ultrasound (US), hyperthermia

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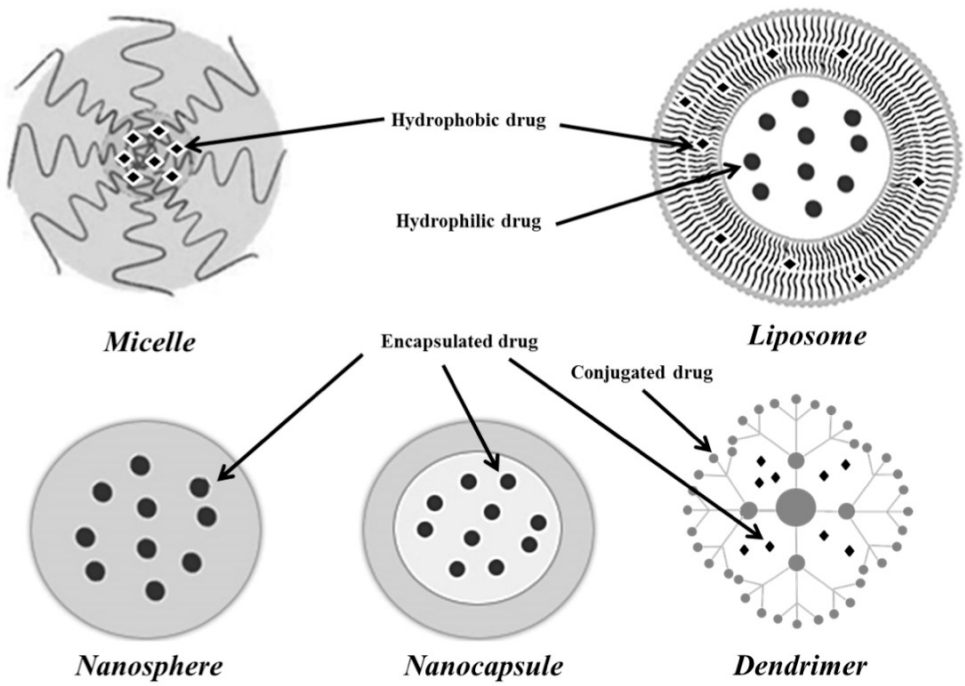

Fig. (1). Different types of nanoparticles used as drug carriers [17].

and magnetic fields, can be used to trigger the drug delivery, a process called *triggered targeting* [17, 18].

Some of the most common drugs used in chemotherapy belong to the anthracycline family, and Doxorubicin (Dox) is widely used with high efficacy against several types of cancer [19]. However, it has several noxious side effects including cardiotoxicity and nonspecific action throughout the body [20, 21].

2. POLYMERIC MICELLES

Micelles, especially polymeric micelles, have been extensively studied as drug carriers nanosystems [6]. The classification of micelles is based on the type of intermolecular forces involved in their formation. In general, there are three types of micelles: amphiphilic micelles with predominance of hydrophobic interactions, polyion complex micelles which mainly have electrostatic interactions, and micelles stemming from metal complexation [22]. Polymeric micelles belong to the first category, and as mentioned, consist of amphiphilic block copolymers that self-assemble into core-shell structures.

The term micelle was first used to describe the self-assembled structures of small surfactant molecules [23]. At low concentrations, these molecules exist as monomers but when concentration increases, their hydrophobic tails tend to avoid water, thus aggregating in structures in which the hydrophobic parts are shielded, while the hydrophilic ones are exposed to water. This process, which is thermodynamically and entropically favorable, is called micellization [24]. The concentration at which micelles are formed is called *critical micellar concentration* (CMC) [25]. The micelle stability is driven by van der Waals forces between the hydrophobic regions [15], and the establishment of hydrogen bonds between the hydrophilic shell and water molecules [26]. Hence, micelles are characterized by a core, resulting from the aggregation of the hydrophobic regions, and a shell composed of the hydrophilic parts, exposed to the aqueous medium. The core of the micelle can solubilize poorly soluble drugs, while polar molecules can be adsorbed on the surface [9].

Polymeric micelles are, similar to low-molecular-weight surfactant-based micelles, characterized by a hydrophobic core - hydrophilic shell/corona structure. These micelles are made of amphiphilic block copolymers, developed as a result of the advances in polymer chemistry that occurred during the last 3 decades of the 20th century. These copolymers allow the formation of a wide variety of nanostructures, and have several applications not only in pharmaceutical sciences, but also in coatings and paint formulations and personal care products, among others [27]. Amphiphilic block copolymers consist of at least two regions with different chemical properties, which allow them to self-assemble into micelles that may serve as nanocarriers to poor water-soluble compounds [28].

By the end of the 1990s, most micellar pharmaceutical research had focused on A-B diblock copolymers. Triblock copolymers with a structure A-B-A, have also been extensively studied in the last few decades, since they also self-assemble into micelles which can be used as nanocarriers [26, 29, 30]. The most used hydrophilic blocks are poly(ethylene glycol) (PEG) and poly(ethylene oxide) (PEO), while the core-forming hydrophobic blocks may be poly(Lamino acids) (e.g., poly(L-aspartate), poly(L-lysine) and derivatives, poly(esters) (e.g., poly(lactic acid), poly(-caprolactone), poly(glycolic acid) or poly(propylene oxide) [26, 28, 31].

Within the family of polymeric micelles, one of the most widely studied, for drug delivery purposes, is the family of Pluronic®-based micelles, which is available commercially [28, 29, 32]. These are triblock copolymers composed of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), PEO-PPO-PEO, which when dissolved in water form a hydrophobic core and a hydrophilic shell, as illustrated in Fig. (**2**). Hence, hydrophobic drugs can be encapsulated inside the core of the micelle [6, 33]. An advantage of using Pluronic® micelles as chemotherapy carriers (compared to other carriers) is their inherent size (10 to 200 nm), which allows them to escape renal excretion and yet still extravasate at the tumor site. Additionally, the incorporation of the drugs inside their core is a simple process due to the hydrophobichydrophobic interactions between the drug and the PPO core [29, 34]. Furthermore, micelles are easy to prepare, their shelf life is long [29] and, at low concentration, they can sensitize multi-drug resistance (MDR) in cancer cells [35-37]. One of their main disadvantages, however, is that they disassociate if diluted below their

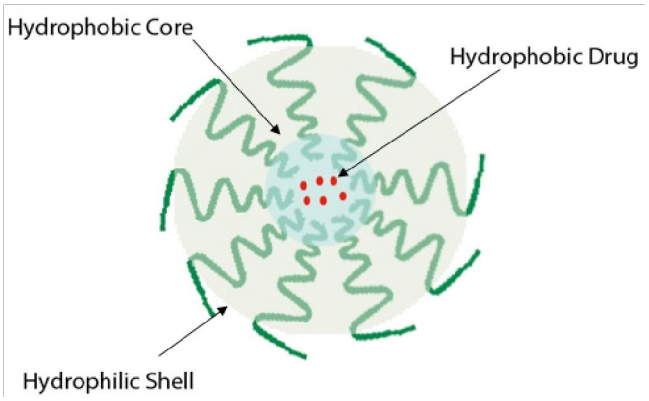

Fig. (2). The structure of a polymeric micelle [33]. The hydrophobic polymer forms the core of the micelle, where the drug is encapsulated, while the hydrophilic chains face the outside, providing stability.

CMC, which makes them unstable when diluted to low concentrations, such as when injected into blood [34, 38].

The literature suggests the importance of determining the micellar stability in order to know if their structure can release and reencapsulate the drugs once the micelles reach the intended site. Also, it is important to study the effect of stability in relation to the rate of elimination of Pluronic® chains from the body after the drug has been released at the tumor site. The stability of Pluronic® micelles can be enhanced by cross-linking their core via emulsion polymerization using poly(N,N-diethyl acrylamide) (pNNDEA) as an example [38, 39]. The stability of these micellar structures can be determined using the fluorescent probe diphenyl-1,3,5hexatriene (DPH) and dynamic light scattering [38, 40].

This review focuses on Pluronic® P105 which is the most common copolymer used in acoustically activated micellar drug delivery. These micellar carriers are ideal for US-triggered drug release because they are sensitive to low-frequency US, releasing the drug, and the drug can be quickly re-encapsulated when the insonation stops [30]. However, other polymeric micelles have been studied as US-sensitive nanoparticles, including the diblock copolymers poly(ethylene oxide)-*block*-poly(2-tetrahydropyranyl methacrylate) (PEO-*b*-PTHPMA), poly(ethylene oxide)-*block*-poly[1(isobutoxy)ethyl methacrylate] (PEO-*b*-PIBMA), poly (ethylene oxide)-*block*-poly[(2-tetrahydrofuranyloxy)ethyl methacry-late] (PEO-*b*-PTHFEMA), poly(ethylene oxide-*block*-poly(methyl methacrylate) (PEO-*b*-PMMA) [41], poly(lactic acid)-*block*poly(ethylene glycol) (PLA-*b*-PEG) [42] and others which are further discussed in Section 5.1.

In Pluronic® P105, both flanking PEO blocks contain 37 repeat units and the central block has 56 units of PPO, i.e., the structure is PEO_{37}-PPO_{56}-PEO_{37}. The features that make Pluronic® P105 a good choice over the other copolymeric micelles are its low toxicity, quick formation once dissolved in water, and stability above the CMC due to its hydrophobic core [6]. In this case, the CMC is defined as the minimum concentration of block copolymer that will result in micelle formation. The stability of these micelles is a function of the CMC, especially when the copolymer is diluted in bodily fluids. At room temperature, the CMC of Pluronic® P105 is close to 1 wt% [34]. The method of incorporating anti-neoplastic chemotherapeutics inside P105 micelles is relatively easy. First, a filtered stock solution of P105 of known concentration is prepared in a phosphate buffered saline (PBS) solution. Subsequently, a solution of the drug is added to the micellar solution, which results in micelles encapsulating the drug at a prescribed concentration [43].

3. ULTRASOUND

Ultrasonics is a branch of acoustics that studies sound pressure waves in a range of frequencies higher than the upper limit of human hearing, which is considered to be 20 kHz [44]. Ultrasonic waves have the same physical properties as other waves, so they can be reflected, absorbed, refracted and focused [44].

Ultrasound has been widely used in medicine mainly as an imaging technique [45], but its use in the last three decades has dramatically increased due to the discovery of possible therapeutic applications, especially in cancer treatment. It has been observed that US has a synergistic effect on chemotherapeutic efficiency, while contributing to a decrease in the side effects of conventional chemotherapy [29]. Ultrasound can be categorized as high-intensity or low-intensity, and high-frequency (> 1 MHz) or low-frequency (20 – 100 kHz). Low-intensity US is used for medical imaging and, to a lesser extent, for therapeutic purposes, while high intensity US is studied as a treatment directed toward many types of cancers. For a given intensity, higher frequency produces better imaging resolution, but the penetration depth of the wave decreases [46]. Recently, high frequency US (HFUS) is gaining more attention in cancer treatment due to the fact that it is easily focused on the tumor in comparison with low frequency US (LFUS) [47-49]. This characteristic of HFUS aids in efficiently controlling the release of chemotherapeutic agents from drug carriers, as will be explained shortly. Furthermore, as already stated, an increase in the US frequency allows the acquisition of clearer images of the tumor, which helps in directing the drug release process without the need for additional instrumentation [50]. Additionally, HFUS enhances the drug uptake in cells and tissues and facilitates the dissolution of blood clots for the treatment of strokes [51]. Another important US advantage is the non-invasive nature of this technique which avoids the disadvantages and complications of surgery [45].

When US is used in DDS it may cause both thermal and nonthermal effects on the body organs and tissues. The thermal effect is usually referred to as hyperthermia and is a result of the energy absorption by tissues and body fluids, causing a rise in temperature that may lead to the death of healthy cells. By selecting the proper US parameters, including its frequency and intensity, hyperthermia can be controlled and used as an effective modality in cancer treatment [6, 12, 52]. Furthermore, in some DDS, the drug carriers are synthesized to exhibit sensitivity to changes in temperature, such as temperature sensitive liposomes. Using US to heat the tumor site, temperature-sensitive drug carriers found in the vicinity of the tumor will be induced to release their content, thus mediating the process of drug delivery [53-55].

The non-thermal effects of US are mostly related to the cavitation of gas bubbles, which is their oscillation and possible collapse in an acoustic field [12, 52]. Several studies attribute the release of molecules from micelles exposed to US to ultrasonic cavitation [6].

There are two main categories of cavitation: *stable cavitation* which is the continuous oscillation of the bubbles without collapsing, and *collapse (or inertial) cavitation*, where the bubbles collapse aggressively, generating shock waves and causing large local temperature and pressure increases. Stable cavitation occurs at low US intensities, while collapse cavitation happens at higher intensities [56, 57]. When a microbubble collapses, a shock wave is generated along with an extreme increase in the local temperature for a very short period. This shock wave propagates in the surrounding medium, where micelles may be found. If micelles happen to be in the vicinity of the shockwave, they get destroyed as the polymeric chains, forming the micellar structure, lose their association due to the high energy generated by the wave. This leads to the release of the encapsulated drug [58]. The effect of a collapse cavitation shock wave on a micelle is illustrated in Fig. (**3**).

The US-triggered release of drugs from micelles can be divided into three phases. The first phase is a rapid release of most of the encapsulated drug. The second phase entails a slower release, while the third and final phase includes a reformation of the micelle and re-encapsulation of some of the drug molecules. The graphic in Fig. (**4**) shows these three phases [59].

A very promising aspect of the use of US as a trigger mechanism is that the drug is quickly re-encapsulated into reformed micelles when the US is turned *off* [39]. The fraction of drug that does not enter nearby cancer cells can re-enter the micelles, hence decreasing its effect on healthy cells in other locations in the body.

4. TARGETING

In micellar systems there are many factors that can be studied and manipulated to achieve the best delivery, including micellar composition, tumor location and the drug itself. Micellar nanocarriers may be used in *passive*, *triggered* and *ligand* (also referred to as *active*) *targeting*. The mechanisms involved in each general category should be understood to obtain and leverage the best treatment results when using nanoparticles in chemotherapy [6, 60].

In *passive targeting* the particles circulating in the bloodstream accumulate in a desired location based on mechanical flow and deposition, not on chemistry. Passive targeting to tumors is based on the increased permeability of the vasculature observed in some tumors. This permeability varies depending on the condition and the type of tissue and tumor. The presence of a leaky vasculature at the site of the tumor facilitates the extravasation of the nanoparticles carrying the chemotherapeutic agent, which will enhance the relative release of drugs to the region and increase the local concentration, resulting in more cellular uptake. This phenomenon of particle collection is known as the *enhanced permeation and retention* (EPR) effect [61, 62].

In addition, tumor-targeting may be enhanced by modifying the micelle surface with ligands that are specific to receptors present on the membrane of the cancer cells [63]. This type of modification, called *ligand targeting*, is an *active targeting* strategy that can be generally defined as the use of targeting moieties or ligands to attach nanoparticles to the target site [64]. Choosing the best targeting moiety is important because it affects nanoparticle properties including circulation half-life, cellular uptake, binding affinity, and extravasation [65]. Several types of moieties have been investigated including small peptides, antibodies (*immunomicelles*), hormones, aptamers and low molecular weight ligands such as folate [66]. Folate (folic acid, vitamin B9), a molecule essential for several processes including cell growth and division, is one of the most studied small molecules used in ligand targeting [67-70]. Its use in drug delivery and targeting is a result of its strong binding to the folate receptor (FR), which is widely expressed on the surface of some cancer cells, including ovarian, brain, kidney, breast, lung and others [71, 72]. Folate binds with high affinity to the FR. Employing folate in a DDS renders it more useful for both imaging [73] and therapeutic purposes [74].

Another approach to active tumor targeting is *triggered targeting*, by which the drug release from micelles to the intended tumor is enhanced by stimuli, including electric field, pH, temperature and ultrasound [75]. The selection of the trigger to be used depends on the type of micelles, the anti-neoplastic agent, and the surrounding environment of the tumor [25]. Ultrasound, which has been previously discussed, is considered an attractive trigger mechanism in drug delivery because it is non-invasive and capable of disrupting micelles and releasing the drug from their core [76].

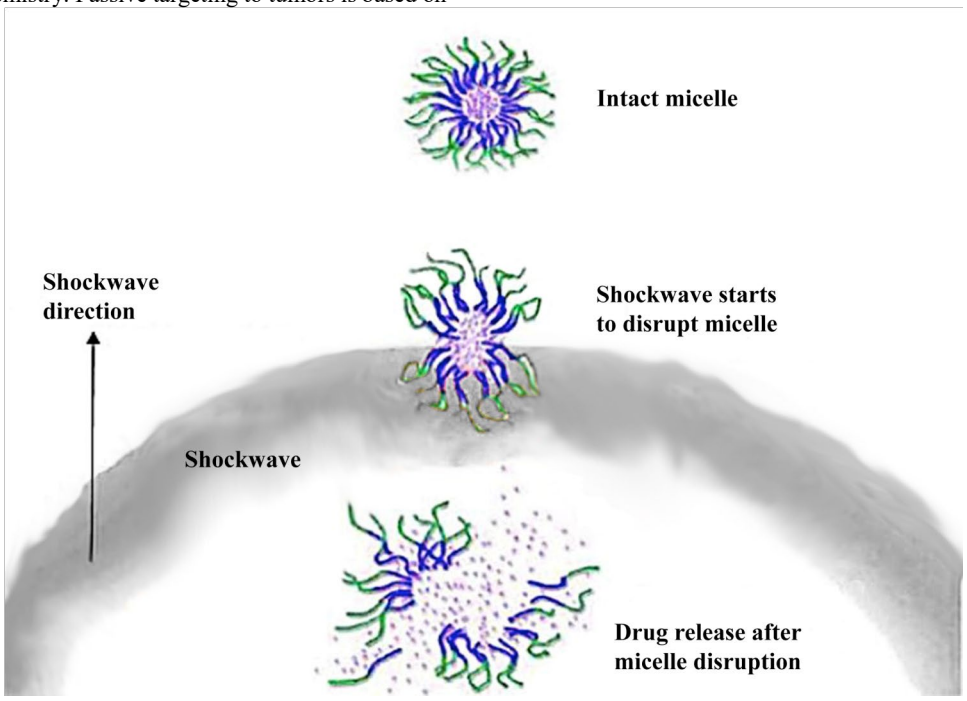

Fig. (3). The effect of cavitation events on the release of drugs from polymeric micelles [29].

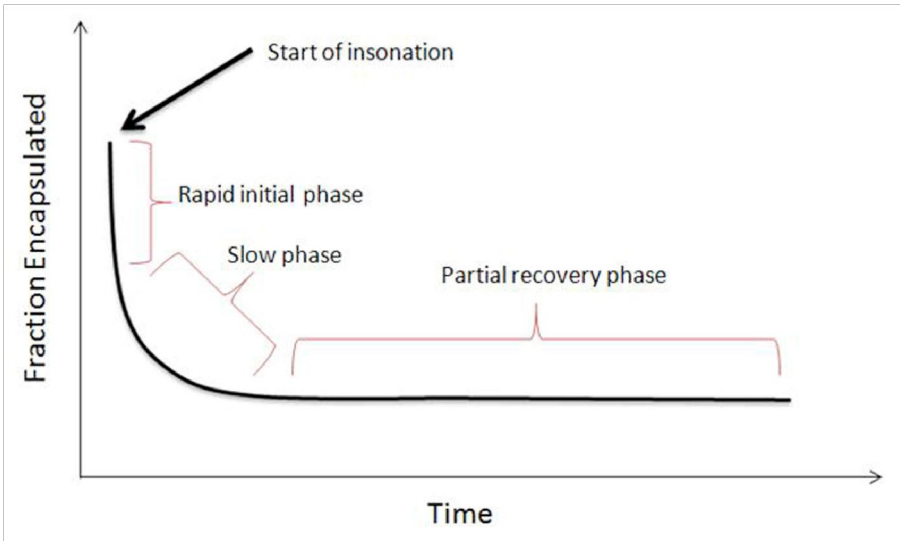

Fig. (4). Phases of drug release from micelles in the presence of US [56]. The graph shows the effect of cavitation events on the release of drugs from a collection of polymeric micelles in a large volume [29].

5. RELEVANT *IN VITRO* AND *IN VIVO* WORK AND CLINICAL STUDIES

There are several scientific reports describing the use of ultrasound to release drugs from polymeric micelles *in vitro* and *in vivo* [6, 12, 29, 30, 76]. This section will focus on some of the most important studies in this area.

5.1. *In Vitro* Studies

Several *in vitro* studies have been published regarding the release of drugs from micelles upon the application of US, and different factors and environments were studied to improve this DDS. Some of these factors are related to US, including power density, frequency and duration of sonication, while others are related to the polymer properties and the drugs used. All of these studies aim to make these systems suitable and safe for human use [12, 30]. Additionally, *in vitro* studies using cell cultures are very useful in order to design *in vivo* experiments.

Many *in vitro* drug release and cancer treatment research studies use fluorescence techniques to quantitate drug release in one of two ways: (i) the *offline* method where the sonication of the sample is done externally and then the fluorescence level is measured afterwards; and (ii) the *online* method which involves the use of a sonication chamber fitted with a fluorescence measurement device [57]. In the sonication chamber, the ultrasonic exposure of the sample happens while simultaneously and continuously monitoring the fluorescence level using some kind of fluorescence detector. Doxorubicin is an example of a fluorescent chemotherapeutic agent, absorbing light at 488 nm and emitting fluorescent light between 530 and 630 nm [77, 78]. Ultrasound is applied to drug carriers using a transducer with a known frequency, applying a power density measured by a hydrophone. Fiber optics guide laser excitation light to the sample, and also collect the fluorescent emissions during the application of US; the data are usually collected, stored and analyzed offline [79, 80]. A simplified diagram of such an US chamber with fluorescence detection is shown in Fig. (**5**).

5.1.1. Factors Affecting Drug Release

The effect of US frequency on the release of drugs from polymeric micelles has been widely studied by Pitt and co-workers (reviewed in [6, 12, 30]), and the results show that the release of drugs encapsulated in polymeric micelles increases as the ultrasonic frequency decreases (at constant intensity). Husseini *et al.* [76] prepared Pluronic® P105 micelles containing either Dox or its paramagnetic analogue Ruboxyl, and exposed them to different frequencies of US, in the range of 20 to 90 kHz. The group also studied the effect of varying the power density (0 to 3 W/cm^2) and observed that, at a constant frequency, an increase in power density caused an increase in drug release. It was also observed that at micellar concentrations lower than 0.1 wt%, the release was higher compared to the higher Pluronic® concentrations. In studies of varying pulse duration, it was observed that, above a threshold of 0.1 s, there was increased release with increasing pulse duration up to 0.5 s. Above this pulse length, the release was not different than with application of continuous wave (CW) US. Furthermore, it was observed that the drug was re-encapsulated between pulses, which suggests that perhaps *in vivo*, released drug that is not taken up by cells may re-enter the micelle and decrease the harmful effects associated with Dox on adjacent non-cancerous tissues. A later study [81] using a fluorescence detection exposure chamber similar to the one described earlier (Fig. **5**) investigated the release and reencapsulation of Dox from Pluronic® P105 micelles upon exposure to 20-kHz US at a power density of 58 mW/cm^2. The results showed that exposure to US for a period shorter than 0.1 s, did not induce release of Dox from the micelles, similar to the result obtained in the previous study [76]. Above this threshold, the release increased with the pulse length, up to 0.6 s. On the other hand, the minimum *off* pulse for the re-encapsulation to occur was 0.1 s. The data were used to develop a mathematical model for this polymeric system and the model showed a zero-order release and first-order re-encapsulation kinetics. The model was further investigated by the same group [59] and four simultaneous mechanisms were proposed using the assumption of four different micelle sizes. These mechanisms were divided into two parts, the first included destroying and re-assembly of the micelle, while the second included the releasing and re-encapsulation of Dox. It was assumed that the micelles' destruction was due to the cavitation events. The kinetic model built for these mechanisms was consistent with the assumption that the collapse cavitation plays a strong role in the release phenomena observed experimentally. Another modeling study by the same group [82] used an artificial neural network (ANN) model to predict Dox release from the same type of micelles, upon exposure to 20-kHz US at different power densities. This model was then used to optimize the US parameters needed to achieve a target drug release at the tumor site.

The fact that polymeric micelles are not stable upon dilution in blood led researchers to design and synthesize stabilized polymeric micelles. Pruitt *et al.* [38] synthesized NanoDeliv™, P105 micelles stabilized with the cross-linking agent pNNDEA, and the same group later [39, 83] compared the release from these stabilized micelles and the unstabilized Pluronic® P105. Using 70-kHz US they observed that the release from both types of micelles was similar [39]. Another study using the same US frequency [83] compared the Dox release from unstabilized and stabilized polymeric micelles (NanoDeliv™ and micelles of PEO-*b*-poly(N-isopropylacrylamide)-*b*-poly(oligolactylmethacrylate) with stabilized cores). The study of the release kinetics showed that the Dox release rates from unstabilized micelles were significantly higher than those from stabilized ones, but that the onset of release occurred at the same ultrasonic power density for all micelles studied.

A different type of stabilized micelles was developed by Ugarenko *et al.* [84], who studied the release of Dox and formaldehyde-releasing prodrugs from stabilized Pluronic®-DSPE-PEG2000 mixed micelles, using 20-kHz US at high power densities (100 W/cm^2). They observed that when micelles were formed, 60% of Dox was encapsulated with a retention half-life of approximately 12 hours. It was observed that at these high US power densities, 7-10% of the encapsulated Dox was released. On the other hand, the formaldehyde-releasing prodrugs were not encapsulated inside the micelles, but they could be used separately to enhance the formation of Dox-DNA adducts in tumor tissues.

Besides Pluronic® P105, other block copolymers have been used to form drug delivering micelles, and the effect of US on the release of encapsulated drugs has been studied. For example, Zhang and co-workers [42] synthesized polymeric micelles using a block copolymer PLA-*b*-PEG, loaded with the Nile Red stain. They used high-intensity focused ultrasound (HIFU) to trigger the release from these nanoparticles. Their results showed that the HIFU can trigger the irreversible release of Nile Red through mechanisms related to transient cavitation. The release could be controlled by tuning the properties of US including intensity, time and the location where it is focused. Recently, Xuan and co-workers [41] studied the effect of 1.1-MHz HIFU in several different polymeric micelle formulations, using PEO-*b*-PTHPMA, PEO-*b*-PIBMA, PEO-*b*-PTHFEMA, and PEO-*b*-PMMA block copolymer micelles. Their results showed that the PEO-*b*-PMMA micelles are more resistant to disruption induced by HIFU, when compared to the other formulations. The same group [85] described a DDS based on 1.1-MHz HIFU and on micelles of a diblock copolymer of PEO and poly(2-(2methoxyethoxy)ethyl methacrylate) ($PMEO_2MA$) with some added units of the 2-tetrahydropyranyl methacrylate (THPMA). The results showed an amplification of the effect of HIFU exposure on the disassembling of the micelles. The exposure to HIFU leads to the hydrolysis of the hydrophobic THPMA groups into hydrophilic methacrylic acid, thus increasing the lower critical solution temperature of the core $PMEO_2MA$ and leading to the disassembly of the micelles.

Wang *et al.* [86] also studied the effect of 1.1-MHz HIFU in the disruption of micelles of a diblock copolymer made of PEO and poly(2-tetrahydropyranyl methacrylate) (PEO-*b*-PTHPMA). The micelle disruption was due to the US-induced hydrolysis of the PTHPMA, which was attributed to the cleavage of tetrahydropyranyl groups.

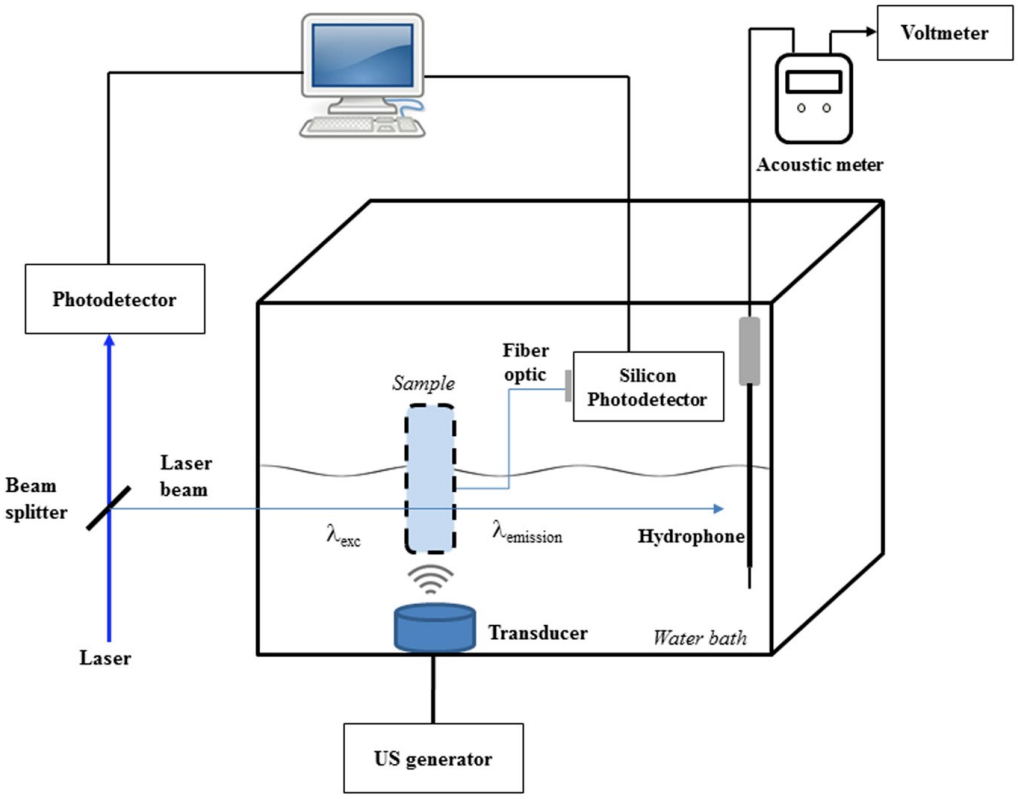

Fig. (5). Ultrasonic exposure chamber [76, 79]. The schematic shows an ultrasonic exposure chamber with fluorescent detection. The sample – micelles with an encapsulated fluorescent drug – is kept in a glass cuvette. The fluorescence of the drug is excited with a laser beam. Drug release is induced by an US probe situated under the cuvette, and is quantified by measuring the fluorescent emission detected by a fiber optic probe, filtered and digitized in a silicon photodetector. The laser power is monitored using a monitor photodetector. Additionally, a hydrophone is used to measure the characteristics of the ultrasound wave, such as frequency and amplitude, by converting it into electric signals.

The HIFU-triggered release of hydrophobic molecules from non-cross-linked and core cross-linked micelles of PEG-*b*-poly[N(2-hydroxypropyl) methacrylamide-lactate] (PEG-*b*-p(HPMAmLac(n))) was recently reported [87]. The results showed that both continuous wave (CW) high frequency US and pulsed 1.5-MHz HIFU triggered the release of the hydrophobic molecule Nile Red from both cross-linked and non-cross-linked micelles. However, the release from the stabilized, cross-linked micelles was lower than that observed from non-cross-linked ones. The results were not attributed to cavitation and/or hyperthermia as the main release mechanisms; instead the authors proposed that the radiation forceinduced shear forces at the walls of the exposure chamber causes the transient destabilization of the micelles, leading to release of their contents. The release from micelles with a densely crosslinked core is lower, since these micelles are stabilized and are more difficult to destabilize than non-stabilized micelles.

Targeted polymeric micelles combined with US have also been recently described as an efficient DDS [60]. Pluronic® P105 micelles conjugated to a folate moiety were synthesized and used to study the relationship between the intensity of 70-kHz US and the kinetics of Dox release. The results suggested, similar to the case of non-targeted micelles, an important role of cavitation in the drug release. Additionally, it was observed that, using the same US conditions, the release from folated micelles was higher than from nonfolated ones [78]. The same author described a model to predict the acoustic drug release from non-targeted and folate-targeted polymeric micelles, using 70-kHz US at several different power densities [88].

5.1.2. Polymeric Micelles and US: Effect on Cell Cultures In Vitro

The first *in vitro* study on the effects of the use of micelleencapsulated drugs on cancer cell cultures in conjunction with US, was described by Munshi *et al.* in 1997 [89], using leukemia HL-60 human cancer cells. The group reported a synergistic effect when using 80-kHz US and Dox encapsulated in Pluronic® P105, noting that the drug IC_{50} decreased in the presence of US.

The same cell line was used by Husseini *et al.* [43] to study the *in vitro* effect of Dox on the cellular DNA. As mentioned Dox is one of the most effective drugs against cancer, but it has severe side effects. This gave rise to the need for more directed DDS, hence the use of targeted micelles. Dox was delivered in two ways: the first was by directly adding it in its free state, and the second was by encapsulating it inside Pluronic® P105 micelles. The structure of the cellular DNA was measured by the comet assay, after experiments with and without exposure to 70-kHz US. No DNA damage was observed in cells exposed to micellar-encapsulated Dox in the absence of US, but free drug produced some DNA damage. However, it was observed that when Dox was delivered encapsulated in micelles under exposure to US, the DNA damage was significantly higher and 96% of the cancer cells were dead after 2 hours of exposure. Importantly, this study also established that the mode of cell death was apoptosis and not necrosis.

Marin *et al.* [90] studied the effect of CW and pulsed 20-kHz US on the uptake by HL-60 cells of free and Pluronic®encapsulated Dox. The results showed that both free and encapsulated drug uptake increased with power intensity, from 1.4 to 33 mW/cm^2. Using pulsed US it was observed that the Pluronic® micelles uptake increased with pulse duration, for 0.1 to 2 s pulses, while there was no significant effect on the uptake of free Dox. For both free and encapsulated Dox, there was no significant effect of the duration of the inter-pulse interval. The authors suggested two mechanisms for the US-induced uptake from Pluronic® micelles: (i) US-induced Dox release from micelles with an increase of free Dox in the medium; (ii) US-induced permeability of the cell membrane, with increase in the uptake of encapsulated Dox.

In another investigation, the relation between drug release and high frequency (1-MHz) US was examined [91]. The tests were conducted on different types of cancer cell lines including HL-60, drug-sensitive ovarian carcinoma and breast cancer MCF-7 cells. The group hypothesized that when US was applied, cavitation events occurred that could be monitored by quantifying the associated free radicals. It was observed that there was formation of radicals with changing frequencies and power densities. The cavitation threshold intensity increased as the US frequency increased, as evidenced by the formation of free radicals at different frequencies. It was noticed that at higher frequencies, although transient cavitation almost ceased to exist, drug release was still observed, indicating that drug release from micelles was not tied to transient cavitation. It was also found that the rate of cellular uptake of Dox was higher even at short time exposures to 1-MHz HFUS.

Howard and co-workers [92] synthesized micelles of methylcapped poly(ethylene oxide)-co-*poly*-(L-lactide)-tocopherol containing encapsulated Paclitaxel. They sonicated the micelles at 1MHz US with 1.7 W/cm^2 power density to study the efficacy of this DDS in killing human breast adenocarcinoma MCF-7 cells. The results showed that drug release from these micelles could be efficiently induced by US and also emphasized the importance of using micelles to encapsulate drugs, which aids in reducing the side effects observed when the drug is introduced in its free form.

Ugarenko *et al.* [84] examined Dox uptake by the breast cancer cell line MDA-MB-231 using Dox and formaldehyde-releasing prodrugs encapsulated in stabilized Pluronic®-DSPE-PEG2000 mixed micelles. The results showed that, in the absence of US, the uptake of the micellar system was reduced when compared to that of free Dox. On the contrary, upon application of 20-kHz, 100 W/cm^2 US, the drug was released from the micelles and the cellular uptake was increased.

Wan *et al.* [93] used a micellar formulation of methoxyPEG*block*-poly(D,L-lactide) (MePEG-*b*-PDLLA) to encapsulate paclitaxel and study the effect of 4-MHz HFUS at 32 W/cm^2 power intensity on the cellular accumulation in drug-sensitive and nonsensitive cell lines. There was an increase in drug uptake in both cell lines, induced by US, and globally the results suggested that US facilitates the uptake and retention of micelle-encapsulated drug.

5.1.3. Mechanism of Drug Release/ Cell Uptake

In vitro release studies suggest three different mechanisms by which US enhances the drug release from micelles and uptake by cells [12]. Although this subject has been extensively studied, different studies have shown different results, suggesting that different cells lines may respond differently.

The first proposed mechanism considers that the drug release induced by US occurs outside the cells, and is followed by the drug entering the cells by a normal diffusion process. Most research done in the area does not support this mechanism.

The second mechanism – sonoporation – states that the cell membrane is transiently perturbed by the application of US, allowing for the transport of released or encapsulated drugs into the cells. Several *in vitro* studies provided evidence that the use of US both releases the drug from the micelles and creates transient pores in the cell membranes through which the drug can enter into the cell cytosol. Tachibana *et al.* [94] and Yamashita *et al.* [95] conducted several experiments to study the effect of US on the permeability of cell membranes. In one of their earlier studies [96], HL-60 cancer cells were sonicated for 30 sec with 255-kHz continuous US, with a power density of 0.4 W/cm^2, in the presence of the fluorescent dye merocyanine 540. Using electron microscopy, the authors observed the formation of pores in the cell membranes that were thought to be

related to cell death. They later concluded that the cell death was caused by both the drug and the US treatment [95]. To confirm their theory, the same group conducted another experiment using the cytosine arabinoside drug with HL-60 cells sonicated at 48-kHz US with a power density of 0.3 W/cm^2 [94]. The results of this study showed that cell death increased upon sonication, when compared to a sonicated control sample that was not exposed to the drug. This suggested that cell membrane permeability increased when subjected to US, allowing for the diffusion of the drug through the transient membrane pores into their cytosol and leading to their death [94, 95]. The effect of US on cell membrane permeability was later studied by Schlicher *et al.* [97, 98]. This group used DU145 prostate cancer and primary human astrocyte (HA) cells. The used US conditions were: 20 pulses of 24-kHz US, each pulse with a duration of 0.1 s, a 10% duty cycle and different acoustic amplitude pressures (0.36, 0.54 and 0.71 atm), which are a measure of the US power [97]. The study concluded that cavitation was responsible for multiple forms of membrane wounding, which increased with acoustic pressure (*i.e.* ultrasonic power density). For the highest pressure used, nuclear ejection through the damaged membrane was observed, followed by cell lysis, which suggested that the size of the pores formed increased with the increase of cavitation events, which was proportional to the sonication intensity. Later, a study done by Zhou *et al.* [99] used the voltage clamp technique to measure the size of the pores formed by the application of 1.075-MHz US (0.2 s, 0.3 MPa) in *Xenopus laevis* oocytes and determined these pores to have a 110±40 nm radius.

A third proposed mechanism considers that endocytosis is the main cause of increased cell death due to the US-induced enhanced chemotherapeutic drug uptake. Several studies showed that there are no significant cell membrane deformations upon application of mild ultrasonication, which excluded sonoporation as the main mechanism of drug release and uptake [100, 101]. Results obtained by Muniruzzaman and co-workers [102] suggested that the uptake of micelles by cells occurs via fluid-phase endocytosis. The aim of their study was to test the effect of the aggregation state on the intracellular uptake of Pluronic® P105 micelles by HL-60 cancer cells. It was observed that, below the CMC, the cell uptake increased with the increasing concentration of micelles in the incubation medium, while above the CMC, the intracellular uptake was less efficient. This suggested that below the CMC the single Pluronic® chains enter the cell via diffusion through the cell membrane, while the micelles enter the cell via endocytosis. Similarly, the studies by Rapoport *et al.* [103] and Sheikov *et al.* [104] provided some evidence of endocytotic events.

In summary, the *in vitro* studies proved the synergism between micelle-encapsulated drugs and US. The application of US increases the release of drugs from the nanocarriers and simultaneously induces transient pores in the cell membranes, which can enhance the uptake of drug by the cells. The endocytosis hypothesis, however, cannot be discarded, and both mechanisms must be considered. A recent review by Lentacker *et al.* [105] states that the mechanism of drug uptake through pores depends on the US parameters and that the US intensity may be modulated to regulate the pore size. Low intensity US may induce the uptake of some drugs (or drug carriers) by endocytosis, while short high intensity US pulses has the capability to induce sonoporation with subsequent drug uptake directly into the cytosol.

5.2. *In Vivo* Studies

In vivo studies are extremely important as preclinical experiments, and the most common animal models used to conduct *in vivo* experiments are mice and rats inoculated with a cancer cell line. The lab animals are then treated with Dox or other chemotherapeutic drugs, encapsulated in micelles or in a free form, to compare the efficiency of the different DDS, with and without exposure to US.

One of the first *in vivo* studies with micelles and US used a colon carcinoma (DHD/K12/TRb cells) in a rat model to investigate the effect of Dox concentration, US frequency, and power density, among other variables [106]. The tumors were grown in the hind legs of the rat by injection with the tumor cells. After the tumors reached a desired size, the rat was treated with Dox at different concentrations, with and without application of US at the tumor site. The Dox was encapsulated into stabilized Pluronic micelles (NanoDeliv) and was administered weekly for a period of 6 weeks. One of the tumors on a leg was exposed to 20 or 70-kHz US, while the tumor on the other leg was not US-treated, corresponding to a negative control. In the group without any drug, the growth of the bilateral tumors was similar and increased exponentially over time. For the groups treated with Dox, it was observed that the higher concentrations of encapsulated Dox were lethal to the animals: 4 and 5.33 mg/kg were lethal within 6 weeks, while 8 mg/kg killed the animals within 2 weeks. The treatment with micelles encapsulating Dox concentrations of 1.33 and 2.67 mg/kg was not lethal to the animals. In these cases, it was observed that the tumors treated with 70-kHz US, 2 W/cm^2 had, on average, a lower growth rate than the non-insonated ones.

The same rat cancer model was used by Staples *et al.* [107] to investigate the effects of Dox encapsulated in stabilized Pluronic® micelles (NanoDeliv) triggered with low (20 kHz) and medium (476 kHz) frequency US. The study compared the results of treating the rats using micelles, with and without US, and it was observed that the tumor growth rate decreased when compared with tumors treated with micelles only (no US). The study also reported that tumor volumes could be fitted to an exponential growth model for both the control and insonated tumors at 20 kHz and 476 kHz. No differences in growth rates were observed for 20-kHz and 476-kHz US (with the same mechanical index, MI, and time-averaged power density). In a similar study [108], the results showed that exposure to 20- or 476-kHz US increased the drug concentration in the tumor, compared to non-insonated micelle-treated ones. However, 12 h after drug injection, no difference in the concentration of Dox was observed between insonated and non-insonated tumors. Additionally, the variation of US frequency did not show any effect on tumor growth: it was observed that frequencies of 20 kHz and 476 kHz (with the same MI and time-averaged power density) produced very similar results with respect to Dox concentration in the tumors and tumor growth rate.

A study by Rapoport and co-workers [109] investigated the advantage of using Dox encapsulated in Pluronic micelles and 1MHz HFUS to treat immuno-compromised athymic *nu/nu* mice, inoculated intraperitoneally with ovarian carcinoma A2780 cells. The Dox fluorescence level after sonication was measured in the tumor, heart, kidneys, liver and spleen and the results were compared to the case when the tumors were not sonicated. The objective of the study was to examine localized drug release controlled by US compared to the non-localized release due to the natural degradation of micelles. It was observed that micellar encapsulated Dox preferentially accumulated in the tumor, and that the tumor uptake was increased by exposure to US. Some fluorescence was detected in the liver but none in the heart. This is in contrast to what was observed when free Dox was used. Upon injection, Dox was found in the liver, spleen and all other organs of the peritoneal cavity. When insonated after injection, free Dox could be found in the tumors, kidneys, and also in the heart. These results confirmed that the cardiac toxicity of Dox can be greatly decreased when using micellar Dox instead of free Dox, and that focused US decreases the drug uptake by healthy tissues, while increasing the drug uptake locally.

Gao and co-workers [110] used unstabilized Pluronic® P105 and stabilized mixed micelles PEG-*b*-PLA (PEG2000diacylphospholipid, and PEG-co-poly(-benzyl-L-aspartate) micelles) to encapsulate Dox and study the intracellular uptake by an ovarian carcinoma A2780 tumor developed in *nu/nu* mice. The exposure to 1- and 3-MHz HFUS

lead to an increase in the intracellular concentration of drug in tumor cells. The combination of US and micellar Dox decreased the tumor growth rate and increased the mice survival rates.

The synergism between the use of encapsulated drugs and US was also revealed in a recent study by Hasanzadeh *et al.* [111] using a mouse model. In this study, adult female mice were inoculated with spontaneous breast adenocarcinoma tumors and were divided into three groups: (i) the tumor was treated with a 1.3 mg/kg dose of free Dox; (ii) the tumor was treated with the same concentration of Dox but encapsulated in Pluronic® P105 micelles; (iii) the tumor was treated using the same concentrations of encapsulated Dox and sonicated at simultaneous frequencies of 28-kHz (0.04 W/cm^2) and 3-MHz (2 W/cm^2) CW US. It was observed that the drug accumulation in tumor cells was enhanced in the third group compared to first and second groups by 8.7- and 2.6-folds, respectively. Another observation was that the uptake by the healthy cells and organs was lower in the third group: 3.4- and 2.5-fold when compared to the first and second groups, respectively. The results of this study clearly support the improvement that US along with micelles can provide when used as a DDS.

Table 1. Polymeric micelles drug delivery systems that have been clinically approved or are under clinical trials [112-114].

Polymer	Drug	Therapeutic indications	Name (clinical trial phase) or commercial name (approval date)	Notes
PEG-PLA	Paclitaxel	Breast, lung, ovarian cancers	Genexol-PM (2007, South Korea)	
NA*	TLC388	Liver, renal	Lipotecan (Phase 1/2)	
NIPAM-VP	Paclitaxel	Advanced breast cancer	Nanoxel (Phase 1)	Approved in India in 2006
PEG-P(Glu)	Oxaliplatin	Solid tumors, lymphoma	NC-4016 (Phase 1)	
PEG-PLA	Cisplatin	Pancreas cancer	NC-6004 (Phase 2/3)	
PEG-P(Asp)	Epirubicin	Solid tumors	NC-6300 (Phase 1)	pH sensitive
PEG-PGA	SN-38	Solid, small cell lung, breast cancer	NK-012 (Phase 2)	
PEG-PAA	Paclitaxel	Gastric, breast cancer	NK-105 (Phase 2/3)	
PEG-PAA	Dox	Solid tumors	NK-911 (Phase 1)	
PEG-PPO	Dox	Advanced adenocarcinoma	SP1049C (Phase 2/3)	Contains Pluronic as ligand

NIPAM, N-isopropyl acrylamide ; PAA, poly(acrylic acid); PEG, polyethylene glycol; P(Asp), poly(aspartate); PGA, polyglycolic acid; P(Glu), poly(glutamic acid); PLA, poly-(L-lactide); VP, vinylpyrrolidone; PPO, polypropyleneoxide.

*NA - not available, property of the company.

5.3. Clinical Trials and Approved Formulations Currently there are several ongoing clinical trials using polymeric micellar systems for drug delivery in cancer patients, as recently reviewed by Wicki *et al.* (Table **1**) [112]. Additionally, micelle-encapsulated Paclitaxel (a product called Genexol) has been approved by the FDA for the treatment of breast cancer in South Korea [25] while Nanoxel, also based on Paclitaxel, has been approved for the treatment of the same cancer in India. However, the application of US to micelle-encapsulated drugs has not reached the clinical trial stage yet.

CONCLUSIONS AND FUTURE DIRECTIONS

Micelles are a convenient carrier for hydrophobic drugs, particularly when the micelles are stabilized so they adequately retain their payload when diluted during infusion into blood or when injected directly into tissue. The Pluronic family of block copolymers has been used in micellar DDS, as well as some specialty synthesized block copolymers.

Ultrasound at low to moderate frequencies and relatively low intensities can by used to release the payload of the micelles. This is useful in drug delivery as the US can be focused on a tumor (or other tissue) and produce localized release. Accumulation of the micelles at the targeted site also increases the local release. Accumulation can be produced passively by the EPR effect observed in some tumors, and by attaching binding ligands to the micelles. The interaction between US and micelles appears to be related to acoustic cavitation, and may be produced by fluid movement or shock waves perturbing the structure of the micelles and releasing some drug. Cells may also be perturbed by US, leading to the increased uptake of drugs or micelles via holes in cell membranes or enhanced endocytosis. Efficacy in treating tumors *in vivo* has been observed using doxorubicin and other drugs, but this technique has not yet been applied in human clinical trials.

There are yet some unfinished research studies that should be done before translation to clinical trials. Perhaps first, and sometimes neglected in lab research, is the fate of the block copolymers forming the micelles. Research needs to be done to show that they are safely eliminated from the body and determine the rate of clearance. Another area needful of research is accumulation of drugloaded micelles in non-targeted tissues, such as the liver, spleen and glomerulus of the kidneys. More research should also be done in the physics of adequately delivering and focusing low and mid frequency US. While high frequency US can be focused well, it cannot penetrate tissues deeply, so it may not be available to treat deep tumors.

Despite these unanswered questions, we feel that the future of controlled delivery using US and micellar carriers is promising. All

avenues that may reduce the mortality and morbidity of cancer and the noxious side effects of conventional chemotherapy should be pursued.

ABBREVIATIONS

CMC	=	critical micellar concentration
CW	=	continuous wave (US)
DDS	=	drug delivery systems
EPR	=	enhanced permeation and retention
Dox	=	doxorubicin

HFUS	=	high frequency ultrasound
HIFU	=	high-intensity focused ultrasound
LFUS	=	low frequency ultrasound
MePEG-*b*-PDLLA	=	methoxyPEG-*block*-poly(D,L-lactide)
MI	=	mechanical index
PBS	=	phosphate buffered saline
PEG	=	polyethylene glycol
PEG-*b*-p(HPMAm-Lac(n))	=	PEG-*b*-poly[N-(2-hydroxypropyl) methacrylamide-lactate]
PEO	=	polyethylene oxide
PLA-*b*-PEG	=	block copolymer of poly-lactic acid and polyethylene glycol
$PMEO_2MA$	=	poly(2-(2-methoxyethoxy)ethyl methacrylate)
pNNDEA	=	poly(N,N-diethyl acrylamide)
PPO	=	polypropylene oxide
THPMA	=	2-tetrahydropyranyl methacrylate
US	=	ultrasound

CONFLICT OF INTEREST

The authors report no conflict of interest regarding the publication of this paper. The authors acknowledge the financial support of the Faculty Research Grant Type 1, from American University of Sharjah (to G.A. Husseini).

ACKNOWLEDGEMENTS

Declared none.

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Received: January 4, 2016 **Accepted: February 16, 2016**

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