Ultrasound-responsive Nanocarriers in Cancer Treatment- a review

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Abstract: The safe and effective delivery of anticancer agents to diseased tissues is one of the significant challenges in cancer therapy. Conventional anticancer agents are generally cytotoxins with poor pharmacokinetics and bioavailability. Nanocarriers are nanosized particles, a few of which have received clinical approval, which increase the selectivity of anti-cancer drugs and genes transport to tumors. They are small enough to extravasate into solid tumors where they slowly release their therapeutic load by passive leakage or by biodegradation. Using smart nanocarriers, the rate of release of the entrapped therapeutic(s) can be increased, and greater exposure of the tumor cells to the therapeutics can be achieved when the nanocarriers are exposed to certain internally (enzymes, pH and temperature) or externally applied (light, magnetic field, and ultrasound) stimuli that trigger the release of their load in a safe and controlled manner, spatially and temporally. This review gives a comprehensive overview of recent research findings on the different types of stimuli-responsive nanocarriers and their application in cancer treatment, with a particular focus on ultrasound.

Keywords: Nanocarriers, Chemotherapy, Ultrasound, drug release.

1. INTRODUCTION

Cancer is the second leading cause of global mortality, with 17 million new cases reported in 2018, and 9.6 million reported deaths in the same year.[1] Given the high incidence of morbidity and mortality, it is essential that more research is directed towards developing more effective methods of prevention, early detection, and treatment of the disease. Despite advances in molecular cancer therapeutics, traditional cancer therapies, such as small molecule chemotherapy, are still widely used to suppress cancer cell proliferation, in efforts to control its progression and metastasis. Many of the small molecule therapeutics used in conventional chemotherapy have poor aqueous solubility resulting in a large volume of distribution throughout the body, causing toxic side effects to normal tissues, while only a very small percentage reaches diseased tissue.[2] These agents, therefore, have a low therapeutic index with a narrow window of concentration between toxicity and therapeutic effect. Raising the dose to achieve an increased therapeutic effect also increases the levels of dose-limiting adverse effects. Since chemotherapeutics have poor selectivity, i.e., poor ability to differentiate between malignant and benign cells (which have high division rates such as those in the bone marrow, hair follicles, digestive tract, and reproductive system) they kill both healthy and cancerous cells. This lack of selectivity results in a number of toxic side effects including nausea, vomiting, fatigue, hair loss, anemia, diarrhea, cardiac problems and sexual dysfunction.

The pathophysiology of some tumors creates a bed of abnormal, highly permeable capillaries that bring oxygen and nutrients to the tumor cells. Nano-sized carriers (nanocarriers), encapsulating anti-neoplastic agents, are small enough to extravasate from the vasculature into the interstitial space surrounding the tumor cells, increasing the delivery of their cargo to the tumor site. Encapsulation significantly reduces the delivery of cytotoxic drugs to healthy tissues, which have normal, poorly permeable vessels. Nanocarriers also improve the solubility of some drugs, prolong their therapeutic lifetime, and increase their therapeutic benefit.[3] Hence, encapsulation of anticancer therapeutics in nanocarriers maintains or improves the efficacy of the treatment, while reducing the side effects to healthy tissues. Some receptors are overexpressed on the surfaces of the tumor cells, and these molecules can be targeted with nanocarriers decorated with targeting moieties complementary to the overexpressed receptors.[4]

As shown in Figure 1, nanocarriers that are designed to encapsulate drug molecules within a membrane are referred to as “nanocapsules”, while other nanocarriers in which the drug molecules are embedded and distributed within a polymer matrix are called “nanospheres”.[5] All types of nanocarriers must encapsulate drugs efficiently and, while the particles are still circulating in the bloodstream, retain the agents without significant release in order to prevent their uptake by healthy tissues. However, upon reaching their desired site of action, these nanocarriers are expected to release their load in an active form.

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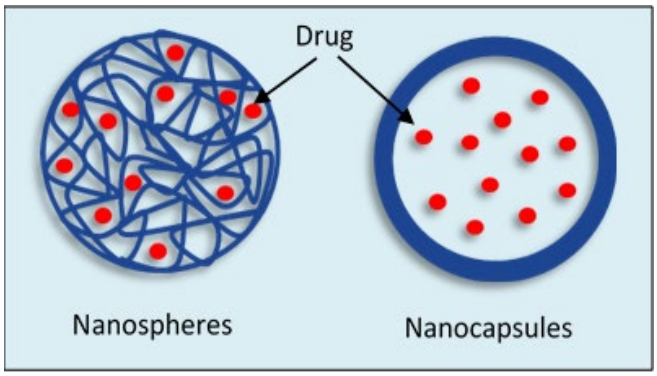

Fig. (1). Schematic representation of nanocarriers encapsulating drugs.

Following their accumulation in tumor tissues, several internal or external stimuli can be employed to trigger drug release from stimuli-responsive nanocarriers (called smart nanocarriers), ensuring a faster and more controllable rate of drug release at the target site. Several release triggers have been investigated. These include light, temperature, enzymes, and pH.[6–9] Ultrasound has been shown to be an effective technique for triggering drug release from nanocarriers.[10] Hence, controlled drug delivery systems with associated triggered release mechanisms can increase drug delivery at desired locations, while significantly lowering toxic effects in healthy cells.[11]

2. NANOCARRIERS USED IN DRUG DELIVERY

A pressing need to improve the pharmacokinetics, biodistribution and therapeutic efficacy of anticancer drugs led to the development (and several subsequent clinical approvals) of a wide range of nanocarriers ranging in size from ~ 10 to 1000 nm. The size of these nanocarriers plays a crucial role in their *in vivo* biodistribution. While particles that are smaller than 5 nm are cleared from the blood rapidly,[12,13] particles larger than 220 nm usually accumulate in the liver, spleen, and bone marrow.[14] Most products in the clinic have sizes of ~ 60 - 200 nm.[15]

Long circulation half-lives are an important attribute, and nanocarriers must evade clearance by biological mechanisms such as the mononuclear phagocytosis. Other important attributes are (1) biocompatibility and safety, (2) efficient drug loading, (3) efficient delivery of the drug to targeted locations in the body, and (4) lack of immunogenicity.[16] The field of nanocarriers has been growing steadily over the past two decades. Several nanoparticles, ranging in size, shape, composition, and physical properties, have been used to treat tumors and infections.[17]

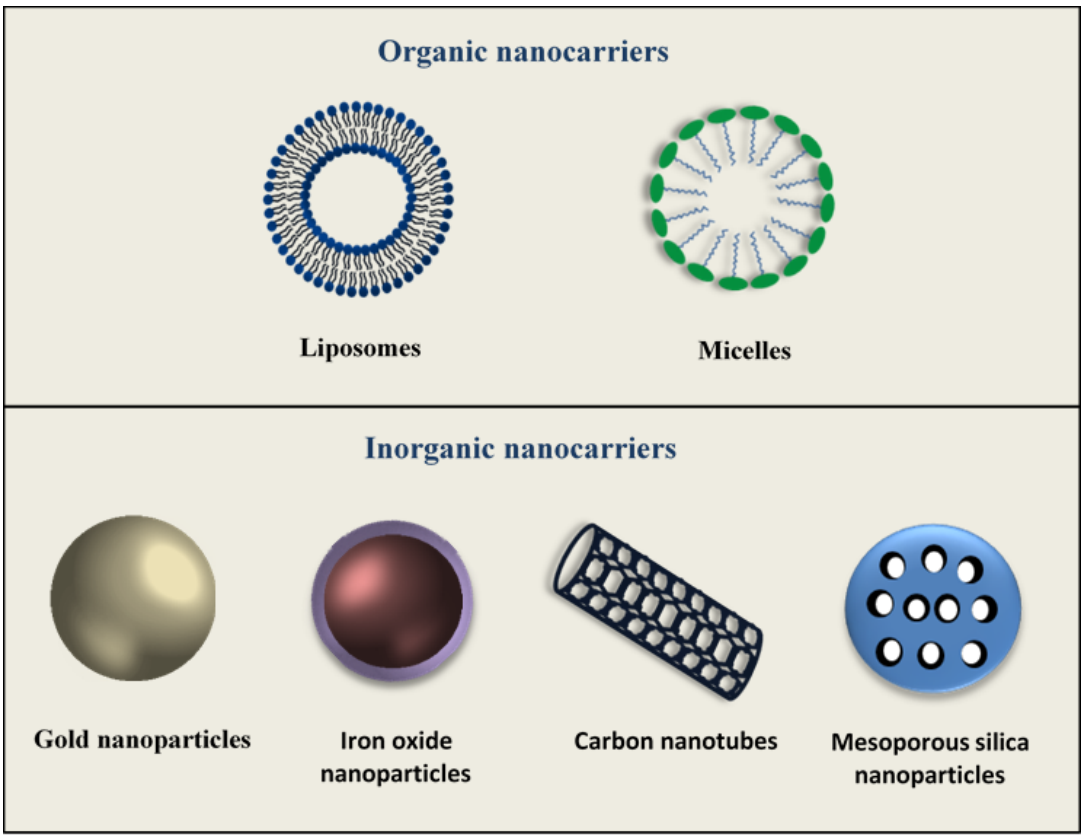

Fig. (2). Organic and inorganic nanocarriers used in drug delivery.

Nanocarriers may be organic in nature (such as micelles, liposomes, and dendrimers), or composed of inorganic materials such as mesoporous silica nanoparticles, superparamagnetic iron oxide nanoparticles, carbon nanotubes, gold nanoparticles, metal-organic

frameworks, and quantum dots.[18] Figure 2 shows several nanocarriers, which will be discussed below. The most successful clinical products to date have been lipidic nanoparticles, such as liposomes.

2.1. Passive targeting of cancer cells by nanocarriers

Nanocarriers have multiple applications in the field of drug delivery, most particularly in the treatment of cancer, inflammation, and infection. The potential of these nanocarriers in cancer treatment is associated with their passive (or non-selective) targeting of solid tumors, via the "enhanced permeability and retention" (EPR) effect. This occurs when tumors develop new vasculature, during the angiogenesis that supplies their high requirement for oxygen and nutrients. The newly formed capillary endothelium in tumor tissues is disorganized and leaky, with imperfect endothelial cell coverage and improper lymphatic drainage.[19] This leads to the aberrant molecular and fluid dynamic behavior in the tumor. The small size of nanoparticles allows them to extravasate through the leaky vasculature and to accumulate at the diseased site (Figure 3) if circulation half-life is sufficiently long.

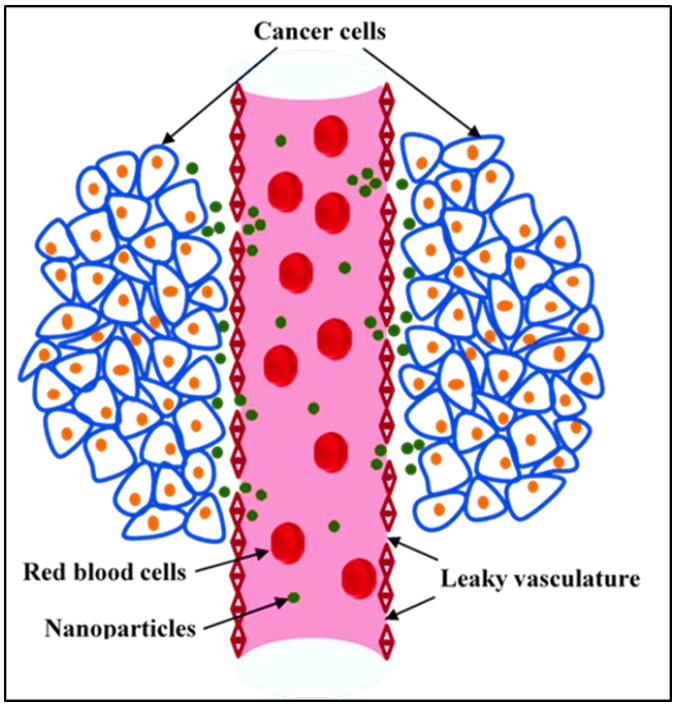

Fig. (3). The leaky blood vessels surrounding the tumor with retarded lymphatic drainage (EPR effect) allows nanoparticles to extravagate through the blood vessels and accumulate inside the tumor.

A commonly used technique to increase their blood circulation times is to graft polymers such as polyethylene glycol (PEG) chains to the particle surface, thus enabling them to reach the tumor circulation and extravasate into the interstitial space at increased concentrations[20] (where there are sufficient angiogenic blood vessels). Some tumors show inherent low drug accumulation by the EPR effect, in others, vessel permeability can be highly heterogeneous.[21]

PEG is non-ionic and highly soluble in water and organic solvents. Conventional liposomes, i.e., those not grafted with PEG or similar polymers, interact with specific proteins (i.e., opsonins) in the bloodstream (a process referred to as opsonization), resulting in their rapid removal from the blood by hepatic and splenic macrophages. However, even with PEGylated particles (so-called 'stealth' liposomes), the distribution of the particles to the disease site via the bloodstream (i.e. 'passive' targeting) does not ensure the uptake of nanoparticles by the cancer cells. Therapeutics released from the nanoparticles in the vicinity of the tumor cells, e.g., by diffusion across the nanoparticle membrane, can be taken up by normal uptake mechanisms. Hence, there is room for improvements over passive targeting mechanisms.

2.2. Ligand-mediated targeting of cancer cells by nanocarriers and triggered release

Tumor-targeting ligands have been used to significantly increase the uptake of nanocarriers into cancer cells. This is accomplished by conjugating targeting moieties that bind to cancer-selective surface ligands to the nanoparticle surface. Ligand-targeted nanocarriers are designed to bind to overexpressed receptors, and to trigger the internalization of the targeted nanocarriers into the cell. Molecules such as proteins, hormones, antibodies, peptides, aptamers, and oligonucleotides are already used to target different types of cancer.[22] The targeted nanocarriers are designed to sequester their drug contents until the carrier and its drug load are delivered to the target site. At that point, a burst release of the payload, triggered by an external stimulus, rather than a slow release of drugs from the nanoparticles by passive diffusion, is the most effective mechanism for achieving a higher drug concentration inside a tumor cell.[23] The triggering mechanism can change a nanocarrier from one with a low release rate to one in an unstable state with a high rate of drug release. This drug release trigger can be achieved by employing the unique characteristics of the tumor's microenvironment.

Intrinsic components of responsive targeted drug delivery include overexpressed tumor enzymes,[24] low tumor pH,[25] and temperature[26] differences between the tumor and healthy tissues. Nanocarriers can also be designed to be responsive to "external stimuli" such as light,[27] magnetic fields,[28] and ultrasound.[29] Figure 4 shows the three main components of nanocarrier-based targeted drug delivery.

3. GENERAL ASPECTS OF ULTRASOUND

Ultrasound consists of pressure waves with a frequency above the audible range for humans, usually defined as being above 20 kHz. Ultrasound can be further classified into low-frequency ultrasound (frequencies lower than 1 MHz), medium-frequency ultrasound (1–5 MHz), and high-frequency ultrasound (frequencies higher than 5 MHz).[30] Ultrasonic waves propagate in soft tissue as mechanical longitudinal waves, causing alternating regions of compression and rarefaction in the medium. These oscillations in density and pressure can cause several biological effects, which are discussed below.

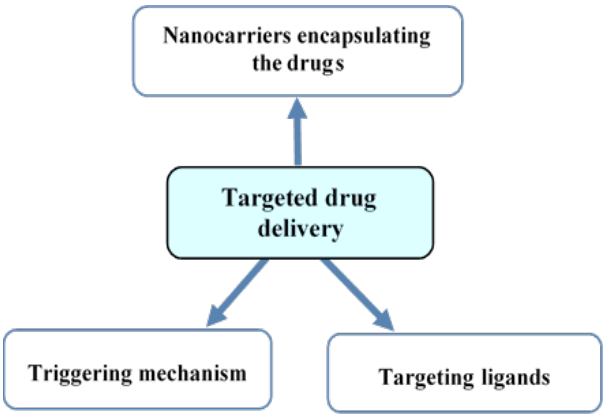

Fig. (4). The key components of responsive targeted nanocarriersdrug delivery.

Ultrasound technology has been widely used in the medical field for both diagnostic and therapeutic purposes due to its cost-effectiveness, safety, non-invasiveness, and simplicity. For diagnostic applications, ultrasound parameters such as frequency and intensity are chosen in such a way that they produce minimal biological effects while allowing the production of images with good spatial and temporal resolution. However, for therapeutic applications, reversible or irreversible biophysical effects are often sought.[31] Although there are many therapeutic applications of ultrasound, this review will focus on the applications of ultrasound in cancer treatment. Two ultrasound parameters, that are often manipulated to induce desired biological effects, are the acoustic frequency and intensity. Decreasing acoustic frequency increases the depth of penetration and the likelihood of cavitation, while increasing the intensity may control the amount of energy that can reach a target site. Other factors, such as the exposure time and pulsing regime (i.e., duty cycle) can also be adjusted to create several useful biological effects.

Frequency. Ultrasound frequencies used in the medical field vary, depending on the required application. For diagnostic applications, the frequency defines the spatial resolution (i.e., increased resolution is obtained at higher frequencies). However, the penetration depth is reduced, and heating is increased with increasing frequencies. Ultrasound attenuation and absorption by the medium is strongly dependent on frequency.[32] At higher frequencies, ultrasonic energy is more readily absorbed and attenuated, reducing penetration into the deep tissues.

The velocity at which the sound wave propagates in the medium (c) is a function of the medium's elasticity (K) and density (ρ):

$$\boldsymbol{c = \sqrt{(K/\rho)}} \quad (1)$$

The velocity, frequency (f) and the wavelength (λ) are related by

$$\boldsymbol{c = f\lambda} \quad (2)$$

Acoustic intensity. As ultrasound waves propagate, regions of high pressure (compression) and low pressure (rarefaction) move through the medium. The amplitude of an ultrasonic wave can be defined as the difference between peak compression and rarefaction values, ($p^+ + p^-$). The pressure wave causes physical displacement of molecules, with an amplitude of $P^+/(\rho c)$, where P^+ is the peak compression pressure. Ultrasound intensity (I), which is a function of pressure, is defined as the amount of power produced by the ultrasound wave divided by the surface area through which it propagates. "I" is measured in W/cm^2, and is directly related to the acoustic pressure (P), the density of the medium (ρ) and the ultrasound propagation velocity in the medium (c) by:

$$\boldsymbol{I = \frac{P^2}{\rho c}} \quad (3)$$

When ultrasound passes through the tissues, it induces mechanical, thermal, and sometimes chemical effects. All these effects have the potential for use in cancer treatment applications.

3.1. Mechanical effects of ultrasound

The two main mechanisms that cause mechanical effects are cavitation and ultrasound radiation force.

3.1.1. Cavitation

Cavities or bubbles either pre-exist in the liquid or are created due to the pressure drop to a value lower than the vapor pressure of the liquid. The formation, oscillation, growth, and collapse of these cavities in an ultrasonic field is called acoustic cavitation. The behavior of the cavitation bubbles varies depending on the pressure, frequency, the radius of the bubbles, and the physical properties of the surrounding fluid (e.g., viscosity, and compressibility). The probability of cavitation occurrence increases as the pressure increases and generally decreases with frequency. Depending upon the behavior of the bubble, oscillations can be classified as stable (non-inertial) cavitation or inertial cavitation (also called transient or collapse cavitation).

Stable cavitation occurs when the acoustic pressure is at a level for which bubble response is generally linear. The bubbles oscillate around an equilibrium radius value in each acoustic cycle (Figure 5). This oscillation results in microstreaming in the surrounding fluid, fluid shear stresses, and localized generation of heat.[33,34] During the negative pressure phase, dissolved gas diffuses into the bubbles and continues to accumulate inside these bubbles leading to an increase in size, with the opposite occurring during the positive pressure portion of the cycle, but to a lesser extent. With continued exposure, the bubbles grow in size with the net transport of gas into these bubbles.

Inertial or collapse cavitation occurs at higher peak negative pressures or when the bubble size grows to its natural resonance size, leading to large amplitude oscillations. Under these conditions, the bubbles oscillate non-linearly, producing subharmonic and higher (ultra-) harmonic oscillations. During the positive pressure phase, the compression by the inward-moving surrounding liquid collapses the gas bubble to the point of a supercritical fluid, creating

pressures exceeding 100 atm (Figure 5). This collapse generates shock waves of very high pressures and produces high local temperatures. If the collapse is not symmetrical, high-speed liquid microjets are generated at the microscopic level.[33,35–37] Cavitation can produce not only mechanical effects but also thermal and chemical effects in adjacent tissues. These physicochemical effects will be discussed below. The Mechanical Index (MI) describes the possibility of the occurrence of cavitation. To avoid tissue damage during diagnostic imaging, the U.S. Food and Drug Administration (FDA) restricts the MI to less than 1.9. The MI is calculated as $MI=P^{-}/\sqrt{f}$, where P^{-} is the peak negative pressure in MPa, and f is the central frequency of the ultrasound wave in MHz.

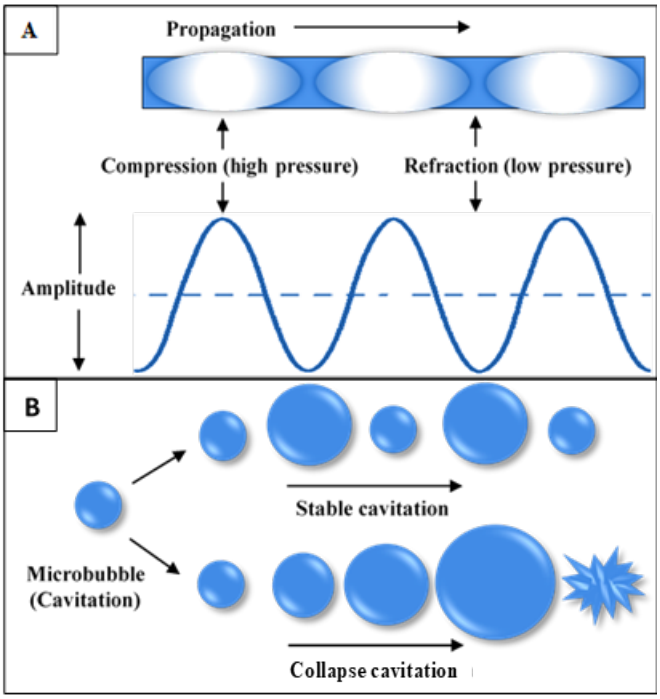

Fig. (5). Schematic representation of the acoustic pressure waves (A), stable and collapsed cavitation of microbubbles (B).

3.1.1.1. Sonoporation

Sonoporation is the process of pore formation in a cell membrane upon exposure to ultrasound. It is a cavitation induced biological effect that is often leveraged for drug delivery. From a therapeutic point of view, sonoporation enables membrane-impermeable compounds to enter the cells through defects generated in the cell membrane. Sonoporation occurs as a result of several mechanisms. Firstly, asymmetric inertial cavitation can produce high-speed microjets that pierce the cell membrane. This asymmetry can occur when the cavitating bubble is near a cell surface.[38] Stable cavitation or inertial cavitation can generate a local steady or transient 'microstreaming' flow that produces holes by shearing[39] or stretching the cell membrane.[40] Wu et al.[41] have shown that by using a mason horn at 21 kHz, shear stress of over 12 Pa on the cell membrane can cause sonoporation. Some of their theoretical analysis has shown that 92 Pa shear stress can be generated by the microstreaming caused by ultrasound at 2 MHz and 0.1 MPa. As the acoustic pressure increased to 0.4 MPa, the shear stress also increased to 1100 Pa.[40] As illustrated in Figure (6), the compression and expansion of the bubble near the cell membrane can cause membrane disruption. In addition, the radiation forces produced by the ultrasound waves may push the bubble towards the cell membrane, eventually disrupting its integrity.[42] Increasing the amplitude of ultrasound leads to a rapid increase in the oscillation amplitude of the bubble. The collapse of the bubble creates shock waves and microjets if the collapse is near to a surface such as a vessel wall, leading to an increase in the size and number of pores created on the cell membrane. Ultrasound parameters have a direct effect on the pore size.[43] Transient pore sizes of up to 4 µm were created in the membrane of MCF-7 breast cancer cells when exposed to moderate ultrasound (0.25 MPa) assisted by the addition of encapsulated 1-µm diameter microbubbles.[44]

3.1.1.2 Free Radical Formation

During inertial cavitation, extremely high temperatures (above 5000 K) and pressures (above 1000 atm) are produced. This leads to the thermal dissociation of the water and, therefore, the formation of hydrogen atoms and hydroxyl radicals.[45] Sonosensitive nanoparticles are often added to create free radicals, which enhances effects such as sonoluminescence and pyrolysis.[46] Light emission ensues immediately following the collapse of the bubbles when the excited chemical species are relaxed, or radicals recombine; the emitted light can then activate certain sensitizers. These sensitizers produce an electron-hole (e-/h+) pair leading to the production of reactive oxygen species (ROS) in an aqueous environment.[47] In pyrolysis, the high temperature generated by the collapse of the cavity chemically excites the sensitizer, producing highly reactive free radicals that form ROS when reacting with other molecules in the aqueous environment.[48]

The most common sonochemical effects are mainly produced by two ROS: hydroxyl free radicals and singlet molecular oxygen. The production of ROS will depend on the sonosensitizer used, the intensity of ultrasound, and the frequency and strength of the cavitation events. The reactive hydroxyl free radicals are formed by the pyrolysis of water occurring as a result of a collapse event. These free radicals can further react with nearby water to produce hydroperoxide radicals or can react with non-volatile solutes such as DNA and modify its purine and pyrimidine bases.[49,50] When reactive oxygen species are not adequately removed from the medium, oxidative stresses on the cells result in severe metabolic dysfunction, including peroxidation of membrane lipids, cytoskeletal disruption, generation of protein radicals, modification to nucleic acids, DNA damage, and cell death.[50,51] The formation of free radicals can happen either in extracellular or intracellular spaces. Those formed outside the cells are short-lived, and their bioeffects are doubtful unless they react with the solutes and produce toxic compounds.[52]

3.1.1.3 Endocytosis

Macromolecules larger than 1 kDa cannot penetrate the external lipid bilayer structure of the cell membranes. The cell possesses a variety of internalization mechanisms, collectively called endocytosis, to internalize these complex molecules. Endocytosis can occur through different mechanisms that can be mediated by, for example, clathrin or caveolae. Although the usual explanation for ultrasound-assisted drug uptake is sonoporation, there is evidence that specific physicochemical stimuli such as mechanical forces or free radicals can promote the internalization of molecules into cells by various endocytic mechanisms. For example, the shear stress created by ultrasound on the cell membranes has been found to induce endocytosis.[39] Both sonoporation and endocytosis were involved in enhancing the uptake of nanocarriers by the cells following sonication.[53,54] Mechanical sensors such as integrins and ion channels sense the mechanical deformation of the cytoskeleton by ultrasound and can convert these signals to activate endocytosis.[55]

Lionetti et al.[56] reported that the application of diagnostic ultrasound (1.6 MHz and 1.2 MI) for 30 mins *in vitro* enhanced caveolae-mediated endocytosis. The study reported that cells treated with GST-Tatt11-EFGP and exposed to ultrasound at a mechanical index of 1.2 for 30 minutes showed increased internalization of the compound over that seen in the control sample with no alterations in cell viability and membrane integrity. Adding free radical generation inhibitors reduced the cellular internalization by 50 %, indicating that endocytosis is partially mediated by ROS formation. Another study by Tardoski et al.[57] showed that low-intensity ultrasound promoted clathrin-mediated endocytosis. It was also demonstrated that following sonication, the transport mechanism of macromolecules happens either through transient pores or through endocytosis, depending on the molecular size.[58]

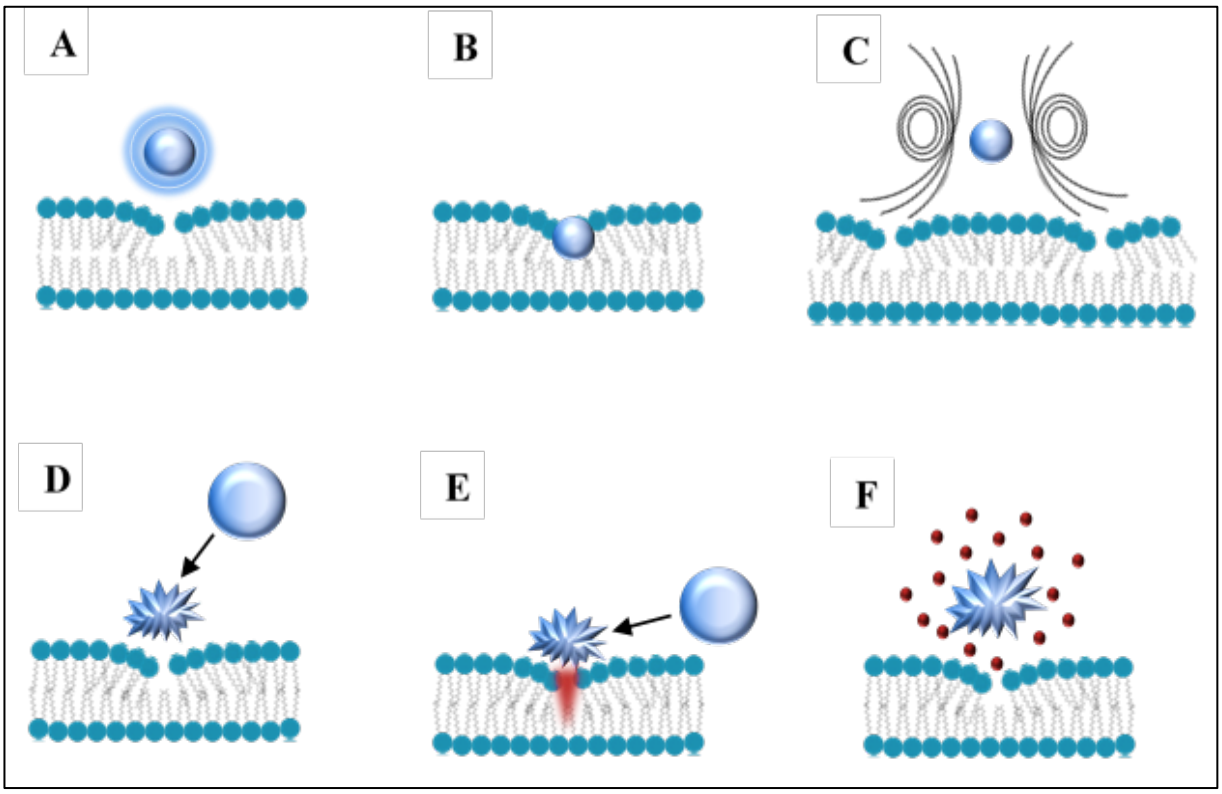

Fig. (6). Different mechanisms by which oscillating microbubbles may disrupt the integrity of cell membranes. A: Stable cavitation produces shear forces that can perturb membrane structure. B: Stable cavitation generates radiation forces that push microbubbles towards the cell and possible through the cell membrane. C: The mechanical stress created by microstreaming around microbubbles during stable cavitation, causes pore formation. D: During inertial cavitation, the shock waves formed by the collapsing microbubbles generate high pressure which disrupts the cell membrane. E: The asymmetrical collapse of microbubble accelerates a microjet toward the cell membrane, forming a pore. F: During inertial cavitation, reactive oxygen species (ROS) can be formed. This leads to the disruption of the cell membrane through lipid peroxidation.

3.1.2. Acoustic radiation forces

Acoustic radiation forces are exerted by ultrasound on an object in the acoustic field. It may cause biological effects. This force is given by W/c, where W is the acoustic power, and c is the speed of sound traveling through the medium. When ultrasound propagates through a medium, absorption of wave energy imparts kinetic energy to the fluid, leading to localized fluid flow, a phenomenon known as acoustic streaming. The flow's velocity (v) is given by:

$$v = \frac{2\alpha W}{\mu p c} \quad (4)$$

where μ is the fluid viscosity, and α is the absorption coefficient.[32] The beam geometry and kinematic turbulence also influence the streaming velocity.[33]

The radiation force, in the case of complete wave reflection, is:

$$F = 2\frac{W}{c} \quad (5)$$

These radiation forces, or the acoustic flow they create, can lead to the displacement of the bubbles and particles in the blood, driving particles into the target tissue, and hence creating reversible structural deformations (Figure 6B).[59,60]

3.2. Thermal Effects of Ultrasound

In addition to mechanical effects, thermal effects are important by-products of acoustic waves. When pressure waves propagate through tissue, they are attenuated due to absorption and scattering. For a sinusoidal ultrasound wave with frequency *f*, and initial pressure amplitude P_o, the pressure amplitude at point *x* from the initial point is given by:

$$P_x = P_0 \exp(-\alpha x) \quad (5)$$

When the acoustic energy is absorbed, it is converted to thermal energy. The amount of energy absorbed by the tissue depends on the absorption coefficient α, which is frequency-dependent with the relationship shown in the equation below:

$$\alpha = \alpha_0 f^n \quad (6)$$

where α_0 is the reference absorption coefficient of the particular tissue, and *n* is a fitted constant. Thus, as ultrasound frequency increases, more of the ultrasound energy will be absorbed by the tissues, producing more heat. Where the temperature-induced is sufficiently high, local ultrasonic mediated heat deposition (hyperthermia) has been shown to kill cells and damage proteins and other cellular structures, while minimizing the adverse effects on adjacent healthy tissues.[60,61]

Raised temperatures have been found to induce apoptotic and necrotic cell death.[60] The main factors that determine the type of hyperthermia-induced cell death are the type of cell, the increase in temperature, and the time for which it is maintained.[62] As the temperature is raised above 43 °C (hyperthermia), changes start to occur, which affect different cellular compartments such as membrane fluidity, thus impeding the function of transmembranal transport protein and receptors located at the cell surface.[63] Cell shape and cytoskeleton changes were observed following heat treatment. Depending on the cell type, cells have been flattened or rounded, while blebbing has also been observed on some cell surfaces.[64–66] Reduction in the expression of membrane proteins and integrins has also been observed,[67] in addition to protein denaturation.[68]

4. ULTRASOUND AND THERAPY

Ultrasound has revolutionized both medical diagnosis and treatment. It has also been used to stimulate and accelerate the healing of bone fractures as well as other injuries to soft tissues.[69] It is also currently used in bone growth stimulation.[31] Diagnostic ultrasound is generally safe and has good tissue penetration with low operational and instrumental costs.[70] As mentioned, several factors control the biological effects of ultrasound: the intensity and frequency of the acoustic waves, the exposure time, and the number of pulses.[71] High intensity focused ultrasound (HIFU) focuses high acoustic energy on a specific focal target, and has been used in cancer treatment. This energy is absorbed by the tissues located at the focal point of treatment, producing an extreme rise in temperature (above 80°C) while not creating cytotoxic temperatures outside the focal zone.[72,73] This results in the total destruction of the targeted cancer cells. HIFU ablation works through the placement of small lesions side by side on the tumor tissues, which requires precision if an entire tumor is to be ablated efficiently.[74] While the thermal effect of HIFU is the main contributor to cancer treatment, non-thermal effects due to acoustic cavitation, such as microstreaming and radiation forces that cause membrane destruction and cell death, are also produced.

HIFU treatment of tumors has been associated with some complications, including second and third-degree skin burns. Additionally, there has been concerns that the rupture of the tumor cells and their surrounding blood vessels may cause metastasis through the bloodstream, although this has not been proven conclusively.[75] The release of inflammatory cytokines may help to stimulate the immune response.[76]

Ultrasound is a major component of a promising cancer treatment known as sonodynamic therapy (SDT). SDT uses low-intensity ultrasound combined with sonosensitizers; agents that facilitate the production of reactive oxygen species (ROS). This technique requires the uptake and retention of sonosensitizers by the cancer cells; ultrasound can then be applied to activate these sonosensitizers producing ROS and other toxic molecules.[77] Cavitation processes activate sonosensitizers from the ground state to the excited state. As they return to their stable ground state, energy is released that is transferred to the surrounding oxygen, producing large amounts of ROS such as oxygen ions, singlet oxygen, and peroxides. ROS are highly toxic to cells and can result in apoptosis.[78] Sonosensitizers have a very low risk of systemic toxicity and are considered to be safe because they only become bioactive after being exposed to ultrasound, which can be focused on the tumor without involving the surrounding tissues.[79]

As discussed earlier, ultrasound plays a role in enhancing cell membrane permeability (sonoporation) via microbubbles generated during the cavitation process. Sonoporation allows the transfer of molecules, including drugs and DNA, from extra- to intracellular compartments. Studies conducted both *in vitro* and *in vivo* have shown that sonoporation enhances the uptake of anticancer drugs,

e.g. bleomycin and Adriamycin.[80,81] Sonoporation has also been found to enhance gene uptake by cells when ultrasound (0.5-4 MHz) was applied.[82] As described above, ultrasound enhances the uptake of different molecules either by forming small pores in the membrane or by enhancing endocytosis.[58,83]

4.1. Ultrasonically triggered nanoparticles for drug delivery and cancer treatment

The energy produced by ultrasound can be used as an external stimulus to trigger drug release from nanoparticles. While other external stimuli such as light penetrate poorly into the body, low-frequency ultrasound penetrates deeply into soft tissues.[84] The ability to focus ultrasonic waves using probes placed on the patient's skin allows the safe non-invasive deposition of significant levels of energy into specific small volumes deep inside the body while sparing the surrounding healthy tissues.[23] The effective focus of the ultrasound can target volumes as small as a few cubic millimeters.[85] As shown in Figure 7, nanocarriers located in the focal zone can be triggered by the ultrasound to release their payload, while nanocarriers positioned outside this zone will not be triggered.[86] Ultrasonically triggered drug release from nanocarriers can occur as a result of the thermal effect of the ultrasound and/or the mechanical effect through cavitation or radiation forces. Subjecting sonosensitive nanocarriers to these physical forces induces structural destabilization and subsequent drug release. In addition, these forces induce a transient increase in blood vessel permeability, thus enhancing extravasation of particles or drugs into tumor interstitial spaces, increasing the eventual cellular uptake of the drug.[32,87]

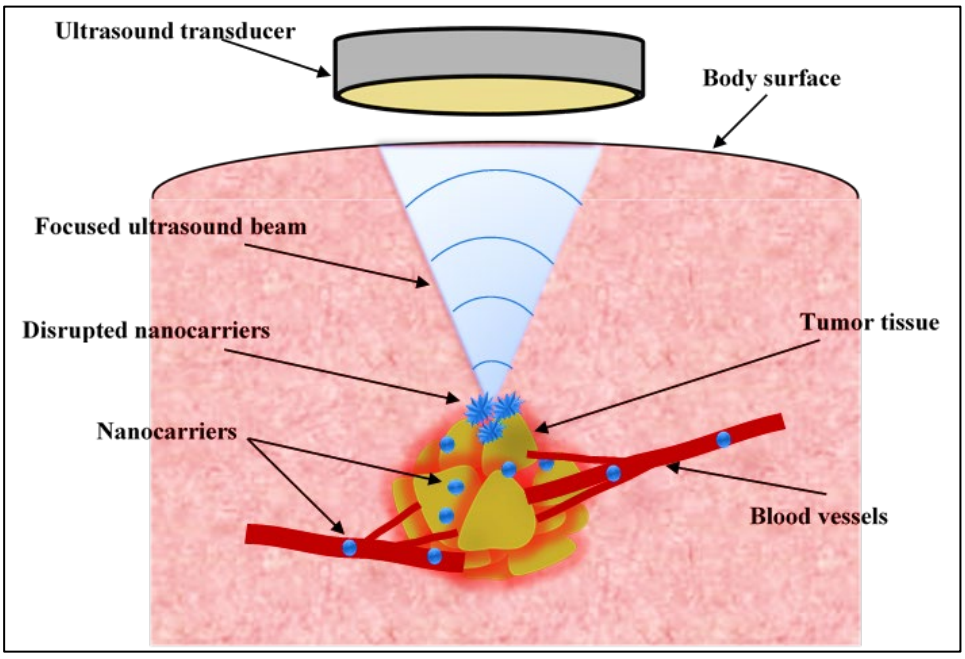

Fig. (7). Ultrasound-triggered drug release from targeted nanoparticles. The controlled ultrasound beam is focused on the tumor tissue, nanocarriers passing through the high intensity focused beam are disrupted or activated.

As mentioned above, inertial cavitation, high temperatures, and pressures are generated in response to the collapse of the bubbles, which in turn generate ROS.[70] Combining hard nanoparticles (metals and metal oxides) with ultrasound has many advantages. In addition to their possible use as drug delivery vehicles, these nanoparticles can act both as sonosensitizers (via their metal oxide surface chemistry) and as cavitation nuclei (via the reduction of the required peak negative pressures to form gas bubbles, thus increasing the number of bubbles and the cavitation rate).[77] Acoustic cavitation can cause structural changes in mesoporous nanoparticles by mechanically fracturing and creating fresh highly-reactive metal oxide surfaces. The ROS produced, due to the effect of both the ultrasound and the nanoparticles cause oxidative stresses that resulted in reduced cell viability due to membrane destruction, fragmentation of the DNA and damage to the mitochondrial membrane.[46,88]

4.1.1. Mesoporous Silica Nanoparticles (MSNs) triggered with US

Mesoporous silica nanoparticles (MSNs) are porous silica nanoparticles with pore diameters ranging from 2 nm to 50 nm. In addition to their potential as cavitation nuclei and sonosensitizers (see above), these MSNs have been extensively investigated for their promise in drug delivery and biomedical applications.[89] MSNs are also biocompatible and biodegradable.[90,91] They have large surface areas due to their high pore volumes, which confers high loading efficiency of drugs. Horcajada et al.[92] reported that the rate of delivery of ibuprofen, encapsulated inside MCM 41 type mesoporous materials, decreased in response to a decrease in pore diameters from 3.6 nm to 2.5 nm. Drug solubility and interactions with pore walls determine the loading and release efficiency of the MSNs.[93] The pore walls can be functionalized with various chemical groups that

control both the loading and the release of the drugs by controlling the time and duration of pore opening or closure. This limits the possibility of premature drug release in the bloodstream, but allows the release of the cargo once the MSNs reach the desired site.[93]

Ultrasound has been used by Paris et al.[94] to trigger doxorubicin (DOX) release from MSNs. In their study, MSNs were grafted with temperature-sensitive copolymers (MEO_2MA and THPMA) able to open the pores (the polymer having a coil-like form during drug loading), and then close the pores (polymer having a collapsed formed during blood circulation). The energy of the ultrasound waves cleaves the hydrophobic tetrahydropyranyl moieties changing the polymer's conformation to coil-like, which then allows drug release. A more recent study by the same group[95] investigated ultrasound-mediated drug release from MSNs grafted with a polyethylene glycol (PEG) shell. When high-frequency ultrasound is applied, the hydrophilic PEG shell detaches from MSNs encapsulating the drug topotecan, and the positive charge of the exposed surface led to their internalization into the human osteosarcoma cells, releasing the drug inside these cells. Li et al.[96] synthesized MSNs coated with the polymer sodium alginate (SA), and carboxyl-calcium (COO^--Ca^{2+}) was used to crosslink the SA on the outer layer (SA-$CaCl_2$), blocking the pores and preventing the release of the encapsulated drug. Upon sonication using both low-frequency (20 kHz) and high-frequency (1.1 MHz) ultrasound, the coordination bonds were destroyed and the drug was released from the MSNs. The group concluded that cavitation was the mechanism behind the disruption of these coordination bonds, and hence the subsequent drug release.

Another group[97] has synthesized multifunctional targeted MSNs capped with β-cyclodextrin (β-CD) and functionalized them with folic acid (FA-β-CD) as a targeting moiety to increase uptake by cancer cells. These MSNs were loaded with the chemotherapy drug paclitaxel (PTX). To enhance their sonosensitivity, gas bubbles were stored in a hydrophobic internal channel. While these nanoparticles had slow drug release in the bloodstream, triggering with low-intensity ultrasound (0.4 to 1 W/cm^2, 1 MHz) significantly enhanced drug release both *in vitro* and *in vivo.* When activated using ultrasound, MSNs act as cavitation nuclei or sonosensitizers, as explained above. The roughness of their surfaces leads to the presence of residual gas bubbles within the pores. In addition, the dissolution of the silicon nanoparticles forms hydrogen bubbles.[98] The synergistic cytotoxic effect of low-intensity ultrasound and MSNs has resulted in the destruction of cancer cells *in vitro.*[99,100]

4.1.2. Super magnetic iron oxide nanoparticles triggered with US

Superparamagnetic iron oxide nanoparticles (SPIONs) are composed of magnetite iron oxide (Fe_3O_4) or its oxidized form maghemite (γ-Fe_2O_3), with diameters ranging between 1 and 100 nm. SPIONs consist of iron oxide cores and, can, therefore, be targeted to a specific location via externally applied magnets. A magnet is placed externally over the targeted area producing a strong magnetic field that attracts the SPIONs to the desired location. SPIONs are promising nanocarriers capable of delivering drugs to the body because they are biodegradable and simple to synthesize.[101]

Different types of functional groups, including carboxyl groups and carbodiimides, can be grafted on the SPION surface. Chemotherapeutic drugs can then be conjugated to these functional groups. However, because drugs are only loaded on their surface, SPIONs have been found to release their drug load soon after injection into the bloodstream (burst effect). The anti-neoplastic agents then fail to reach their therapeutic levels at the desired site. To reduce their premature drug release, biocompatible polymers are usually used to coat the metal cores. Various polymers, including PEG, polylactic-co-glycolic acid (PLGA), polyethylene-co-vinylacetate, and polyvinylpyrrolidone (PVP), have been used as coating materials in aqueous solutions.[102] These coatings protect the magnetic core and allow drug binding by forming covalent bonds, adsorption or entrapment on the particles.[103] For example, coating the iron oxide core with the cross-linked polymer, poly (ethylene glycol)-co-fumarate (PEGF), caused a significant reduction in premature release (21 %) compared to the non-coated SPIONs.[104] To increase their targeting abilities, the surfaces of the SPIONs can be crafted with targeting molecules such as folic acid, RGD, proteins, transferrin, and hyaluronic acid.[104] Several *In vitro* studies have shown no, or low, cytotoxic effects of SPIONs on cell cultures.[105–107] However, other *In vivo* studies showed controversial toxicity patterns of SPIONs; from negative[108,109] to positive toxicity[110,111] in several pre-clinical animal models. Generally, the toxicity of SPIONs depends on their size, dose, surface coating, and species.[112]

Fard et al.[113] investigated the effect of exposing Fe_3O_4 nanoparticles to ultrasound (1 MHz and intensity of 2 W/cm^2) on the viability of cancer cells. The study showed that the level of reactive oxygen species (ROS) in the cells was enhanced following sonication, resulting in cell destruction. Furthermore, the study also reported that high concentrations of Fe_3O_4 nanoparticles (above 100 mg/ml) behave as sonosensitizers generating ROS (synergic effect). Coating Fe_3O_4 nanoparticles with sonosensitizers, e.g., titanium dioxide (TiO_2), is a promising technique for enhancing ultrasound-assisted stimulation by inducing the formation of ROS, including hydrogen peroxide, hydroxyl radicals, and superoxides.[114] A study by Shen et al.[115] investigated the cytotoxicity of DOX-loaded titanium dioxide-encapsulated Fe_3O_4 nanoparticles (Fe_3O_4-TiO_2 NPs) coupled with ultrasound (1 MHz, 1 W/cm^2). This study reported that these nanoparticles produced ROS following the exposure to ultrasound. Incubation of cancer cells with Fe_3O_4-TiO_2-DOX followed by sonication showed higher toxicities compared to the treatment with free DOX or Fe_3O_4-TiO_2-DOX alone.

4.1.3. Gold nanoparticles triggered with US

Gold nanoparticles (GNPs) have intriguing potential as cancer drug nanocarriers. GNPs possess unique optical (energy absorption), physical (size and stability), and chemical (reactive with thiols) characteristics, which give them a highly multifunctional platform for imaging, guiding surgical procedures, and drug delivery.[75,116–118] A study by Chithrani and Chan[119] reported that cellular uptake of the GNPs is mainly through the receptor-mediated clathrin-dependent endocytosis pathway. The study also reported that the small diameter of the nanocarriers (≤50-nm) leads to faster uptake and higher concentrations of the GNPs inside the cells, relative to larger

carriers. GNPs were found to interact with heparin-binding glycoproteins, including vascular permeability factors and basic fibroblast growth factors that mediate angiogenesis. Inhibition of angiogenesis reduces tumor growth. Although GNPs themselves could be used as therapeutic agents to destroy tumor cells, they have also been used in the field of drug delivery. GNPs, with diameters of 10 to 100 nm, can be conjugated to chemotherapeutic drugs, making them potential drug delivery carriers.[118] The conjugation is achieved by simple physical adsorption and by using alkanethiol linkers.[120] To enhance their solubility and stability, GNPs can be decorated with PEG thiols (pegylation) to reduce their uptake by the reticuloendothelial system (RES). In addition, GNPs can be designed to target tumors by conjugation with various ligands such as transferrin (TF) and folic acid.[121,122] Once at the desired location, drug release from the GNPs can be stimulated using external (light, ultrasound, laser) or internal triggers (pH, redox condition, matrix metalloproteinase, heat).[18]

When ultrasound is applied, gold particles in the liquid medium act as nucleation agents, which enhances the cavitation processes by reducing the latter's threshold. When added to the culture media, GNPs were found to enhance the inertial cavitation of ultrasound.[123] Brazzale et al.[124] synthesized gold nanoparticles containing PEG as a polymer coating and folic acid as a targeting moiety (FA-PEG-GNP). Incubation of these nanoparticles with two cancer cell lines (KB and HCT-116), followed by exposure to ultrasound (8×10^{-6} J cm^{-2} and 8×10^{-5} J cm^{-2}, for 5 min at 1.866 MHz) significantly reduced cell growth and proliferation together with the significant generation of ROS species. Another study by Kosheleva et al.[125] reported that lung cancer cells (A549) showed higher sensitivity to the treatment with GNPs combined with 4 MHz HIFU, than to the treatment with the GNPs only.

Furthermore, combining GNPs with ultrasound (1 and 3 MHz, 2 W/cm^2) and an intense pulse of light was found to significantly improve the therapeutic effect in colon tumors in mice.[126] A study that was conducted *in vivo* by Shanei et al.[127] found that ultrasound (1.1 MHz, 2 W/cm^2) applied to GNPs conjugated to the sonosensitizer PpIX (Au-PpIX), resulted in greater inhibition of the colon cancer (CT26) than when using PpIX alone. This was attributed to GNPs acting as nucleation agents, leading to more collapsing cavities in addition to improving uptake of the Au-PpIX by the tumor cells.

4.1.4. Carbon nanotubes (CNTs) triggered with US

Over the past 20 years, many studies have shown the potential of employing carbon nanotubes (CNTs) as drug and gene delivery systems that benefit from their unique physical properties including their low mass density, large surface area, wide range of diameters (from a few to hundreds of nanometers), in addition to their non-immunogenicity, biocompatibility, and high drug cargo-carrying ability.[128] A CNT is a carbon allotrope consisting of a single graphene sheet rolled up to form a single-walled carbon nanotube (SWCNT).[129] Multi-walled CNTs carbon nanotubes (MWCNTs) contain more than one rolled sheet arranged in a concentric form.[128] CNTs dissolve poorly in aqueous media and tend to form aggregates. To overcome these issues, CNTs surfaces can be modified using physical or chemical methods, including electrostatic, hydrophobic, or covalent and non-covalent bonding.[129] Considerable research has been conducted on their purification, carboxylation, acylation, amidation, esterification, PEGylation, and surface modification with polymers. In addition, their surfaces can be conjugated to targeting molecules including carbohydrates, proteins, antibodies and folic acid, making them suitable for targeted drug delivery.[128,130,131] The potential of CNTs in cancer treatment through both photothermal therapy and photodynamic therapy have been investigated. CNTs have been found to be capable of transforming near-infrared light into heat which established their role in photothermal therapy through promoting hyperthermia (thermal ablation) leading to enhanced tumor suppression.[132] Combining CNTs with photosensitizers (PS) that generate ROS upon illumination resulted in a noticeable tumor reduction in mice.[133]

In addition to their drug loading capabilities, CNTs can be engineered to act as excellent adjuvant contrast agents for use in imaging.[129] For example, the hyperechogenicity of the MWCNTs promoted their use as ultrasound contrast agents.[134,135] A study by Delogu et al.[136] reported that functionalized MWCNTs have long-lasting ultrasound contrast properties. Pagani et al.[137] reported that the cavitation formed during sonication results in the dispersion of CNTs in liquids by exfoliating CNTs bundles into individual units, in addition to the breaking and shortening of the individual CNTs. Spectroscopic and morphological analysis of CNTs subjected to sonication using an ultrasonic bath (water volume 2.5 L, power 260 W) revealed that as sonication time increased, the length of CNTs' decreased dramatically.[138] In addition, as the length of CNTs decreased due to the increase in sonication time, the structures of CNTs also changed from solid-like to liquid-like structures.[139] An earlier study by Ruan and Jacobi[140] reported that the ability of MWCNTs to conduct heat increased nonlinearly with an increase in sonication specific energy input.

The ability of ultrasound to successfully disperse CNTs, releasing their load in a controlled manner, makes ultrasound-assisted CNT drug delivery a promising technique in cancer treatment. In addition, the increased thermal conductivity of MWCNTs following sonication shows the potential of combining MWCNTs with HIFU ablation. More research is certainly needed to develop the role of ultrasound-assisted CNTs in drug delivery and to optimize suitable sonication parameters to ensure the successful outcome pertaining to the desired ultrasound-mediated effect. However, there is considerable concern about the biocompatibility and toxicity of MWCNTs and they have recently been added to the SIN List as a nanomaterial of very high concern.[141]

4.1.5. Polymeric micelles triggered with ultrasound

Polymeric micelles are nanocarriers synthesized from amphiphilic copolymers forming a hydrophobic core and a hydrophilic shell. Poly-ethylene glycol (PEG) is widely used to form the hydrophilic outer shell while polyesters, polyethers, or polyamino acids are used as the blocks forming the hydrophobic core.[142] Polymeric micelles spontaneously self-assemble when in aqueous solutions to form a stable and unique nanoscale structure with many physicochemical and biological advantages, making them efficient drug delivery

vehicles.[143] The hydrophilic shell increases the circulation time in the blood while evading the uptake of the polymeric micelles by the immune cells, thus reducing their clearance from the blood. The hydrophobic core ensures that drugs insoluble in aqueous solutions can easily be encapsulated and delivered to the cancer site.[143] In addition to the physical entrapment of the drugs inside their cores, drugs can be loaded into polymeric micelles through chemical conjugation and ionic bonding. Micelles can also be crafted with a number of targeting moieties, including antibodies, folic acid, growth factors, and transferrin.[144] Upon reaching the desired site, Intrinsic or extrinsic stimuli can be used to trigger drug release from micelles with temporal and spatial control.

Different studies have explored ultrasound to trigger the controlled release of active agents from polymeric micelles, mainly Pluronic micelles.[145–147] Ultrasound can trigger irreversible drug release from polymeric micelles through ester hydrolysis which is usually associated with HIFU (1.1 MHz); low-frequency ultrasound (20–90 kHz), on the other hand, triggers reversible drug release through inertial cavitation.[148] Xuan et al.[149] showed that the effect of HIFU could be boosted through the incorporation of a small amount of HIFU-labile 2-tetrahydropyranyl methacrylate (THPMA) comonomer units into the core of the micelles to enhance their thermosensitivity, thus releasing more encapsulated drugs with HIFU irradiation. Cavitation triggers the release of the drugs from the core of polymeric micelles by disrupting the assembled polymeric micelles, releasing their payload. When ultrasound is paused, polymeric micelles have the ability to reassemble and successfully re-encapsulate the drugs inside their cores. Therefore, the reversible disordering of the micelles can produce reversible drug release.[146,147] Wu et al.[150] recently reported that P123/F127 mixed micelles, loaded with curcumin, circulated in the blood for longer periods, showing higher uptake by cancer cells compared to free curcumin. The study reported the cleavage of the Cu(II)–terpyridine dynamic bond in the copolymer chain when exposed to HIFU (3 W, 1.90 MHz) for 3 min, resulting in the disruption of the micellar structure and subsequent drug release. Salgarella et al.[148] investigated micelles prepared using five different types of poly(2-oxazoline) block copolymers and found that sonication (40 kHz, 20 W, 10 min) enhanced the release of the encapsulated dexamethasone from 6 % to 105 %. The study found that the type of copolymer used had an effect on the amount of drug released. Table 1 below shows a summary of some studies investigating the ultrasound-mediated drug release from polymeric micelles.

Table 1. Studies investigating the acoustically triggered drug release from polymeric micelles.

Reference	Study type	US parameters	Encapsulated substance	Effect
Liang et al.[151]	*in vitro* study	1.1 MHz. (0−150 W)	Pyrene/Nile red	The encapsulated agents are quickly released from the (PPG-[Cu]-PEG) micelles following sonication using HIFU.
Staples et al.[152]	*in vivo* study using DHD/K12/TRb colorectal epithelial cancer	20 kHz (1.0 W/cm^2) or 476 kHz (23.61 W/cm^2)	DOX	US increased DOX concentration by about 50% in the tumor. Varying the frequency had no statistical difference on the treatment.
Hasanzadeh et al.[153]	*in vivo* using murine spontaneous breast adenocarcinoma	3 MHz (2 W/cm^2) and 28 kHz (0.04 W/cm^2)	DOX	Both frequencies used triggered drug release in addition to enhancing the uptake of the drug by the cancer cells
Rapoport.[154]	*in vitro* study using Ovarian carcinoma A2780 and MDR cells	(1, 3 MHz) and (20 kHz–3 MHz) power densities (0.058, 6 and 0-0.2 W/cm^2)	DOX	Sonication enhanced drug release from micelles, as well as enhancing the intracellular uptake of the drug.
Nelson et al.[155]	*in vivo* study using Colon tumor (DHD/K12/TRb)	20 and 70 kHz (0.048, 1 and 2 W/cm^2)	DOX	US enhances DOX release from micelles. US may assist drugs/micelles uptake by the cancer cells.
Husseini et al.[156]	*in vitro* study	20 to 90 kHz (0 to 3 W/cm^2)	DOX and Ruboxyl	Cavitation disrupted the micelles releasing their encapsulated drugs. DOX release was higher than that of Ruboxyl.

Munshi et al.[157]	*in vitro* study using HL-60 human leukemia cells	80 KHz (3.6 W/cm^2)	DOX	Sonication resulted in lowering DOX IC_{50} from 2.35 µg/ml to 0.19 µg/ml.
Zeng and Pitt[158]	*in vitro* study	Low-frequency US (70 kHz)	DPH/ DOX	DOX release increased as the temperature increased from 25 °C (2 %) to 37 °C (4 %). Dox re-encapsulation occurred when sonication was paused.
Marin et al.[159]	*In vitro* study using HL-60 Human Leukemia, breast cancer (MCF-7) and MDR cells	20-kHz (0.058 W/cm^2), 67-kHz (2.8 W/cm^2) and 1.0-MHz (7.2 W/cm^2) .	DOX	DOX release was increased at higher power densities.
Marin et al.[160]	*In vitro* study using HL-60 Human Leukemia cells	20 kHz (1.4, 14 and 33 mW/cm^2	DOX	Sonication resulted in enhancing DOX uptake by the cancer cells.
Gao et al.[161]	*in vivo* study using Ovarian carcinoma A2780 cells	1 MHz or 3 MHz (3.4 W/cm^2)	DOX	Sonication of the drug-loaded micelles inhibited tumor growth.
Husseini et al.[162]	*in vitro* study	70 kHz (0.28 W/cm^2)	DOX	Transient cavitation disrupted and disassembled the micelles, thus releasing the encapsulated drug.
Husseini et al.[163]	*in vitro* study using HL-60 human leukemia cells	70 kHz, (1.3, 2.3 W/cm^2)	DOX	US-mediated drug release from micelles resulted in cell death through apoptosis.
Zhang et al.[164]	*in vitro* study	High-intensity US	Nile red	Collapsed cavitation occurring at the focal point of HIFU resulted in the irreversible release of Nile Red from micelles.
Husseini et al.[165]	*in vitro* study using HL-60 human leukemia cells	70 kHz, (1.3 W/cm^2)	DOX	US triggered DOX released from Pluronic P105 micelles damaging the DNA of HL-60 cells in the process.
Pruitt and Pitt[166]	*in vitro* study using HL-60 Human leukemia cells	70 kHz (1.5 W/cm^2)	DOX	US enhanced DOX toxicity by extravasation, perturbation of the cell membrane, and permeabilization of micellar membranes.
Husseini et al.[167]	*in vitro* study using HL-60 cells/HeLa cells	70 kHz, (1.2 W/cm^2)	DOX	The application of US enhances drug uptake through the cell membrane, not through the acidic vesicles.
Husseini et al.[146]; Husseini et al.[168]	*in vitro* study	70 kHz (0.53 W/cm^2-6 W/cm^2)	DOX	Maximum DOX release (14%) was measured at 5.4 W/cm^2 following a zero-order model
Tong et al.[169]	*in vitro* study	1.1 MHz (0−150 W)	Pyrene/DTT	Combining the effect of HIFU and redox enhanced drug release from copolymer micelles.
Husseini et al.[170]	*in vitro* study	70 kHz (0.27 W/cm^2)	DOX	Micelles are triggered to release their load as a result of the shearing events produced by cavitation.

4.1.6. Liposomes triggered with ultrasound

Liposomes are nano-sized carriers that contain central aqueous compartments surrounded by a lipid bilayer shell. Liposomes are able to efficiently entrap hydrophobic drugs within the lipid bilayer and encapsulate hydrophilic drugs in their cores.[171] Monodisperse liposomes with an optimized diameter (less than 200 nm) can be manufactured, which allows them to extravasate through the leaky vasculature of solid tumors. Surface modification of the liposomes with polymers such as polyethylene glycol (PEG), known as Stealth® liposomes, have reduced uptake into the cells of the RES, and longer circulation times than liposome lacking this modification.[150,172] Drug encapsulation inside the liposomes can significantly increase the accumulation of the loaded drugs at the sites of solid tumors showing a 50- to 100-fold increase compared to the free drug.[173,174] However, this does not mean an equally significant increase in therapeutic effects, as the drugs have to be released from the carriers before they can act on the surrounding cells.

Drug delivery of anthracycline-containing liposomes have been studied widely in the literature since several of these are already approved by the FDA including liposomal doxorubicin (Myocet™), pegylated liposomal doxorubicin (Doxil® also known as Caelyx™), liposomal daunorubicin (DaunoXome®)[175] and one generic liposomal doxorubicin Lipodox®. For tumors with highly permeable vasculature, e.g., Kaposi's sarcoma, these anthracycline-liposomes are capable of selectively delivering high dosages of anthracyclines to tumor tissues without harming healthy, drug-sensitive tissues such as heart tissue. This is due to their high encapsulation and retention efficacy, as well as their prolonged circulation time in the bloodstream.[176,177] In addition, the small size of the liposomes allows them to escape through the defective vasculature surrounding the tumor (EPR effect) and accumulate in large numbers at the diseased location.[178] However, as long as DOX is encapsulated inside the liposomes, it remains in a crystalline form and is not bioavailable, so the concentrations of free (bioavailable) drugs at the tumor site will be sub-optimal.[179]

Ultrasound has been combined with liposomes in several studies. Low-frequency ultrasound (LFUS) enhances the permeability of cell membranes, thus enhancing drug and gene uptake.[180] As the phospholipid bilayer structure surrounding the liposomes is similar to that of the cells, LFUS application also enhances the permeability of liposomes, thus increasing the rate of content release.[144] Several studies, conducted *in vitro*, have reported that LFUS (20 kHz) enhanced drug release from liposomes.[181,182] *In vivo* studies have reported enhanced therapeutic benefits of combining US and liposomal cytostatic drugs in tumor treatment.[183,184] Liposome sensitivity to the acoustic waves is affected by their lipid composition. For example, the sonosensitivity of HSPC-based (solid) liposomes is lower than that of DOPE-based (fluid) liposomes, the latter being highly sonosensitive, releasing their payload efficiently due to the irreversible disruption of the membrane during sonication.[183] Furthermore, DSPE-based liposomes released more DOX compared to DSPC-based liposomes (69% and 9% respectively) following 6 min of insonation.[182] Liposomes prepared from DOPE/DSPC/DSPE-PEG2000/cholesterol (25:27:8:40 mol%) have extended circulation times and released 70% of their contents following 6 min of sonication.[185]

The sonosensitivity of liposomes can be further enhanced by the internal incorporation of a gas. Emulsion liposomes (eLiposomes) can encapsulate perfluorocarbon nanodroplets.[186] The energy of the US wave causes the evaporation and expansion of the gas, thus rupturing the liposomes (drug release) and promoting sonoporation (drug uptake). Emulsion liposomes are stable at physiological temperatures.[187] Sonication for 15 min (1 W/cm²) has resulted in the total destruction of eLiposomes.[188]

Following sonication, free DOX injected together with eLiposomes significantly inhibited the growth of osteosarcoma cells achieving similar therapeutic effects to that of free DOX but at a much lower dose (1/5 the dose of free DOX).[189]

Thermo-responsive liposomes are sensitive to increases in temperature and can be triggered to release their load when exposed to mild hyperthermia. Thermodox®, temperature-sensitive liposomes (TSLs) encapsulating DOX, reached Phase III clinical trials but failed to meet the primary endpoint of a 33% improvement in progression-free survival.[190] TSLs release their payload when the local temperature reaches their membrane solid to fluid transition temperature (T_m), at which point their fluidity changes from a solid-like phase/structure to a liquid-crystalline phase/structure.[191] As tumors are often warmer than healthy tissue, this is a form of triggered release.

There is interest in applying US with TSLs that have a T_m slightly higher than physiological temperatures for controlled drug release. Usually, a mild rise in local heat (mild hyperthermia) is achieved using medium frequency ultrasound; overheating can be avoided by controlling the duty cycle.[192] Farr et al.[193] reported that temperature-sensitive liposomes encapsulating DOX (TSL-DOX), triggered by the mild hyperthermia, produced by HIFU, increased DOX uptake by mouse pancreatic tumor tissue by 23-fold compared to TSL-DOX alone. Temperature-sensitive liposomes encapsulating DOX combined with US and microwave triggering were used to treat breast cancer patients, showing significant improvement (48 %) in local response.[194] A recent clinical trial[195] used thermosensitive liposomes combined with focused ultrasound to deliver DOX to patients with liver tumors. The trial concluded that while HIFU-triggered TSL-DOX enhanced drug delivery to the tumor, no adverse effects were observed as a result of the thermal ablation produced by HIFU. Table 2 below shows a summary of studies investigating the acoustically triggered drug release from liposomes.

Table 2. Studies investigating the acoustically triggered drug release from liposomes.

Reference	Study type	US parameters	Encapsulated substance	Effect
Rizzitelli et al.[196]	*In vitro/in vivo* study using TS/A (mammary) cells	3 MH (3.3 W/cm^2)	DOX	Enhanced therapeutic outcome due to the improved drug diffusion in the tumor following sonication.
Dromi et al.[197]	*In vitro*/*in vivo* study using murine adenocarcinoma tumors	1.189 MHz (4 W/cm^2)	DOX	Combining HIFU with TSLs enhanced DOX delivery.
Kheirolomoom et al.[198]	*In vivo* study using Murine breast cancer	1.54 MHz	DOX	Tumor elimination following the treatment with ultrasound triggered liposomal copper-DOX, CuDOX-LTSLs.
Lattin et al.[199]	*In vitro* study	20 kHz (5 W/cm^2)	Calcein	Following sonication, calcein release was significantly higher from e-Liposomes compared to conventional liposomes.
Gray et al.[195]	Phase I Trial	0.96 MHz	DOX	TSL combined with focused ultrasound hyperthermia is feasible and safe.
Um et al.[200]	*In vitro* study using EMT6 mouse mammary tumor cells	15 kHz (20 W/cm^2, 10 W/cm^2 and 10 µW/cm^2)	DOX	Similar enhancement of DOX release from thermosensitive liposomes triggered with both thermal and non-thermal acoustic treatment.
Santos et al.[201]	*In vivo* study using Human FaDu squamous cell	1.2 MHz	DOX	Combining TSL and Focused US improved drug delivery.
Awad et al.[202]	*In vitro* study using breast cancer cell lines (MDA-MB-231 and MCF-7)	20 kHz (6,7 and 12 W/cm^2) and 40 kHz (1 W/cm^2)	Calcein	Calcein uptake by cancer cells was enhanced following sonication.
Lentacker et al.[203]	*In vitro* study using Melanoma cells	1 MHz (2 W/cm^2)	DOX	Following the exposure to US, DOX-liposomes containing microbubbles were more effective in tumor elimination.
Yang et al.[204]	*In vivo* study using (GBM) 8401 glioma cells	1 MHz (2.9 W/cm^2)	DOX	Sonication enhanced targeted drug delivery of AP-1 Lipo-DOX, resulting in the inhibiting the brain tumor growth.
Novell et al.[205]	*In vitro* study	1 MHz	Calcein	The mechanical stresses generated by the focus US enhanced drug release from TSLs.
Treat et al.[206]	*In vivo* study using 9L gliosarcoma cells	1.7 MHz	DOX	Combining the US-mediated BBB disruption increased the therapeutic effect of liposomal DOX in the brain.
Park et al.[207]	*In vivo* study using HeLa cells	1 MHz	DOX	HIFU combined with the TSL liposomes significantly inhibited tumor regression.
Pitt et al.[208]	*In vivo* study using Colorectal cancer cells (DHD/K12/TRb)	20 kHz (1 W/cm^2)	DOX	US combined with DOX-liposomes showed a higher reduction of tumor growth compared to DOX delivered from micelles.

Ahmed et al.[29]	*In vitro* study	20 kHz 1, and 3 MHz with different power densities	Calcein	Calcein release was higher at the low frequency (20 kHz) investigated.
Eggen et al.[209]	*In vivo* study using Prostate cancer cells	1 MHz and 300 kHz	DOX	Both frequencies used led to an increase in tumor uptake of DOX from sonosensitive liposomes.
Ranjan et al.[210]	*In vivo* study using Vx2 tumor cell	MR-HIFU	DOX	Following sonication, DOX delivery from LTSL to Vx2 tumors in rabbits was 3.4-fold higher than that of LTSL alone.
Salkho et al.[211]	*In vitro* study using breast cancer cell lines MDA-MB-231 and MCF-7	20 kHz, 1.07 and 3.24 MHz	Calcein	The exposure to LFUS revealed an enhanced calcein uptake by the cells.
Shen et al.[115]	*In vivo* study using human glioblastoma cell line U87 MG	1.1 MHz	Paclitaxel	Combining PTX-liposomes with US in the presence of microbubbles enhanced drug efficacy towards glioblastoma.
Aryal et al.[212]	*In vivo* study using rat gliosarcoma cells	690 kHz	DOX	US enhanced the delivery of liposomal DOX from pegylated liposomes.
Afadzi et al.[53]	*In vitro* study using HeLa cell line (cervical cancer)	300 kHz	DOX	Sonication of DOX liposomes and microbubbles enhanced DOX uptake by cancer cells.
Williams et al.[213]	*In vitro* study using KB-V1 (Dox resistant) and KB-3-1 (Dox sensitive) cell lines	20 kHz (1 W/cm^2)	DOX	US released 78% of the encapsulated DOX from folate eLiposomes (feLD). US had no effect on the viability of both cell lines following the treatment with feLD.
Centelles et al.[214]	*In vitro/in vivo* study using IGROV-1 (ovarian cancer) cells	1.3 MHz	Topotecan	Enhanced uptake of both Topotecan and TSLs due to the focused US treatments.
Ueno et al.[189]	*In vivo* study using murine osteosarcoma cell line LM8	1 and 2 MHz (0.5 and 2 W/cm^2)	DOX	The therapeutic effect of DOX was significantly improved following sonication in the presence of bubble liposomes.
Lin et al.[215]	*In vitro* study using HeLa cell line	20 kHz (1 W/cm^2)	DOX	Higher DOX release from eLiposomes was observed following sonication when compared to liposomes without emulsions.
Hamano et al.[216]	*In vitro* study using 293T human embryonic kidney carcinoma cells	2 MHz (0.25-1 W/cm^2)	DOX	The combination of DOX-liposomes with bubble liposomes showed a US-enhanced drug release.
Awad et al.[217]	*In vitro* study	20 kHz (6, 7 and 12 W/cm^2)	Calcein	HSA-PEG and Tf-PEG liposomes were more sonosensitive compared to the control pegylated liposomes upon exposure to LFUS.

4.1.7. Liposomes-microbubble conjugates triggered with US

Contrast agent microbubbles are widely used in diagnostic imaging. These consist of an outer shell prepared from a lipid monolayer or albumin surrounding a low-solubility gas, thus enhancing blood-to-tissue contrast.[218] Microbubbles have a therapeutic potential in the field of gene therapy and drug delivery.[219,220] Microbubbles are 1-2 µm in diameter and usually contain a perfluorocarbon (PFC) or SF_6 gas. The density of the encapsulated gas is very different from that of the surrounding water, which makes the lipid-shelled microbubbles more sensitive to the pressure generated by the ultrasound waves and highly echogenic, allowing the easy manipulation of the microbubbles using the acoustic radiation force.[221] Subjecting the microbubbles to the pressure produced by ultrasound (MI>0.3-0.6) will generate mechanical stresses as the microbubbles rapidly expand, contract or collapse. This oscillation or collapse creates micro-streaming or a local microjet, resulting in membrane permeability via sonoporation. This will significantly enhance drug and gene delivery to the cells.[222]

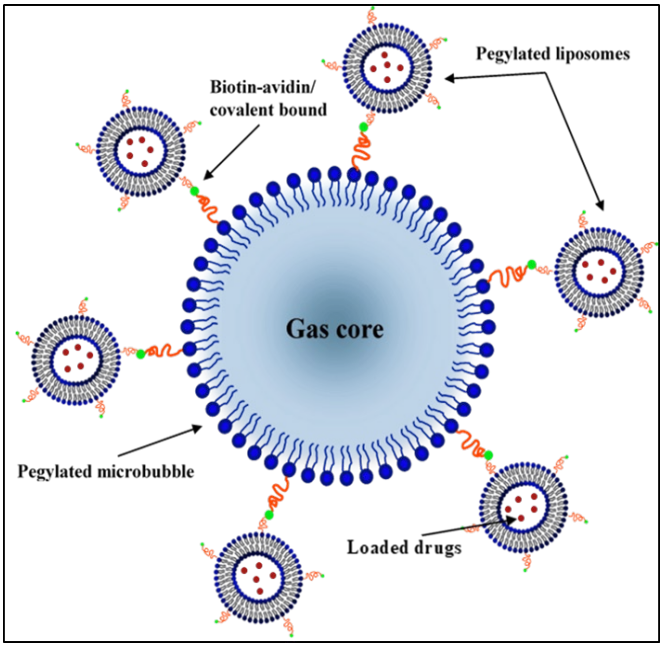

Fig. (8). Microbubble-conjugated liposomes.

Microbubbles can be used to deliver hydrophobic drugs within the protective lipid monolayer that covers them.[223] Generally, however, the space available for the drugs within the surrounded shell, as well as the gaseous core, is restricted which limits the efficiency of using microbubbles in drug delivery. To enhance their load capacity, microbubbles can be combined with other nanocarriers loaded with therapeutics. For example, liposomes encapsulating drugs can be conjugated to the surfaces of the microbubbles (Figure 8). These liposome-microbubble conjugates are promising drug delivery vehicles when combined with ultrasound-triggered drug release under ultrasound imaging guidance to treat solid tumors. At low intensity, microbubbles can be positioned spatially using radiation forces.[40] As the intensity of the ultrasound increases, the oscillation of the microbubbles increases, leading to the total collapse and destruction of the microbubble. These physical effects increase the permeability of cancer cells and their surrounding vasculature, leading to the enhanced uptake of drugs released in the vicinity of the target cells.[23] Usually, the pressure produced from the collapsing microbubble disrupts the structure of the conjugated liposome located within 40 µm.[23]

Drug-loaded liposomes can be conjugated to the surface of microbubbles using biotin-avidin linkers or covalent thiol-maleimide linkages.[223–225] Once injected into the bloodstream, microbubbles can be traced using conventional ultrasound contrast imaging modes. Following the accumulation of the liposome-microbubbles at the tumor site, high-intensity therapeutic ultrasound initiates microbubble destruction, drug release, and the sonoporation of the cells in its acoustic field.[224,226]

A recent *in vivo* study by Zhang et al.[227] reported that iRGD-targeted paclitaxel-loaded liposomes were conjugated to the surface of microbubbles using biotin-avidin linkages. The prepared iRGD-PTX liposome-microbubble complex retained ultrasound imaging capabilities and showed active ultrasound-triggered drug release. Histological examination showed elevated drug concentrations inside the cancer cells together with an increased number of apoptotic tissues in tumor xenografts leading to the inhibition of tumor growth. Another *in vivo* study by Yan et al.[228] reported enhanced antitumor efficacy as a result of breast tumor sonication following prior injection with paclitaxel-loaded liposomes conjugated to microbubbles, when compared to both the non-sonicated tumors and the non-conjugated liposomes. Yu et al.[229] reported that non-inertial ultrasound exposure released ~70% of the DOX encapsulated inside liposomes conjugated to polymer microbubbles. The study found that ultrasound exposure increased DOX uptake by the cancer cells and subsequently reduced their proliferation at 48 h.

5. Challenges and future prospectives of nanocarriers in cancer treatment

The development of a wide range of nanocarriers has revolutionized drug delivery in cancer treatment. Conventional chemotherapeutic drugs are faced with a number of challenges such as insolubility, short half-life, drug resistance, as well as their lack of specificity, which results in delivering toxic doses to healthy tissues. The developed nanocarriers are designed to circumvent these challenges by taking control of the drugs' circulation, distribution, and release, enhancing drug efficacy while reducing toxicity and the multidrug resistance (MDR) developed by the cancer cells. MDR is a considerable barrier in cancer treatment, and numerous studies have been dedicated to better understand MDR and develop strategies to

overcome this biological phenomenon. The proposed strategies include nanocarriers, RNA interference (RNAi) therapy, and physical approaches. The potential of nanocarriers in overcoming MDR lies in their ability to increase drug uptake and accumulation in tumor cells, suppress MDR proteins (such as the membrane bound drug transporter P-gp), and induce apoptosis. [230] RNAi therapy involves the use of various small interfering RNAs (siRNAs) to overcome MDR by silencing MDR genes such as P-gp/MDR1, MRP1, Bcl2, BCRP. However, the standalone therapeutic efficacy of these constructs was not satisfactory because RNAi mediators, such as siRNAs, are highly susceptible to rapid renal clearance, degradation by endogenous RNAses, and recognition by innate immune receptors. [231] Therefore, combining nanocarriers with RNAi therapy is currently one of the most promising methods to reduce drug resistance. The success of this approach is attributed the ability of nanocarriers to prevent the rapid degradation of siRNA molecules in serum, increase target selectivity, and increase cellular uptake. [230,232] A few siRNA-based anticancer drugs have entered early phase clinical trials, and the majority of these studies utilized moiety-free lipid nanoparticles (LNPs) and polymeric NPs. [233,234] The conducted studies have shown that the developed therapeutics were relatively well tolerated, cytokine release and infusion-related side effects were manageable using supportive treatments; in addition, none of the developed systems showed any major signs of antibody-mediated rapid clearance after multiple dosing.[231,233] Another approach in RNAi therapy involves combining chemotherapeutics with suitable siRNAs to overcome MDR and enhance the therapeutic efficacy of chemotherapeutic agents.[230,231]

With respect to physical strategies, temperature and ultrasound are external stimuli that have shown great promise in enhancing the delivery of numerous nanocarriers.[230] Effects mediated by hyperthermia include protein denaturation, activation of apoptotic pathways, alterations in the tumor microenvironment, modifications in oxygen and nutrient distribution at the tumor site, increase in the drug uptake, and reduction in cell membrane localization of MDR proteins. [230,232] Ultrasound, on the other hand, can be used either to induce hyperthermia or to reverse MDR by increasing drug diffusion, increasing drug-release from nanocarriers, and increasing the cellular accumulation of therapeutic agents at the tumor site. [230,235] Because ultrasound has shown great promise as a triggering mechanism, a great deal of attention has been placed on the optimization of ultrasound parameters, namely frequency, intensity, and exposure duration. Some studies reported that frequencies in the order of kHz induced more release than those in the order of MHz, this was attributed to the higher attenuation of ultrasound waves experienced at higher frequencies.[235] Other studies have shown a linear dependence of drug release on ultrasound intensity, highlighting the importance of inertial cavitation in drug release. [156,208,236] With regard to duration, it has been concluded that drug release increased with increasing exposure time.[235,237] This was ascribed to the self-sealing properties of liposomes; as some reports suggest that lipid membranes reseal rapidly following sonoporation, but the longer the pulse duration the more time it takes for the membrane to reseal itself.[238] Despite the breakthroughs in research investigating ultrasound-mediated liposomal release, there is still a need for further investigations into the optimization of ultrasound parameters in order to enable this technology to reach clinical settings.

Tumor metastasis is another major hurdle in cancer treatment. Many efforts are dedicated to developing nanocarrier-enabled tumor metastasis treatments. So far, the devised strategies can be divided into primary cancer targeting drug delivery and cancer metastasis-targeting drug delivery.[239,240] Primary cancer targeting drug delivery depends on preventing metastasis by inhibiting the growth of the primary tumor.[240,241] An emerging approach in this field targets cancer stem cells (CSCs). CSCs are a group of cells with high self-renewal abilities, which is believed to promote tumor development, recurrence and metastasis.[242,243] Studies have reported that reducing CSC-like properties of cancer cells or reducing the number of CSC cells can kill primary tumors and inhibit metastasis.[240,242] Another strategy involves targeting transcription factors (e.g. Oct-4, Nanog, KLF4, and MYC), intracellular signalling pathways (e.g. NF-κB, Hh, JAK-STAT, PI3K/AKT/mTOR, TGF/Smad, and PPAR), as well as extracellular factors (e.g. the vascular microenvironment, tumor associated macrophages, cancer-associated fibroblasts and mesenchymal cells, and the extracellular matrix) that regulate CSCs to inhibit tumor metastasis.[239,240,242] However, some issues need to be resolved before CSCs can be effectively used in cancer therapy. For instance, the characteristics of many CSCs specific to certain types of tumors are not well identified, most studies are performed in immune-deficient mice which does not mirror the biological complexity of tumors encountered when treating cancer patients. CSCs reside in niches (anatomically distinct regions within the tumor microenvironment) which are not well understood at the moment. In addition, most studies use isolated CSCs, which lack their specific niche that sustains their survival, which again does not reflect the conditions encountered when transitioning to clinical experiments. [242–244]

The most common approach in cancer metastasis-targeted drug delivery is the functionalization of nanocarriers with multiple targeting ligands. Another promising approach is the use of biomimetic NPs. Biomimetic NPs are a class of NPs that combine the functionality of biomaterials with the versatility of NPs to navigate complex biological systems.[245] NPs camouflaged in cell membranes are a novel class of biomimetic nanocarriers that can mimic some of the membrane functions of the cells from which these membranes are derived. Various cell membranes derivatives have been tested including those from red blood cells (RBCs), immune cells, macrophages, and cancer cells. Cancer cell membrane coated nanoparticles (CCMCNPs) consist of a NP core coated with a cancer cell plasma membrane. The NP core can be loaded with imaging and/or therapeutic agents, while the outer coating can carry tumor-specific receptors and antigens for cancer targeting.[240,245,246] CCMCNPs operate by actively reducing the ability of fibroblasts to attract cancer cells, therefore significantly reducing metastasis.[245] Other techniques investigated for cancer-metastasis targeted drug delivery involve targeting pre-metastasis niches.[240] Tumors can alter microenvironments in distant organs before their arrival at these sites, creating environments favorable for their survival and outgrowth. Pre-metastasis niche formation includes the induction of vascular leakiness, remodelling of the cell stroma and extracellular

matrix, as well as systemic effects on the immune system. The use of nanocarriers to identify such pre-metastasis niches could revolutionize cancer treatment and lead to the development of treatments that can pre-empt metastasis. [240,247]

Tumors vary in their biology and physiology from one patient to another, thus adding to the complexity of their treatment.[248] Precision and personalized medicine (PPM) is a novel approach in which treatments are tailored specifically for each individual.[249] In cancer therapy, PPM can be used to create individualized treatments based on the patient's tumor molecular profile.[250,251] Another aspect of PPM that may lead to improved disease outcomes is the use of multifunctional nanocarriers. These nanocarriers can be employed in the early detection of cancer by acting as imaging agents while delivering the drugs at the same time. The use of nanocarriers will provide the imaging of deep tissues allowing the early detection of small-sized tumors; as well as an assessment of the tumor microenvironment (namely vasculature and hypoxia) which provides information that may help predict drug behavior and allow better drug selection. [252,253]

Despite the promising opportunities presented by nanocarriers and their fast progress in the field of drug delivery, their clinical approval is comparatively slow, and only a few nanocarriers are commercially available such as liposomes and micelles. The future of nanocarriers depends on addressing some challenges which the technology is still facing. The efficient characterization of the different types of nanocarriers, as well as their successful reproducibility, large scale production and addressing their safety concerns are essential for their clinical development.[254]

The use of nanocarriers for drug delivery is relatively new compared to conventional chemotherapy. Therefore, the long term side effects/toxicities are still unknown. *In vitro* studies are essential in establishing proofs-of-concept but are not able to mimic the conditions inside the body. *In vivo* studies involving nanocarriers are extremely complex which makes analyzing their toxicity to the different parts of the body complicated.[255] Nanocarriers are readily taken up by the living cells. However, the intracellular reactions and pathways of nanocarriers are not yet fully understood.[256] In addition, nanocarriers vary in their materials, shape, sizes, surface, charge, coating, matrix, dispersion agglomeration, and aggregation. Thus, different nanocarriers will have different reactions and toxicity levels. Therefore, their toxicity cannot be measured unless the nanocarriers are fully characterized.[18] Generally, the smaller the size of nanocarriers, the higher the toxicity.[257] Changing the shape of gold nanocarriers for example from spherical to the rod shape increased their toxicity.[258] In addition, nanocarriers with a positively charged surface are more toxic than negatively charged nanocarriers, the presence of a coating layer or a shell will result in a significant reduction in nanocarriers' toxicity.[259] Furthermore, while circulating in the blood, some of the nanocarriers may accumulate in specific organs of the body, resulting in toxic effects.[260] Despite these safety concerns, the research of developing nanocarrier technology promises to overcome these challenges and provide effective and safe cancer treatments.

Conclusions

This paper reviews the growing technology of combining ultrasound with different nanocarriers to treat cancer. We have reported studies outlining the efficacy of ultrasound-responsive nanocarriers in *in vitro* and *in vivo* studies. However, several obstacles need to be addressed to ensure the success of ultrasound-responsive nanocarriers in clinical settings. More studies are needed to closely monitor the biodistribution and pharmacokinetics of ultrasound-responsive nanocarriers when ultrasound is applied *in vivo*. There is a need for continuous technological improvements to develop more biocompatible and effective ultrasound-responsive nanocarriers. Both the ultrasound parameters and nanocarrier specifications need to be optimized to ensure their successful use *in vitro*, *in vivo,* and future clinical trials**.**

Conflicts of interest

There are no conflicts to declare.

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