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REVIEW ARTICLE

Liposomes as a Promising Ultrasound-Triggered Drug Delivery System in Cancer Treatment

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Abstract: The initial uses of ultrasound waves in the medical field were limited to the thermal ablation of solid tumors and as a diagnostic tool. Recent advances at the preclinical stage have allowed the use of ultrasound as a powerful tool to improve drug delivery when the agent is administered encapsulated inside a nanoparticle. This spatial and temporal control of drug release, using a non-invasive modality, is a promising approach to decrease the side effects of conventional chemotherapy in cancer treatments, as it reduces the interaction of the anti-neoplastic agent with healthy tissues.

In this review, we explain the physics of ultrasound, introduce and discuss several examples on the use of nanoparticles as drug carriers, with a focus on liposomes. Examples of *in vitro* and *in vivo* studies are presented and discussed.

Keywords: Cancer, liposomes, drug delivery, ultrasound, drug release, anti-neoplastic agent.

1. INTRODUCTION

The development of effective cancer therapy alternatives remains an unpaired challenge. The World Health Organization reported 8.2 million deaths related to cancer in 2012, and predicts a rise of about 70% in new cases the next two decade [1]. Thus, the development of novel biomedical technologies and the improvement of cancer physiopathology knowledge are vital.

Many treatment methods are commonly used and known, *i.e.*, chemotherapy, surgery (in early cancer stages), hormonal therapy (to decrease or completely impair the production of hormones that stimulate the tumor growth) and targeted therapy (the focus of this paper). Traditional chemotherapeutic drugs are largely involved in the inhibition of cell division and their side effects include damaging normal/healthy cells that divide rapidly and are thus sensitive to anti-mitotic drugs (for example, cells in the bone marrow, digestive tract or hair follicles). The low efficiency of drug delivery to the tumor tissues is also an issue which increases toxicity to healthy cells and remains hampered by the difficult penetration of the drug in the vicinity of the cells that cause the disease. In fact, the pharmacokinetics of the chemotherapeutical drug is usually very poor, with a low percentage of the total amount of administrated drug reaching the tumor tissue. The main reason for this is the poor and heterogeneous vascularization of tumors and the high fluid pressure of the interstitial tissue of the tumors [2].

In an attempt to decrease the side effects of chemotherapy on healthy tissues, researchers developed nanoparticles to carry drugs and preferably extravasate into tumor tissue due to the enhanced permeability and retention (EPR) effect [3].

Because of its specificity, low toxicity, solubility in biological fluids, and immunostimulatory properties, targeted therapy using synthetic polymer nanoparticles has fewer side effects than traditional therapy with the additional advantage that various drugs can be encapsulated for all types of cancer [4].

Several nanoparticles have been studied for this purpose, such as micelles, dendrimers, solid lipid nanoparticles, and liposomes, among others. Liposomes offer the advantage that they are similar to the cellular membrane, being composed of a lipid bilayer surrounding an aqueous core where hydrophilic drugs can be encapsulated [5]. This reduces undesired effects such as being captured by the immune system and it also allows the fusion of the liposomal and the cell membranes, and the release of the anti-neoplastic drugs intracellularly.

Additionally, it is possible to enhance the specificity of these nanoparticles to cancer cells, by attaching ligands to their surface, and making use of the fact that cancer cells usually overexpress receptors for several ligands such as folic acid [6]. This results in a preferential targeting of these drug carriers to the cancer cells and reduces their impact on healthy tissues and organs.

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Another layer can be added to control the time and space of drug release from these highly specific nanoparticles. The combined use of a trigger, such as heat, change in pH, magnetic field, or ultrasound, and nanoparticles that are designed to be sensitive to one or more of these stimuli, has been proved to be an excellent strategy to control where and when the drug will be released [7]. Ultrasound has been widely studied as a release trigger, offering several advantages, such as the fact that it is a non-invasive technique, can be focused on the tumor tissue and its parameters can be finely tuned to reach an optimal release [8].

This review paper focuses on the use of liposomes as nanoparticle drug carriers in association with non-invasive and non-destructive ultrasound, and how this alliance can contribute to the development of new biomedical solutions in drug delivery.

2. ULTRASOUND

2.1. Introduction to Ultrasound

Ultrasound (US) is composed of oscillatory sound pressure waves with frequencies higher than the audible limit of humans (*i.e*., >20 kHz) [9]. Pressure waves, also known as stress waves, require a medium to propagate because their transmission occurs by direct contact of physical masses [10]. Additionally, these waves depend on the elastic nature of the medium, which plays a key role in sustained vibrations, and hence stress waves are also known as elastic waves [9]. These waves can be induced by vibrating piezoelectric transducers, which became a prominent research topic during World War I (1918), when a French scientist, Paul Langevin, suggested the use of a piezoelectric receiver [9]. Many scientists, from different disciplines, have contributed to the development of the science of acoustics: Sir Isaac Newton (1642-1727) derived the velocity of the sound wave in air, Jean Fourier (1768-1830) introduced a mathematical series characterizing ultrasonic waves, and it was the observation of the Italian biologist Lazzaro Spallanzani (1729-1799) that triggered the idea of SONAR when he discovered that bats used US to navigate in the dark [9]. Thorough research in acoustics, accompanied by technological advancements and progress in theoretical analysis and computer modeling in the 1970s, allowed the subsequent use of US in a wide range of fields, including aerospace, defense, nuclear, engineering, materials science, metrology, biology and chemistry [9].

Ultrasound waves can be classified, according to their intensities, into low-intensity and high-intensity waves, which have different applications. For example, applications such as nondestructive characterization of materials, medical diagnosis and the area of sensors use low-intensity US waves which require only transmission of energy through a medium without altering it. However, when US waves are meant to impose an effect on the medium being propagated through, then high-intensity US is the suitable choice. Examples of this include, kidney stone shattering, tumor ablation, cell lysis, emulsification, atomization of liquids and welding plastics or metals [9, 10]. Ultrasound waves with high intensity are often associated with thermal or mechanical effects which can be used to induce cavitation events which can also be applied to nanoparticles [9]. Drug delivery using nanoparticles is an evolving area of research, and this paper focuses on the use of US as a triggering mechanism by inducing mechanical and/or thermal effects on nanocarriers, especially on liposomes. Below, further details on the nature of US and its properties are presented.

2.2. Generation of Ultrasound

Ultrasound waves can be generated in three ways: the Galton's whistle, magnetostriction, and the piezoelectric method [11]. Francis Galton invented a special type of whistle that generates US waves that can be used to train animals. Such whistles are capable of producing sound waves with frequencies of up to 30 kHz [11]. Magnetostriction is a phenomenon utilized to generate US and was first discovered by James Joule in the early 1840s [12]. Joule describes magnetostriction as a change in the dimensions of a ferromagnetic material (*e.g*., iron, nickel) with a rectangular-bar shape when a magnetic field is applied along its axis. If the field is non-oscillating, it will result in a minor increase in the bar length (10^{-6} of the original length for a nickel bar) [13]. However, when an oscillating field is applied, it will cause a significant increase in the bar length since the elasticity of the material can no longer counteract the change imposed [13]. Usually, magnetostriction is used to generate US with low frequencies (since high frequency requires shorter transducer), thus another method is required to generate US waves with higher frequencies [13]. In 1880, the brothers Pierre and Jacques Curie discovered the piezoelectric phenomenon. They noticed that specific crystals like quartz, Rochelle and tourmaline salt accumulate an electric charge on their surface, upon exposure to a mechanical pressure/tension [13]. In a reversible manner, a piezoelectric material vibrates when an electric charge is imposed on its surface. To further explore this phenomenon, let us consider a cylindrical bar of ceramic after polarization. When an electric field (*i.e*., voltage difference) is applied in the same direction of the poling voltage, the resultant effect will be an elongation of the bar. On the contrary, when the voltage direction is reversed, the bar will undergo a compression process [14]. The other scenario is to translate a mechanical stress (tension/compression) into electrical energy as shown in Fig. **1**.

Generally, the main components required to construct a device that produces US waves are a transducer, a pulse generator and an amplifier. The transducer contains the piezoelectric material that translates electric pulses into mechanical vibrations. In medical scanning devices, a transducer also operates in the reverse direction receiving echoes (mechanical

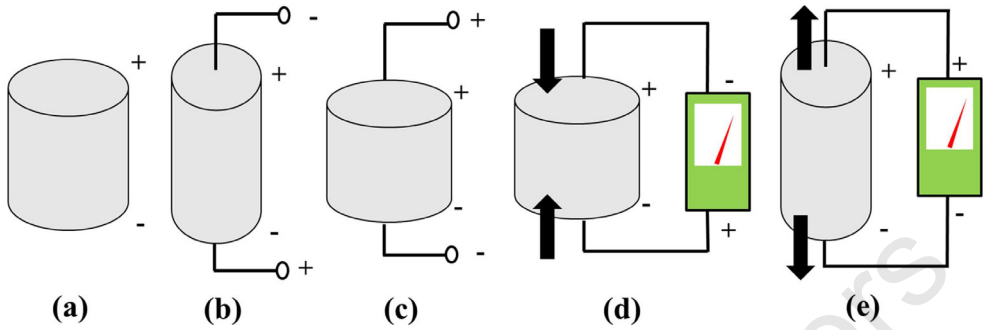

Fig. (1). Piezoelectric effect. (**a**) Polarized segment, (**b**) stretched segment and (**c**) compressed segment due to voltage difference imposed, voltage produced due to (**d**) compression and (**e**) tension applied on the segment [14].

waves) and generating electric signals as a characteristic of the scanned medium. The pulse generator, as the name implies, generates regular electric pulses to be applied on the transducer and allows the user to control pulse frequency and amplitude, among other features. Finally, amplifiers are utilized in circuits to magnify the size of an electric signal. Other accessories may also be added to the circuit depending on the application.

2.3. Physical Properties of Ultrasound

The energy of US waves propagates through a medium by collisions of oscillatory particles but with no net displacement [15]. These waves can be focused, reflected and refracted [9]. Ultrasound waves are sinusoidal waves with a given frequency, but when two waves interfere, their amplitudes are added/subtracted (depending on their phase) and the resulting frequency will be the sum of the individual frequencies of each wave. Consequently, the superposition of waves can cause beats, and hence the wave is known to have a beat frequency which is coupled with the Doppler effect in many applications [9]. Once a US wave propagates through a medium, its amplitude diminishes, a phenomenon known as attenuation. Attenuation occurs due to several factors such as the absorption of waves as a result of the conversion of mechanical energy into heat, and the reflection and scattering of waves by irregular surfaces/interfaces. The intensity of the US beam is selected depending on its application, as mentioned earlier. High-intensity US generates intense heat that is sufficient to melt steel. In liquids, high-intensity US is associated with a phenomenon known as cavitation which is used in cleaning processes [9].

Ultrasound waves are characterized by their frequency, propagation speed and amplitude. When a US wave propagates from one medium to another, both amplitude and velocity are affected, but the shape of the wave remains unchanged [16]. The velocity of a wave depends on the nature of the medium (its density and elasticity) and the type of the wave, while its amplitude depends on the impedance ratio of both mediums [9, 16]. Additionally, there are several modes of US vibration - longitudinal, transverse, torsion, shear, surface, flexural and Rayleigh-that can be utilized in ultrasonic applications [9]. Longitudinal waves (also known as compressional waves) are characterized by the vibration of molecules in parallel to the direction of energy transfer, while transverse waves are described by molecular vibrations that are orthogonal to the direction of energy transfer [17]. Transverse waves can only propagate through a solid medium. On the contrary, gases can only transfer longitudinal waves, whereas liquids can transfer both longitudinal and surface waves [17]. In biological-interaction systems, longitudinal waves are of special interest due to the favorable sequence of compressions and rarefactions they create [17].

2.3.1. Acoustic Impedance

When an acoustic wave propagates through a fluid, the particles of that medium are forced to displace around their original position with a velocity known as the acoustic particle velocity [18]. However, in any medium, there is a resistance to acoustic wave propagation, which is called acoustic impedance (with SI units of Pa.s/m^3). Acoustic impedance is a key feature in determining the proportion of acoustic energy transmitted and reflected [19]. When the medium is characterized by closely-packed particles (*i.e.*, dense material, high specific acoustic impedance), the particles require high pressures to move at a given velocity compared to a lower pressure requirement for loosely-packed materials (*i.e.*, low specific acoustic impedance) at the same velocity [20]. The equation that relates the pressure of an acoustic wave (P), the speed of sound in the medium (c), the particle velocity (v) and the density of the medium (ρ) for which the wave is propagating through is [18]:

$$P = \rho.c.v = Z.v \quad P = \rho.c.v = Z.v \qquad \text{Eq. (1)}$$

Based on the equation above, the specific acoustic impedance (Z) for a substance (Pa.s/m or rayl) depends on the density of the substance and the velocity of the acoustic wave. Table **1** lists the specific acoustic impedances for water and some tissues [18].

2.3.2. Reflection of Waves

When a wave strikes a boundary (*e.g.*, a bone-tissue interface), the characteristic acoustic impedances of both media determine the fraction of the wave's energy reflected as an echo [19]. The difference between two impedances is known as acoustic impedance mismatch [20]. The greater the difference

Table 1. Characteristic acoustic impedance for selected biological tissues [18].

Tissue	Characteristic acoustic impedance (Rayl)
Water (20 °C)	1.48×10^6
Muscle	$1.65 - 1.74\times10^6$
Fat	1.38×10^6
Skin	1.7×10^6
Cortical bone	$4 - 8\times10^6$

between the impedances, the more the acoustic energy is reflected from the interface, hence less energy is transmitted into the second medium. If the impedances of both mediums are identical, then there will be a complete transmission with no reflection [20]. Besides the mismatch factor, the angle of the wave incidence also plays a role in determining the proportion of energy to be reflected and transmitted [19]. When a beam is propagated on a surface at an orthogonal angle, the fraction of the reflected (R) and transmitted (T) energies for longitudinal waves can be calculated by the following equations [18]:

$$R = (Z_1 - Z_2)^2/(Z_1 + Z_2)^2 \quad \text{Eq. (2)}$$

$$T = 4\,Z_1Z_2/(Z_1 + Z_2)^2 \quad \text{Eq. (3)}$$

where Z_1 and Z_2 are the impedances of materials 1 and 2, respectively. Based on the two previous equations, the sum of reflection and transmittance is equivalent to unity (R+T=1), hence energy is conserved (lossless case) [18]. The reflection of waves is used in medical imaging to visualize tissues and organs. When there is a strong acoustic mismatch, most of the waves are bounced back from the interface as a strong echo while the rest of the energy transmitted into the second medium cannot be used to produce images for inner organs and tissues. This is the reason why a transducer cannot acquire an image when there is a gap of air between the transducer and the patient's skin (case of total reflection of acoustic waves due to impedance discontinuity). Thus, a material with an intermediate acoustic impedance (gel or oil) must be placed between the transducer and the skin while imaging. This justifies why air-filled organs block the tissues and organs underneath from imaging [18].

2.4. Cavitation Phenomena: Inertial and Stable

The energy of US when focused on a specific area will usually dissipate in heat, acoustic cavitation, and radiation forces, as shown in Fig. **2** [21].

Cavitation is defined as the formation, oscillation and collapse of bubbles in a liquid medium exposed to US waves [22]. The bubbles are either originally present in the liquid, or may be newly formed when the pressure is lowered below the vapor pressure of the liquid [10]. The bubbles can be classified into free

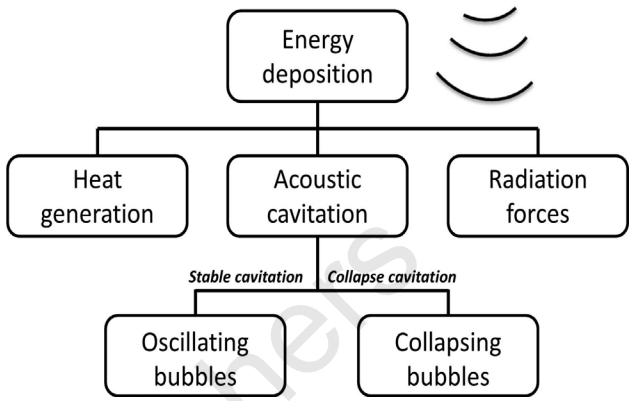

Fig. (2). Schematic representation of US energy deposition [21].

bubbles and encapsulated microbubbles (EMB). Free bubbles are voids or cavities that are filled with gas and do not have artificial boundaries to prevent leakage of gas, unlike the EMB. The gas bubbles expand at low pressure and contract at high pressure. There are two types of cavitation depending on the bubble size stability: stable and transient cavitation [23]. Stable or non-inertial cavitation creates a circulating fluid around the bubble with velocities and shear rates proportional to the oscillation amplitude. This type of cavitation persists for a large number of acoustic cycles without collapse. On the other hand, at low frequency US (LFUS) with very high intensity and microbubble concentration, collapse cavitation (also called inertial or transient) occurs [24, 25]. In this case, the gas bubbles expand rapidly, become unstable, and finally collapse. The bursting bubbles in transient cavitation generate a short-lived intense local heating, which may reach up to 5000 K and is accompanied by high pressures, which can be as high as 1000 atm [10].

The size of the bubble is affected by the applied frequency of the US. As the frequency increases the bubbles decrease in radii. For instance, at 20 kHz the air bubble radius in water is 40 μm [26], while at 3 MHz it is only 1 μm [27].

The mechanical index (MI) is a value that estimates the probability of an adverse mechanical effect (cavitation) in a subject exposed to diagnostic US [28]. The MI has an inverse relationship to the square root of the US frequency f. Hence, at LFUS, a high MI value is reached, thus there is a higher probability to generate cavitation.

2.5. Medical Applications of Ultrasound

Ultrasound waves have various medical applications such as imaging, kidney stone disruption, blood flow analysis, drug delivery, and tumor ablation [10]. An emerging application of US in biotechnology is the use of EMB in diagnostic US imaging. These EMBs are called US contrast agents (UCAs) due to their acoustic impedance being different from that of the soft tissue [28]. Ultrasound frequencies used for such applications typically range between 0.8 and 3 MHz [29].

In targeted drug delivery, US can be employed due to two phenomena: hyperthermia (temperature effects) and cavitation (mechanical effects). Hyperthermia occurs when the US beam is focused on a small tumor tissue area and hence the power/area ratio (called power density) becomes very high resulting in local heating, which can be locally used to ablate tissues [24]. It has also been reported that, at lower intensities, thermal energy from heating can be employed to release drugs encapsulated in heat-sensitive nanoparticles: when the temperature increases beyond the phase transition temperature (T_m) of the lipid bilayer of a nanocarrier (*e.g.*, liposomes), it allows its destruction, thus releasing the drug [24, 25]. Furthermore, in drug delivery, US is used to control the release of specific drugs *via* mechanical effects due to the oscillating pressure waves. The encapsulated drug released by the effect of stable cavitation as a result of the convective flow around the oscillating bubble is called microstreaming. Microstreaming has the ability to shear liposomes open, thus releasing their therapeutic contents [25]. It has also been hypothesized that intense cavitation increases cell membrane permeability [30]. On the other hand, at very high intensity and microbubble concentration, inertial cavitation occurs [24]. In this case, the gas bubble collapses causing the release of a liquid jet at a sonic speed capable of piercing the cell endothelial layer [30]. The consequences of this cavitation phenomenon may be detrimental to tissues and adjacent cells due to the huge shear stress, the shockwave produced and the free radicals generated at elevated temperatures which may interfere with biochemical processes [25]. Stable cavitation, on the other hand, has no negative biological attributes and can be applied to enhance the convection of oxygen and nutrients into normal cells [25]. In liposomal drug release, stable cavitation is not as effective as transient cavitation. In order to use transient cavitation in drug release, drawbacks of using this method should be minimized by selecting the suitable parameters of US. The key is to produce a bubble activity that effectively ruptures the liposomal membrane without damaging the adjacent endothelial cells or causing thrombosis [25]. In this case, the release of drugs to the patient's infected tissues is controlled which reduces the side effects on nearby healthy cells.

3. NANOPARTICLES

Nanotechnology refers to the scientific, technological and/or engineering use of particles which are developed at the nanometer scale. Although these particles can be conjugated or pieced together to form structures which can extend into the micrometer range, they are initially designed at the nanometer level which imparts specific qualities and functions upon them which differ from when they are "seen in bulk scale" [31]. Some of these qualities, including an improved solubility due to their small size and having a customizable surface, render these particles ideal for biomedical applications [32]. In this review, we focus on the applications of nanoparticles in drug delivery systems (DDS) used in cancer chemotherapy. Several nanoparticles are currently being researched to designing novel DDSs (Fig. **3**), the most important of which will be discussed in the following sections.

3.1. Micelles

Micelles are self-assembling structures of amphiphilic molecules, *i.e.*, molecules that have both hydrophobic and hydrophilic regions [33].

Micelles can be formed from small surfactant molecules, including phospholipids, the latter being the main component in the synthesis of liposomes as well as biological membranes. However, whereas liposomes arrange to form a bilayer with the hydrophobic tails sequestered between two hydrophilic layers, micelles form a monolayer with a hydrophilic exterior shell (corona) and a hydrophobic interior (core) [34]. This quality renders micelles particularly useful in the delivery of hydrophobic drugs to tumors but less so in the case of hydrophilic drugs.

Micelles can also be formed from amphiphilic block copolymers, which are much larger than surfactant molecules. These are called polymeric micelles and they have important properties which make them valuable DDS nanoparticles. For example, the polymeric micelles of Pluronic® P105 have been widely studied as drug-encapsulating nanoparticles, with the capability of being used in conjunction with ultrasound, which acts as a trigger to induce drug release from the micellar core [35].

There are several advantages associated with micellar drug delivery, perhaps the most significant of which is their small size, which allows them to undergo extravasation at the site of tumor formation, and also prevents them from being excreted by the kidneys through absorption and secretion [35]. Additionally, they can have their surface modified with ligands allowing for ligand-targeted drug delivery [36] (see section 4.5).

3.2. Dendrimers

Dendrimers are globular shaped macromolecules which consist of three main regions: core, branches emanating from the core and surface functional groups [37]. Dendrimers are synthesized stepwise from branched monomer units, hence it is possible to precisely control dendrimer size, shape, dimension, density, polarity, flexibility and solubility, by choosing different building/branching units and surface functional groups [37]. The fact that all of these important variables can be controlled makes dendrimers one of the most attractive nanoparticles for use in DDS. For example, they are superior to micelles in that they are easily adaptable for the transport of both hydrophilic and hydrophobic drugs [38].

3.3. Nanoemulsions and Solid Lipid Nanoparticles

An emulsion is a mixture of two phases where one component of the fluid is dispersed as small vesicles

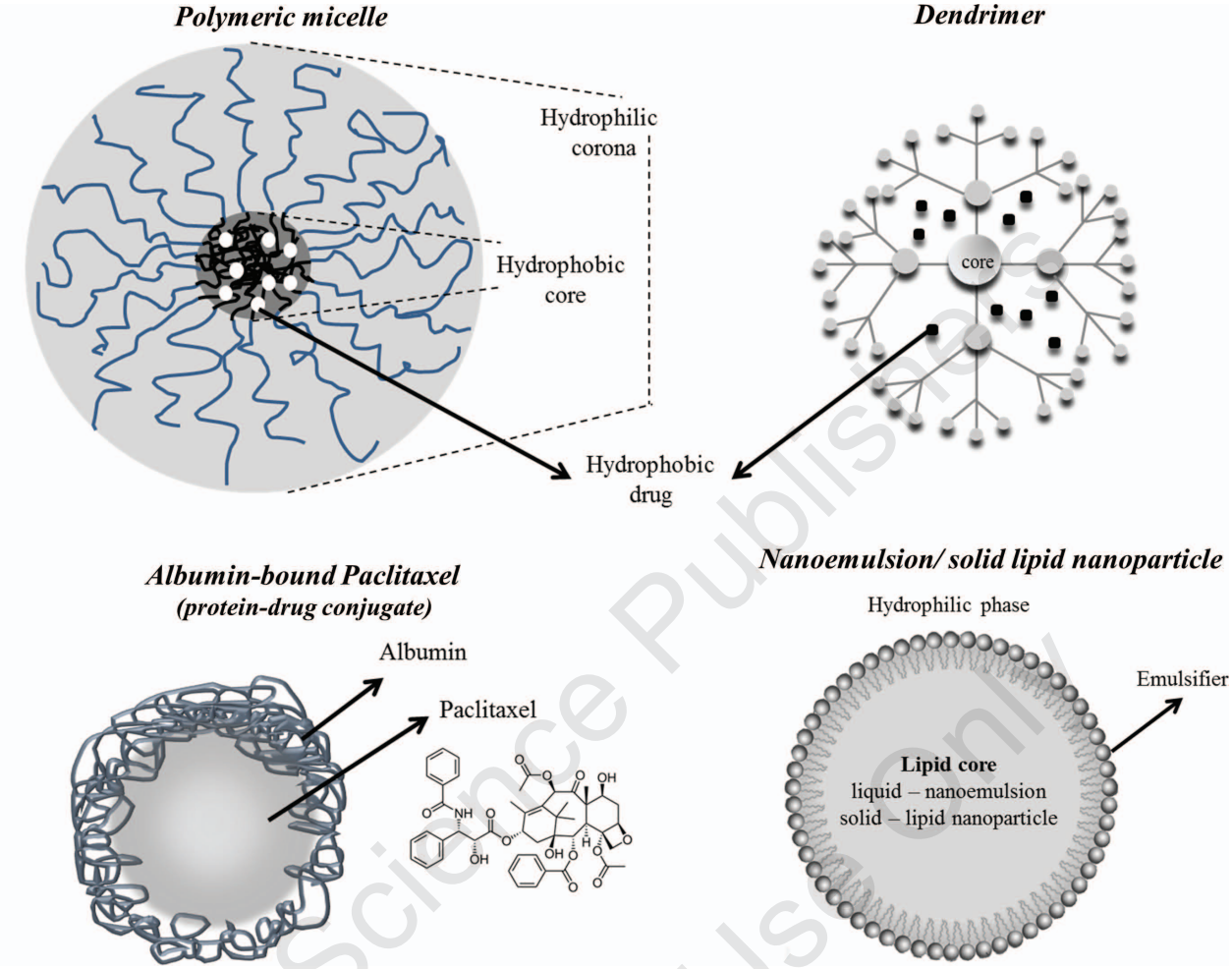

Fig. (3). Some examples of nanoparticles used as drug delivery systems.

(*dispersed phase*), but not completely dissolved, within the other (*continuous phase*) [39]. Nanoemulsions are emulsions prepared in the submicron size range that can act as drug carriers [40]. A nanoemulsion is composed of a lipophilic phase, such as an oil droplet, where the drugs are usually encapsulated, and a hydrophilic aqueous phase where the lipophilic phase is dispersed. This arrangement is thermodynamically unstable, hence an emulsifying agent - an interphase - is needed to prevent the two phases from separating into distinct layers [39, 40]. The size of the interphase can be controlled, and this affects the size of the dispersed phase droplets: if they are small enough (*i.e.*, within the range of 20-600 nm) the emulsion is considered a nanoemulsion [39, 40]. There are several advantages associated with the use of nanoemulsions some of which is the large surface area provided by the small size of droplets, which allows them to be absorbed quite easily by target organs during extravasation, thus improving the bioavailability of the drug [40]. Another advantage is that, by virtue of their small size, they are less likely to cause sedimentation within the body which prevents the occurrence of blockages and other complications. Additionally, they are non-toxic, have improved physical stability and can be formulated as foams, creams, liquids and sprays [40]. These advantages make nanoemulsions very important in the field of DDS alongside liposomes, dendrimers and micelles.

Solid lipid nanoparticles (SLN) are similar to nanoemulsions but the core of the hydrophobic phase is solid lipid (instead of liquid lipid) [41]. The encapsulation of the drug inside a solid matrix decreases its mobility, and enhances the release control compared to nanoemulsions. Other advantages include an increased permeation through biological barriers, chemical stability, the possibility of surface modification and the response to drug-releasing stimuli [42]. The work of Mehnert and Mäder extensively reviews these nanoparticles [43] and a recent review [42] updates the use of SLN as drug delivery systems.

3.4. Protein-Drug Conjugates

Anti-cancer drugs can be conjugated to proteins, yielding protein-drug conjugates, a new class of targeted therapeutics that combines the specificity of the protein moiety with the cytotoxic effect of the drug [44]. The specificity is particularly significant when the protein is a monoclonal antibody, and some antibody-drug conjugates have already been successful in cancer treatment (see [45] for a recent review on this subject).

Here, we chose protein-bound Paclitaxel as an example of a protein-drug conjugate. Paclitaxel (also known as Taxol) is an anti-cancer drug which was isolated from the bark of yew trees in the Pacific. However, this process is slow, inefficient and destroys yew plantations, hence Paclitaxel is currently being synthesized chemically and is used to treat a wide variety of cancers including ovarian, breast, lung and others [46]. However, a significant drawback associated with the use of Paclitaxel is its non-specificity and the excessive toxicity in patients [46]. Because of these dangerous side-effects, research has focused on developing an alternative mode of delivery to preferentially target tumors. In this regard, protein carriers have significantly increased Paclitaxel's effectiveness and targetability against tumors. In particular, the use of albumin as a protein carrier avoids solvent-based toxicity and allows the use of albumin targeting pathways, which result in higher intratumor concentrations of the drug [46].

4. LIPOSOMES

4.1. Brief Introduction to Liposomes

Liposomes are defined as self-assembling spherical vesicles consisting of amphipathic lipid bilayers, which can encapsulate molecules smaller than themselves [47-49]. In general, the essential components of liposomes are phospholipid molecules containing a polar head and two long hydrophobic tail groups, in addition to other molecules such as cholesterol. Because these chemical groups can vary in size and length, the total size of the liposomes is highly variable, ranging from 25 nm to several micrometers in diameter [47, 50]. In aqueous solutions, they tend to be oriented such that their polar head is facing towards the hydrophilic inner and outer environments while the hydrophobic tails are sheltered between the polar heads in a hydrophobic leaflet. The spontaneous behavior of the liposomes in this manner is attributed to the hydrophobic effect which aims to minimize entropically unfavorable interactions between hydrophilic and hydrophobic moieties and is an important property with regards to their function (Fig. **4**).

Liposomes were first discovered by the British biophysicist Alec Bangham who noted that distinct globular aggregates were formed when lecithin, an egg protein, and water were mixed [51]. Upon closer inspection with an electron microscope, the globular structures were revealed to be vesicles that formed spontaneously when certain fats (specifically those with amphipathic/amphiphilic properties) were mixed in aqueous solutions. Subsequently, these vesicles were termed liposomes. They bear a striking resemblance in terms of chemistry and structure to the plasma membranes that characterize cells of living organisms. Building on this fact, an important property of liposomes is their ability to fuse with other membranes which are similar in composition, such as plasma membranes, and release their contents inside [52]. As a result, their clinical applications have been widely investigated and liposomes have proven their utility as drug delivery vehicles [53].

4.2. Classification of Liposomes

Although there are different ways of classifying liposomes, perhaps the most established method is based on size, which is related to the number of bilayers they possess (Fig. **5**) [47]. As mentioned previously, the size of liposomes is highly variable, which can contribute to a differentiated pharmacokinetic and bioavailability. While smaller liposomes can circulate more effectively through the bloodstream and for longer durations, larger ones can be useful due to their ability to encapsulate larger molecules and to deliver them to the desired cells [54].

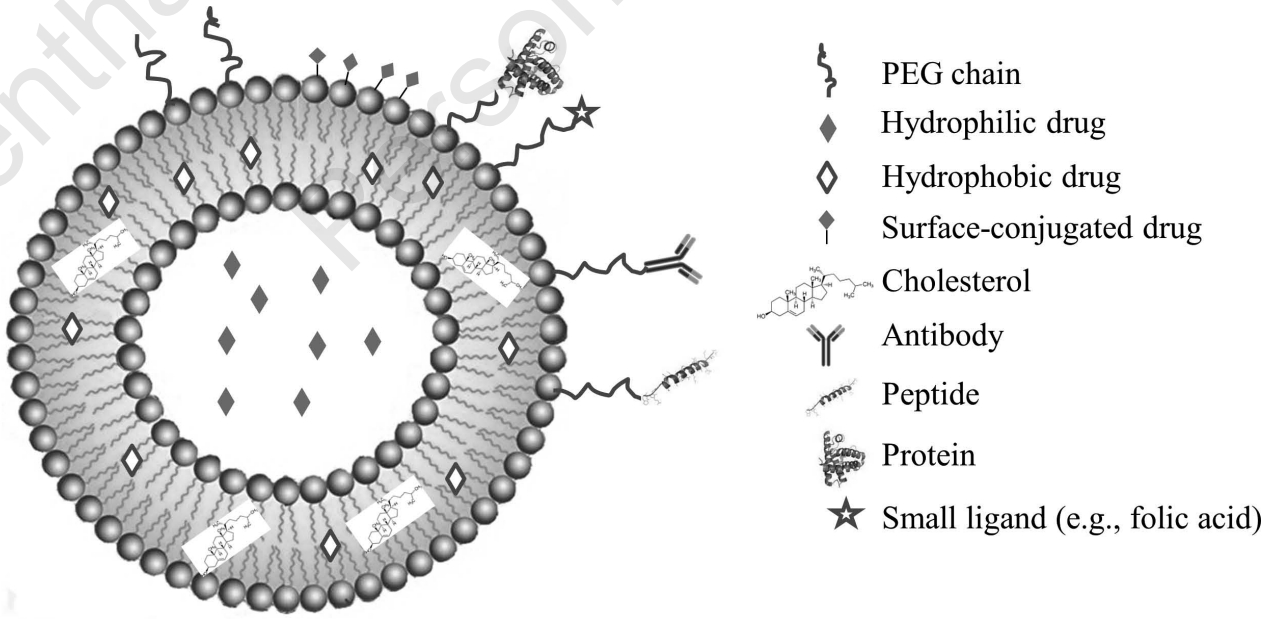

Fig. (4). General structure of a liposome and possible modifications of its surface.

Smaller liposomes could also be used for macrophage-targeted phagocytosis as they are small enough to be engulfed by these cells [55]. This would be desirable in situations where macrophages and monocytes, which can contribute to the progression of a disease and its symptoms through inflammation, must be neutralized. Hence, it is clear that size is an important factor to consider when selecting liposomes for a specific clinical application.

Another important property that can be used to categorize liposomes is the number of bilayers they possess, which is dependent on the method used in their preparation [8]. Liposomes with more than one fluid compartment separated by multiple lipid bilayers are called *multivesicular* vesicles (MVVs), if they are made of non-concentric internal aqueous chambers separated by a network of phospholipid bilayers [56], and they are called *multilamellar* vesicles (MLVs) if the chambers are concentrical, with an onion-like structure [8, 47]. On the other hand, liposomes that have a single fluid core with a diameter of 25 nm - 1 μm are designated as *unilamellar* vesicles (ULVs) [57] (Fig. **5**). Depending on the size, ULVs can be further classified as *small unilamellar* vesicles (SUVs) or *large unilamellar* vesicles (LUVs) [8, 47]. The significance of the number of bilayers lies in that they influence vital parameters with regards to drug delivery such as their pharmacokinetics, the encapsulation efficiency of the drug and the rate at which it is effused to target cells [58]. For example, although MLVs vesicles (which are typically larger than their ULVs counterparts) could be easily targeted, SUVs are more effective at delivery because they can encapsulate higher concentrations of the drug [59].

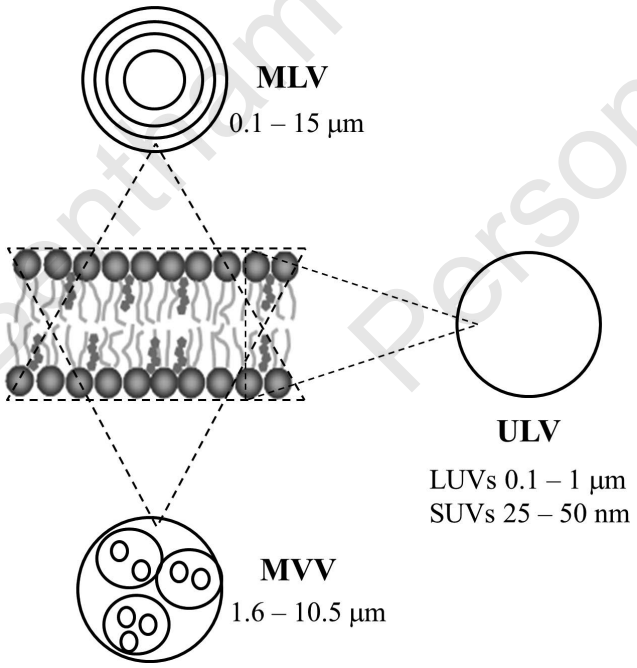

Fig. (5). Classification of liposomes based on size and the number of bilayers [57]. ULV - unilamellar vesicle, LUV - large unilamellar vesicles, SUV - small unilamellar vesicles, MLV - multilamellar vesicles, MVV - multivesicular vesicle.

One of the most crucial considerations in modern medicine is the necessity of specifically targeting the diseased tissues. To this end, different types of liposomes have been designed to release their contents once they reach the target and when exposed to an external stimuli, such as heat, light, US, etc. (see section 4.6) [34]. In this review, we will focus on drug release from liposomes triggered *via* acoustic power. Liposomes containing microbubbles are characterized by their susceptibility to cavitation induced by ultra-sonication [60]. eLiposomes, another type of sono-sensitive liposomes, contain enclosed nanoemulsion droplets [61]. This sonosensitivity allows further control of the drug release from these nanoparticles, upon the application of an external US source. In section 4.6 we further explore this subject, briefly introducing some of the triggers used in drug delivery, and focus our attention on US-sensitive liposomes.

4.3. Methods for Liposome Preparation

Liposomes have a vital role in drug delivery, thus many researchers have been developing various techniques to form liposomes with desired characteristics. In general, the drug is loaded into the liposomes either by passive or active methods [62]. The main variables to be considered in drug encapsulation are trapping efficiency, drug retention and drug-to-lipid ratio [62]. Trapping efficiency favors procedures that achieve high drug encapsulation (> 90%), while drug retention is significant for storage purposes and drug release during treatment [62]. Passive loading includes techniques where drug and lipids are both dispersed in an aqueous buffer, hence drug entrapment occurs during liposome preparation [62]. On the other hand, active loading methods involve drug encapsulation after forming liposomes, by establishing a membrane potential or transmembrane pH gradient [62]. The choice of the liposome preparation method depends on many factors including [63]: (i) the medium used to disperse the lipids; (ii) the characteristics of the substance/agent to be entrapped/encapsulated; (iii) the constituents used in the liposomal formulation; (iv) the concentration of the substance to be encapsulated; and (v) the physical properties desired for the synthesized liposomes including size, polydispersity and the shelf-life of vesicles.

In general, liposomes are classified into three main categories, according to their method of preparation: (i) mechanical dispersion, (ii) solvent dispersion, and (iii) detergent removal [63] (Table **2**). A few of these methods will be further discussed in the following sections.

4.3.1. Lipid Film Hydration

To prepare liposomes according to the lipid film hydration method, lipids are first dissolved in an organic solvent or mixture of organic solvents (*e.g.*, chloroform or chloroform/methanol 2:1 (v/v)) in a round bottom flask or vial, to obtain a homogenous mixture

Table 2. Liposome preparation methods according to passive loading techniques [63].

Mechanical dispersion	Solvent dispersion	Detergent dispersion
• Hand-shaken and non-hand shaken lipid film hydration, freeze drying • Sonication • Membrane extrusion • French press • Micro-emulsification • Freeze-thaw • Dried reconstituted vesicles	• Ethanol or ether injection • Reverse phase evaporation • Double emulsion • Stable pluri lamellar vesicles	• Detergent removal from mixed micelles by dialysis, column chromatography or dilution

with a concentration of 10-20 mg lipids/ml of solvent [63, 64]. Afterward, the organic solvent is evaporated by either purging the sample with nitrogen or argon or using a rotary evaporator, for large volume samples [63], an operation performed at a temperature above the T_m of the lipids [64]. The lipid film is then hydrated with an aqueous medium (water or buffered solution) at the same temperature, by rotating the round bottom flask in a water bath, for up to one hour [63, 64]. The product of this synthesis includes a mixture of milky-like MLVs (Fig. **5**), that can be furthered downsized into SUVs using a mechanical method, such as sonication [64]. Sonication may be performed in a sonicator bath or by immersing a sonicator probe into the sample [64]. Usually, sonicator baths are preferred, since ultrasound tips deliver very high energy, which may induce local heating that can de-esterify the lipids, and may also contaminate the sample with metals (*e.g*., titanium) [63, 64]. During sonication, the suspension will change from a milky-like to an opalescent solution [63, 64]. Vesicles with small diameter (<40 nm) produced after sonication are metastable, *i.e*., due to the high curvature energy, they tend to fuse with others to form bigger vesicles (d= 60-80 nm) that are more stable [64].

4.3.2. Reverse-Phase Evaporation (REV)

The REV method introduced by Szoka and Papahadjopoulos in 1978 [65], was one of the most significant achievements in a liposomal preparation. At the time of its implementation, this was the first technique allowing for the high encapsulation efficiency in aqueous media [64]. Additionally, REV is applicable to various lipids, including cholesterol, and can achieve an aqueous volume-to-lipids ratio up to 30 times higher than that obtained from SUVs prepared by sonication and four times of MLVs achieved by the lipid film hydration method [64]. The major drawback of REV arises when the molecules to be encapsulated are proteins, due to their possible denaturation upon mixing with an organic solvent [64, 65].

The protocol for REV includes, first, the formation of inverted micelles [64]. The lipids are dissolved in chloroform and dried in a rotary evaporator. Afterward, the lipids are dissolved in an organic phase, *e.g*., diethyl ether, followed by the addition of an aqueous medium containing the molecules to be encapsulated. The ratio of the organic phase-to-aqueous medium should be 3:1 (v/v) so that an optimum encapsulation efficiency can be achieved. To form inverted micelles, the two-phase solution is then sonicated 2 - 5 min in a sonicator bath, at a temperature below 10 ^{o}C to avoid the separation of dispersed micelles from the organic phase, until the mixture becomes an opalescent one-phase solution. After sonication, diethyl ether is evaporated at room temperature under reduced pressure in a rotary evaporator and after evaporation, inverted micelles become viscous and some of them will disintegrate to build up a second layer around the remaining inverted micelles forming what is known as REV liposomes. Liposomes formed by this method are mostly unilamellar with a heterogeneous size distribution (100 nm - 1 μm), and they can be purified by centrifugation at 20,000x*g* or by passing them through a Sepharose 4B column.

Protocols can be further modified to prepare liposomes with certain characteristics that improve their targetability, pharmacokinetics and the bioavailability of the drug they encapsulate as shown in the following two sections.

4.4. Modification of Liposomes

The attachment of polyethylene glycol (PEG) to the phospholipid bilayer of liposomes has been shown to increase their biological half-life by allowing them to circulate longer within the body [66]. Similarly, attaching albumin, an endogenous globular protein involved in maintaining osmotic pressure in the blood, to liposomes has shown to reduce their interactions with a certain opsonin and thus their elimination from the body by phagocytosis [67]. Liposomes formed by such modifications are known as *stealth liposomes* due to their ability to evade the immune system and remain within the body for longer periods of time [34]. Additionally, the conjugation of specific moieties to the liposome surface allows for *active* targeting (see section 4.5.3). These moieties interact with specific receptors present on the surface of cells, which are often overexpressed on the surface of cancer cells. These moieties include small ligands such as folate, peptides, proteins, antibodies (used in *immunoliposomes*), and others [68].

Not all moieties used to modify liposomes are necessarily attached to the phospholipid bilayer, but they can also be embedded in it, such as in the case of liposomes containing cationic lipids (*i.e*., lipids with a positively charged head group) [69]. Substances used for treating diseases at the molecular level, such as miRNA and siRNA, are negatively charged which, due to electrostatic repulsion, makes it difficult for them to

penetrate cells through the negatively charged cell membrane [69]. However, liposomes containing cationic lipids bypass this issue and are more easily integrated into the cell, thus increasing the efficacy of treatment.

Another important modification to mention here is the addition of cholesterol, which increases the liposome stability both *in vivo* and *in vitro* [70]. This effect can be attributed to the fact that cholesterol restricts the mobility of the phospholipids in the bilayer, which prevents loss of lipoprotein hence the loss of the entrapped substances [70]. Cholesterol also influences the permeability of membranes by increasing fluidity through disruption of hydrophobic tail packing which could increase the fusion of liposomes with target membranes due to their reduced entropic stability.

4.5. Passive *Versus* Active Targeting

4.5.1. Drug Targeting

An active pharmaceutical ingredient (API), when administrated to a patient, is distributed throughout the patient's body. During its journey to the desired location, the API will cross many organs, tissues and cells, where it can be deactivated or taken up by healthy tissues and organs leading to undesirable effects at sites not intended to receive the chemo-therapeutic treatment. Thus, to ensure that the drug reaches the target site at a therapeutic concentration, it has to be administrated in large amounts. This will cause many negative effects on healthy cells, especially if the drug is cytotoxic [71]. This highlights the importance of developing methods for targeting drugs, such that they arrive at only the tissue of interest, avoiding interactions with healthy tissues particularly ones critical for life.

Drug targeting can be defined as an increase in selective and quantitative accumulation of an API in a certain tissue or organ in the body, thereby reducing side effects of unspecific drug accumulation [71].

In the case of the lipid-based nanoparticles, the EPR effect increases the retention of the particles smaller than 0.5 μm at the tumor site which improves the bioavailability of the loaded API. Due to the anatomical and physiological properties of tumors, the EPR effect is crucial on the selectivity and accumulation of a chemotherapeutical drug.

However, the efficiency of passive targeting is compromised in several tumor types because of the irregular vasculature and the high tumor interstitial pressure. To improve therapeutic versatility and cell specificity, active targeting was introduced. The specific conjugation of targeting moieties intended to induce receptor-mediated endocytosis of derived nanoparticles will be discussed below. Ligand-mediated targeting increases the uptake of nanoparticles by the targeted cells but the accumulation of the API in the desired cells depends not only on the receptor density and ligand affinity but also on the vasculature permeability and tumor penetration. For many active-targeting DDS, ligands conjugated to the surface of liposomes resulted in little or no therapeutic improvement over passive targeting, due to the non-internalization, premature content leakage, poor penetrability, or low receptor density [5].

To improve the targetability of liposomes, nanoparticles can also be designed to release the API in the presence of a stimulus. External stimuli, such as magnetic fields or US, and internal ones, including pH or temperature, could trigger the delivery of the drug from liposomes, as will be discussed later [34]. Passive and active targeting are further discussed in the following sections.

4.5.2. Passive Targeting (The EPR Effect)

The vasculature of tumor cells differs from that of healthy cells in their functionality and morphology. In tumors, the blood vessels have defective architecture characterized by irregular shapes and the lack of both a smooth muscle layer and endothelial cells organization (Fig. **6**). Additionally, these tissues are dilated and leaky. The basement membrane in the tumor is usually abnormal or even absent. Moreover, tumors have impaired lymphatic drainage of macro-molecules and lipids [3, 72, 73]. The aforementioned characteristics of tumor tissues triggered the design of nanocarriers that are capable of utilizing the EPR effect thus allowing the extravasation of drug-loaded nanoparticles into tumor cells [74]. The EPR effect can be observed with molecules that have long plasma half-lives, with an apparent size higher than 50 kDa (above the kidney clearance threshold (5 nm)) and smaller than 0.5 μm (allowing the extravasation to the tumor) [3, 75]. Moreover, the drug should be neutral or anionic (not cationic), because the inner surface of blood vessels is highly negatively charged thus able to adsorb cationic molecules, decreasing their half-lives [3].

The EPR effect is usually characterized by imaging the tumor blood volume and flow [75]. When a DDS relies only on the pathophysiological properties of the target cancer tissue, it is referred to as *passive* drug targeting [75].

One of the most successful examples of passive targeting approved for clinical use is Doxil®, which is composed of PEGylated liposomes loaded with Doxorubicin (Dox) and used in the treatment of many cancer types. Other examples include Myocet (non-PEGylated liposome Dox) and Daunoxome (non-PEGylated liposomal daunorubicin) [76]. Even with PEGylated liposomes, less than 5% of the administered drug accumulates in tumor cells by passive targeting [74]. While most of the drugs have a plasma half-life of 20 minutes in humans and mice, it takes around 6 hours of circulation for any carrier to benefit from the EPR effect [3]. Thus, the optimization of passive targeting of liposomes is still a challenge in the nanomaterial research field.

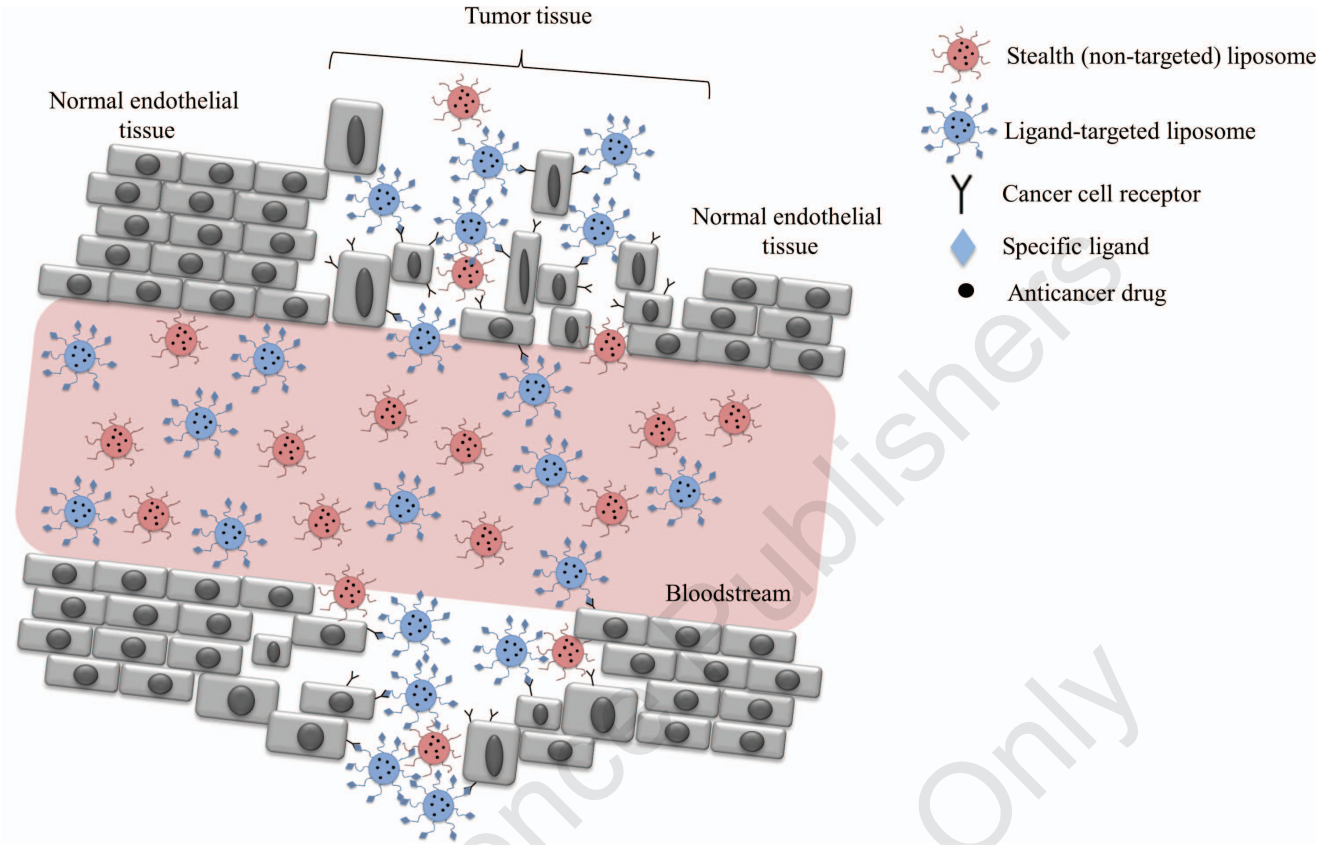

Fig. (6). Passive and active targeting in drug delivery using liposomes.

4.5.3. Active Targeting

Active targeting is achieved by conjugating the carrier with another molecule (called *ligand* or *targeting moiety*) to actively deliver the drug to specific tumor cells. It is particularly important when the affected area's vascular permeability, pH and temperature do not significantly differ from normal tissues properties [71].

In the case of liposomes in cancer therapy, the nanoparticles are modified *via* the conjugation of the targeting moiety or ligand that should be able to recognize certain binding sites (receptors) on the tumor cell surface, such that the carrier remains attached to the cell surface (Fig. **6**) where it releases its loaded drug [77].

Traditionally, the delivery of the content of liposomes to cells is described through various mechanisms including membrane fusion, endocytosis and extracellular release, as depicted in Fig. **7** [78]. Liposomes may adsorb to the surface of the cell, and either breakdown releasing the encapsulated drug outside, which is followed by the diffusion of the drug into the cell (mechanism (a1)), or fuse with the cell membrane to deliver their content intracellularly (mechanism (a2)). Non-specific uptake mechanisms include phagocytosis (mechanism (b)) and pinocytosis (mechanism (d)) are mainly used to take up particles larger than 150 nm. Mechanism (c) represents specific receptor-mediated endocytosis, where liposomes bind to the cell surface receptors and are then drawn into clathrin-coated pits to form vesicles. After invagination, an endosome is formed and diminishes later. Then, liposomes fuse with lysosomes where lipids are degraded and the drug is released [78]. The enhanced uptake of ligand-targeted lipo-somes in diseased tissue in comparison to non-targeted liposomes is well-documented in literature [6, 79].

4.6. Triggering Techniques

After a liposome reaches its designated target site, it is possible to control the time of the release, using a stimulus to trigger the release of the encapsulated drug. This is called *actuated targeting* and involves the use of internal or external triggers, such as magnetic fields, light, enzymes, US, changes in pH or in temperature [71].

pH-sensitive liposomes have been widely used as nanocarriers for anticancer drugs, antibiotics, antisense oligonucleotides, proteins and peptides to *in vitro* cell cultures or *in vivo* [80]. pH-sensitive liposomes are designed to be stable at physiological pH of 7.4, and to undergo destabilization at lower pH (acidic), thus releasing the encapsulated molecules. Such liposomes are pH-sensitive because they contain a combination of phosphatidylethanolamine (PE) and compounds containing an acidic group that act as a stabilizer at neutral pH [80]. Other mechanisms use pH-sensitive lipids, or pH-sensitive polymers with liposomes to trigger their pH sensitivity [80]. It is important to point

out that pH sensitivity decreases with the incorporation of PEG on the liposomes lipidic bilayer [80].

Temperature sensitive liposomes (TSLs) are another example of nanocarriers that have been designed to deliver drugs to tumors efficiently and in a controlled manner. They release the encapsulated drug at the melting phase of the lipid layer that occurs at or close to the transition temperature [81]. TSLs have been studied using different heating sources including needle-based radiofrequency, water baths, light sources, and catheters. Grüll and Langereis [81] studied the use of high intensity focused US (HIFU) as a non-invasive heating source to induce the release of encapsulated drugs and they reported promising results.

Using ferromagnetic properties of nanoparticles, the magnetic field can be used as another triggering mechanism. For example, Shinkai and co-workers [82] prepared magnetite cationic liposomes by including Fe_3O_4 in the lipid bilayer. However, some studies (*e.g.*, Liburdy *et al.* [83]), showed that even without the use of magnetic materials, passive targeting could be enhanced when applying a magnetic field to liposomes. In their experiments, liposomes were exposed to a magnetic field for 1 min, which enhanced drug release by 30% when compared to unexposed liposomes.

Immuno-enzymosomes are actively targeted liposomes coupled with antibodies and enzymes on their surface. The presence of enzymes helps convert the prodrug into an active drug. For example, Vingerhoeds *et al*. [84] studied immuno-enzymosomes using β -glucuronidase. A significant increase in the prodrug cytotoxicity was found when ovarian cancer cells were pretreated with the immuno-enzymosomes prepared. Furthermore, the antibodies placed on the surface of these liposomes mediate their binding to specific antigens which could be overexpressed in cancer cells, hence improving specificity and reducing off-target effects.

Our focus in this review is on sonosensitive liposomes (liposomes that release their contents upon exposure to US). For more information on other stimuli, please see the recent review by Moussa *et al.* [85].

4.7. Ultrasound-Sensitive Liposomes

In targeted drug delivery, US can be employed due to two phenomena: hyperthermia and cavitation, as explained previously. Ultrasound waves can be focused, refracted, and reflected, and they can be absorbed differently according to the medium they are present in [25]. Ultrasound has been widely researched as a trigger mechanism for US-sensitive liposomes, called *echogenic* or *acoustically active liposomes* (AAL). For instance, Holland and Huang developed the preparation protocols for such liposomes through extensive research and experimental work [86, 87]. Two approaches are available to introduce gas (usually air) into liposomes after their downsizing by sonication (see section 4.3.1). The first protocol involves 3-5 freeze-thaw cycles and lyophilization using mannitol (weak cryoprotectant) which, as hypothesized, creates bilayer defects that will be filled with air later during reconstitution [88, 89]. The second protocol employs freezing of liposomes at an elevated pressure of gas [88].

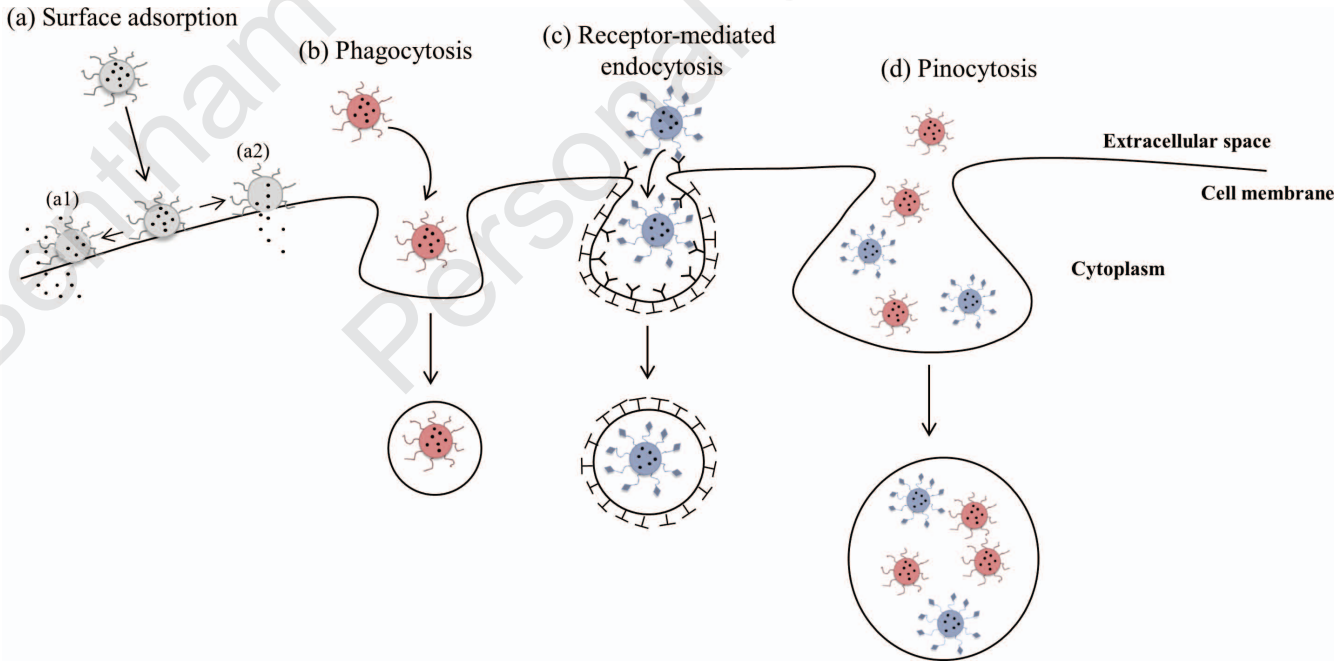

Fig. (7). Mechanisms of interaction between cells and liposomes. (**a**) adsorption of the liposome to the cell surface, followed by liposome breakdown and external release of the drug (a1) or fusion with the cell membrane and intracellular drug release (a2); (**b**) phagocytosis; (**c**) receptor-mediated endocytosis; (**d**) pinocytosis [78].

Ultrasound as a triggering technique works best when gas bubbles are present [90]. However, the incorporation of microbubbles in drug carriers in order to enhance their response to US has been found impractical since microbubbles cannot be formed at sizes below 1 μm [90].

An innovative strategy to overcome the limitations of microbubbles is to include emulsion droplets of high vapor pressure at the normal body temperature inside liposomes, and those liposomes are termed *emulsion liposomes* (eLiposomes) [61, 90, 91]. Perfluorocarbons (PFCs) (*e.g*., perfluorohexane (PFC_6), perfluoropentane (PFC_5)) are suitable to form emulsions in liposomes due to their non-toxicity and sensitivity to US, in addition to their hydrophobicity [92, 93]. The size of eLiposomes as reported by Lin *et al.* is between 200 to 400 nm, which is appropriate for vascular penetration into tumor cells [93].

Emulsion droplets are utilized to shear liposomes and release their content through the acoustic droplet vaporization (ADV) phenomenon, which is based on vaporizing liquid droplets by applying a series of pressure waves (*e.g*., US) [93]. During the rarefaction phase, the pressure drops below the vapor pressure of the liquid which induces boiling; upon vaporization, the formed bubbles are collapsed and as a consequence, the surrounding fluid is propelled at a very high velocity that ruptures the membrane of the liposome [93].

Lin *et al.* reported that the drug release from eLiposomes is better achieved at low frequencies. The same study showed that 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) eLiposomes with PFC_5 and loaded with Dox have a higher release (~80%) as compared to conventional liposomes without emulsion (~50%) after 60 seconds of insonation at 20 kHz with an intensity of 1 W/cm^2 [93].

Using US as a trigger, it is possible to control several factors that influence the release of the drug, such as the power input, ultrasonic intensity, the duration of sonication, and the position of the US source [94].

4.8. Liposomal Drug Delivery Challenges

Although in theory liposomes present a simple and practically effective method for delivering specific compounds to predetermined target sites in the body, there are numerous challenges in the process which must be addressed. For example, cancerous tissues often reside in a micro-environment called the tumor stroma which contains various cells including fibroblastic cells, the latter responsible for secreting the extracellular matrix [95]. The fibroblastic cells develop abnormally which results in the excessive secretion of proteins such as collagen, making the extracellular matrix increasingly rigid and impenetrable [95]. As a result, even if liposomes reach the cancerous region, drugs will not be able to carry its therapeutic effect on the cells as they will not be able to breach the extracellular matrix. Ultrasound has the capacity to remedy this issue as it improves the permeability of cells through the "sonoporation" effect where cell membranes, due to heat and agitation from the US application, become more porous and allow increased uptake of DDS. Furthermore, this obstacle can be surmounted by the use of tumor penetrating peptides such as extracellular matrix recognizing receptors and integrin-binding peptides such as the tripeptide arginylglycylaspartic acid (RGD) [96].

Another issue concerning the use of liposomes as drug delivery carriers is their removal by the reticulo-endothelial system during their circulation time. Although in some situations the uptake of liposomes by macrophages and monocytes is desired, in cancer treatment this is a drawback, since it results in less carriers and hence less agent reaching the diseased location [95]. This issue can be remedied by altering the properties of liposomes as discussed previously in section 4.4. However, size is also an important parameter which should be addressed, as macrophages will have different affinities towards liposomes of different sizes and number of bilayers.

4.9. Clinically-Available Liposomal Formulations and Clinical Trials

Extensive research on liposomes has been done, which includes numerous clinical trials not only in the oncology field, but also as delivery systems optimized to encapsulate genes, anesthetics or anti-fungal, antibiotic and anti-inflammatory drugs [5, 97]. For cancer therapy, there are five drug conjugated liposomes which have been approved by the FDA [53, 98]: (i) Pegylated liposomal Dox (Doxil® or Caelyx®), first approved by the FDA in 1995 for the treatment of chemotherapy refractory acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma as well as ovarian and breast cancers; (ii) non-pegylated liposomal Dox (Myocet®), utilized for the treatment of metastatic breast cancer tumors and often applied in conjunction with cyclophosphamide, another anti-cancer drug; approved by the European Union for use in 2000; (iii) non-pegylated liposomal daunorubicin (DaunoXome®), approved by the FDA in 1996 for use against blood tumors; it is directly injected into the blood stream where it immediately acts against the tumors; (iv) non-pegylated liposomal cytarabine (DepoCyt®), approved by the FDA in 1999 for use against lymphomatous meningitis which afflicts the central nervous system; (v) vincristine sulfate liposomes (Marqibo®), recently approved by the FDA in 2012 for use against the rare Philadelphia chromosome negative (Ph-) acute lymphoblastic leukemia.

It is important to note that none of the above-approved formulations are targeted to specific tissues [53]. Hence, the encapsulated drugs may exert unwanted effects on other parts of the body [99]. As a result, one avenue of research which aims to improve the action of these liposomes proposes the use of antibodies targeted against extracellular growth factors. In fact, *in vivo* mouse trials with Dox encapsulated in such targeted liposomes revealed not only an accurate targeting of the tumor tissues, but also resulted in a

significant tumor size decrease, in comparison to treatment with non-targeted Dox liposomes [53].

Ultrasound may also be used to solve the issue of non-selective tissue targeting. The susceptibility of US-sensitive liposomes to sonication makes it possible to induce drug release once they arrive at the specific tissues and organs which require treatment. Highlighting their potential to dramatically change and improve cancer treatment methods, the use of US and liposomes is increasing in clinical trials since the FDA approved their testing. Although many of these studies have not proceeded to Phase 3, which involves thousands of subjects, and tend to suffer from a low number of participants, the initial findings have been very promising [97].

Dimcevski and co-workers [100] performed a Phase 1 human trial to evaluate the use of low-intensity US, microbubbles and the chemotherapeutic drug gemcitabine in inoperable pancreatic cancer. Although no nanoparticles were used in this study, the results were promising when compared with gemcitabine treatment alone: patients were able to tolerate more cycles of treatment without an increase in side effects, in half of them the tumor diameter was reduced and the survival time increased twice. The beneficial effects of ultrasound were attributed to the sonoporation effect and increased uptake of the drug by the tumor, usually made difficult by the dense tumor stroma.

Another study, by Zagar *et al.* [101] actually combined heat-sensitive liposomes encapsulating Dox, and US and microwave as triggers. The treatment was used in breast cancer patients and showed an amazing 48% improvement in local response.

Taken together, these results emphasize the potential of ultrasound and liposomes to vastly improve the prognosis of patients suffering from various different cancers and increase their chances of survival.

5. RELEVANT RESEARCH STUDIES

When liposomes are studied, several topics are investigated, including the effect of lipid composition on stability and drug release, mechanism of release, encapsulation efficiency, the effect of different parameters of US on drug release, the effect of the targeting moiety, etc.

Ultrasound, which has been used as an imaging/diagnostic technique for decades, is now a topic of intense research to promote the therapeutic efficacy of nanoparticles. *In vitro* and *in vivo* studies have shown that the mechanical and thermal effects of US waves improve cellular uptake of several agents encapsulated in nanoparticles. The mechanical effects of US waves do not only enhance the release of the drug from nanocarriers but also have the capability to cause sonoporation, *i.e*., the formation of holes/pores in the membrane of cells already targeted by the DDS [102-106].

5.1. The Effectiveness of the Incorporation of Targeting Moieties

From all the targeting ligands studied in DDS (antibodies, peptides, aptamers, hormones and several low weight compounds) folic acid is the most commonly used due to the overexpression of its receptors (folate receptors, FR) in a broad variety of cancer cells and activated macrophages, the high affinity between folic acid and FR and the relatively low frequency of FRs in normal tissues [107]. FRs are overexpressed in certain ovarian, breast, lung, colon, kidney and brain tumors [107, 108]. The enhancement in cellular uptake of folate-targeted nanoparticles has been confirmed *in vitro* [108-110]. When combined with US, the use of folate-targeted nanoparticles clearly enhances the anti-tumor activity of the chemotherapeutic drug. A recent *in vivo* study with oridonin (ORI), a cytotoxic drug prevenient from a Chinese medicinal herb, showed better results for the derived ORI nanoparticle than for the free drug or the non-targeted nanoparticle. After 14 days, the tumor inhibition rate for folate-targeted liposome microbubbles loaded with ORI upon US exposure was 87.6%, higher than for liposome microbubbles loaded with ORI (71.5%), liposomes loaded with ORI (64.3%) and free ORI (43.4%) – all the samples being subjected to the same US exposure conditions [111].

Other ligands have also been used to modify nanoparticles, and their ability to increase tumor cell uptake and cell toxicity of conventional chemotherapeutical drugs has been reported. Hamano *et al.* [112] investigated the cytotoxicity of US-imaging gas-entrapping liposomes, also known as *bubble liposomes*, encapsulating Dox and targeted with AG73 peptide. Based on viability studies using the 293T human embryonic kidney carcinoma cell line, the authors reported that higher cytotoxicity was achieved by targeted liposomes as compared to non-targeted liposomes. The study also showed that bubble liposomes modified with AG73 peptide in the presence of US did not enhance the cellular uptake of Dox but promoted the Dox release from the liposomes in the cytoplasm. Kirpotin *et al.* [113] conducted a flow cytometry study, and found that immunoliposomes, targeted with a monoclonal antibody, achieved a 4-fold increase in accumulation in tumor cells as opposed to host stromal cells. On the other hand, non-targeted liposomes showed no preference for tumor cells.

The Arginylglycylaspartic acid peptide (RGD) is another ligand widely studied in a broad range of nanoparticles due to its ability to target αVβ3 integrins that are overexpressed on the actively proliferating endothelium of tumor tissues and are determinants on angiogenic endothelium [114-117]. Several studies reported the successful use of RGD-targeted liposomes as DDS [118-124]. For example, Wang and co-authors [123] reported a significantly higher cellular uptake and higher cytotoxicity for cyclic arginine-glycine-aspartic acid-tyrosine-lysine peptide (cRGDyk)-conjugated liposomes loaded with cisplatin when compared with free cisplatin or cRGDyk-free

liposomes. Additionally, *in vivo* results were promising in the treatment of bone metastases. Yu *et al.* [124] developed a cRGD-anchored nanoparticle that exhibited a significantly greater affinity for pancreatic adenocarcinoma BxPC-3 cells compared to the non-targeted nanoparticle and free gemcitabine (drug highly used on pancreatic cancer treatment). The cRGD-nanoparticles also showed the strongest inhibitory effect against the BxPC-3 cells.

The challenge of improving cellular uptake and drug release from targeted liposomes by US was surmounted when liposomes co-modified with single stranded DNA aptamers were prepared with a copolymer of N-isopropylmethacrylamide and N-isopropylacrylamide (poly(NIPMAM-co-NIPAM)) as the thermosensitive polymer (TSP) activates these liposomes when there is an increase in temperature. These TSP-liposomes recognized platelet-derived growth factor receptors overexpressed in breast cancer cells and when loaded with DOX decreased the viability of MDA-MB-231 cells 24h after ultrasound irradiation (1 MHz at 0.5 W/cm^2 for 30 s) [122].

5.2. The Improvement of Physical-Chemical Properties of Liposomes

The uptake of liposomes by tumor angiogenic vessels is an important issue prior to triggering drug release by a stimulus. Krasnici and coworkers [125] investigated the effect of the liposomal surface charge on the uptake of liposomes by tumor angiogenic vessels of an amelanotic hamster melanoma A-Mel-3. The study found no statistically significant difference in uptake between normal and tumor tissues for both neutral and anionic liposomes. However, cationic liposomes showed a significantly 3-fold higher accumulation in tumors compared to normal tissues. These results are consistent with those reported by Thurston *et al.* [126] and Nomura *et al.* [127].

Evjen *et al.* [128] studied the effect of lipid composition on the circulation half-life and the sonosensitivity of liposomes *in vitro*. Authors found that dioleoylphosphatidylethanolamine (DOPE)-based liposomes with higher cholesterol content (40 mol%) and lower DOPE levels (32 mol%) showed an increased stability in blood (20 ± 3 hrs) as compared to liposomes with 20 mol% of cholesterol and 62 mol% of DOPE (5 ± 1 hr). The authors also reported no statistically significant difference between drug release in the above-mentioned conditions. Another study by Evjen *et al.* [129] investigated the effect of the lipid type on liposome sonosensitivity. The study showed that Dox release from 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)-based liposomes was higher than that from 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)-based liposomes (69% compared to 9%) after 6 minutes of insonation [129].

Park and co-workers [130] studied a novel TSL formulation, where elastin-like polypeptide (ELP) was utilized as a heat triggered moiety, and compared it to conventional lysolipid-based temperature sensitive liposomes (LyTSL). The results showed that the ELP-TSL were more sensitive to an increase in temperature than the LyTSL and also more stable at physiological temperatures.

Another modified liposome has shown promise recently, since it conveys a higher morphological stability: liposomal nanohybrid cerasome. Liang *et al.* [131] studied the combination of high intensity focused ultrasound (HIFU) and temperature sensitive cerasomes (HTSCs) formed from a cerasome-forming lipid (CFL) which acts as the host lipid (and increases the stability of the carrier). The HTSCs were prepared using four types of lipids including CFL, and three lipids commonly used in LTSLs: DPPC, 1-myristoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (MSPC) and DSPE-PEG-2000. Each lipid has an important role in the liposome enhancement: CFL enhances the stability, DPPC conveys thermal sensitivity, MSPC enhances permeability and DSPE-PEG-2000 allows long circulation of HTSCs in the body. The authors reported that the HTSCs could encapsulate both hydrophilic and lipophilic drugs and that the release rate could be tuned by varying the molar ratio of CFL to DPPC. Conveniently, the liposomes were stable at physiological temperatures but released more than 90% of encapsulated calcein when exposed to 1 min of HIFU, due to hyperthermia (42 °C). Additionally, *in vivo* studies using MDA-MB-231-bearing mice, showed that exposure to HIFU following the injection of HTSCs loaded with Dox, lead to a significant inhibition of the tumor growth.

Huang and McDonald [132] studied the effect of air bubbles on drug release from liposomes. The authors compared the release of AAL, containing both air bubbles and calcein, with control liposomes. They also examined the effect of HFUS on triggering calcein release from their AAL. To test their liposomes, 1-MHz HFUS, and several intensities and duty cycles were used. The highest release was observed at 2 W/cm^2, and 100% duty cycle for 10 s. Under these conditions, AAL released about 30% of the encapsulated calcein, while non-acoustically sensitive liposomes released only 3% [132].

Lattin *et al.* [133] studied the effect of encapsulating nanoemulsions (PFC_5 and PFC_6) inside liposomes on calcein release and compared it to release from conventional liposomes. In both liposomes, calcein release increased with increasing US power densities and duration of exposure. The study also reported significantly higher release percent of calcein from eLiposomes than from conventional liposomes. For instance, at 5 W/cm^2, the release from emulsion liposomes with small PCF_5 emulsion droplets, large emulsion droplets, and control liposomes was 39%, 31%, and 10%, respectively [133].

5.3. The Effect of US on Drug Release

Numerous studies have investigated the interaction between drug carriers and US waves in drug delivery. Parameters including US wave frequency, intensity, and exposure time were studied to assess the efficiency of drug release. US was found to enhance

drug release from liposomes *via* various mechanisms including cavitation, acoustic streaming and hyperthermia, depending on US wave parameters and the chemical composition of liposomes [8, 10, 34, 85].

The frequency of US waves is crucial in drug delivery. Low frequency US (LFUS) waves were found to be superior for drug release due to mechanical effects that promote cavitation. Schroeder *et al.* [134] examined the release of different drugs from sterically stabilized liposomes with a uniform size of ~100 nm upon exposure to different amplitudes of LFUS (20 kHz) ranging from 0 to 7 W/cm^2. The liposomal formulations were irradiated with LFUS for 60 s at 37 ^{o}C. The article reported a direct linear relationship between US amplitude and drug release, and the authors concluded that cavitation was achieved above ~1.3 W/cm^2 for a liposomal methylprednisolone hemisuccinate (MPS) formulation. The same study also reported an increase in drug release with increasing irradiation times at 20 kHz and 3.3 W/cm^2, and an ~80% drug release from the liposomal MPS formulation within 150 s. Hence, it was observed that the majority of the drug was successfully released in a short period of time at LFUS, and researchers termed this fast drug release the "dumping" effect. Additionally, researchers found that drug release did not change when the sample was tested immediately after irradiation and when it was left for 72 h after stopping US exposure, hypothesizing that the permeability of membrane is transient and increases only during irradiation time. The group of Schroeder [135] further studied the release of Doxil® at different US frequencies. At LFUS (20 kHz) the release in human plasma was 61%, while at HFUS (1 MHz) the release rate was 5% after a total exposure time of 30 min. They explained the difference in release levels at HFUS and LFUS by the low intensity requirement to induce transient cavitation for low-frequency waves as compared to higher frequencies.

Drug release from TSLs by HIFU-induced hyperthermia (40-45 ^{o}C) has been studied, and recently has been coupled with magnetic resonance (MR) image-guided drug delivery by incorporating a paramagnetic MRI (magnetic resonance imaging) contrast agent (*e.g.*, Gd(HPDO3A)(H_2O)) in the lumen of TSLs [81, 136]. The delivery of Dox encapsulated in the LTSL formulation to Vx2 tumor in rabbits, triggered by MR-HIFU, was 3.4-fold greater than that of LTSL treatment alone and 7.6-fold greater when compared to free Dox treatment [137]. Another study conducted by Kheirolomoon and co-workers [138] investigated the role of multiple treatments in suppressing cancer growth. A pH-sensitive complex of Dox and copper (CuDox) was encapsulated in the core of LyTSLs and was tested *in vivo*, in a breast cancer model, with and without the use of US. Pulses of US were used with 100-cycle bursts at 1.54 MHz and a peak negative pressure of 1.1 MPa. Insonation was applied at 42 ^{o}C for 5 minutes prior to IV injection and 20 minutes after the injection. The researchers found that the Dox fluorescence was higher in tumors treated with CuDox-LyTSLs and 20 min of US, as compared to tumors treated with CuDox-LyTSLs only. Additionally, the fluorescence showed a 1.7-fold increase when the post-injection insonation was increased to 40 minutes. Further *in vivo* studies conducted by Kheirolomoom *et al.* on NDL-tumor bearing mice showed a tumor regression when treated with CuDox-LTSLs combined with US as opposed to tumors treated with CuDox-LTSLs solely and control groups. After 28 days of treatment with 2 doses weekly of CuDox-LTSLs and US exposure for 5 min prior to injection and 20 min after the injection, tumor size was successfully regressed to 30% of its initial volume and tumor disappeared completely after 53 days of treatment, which could not be accomplished with hyperthermia alone or CuDox-LTSLs without US.

Table 3. Factors affecting drug release from sonosensitive liposomes.

Property	Description	Reference
Ultrasound Frequency	Drug release is achieved by mechanical effects (cavitation) at LFUS, and thermal effect by HFUS waves (≥ 1 MHz) Drug release is more efficient at LFUS than that at HFUS. This can be explained by the lower collapse cavitation threshold required to induce transient cavitation for LFUS irradiation	[10, 135, 139, 140]
Ultrasound Intensity	Some researchers argued that hyperthermia is the main mechanism of HIFU in drug release from nanocarriers. Others believe that high shear forces are the mechanism of HIFU.	[85, 141-143]
PEG	The general trend shows an increase in drug release from stealth liposomes upon exposure to LFUS since PEG headgroups improve the absorption of US waves	[10, 144, 145]
Surfactants	The inclusion of phospholipids with unsaturated acyl chain disturbs the packing of the liposomal membrane which increases the sensitivity of liposomes to ultrasound and hence the drug release	[10, 144]
Bilayer Physical State	The phase of the lipid can be classified into liquid-disordered (LD), liquid-ordered (LO) and solid-ordered (SO) with LD phase being more permeable than the latter two. Ultrasound absorption is maximum near the transition temperature of lipids. Liposomal membrane is more permeable at the phase transition temperature due to large defects rising from the coexistence of two phases (solid-ordered and liquid- disordered).	[143, 146, 147]

Table **3** summarizes some of the major factors that affect drug release from liposomes when using ultrasound as the triggering mechanism.

CONCLUSION

Several lipid-based nanoparticles were shown to enhance the cytotoxicity of loaded chemotherapeutical drugs in tumor cells. Also, the injection of some formulations led to an increase in the survival times of animals with oncological disease. For this reason, several liposomes are in clinical trial and some are already in the market with considerable clinical acceptance even with slightly better therapeutic benefits compared with the administration of the free drug. This process however, is just the beginning of the establishment of the nanomedicine delivery systems as a secure, sophisticated, and more prompt therapeutic alternative.

The therapeutic activity of all types of drug delivery systems, including liposomes, is dependent upon several factors such as the appropriate drug release rate in the target tissue. The proper co-utilization of US (at low to moderate frequency and relatively low intensity) and the US-active lipid nanoparticles could lead to a tunable release rate according to the requirement of the therapeutic application. This bioengineering concept is already being explored with promising results. The activation of lipid nanoparticles, such as liposomes, by acoustic waves, leads to an increase in the release rate of the cargo, in an environment already activated by the US. Therefore, the cells of the irradiated region have an augmentation in the internalization due to 'sonoporation' or other conventional mechanisms of internalization of drugs, like diffusion. As discussed in this review, several combinations of US application (with proper physical parameters) and the adequate lipid nanoparticles already demonstrated considerable tumor regression. These promising results direct us to the challenge of building a biophysical/medical technology that should give rise to new oncological solutions.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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