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The combined application of exosomes/exosome-based drug preparations and ultrasound

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Exosomes are small extracellular vesicles with a diameter of 30–150 nm, secreted by a variety of cells and containing various active substances such as nucleic acids, proteins and lipids. The use of exosomes as drug carriers for targeted delivery of therapeutics has been studied for a long time. Ultrasound is recognized as a non-invasive diagnostic and therapeutic method for assisting drug loading and targeted delivery, cellular uptake and therapy. In this review, we summarize the applications of ultrasound in assisting drug loading into exosomes, targeted delivery of exosome-based drug formulations, cellular uptake, and therapy, and explore the prospects for the combined application of exosomes/exosome-based drug formulations and ultrasound.

Introduction

Extracellular vesicles are membrane structures derived from cells, which include different groups such as exosomes, apoptotic bodies, and microvesicles.[1] Exosomes are produced in the form of multivesicular bodies (MVBs), which bud inward and fuse with the plasma membrane (PM) to be released into the microenvironment.[2] They are small extracellular vesicles with a diameter of 30–150 nm. It is believed that exosomes serve as mediators to transport proteins, lipids, nucleic acids, or other components to neighboring or distant cells, thereby facilitating intercellular communication.[3] Consequently, researchers have developed exosome-based drug delivery systems. As natural carriers, exosomes have their own advantages, such as low immunogenicity, high stability in the bloodstream, and the ability to deliver drugs directly into cells.[4] Additionally, exosomes possess the capability to cross biological barriers, such as the blood–brain barrier, intestinal barrier, and placental barrier.[5] In existing related studies, it is well known that drugs (including gene drugs) can be loaded into exosomes primarily through methods such as ultrasonication, electroporation, and incubation. Exosome-based drug formulations could be used for a wider range of diseases, including cancer, various infectious diseases, cardiovascular diseases, and neurodegenerative diseases.[6]

Low-intensity ultrasound has been widely used to promote the disintegration, degradation, and destruction of cell structures of separated extracellular vesicles (EVs) before analysis, induce cavitation in microbubbles, and improve chondrogenesis and cartilage repair through the regulation of autophagy.[7–10] Ultrasound-targeted microbubble destruction (UTMD) has become a new method for region- or tissue-specific gene delivery. After the *in vivo* injection of a mixture of microbubbles containing gene drugs and exosomes, the microbubbles can be destroyed by ultrasound beams, facilitating the delivery of gene drugs through the cavitation effect in the microvasculature of the target tissue, which is particularly advantageous during the delivery process, especially in localized tissues with biological barriers such as the blood–brain barrier (BBB).[11–13] Focused ultrasound (FUS) can produce various physical and biological effects in cells or tissues, such as FUS hyperthermia, which can be achieved by adjusting acoustic parameters.[11] The combination of focused ultrasound (FUS) and microbubbles can instantaneously open the blood–brain barrier (BBB) locally, thereby assisting in the delivery of therapeutic drugs across the BBB.[13] Based on the various effects of ultrasound, it is widely applied in the preparation of exosomes or exosome formulations, targeted delivery, cellular uptake, and therapy.

Using ultrasound for preparing exosomes

Natural exosomes can be secreted by various cells and are widely present in body fluids such as saliva and urine. The exosomes obtained in experiments mainly come from *in vitro* cell culture media, such as tumor cell culture media, stem cell culture media, and immune cell culture media. The content of exosomes secreted by cells in *in vitro* culture media is very limited, resulting in low yields of exosomes, which somewhat restrict their clinical applications. Therefore, there is a need to seek methods to increase exosome content. Alec J. Batts *et al.*[11]

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found that focused ultrasound hyperthermia can promote the release of extracellular vesicles from glioma cells without changing the size of the vesicles. Zubair Ahmed Nizamudeen *et al.*[7] discovered that low-power ultrasound increases the number of smaller particles in extracellular vesicle samples, particularly those with a particle size of less than 50 nm. Research by Yuana Yuana *et al.* reported that microbubble-assisted ultrasound can trigger the release of extracellular vesicles (EVs), with EVs having an apparent diameter of less than 200 nm typically considered representative of the exosome population.[8] It can be inferred that ultrasound can make cells secrete more exosomes. *In vitro* experimental results by Xia *et al.*[14] indicate that LIPUS increases the release of exosomes derived from bone marrow mesenchymal stem cells by activating autophagy, and their findings show that LIPUS does not significantly affect the morphology and size of the exosomes secreted.

Zhiting Deng *et al.*[15] conducted multiple ultrasound stimulations on human astrocytes using the following ultrasound parameters: a working frequency of 1 MHz and a duty cycle of 20%, with a spatial peak temporal average intensity (ISPTA) of 280 mW cm^{-2} (Table 1). Their research results showed that with the help of ultrasound, the number of exosomes released by human astrocytes (HA) increased nearly fivefold, and the ultrasound did not induce the proliferation of astrocytes. Zhao *et al.*[16] conducted ultrasound treatment on A2780 cells with varying intensities and durations. The results indicated that low-intensity ultrasound (LIUS) at 0.5 w cm^{-2} for 60 minutes led to the highest secretion of exosomes from A2780 cells (Table 1), with no significant changes observed in the morphology, size, or volume distribution of the produced exosomes. Their research also showed that LIUS increases the quantity of exosomes secreted by cells by affecting the expression of genes related to exosome biogenesis (such as CHMP28, CHMPS, YKT6, *etc.*). Table 1 summarizes the ultrasound parameters used in the literature for the preparation of exosomes, assisted targeted delivery, and therapy. Some scholars believe that the mechanism by which ultrasound stimulates cells to release exosomes may involve influencing the molecular mechanisms in the biosynthetic pathways of exosomes (including the ESCRT complex, Rab GTPases, and TSAP genes).[17]

In summary, ultrasound can increase the number of exosomes secreted by cells, potentially by enhancing the expression of genes related to exosome biogenesis, which in turn may lead to an increase in the purity of exosome extraction. Therefore, ultrasound-assisted exosome release strategies could be employed for the large-scale production of exosomes in bioreactors.[27]

Exosomes contain mRNA, microRNA (miRNA), lipids, and proteins. Therefore, exosomes can mediate intercellular communication. Studies have shown that the expression levels of contents such as proteins and mRNA in exosomes increase after ultrasound treatment. Research by Yuana Yuana *et al.*[8] found that exosomes derived from FaDu cells treated with ultrasound microbubbles (USMBs) contained higher levels of CD9 and CD63. Xuefeng Li *et al.*[18] discovered that exosomes produced by bone marrow dendritic cells (BMDCs) treated with low-intensity pulsed ultrasound (LIPUS) contained more miR-16 and miR-21 compared to those from untreated BMDCs, which play a role in anti-inflammation. Additionally, Zhiting Deng *et al.*[15] found that exosome marker proteins such as CD63, HSP70, CD9, and Tsg101 significantly increased after ultrasound stimulation. Xia *et al.*'s experimental results showed that compared to LIPUS stimulation for 3 days, the expression of CD63, ALIX, and TSG101 proteins in exosomes isolated from MSC culture medium significantly increased after 7 and 10 days of LIPUS stimulation ($P < 0.05$).[14] The aforementioned experimental results indicate that the exosomes released by cells after ultrasound treatment can significantly increase, and the expression of certain contents may also increase accordingly. Therefore, is it possible to prepare exosomes containing more of the desired contents through ultrasound? Further research may be warranted in the future.

The preparation of ultrasound-assisted exosomes has certain limitations. First, there are relatively few reports clearly indicating an increase in exosome yield due to ultrasound, and the mechanisms by which ultrasound increases the number of exosomes secreted by cells remain controversial. Yang *et al.*[28] suggested that ultrasound triggers signal transduction through physical effects such as thermal effects, shock waves, and shear forces, inducing the biosynthesis and docking of exosomes. Some scholars believe that ultrasound increases the secretion of exosomes by affecting genes related to exosome biogenesis, while others argue that ultrasound mediates the release of exosomes by regulating autophagy. Secondly, the ultrasound treatment process may introduce exogenous contaminants, affecting the yield and quality of exosomes. Finally, the parameters of ultrasound conditions are crucial for exosome preparation, as inappropriate parameter settings may lead to toxic reactions, causing the dissolution of exosomal contents or loss of function, thereby damaging cells and impacting exosome quality. The optimal ultrasound parameters for different cells may vary, and current research on the safe range of ultrasound parameters for various cells is relatively insufficient.

Using ultrasound for loading drugs or genes

Exosomes can evade clearance by the immune system. Exosome-based nanocarriers, like exosomes, also possess excellent biocompatibility, low immunogenicity, low toxicity, and the ability to cross various physiological barriers. Therefore, in research, exosomes are often used to load chemotherapeutic drugs (such as paclitaxel), protein drugs (such as peroxidase), traditional Chinese medicines, and genes (such as miR-21), among others. It is well known that drugs or genes are typically incorporated into exosomes through methods such as incubation, electroporation, and ultrasound treatment. Studies[29,30] have shown that mild ultrasound-assisted drug loading methods do not affect the protein and lipid content of exosomes. The recombination process of the exosomal membrane under ultrasound promotes drug diffusion and results in high loading capacity.[29,30]

Table 1 Summary of the ultrasound parameters used in the literature for the preparation of exosomes, assisted targeted delivery, and therapy

		Ultrasound parameter										
Usage	Ultrasound types	frequency pulses	Pulse width	output power	Pulse repetition frequency	Average intensity	duty cycle	mechanical index	Exposure time	Model or cell	Product or result	Ref.
Exosome Preparation	LIPUS	1.5 MHz	200 μs	—	1 kHz	30 mW cm^{-2}	—	—	12 h	BMDCs	Exosomes(containing high levels of miR-16 and miR-21)	18
Exosome Preparation	LIPUS	3 MHz	—	—	—	50 mW cm^{-2}	—	—	20 minutes per day	MSCs	LIPUS enhances the cartilage repair effect of mesenchymal stem cells in osteoarthritis by regulating autophagy-mediated exosome release	14
Exosome Preparation	—	1 MHz	—	—	—	280mW cm^{-2}	20%	—	3 minutes	Astrocytes	Ultrasound increases the release of exosomes from astrocytes by nearly five times.	15
Exosome Preparation	LIUS	—	—	—	—	0.5 W cm^{-2}	—	—	60 minutes	A2780 cell	LIUS significantly promotes the secretion of exosomes in A2780 cells.	16
Exosome coating	Microfluidic Sonication	80 kHz	—	—	—	—	—	—	—	—	EM-PLGA NP	19
Targeted delivery-*In vivo*	UTMD	0.66 MHz	—	—	—	—	50%	1.6	1 minutes	male C57Bl6 mice	UTMD significantly enhances the delivery of exosome-mediated miRNA to the heart.	12
Targeted delivery	UTMD	0.66 MHz	—	—	—	0.22 to 1.80 W cm^{-2}	—	—	0.5 to 3 minutes	Male C56BL/6 mice	UTMD promotes the delivery of exosomes in refractory tissues.	20
Targeted delivery-*In vivo*	LIFU-guided-ultrasound (US1)	20 kHz	—	—	—	—	—	—	3 minutes	BALB/c mice	Promote the localized accumulation of EXO-DVDMS in tumor regions.	21
Targeted delivery-*In vitro*	—	1	—	—	—	0.1 W cm^{-2}	20%	—	30 s	RAW264.7	The SmartExo system has the ability to evade phagocytosis at non-target sites and to release drugs in a controlled manner at target organs.	22
Targeted delivery-*In vivo*	—	1 MHz	—	—	—	2 W cm^{-2}	Continue	—	180 s	Mouse	Using the SmartExo system, Bmp 7 was successfully delivered to the targeted site under ultrasound irradiation in the abdominal region.	22
augmented targeting and therapeutic	—	1 MHz	—	1 W cm^{-2}	—	—	30%	—	3 minutes/2 day	arthritic mice	Ultrasound-enhanced AI-Exo has a significant targeted anti-inflammatory treatment effect.	23
Drug Release - *In vitro*	—	1 MHz	—	—	—	0.3 W cm^{-2}		—	60 s	hDFB cells	ultrasound-enhanced FA-ExoICG SDT	24
therapeutic effects-*In vivo*	—	1 MHz	—	—	—	0.5 W cm^{-2}	—	—	3 minutes per 2 day	Tumor xenograft mice	FA-ExoICG can serve as an effective and safe targeted nanosensitizer for cancer therapy.	24
therapeutic effects-*In vivo*	—	1 MHz	—	2 W cm^{-2}	—	—	20%	—	5 minutes	male C57BL/6 mice	ultrasound-enhanced Exos SDT	25
therapeutic effects-*In vivo*	LIFU-thera-peutic-ultrasound (US2)	30 kHz	—	—	—	—	—	—	3 minutes	BALB/c mice	ultrasound-enhanced EXO-DVDMS SDT	21

Table 1 (*continued*)

Usage	Ultrasound types	Ultrasound parameter							Model or cell	Product or result	Ref.
		frequency pulses	Pulse width	output power	Pulse repetition frequency	Average intensity	duty cycle	mechanical index	Exposure time		
therapeutic effects-*in vivo*	LIPUS	1.5 MHz	—	0.026 W	1 kHz	30 mW cm^{-2}	20%	—	20 minutes per day	arthritic mice	The promoting effect of LIPUS-enhanced exosomes derived from BMSCs on cartilage regeneration in osteoarthritis.	26

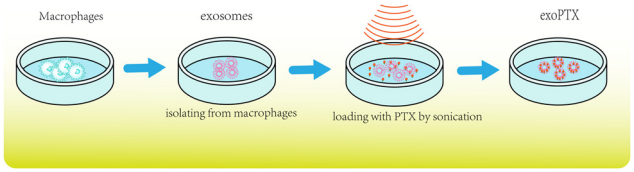

Fig. 1 The process of obtaining drug-loaded exosomes (exoPTX) by treating macrophage-derived exosomes mixed with PTX through ultrasound (parameters: 20% amplitude, 6 cycles of 30 seconds on/off, lasting 3 minutes, with a 2-minute cooling period between each cycle). First, exosomes are isolated from macrophages, then mixed with the drug PTX, followed by ultrasound treatment of the mixture, and finally, exosomes loaded with the drug exoPTX are obtained after further separation.

Current research on the use of ultrasound for drug or gene delivery mainly falls into two areas.

On the one hand, ultrasound is used to load drugs or genes into exosomes. Salarpour *et al.*[31] compared two methods of drug loading into exosomes: incubation and ultrasound irradiation. The results showed that the drug loading rate of exosomes treated with ultrasound (0.92%) was higher than that of the room temperature incubation method (0.74%), and the particle diameter of drug-loaded exosomes treated with ultrasound was larger than that of those incubated at room temperature. Similarly, the research by Myung Soo Kim *et al.*[30] indicated that ultrasound could maximize the loading of PTX (paclitaxel) in exosomes compared to incubation and electroporation (Fig. 1). In their study, the exoPTX particles obtained through ultrasound treatment had the largest diameter, followed by electroporation, while the diameter of exoPTX particles obtained through incubation was the smallest. Furthermore, the formulation exoPTX obtained through ultrasound treatment demonstrated high loading capacity both *in vivo* and *in vitro* compared to PTX. Li *et al.*[32] compared the drug loading methods of ultrasound and incubation, finding that the ultrasound treatment had a higher loading amount (11.68 + 3.68%), while the incubation had a lower loading amount (2.79 + 0.72%). The size of the drug-loaded exosomes obtained through ultrasound treatment also slightly increased. In another study, Myung Soo Kim *et al.*[29] mixed exosomes, PTX, and DSPE-PEG-AA in PBS and used ultrasound irradiation to assist in loading PTX into exosomes, resulting in AA-PEG-exoPTX. Their experimental results showed that ultrasound irradiation significantly increased the amount of PTX loaded into exosomes, and the size of the exosomes obtained through ultrasound treatment increased as well. Additionally, the expression of related proteins (TSG 101 and flotillin) in non-carrier exosomes and carrier exosomes loaded with PTX increased after ultrasound treatment. Similarly, Wang *et al.*[33] used mild ultrasound to load PTX into exosomes derived from M1 macrophages, resulting in a slight increase in size for PTX-M1-Exo (172.8 nm) compared to the size for untreated M1-Exo (75.3 nm), although their morphology and marker protein expression remained unchanged. The study by Sun Wenqi *et al.*[12] demonstrated that with the assistance of UTMD (ultrasound-targeted microbubble

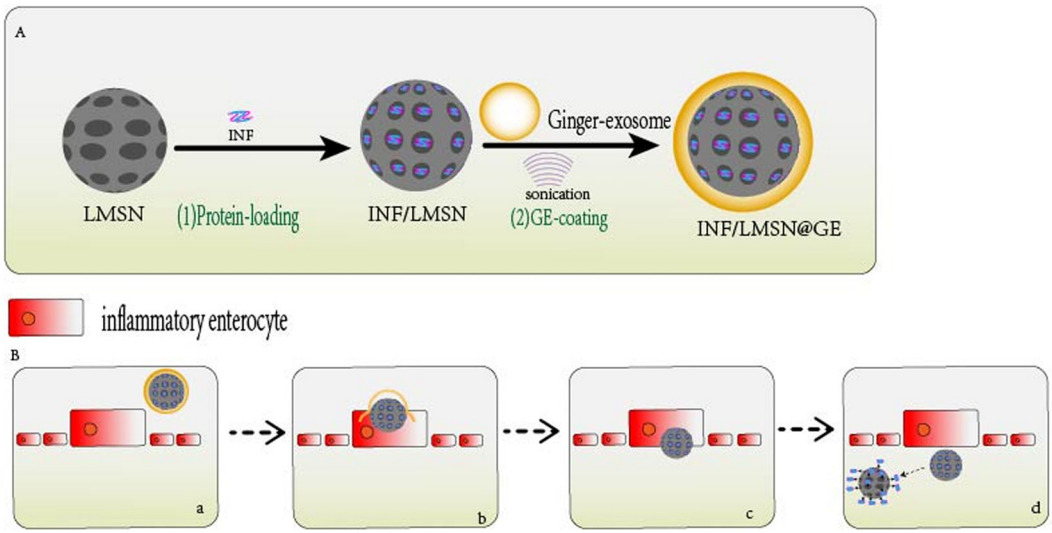

Fig. 2 (A) Schematic illustration of the steps to assemble an exosome membrane core–shell nanocomposite of INF/LMSN@GE. (B) Schematic illustration of the GE-coating protecting the loaded protein drugs released in a specific region (a–d: INF/LMSN@GE transmembrane transports across the epithelium, reaching at the colon lamina propria in the form of INF/LMSN. Then in colon lamina propria, INF releases from the LMSN slowly).

Table 2 Summary of the ultrasound parameters used in the literature for ultrasound-assisted exosome drug loading

		Ultrasound parameter						
Usage	Ultrasound types	amplitude (%)	cycles of on/off	Loop duration (minutes)	cooling period between each cycle	Drug	Product	Ref.
Exosome drug loading	Model 505 sonic dismembrator	20	6 cycles of 30 s on/off	3	2 minutes	PTX	exoPTX	30
Exosome drug loading	UP100H ultrasonicator hielscher	20	6 cycles of 30 s on/off	3	2 minutes	PTX	—	31
Exosome drug loading	JY92-IDN sonic dismembrator	20	3 cycles of 90 s on/off	—	30 s	GEM	ExoGEM	32
Exosome drug loading	Model 505 sonic dismembrator	20	6 cycles of 30 s on/off	3	2 minutes	PTX	PTX-M1-Exos	33

destruction), the gene miR-21 could be effectively integrated into exosomes without altering their morphology.

On the other hand, ultrasound can be used to encapsulate drugs or genes into nanoparticles (NPs), which are then enveloped by biological membranes (such as exosome membranes and cell membranes) and lipids. Research indicates that the destructive force caused by ultrasound and extrusion can disrupt the extracellular matrix (EM) structure and reassemble the EM around the NPs to form a core–shell structure.[34] Studies have shown that during *in vivo* circulation, the rapid clearance of synthetic nanoparticles (NPs) by the mononuclear phagocyte system (MPS) reduces the delivery efficiency of drug-loaded NPs to tumor sites. Using natural membranes to wrap NPs can decrease the clearance of drug-loaded NPs by the immune system and enhance their tumor-specific targeting. Cancer cell membranes (CCMs) exhibit immune evasion and homologous targeting due to the presence of specific antigens. However, compared to cell membranes, cell-derived exosomes can serve as better membrane materials for creating biomimetic NPs.[35] Chao Liu and colleagues[19] reported a microfluidic ultrasound method that can directly prepare exosome membrane (EM-), cancer cell membrane (CCM-), and lipid-coated PLGA (poly(lactic-*co*-glycolic acid)) NPs (nanoparticles) in one step. In their study, the EM successfully covered spherical PLGA cores with the assistance of ultrasound. Most (approximately 90.5%) of the EM-PLGA NPs were surrounded by a typical core–shell structure. In contrast, when ultrasound was not used in the microfluidic device, only 47.3% of the NPs were membrane-coated. The study also found that compared to CCM-PLGA NPs and similarly sized lipid-PLGA NPs prepared by the same method, EM-PLGA NPs exhibited higher homologous targeting and lower monocyte uptake in both *in vitro* and *in vivo* models. Compared to traditional methods, microfluidic ultrasound offers advantages such as high encapsulation efficiency and rapid formation of core–shell NPs, enabling the generation of biomimetic NPs with consistent size and core–shell structure. Research by Yuling Mao *et al.*[36] also reported the application of ginger exosomes (GE) in biomimetic NPs. The low drug loading capacity and poor stability of exosomes limit their application in macromolecular drug delivery therapies. Yuling Mao and colleagues first loaded the macromolecular drug INF into porous nanostructures—large mesoporous silica nanoparticles

(LMSNs)—which can efficiently load macromolecular drugs, resulting in INF/LMSN nanocomposites. The limited space within the pores can resist conformational changes of the macromolecular drug. Then, through the action of ultrasound, the INF/LMSN was completely coated with GE, resulting in the biomimetic nanocomposite INF/LMSN@GE, which inherits the membrane proteins of GE (Fig. 2A). With the assistance of ultrasound, the pores exposed on the LMSN surface were successfully blocked by the GE coating layer, thereby protecting the loaded protein drug from hydrolysis and preventing its premature release in the gastrointestinal tract (Fig. 2B). Table 2 summarizes the ultrasound parameters used in the literature for ultrasound-assisted exosome drug delivery.

Multiple studies have shown that compared to incubation and electroporation, the diameter of drug-loaded exosome particles obtained through ultrasound treatment is the largest. However, the mechanism behind this remains controversial. Wang *et al.*[33] suggested that this size change may be partially attributed to the loading of PTX into the lipid bilayer of the exosomes, specifically due to surface adsorption caused by hydrophobic interactions. Salarpour *et al.*[31] attributed it to the effects of cytotoxicity. Myung Soo Kim *et al.* believed that the increase in exosome size is due to the recombination of exosomes under ultrasound action. Ultrasound-assisted drug loading of exosomes is efficient,[30] and the resulting exosome formulations exhibit long-term stability,[37] not only preventing nucleic acid aggregation but also allowing for sustained drug release, especially of hydrophobic drugs, while also protecting against proteolytic degradation. However, there are certain limitations. First, the ultrasound may lead to the aggregation of exosomes, thereby affecting their immunological activity. Second, there are high instrument requirements when using ultrasound-assisted drug loading of exosomes. Finally, ultrasound may damage the membrane structure of the exosomes, causing drug leakage and resulting in insufficient drug loading. Prolonged ultrasound treatment may also lead to nucleic acid degradation.

Ultrasound-assisted targeted delivery of exosomes

Exosomes, especially those derived from tumor cells, are considered to have an advantage for targeted drug delivery to tumors due to their homotypic nature.[38] Additionally, exosomes from tumor cells express high levels of CD47 on their membranes, which confers resistance to phagocytosis by monocytes and macrophages.[35,39] Therefore, therapeutic agents, including small molecules and nucleic acid drugs, have been loaded into exosomes to achieve targeted drug delivery.[40] Research has shown that ultrasound can enhance the efficiency of exosome-targeted delivery to tissues and cells by increasing the permeability of blood vessels and cell membranes through mechanical effects, thermal effects, cavitation effects, and other mechanisms.

Ultrasound-targeted microbubble destruction (UTMD) is a non-invasive targeted drug delivery technique. The ultrasound microbubble-mediated delivery using UTMD has advantages for cardiac diseases.[41] Sun Wenqi *et al.*[20] investigated the effects of using UTMD to deliver exosomes in refractory tissues. Their research findings indicated that UTMD enhanced the permeability of cell membranes and blood vessels through cavitation effects, enabling stable and localized targeted delivery of exosomes to refractory tissues such as the heart, adipose tissue, and muscle. In another study, Sun Wenqi and colleagues loaded the gene drug miRNA into exosomes, and with the assistance of ultrasound-targeted microbubble destruction (UTMD), the delivery of exosome-mediated miRNA to the heart was significantly increased.[12] After *in vivo* injection, the microbubbles were destroyed by ultrasound, and the cavitation effect within the microvasculature of the target tissue facilitated drug delivery. The promoting effect of UTMD on exosome delivery is transient, which further enhances the safety of UTMD in facilitating targeted delivery of exosomes.

Sonodynamic therapy (SDT) can combine ultrasound, sonosensitizers, and exosomes to achieve targeted delivery and non-invasive treatment of diseases. The following section will discuss its role and mechanisms in treatment in more detail. Exosomes, as natural carriers, can be used for the targeted delivery of sonosensitizers. Some studies have loaded sonosensitizers and drugs into exosomes and achieved safe and effective targeted delivery through ultrasound, with the mechanism possibly attributed to the cavitation effect generated by SDT. Thuy Giang Nguyen Cao *et al.*[24] and Wang *et al.*[25] incubated sonosensitizers in exosomes, which were then injected into mice *via* the tail vein. Ultrasound treatment was applied at the tumor site to stimulate the sonosensitizers to produce reactive oxygen species (ROS) and assist in the targeted delivery of exosomes, achieving the goal of cancer treatment. Liu *et al.*[21] also loaded sonosensitizers into exosomes, but with the difference of performing two ultrasound treatments and using contrast agent microbubbles to assist targeted delivery during the first ultrasound treatment. The first ultrasound (US1) served as a guiding ultrasound, primarily promoting the local accumulation of exosomes loaded with sonosensitizers in the tumor region, thus assisting in the targeted delivery of exosomes. The second ultrasound (US2) was therapeutic and will be mentioned later. Guo *et al.*[22] loaded the sonosensitizer Ce6 into exosomes through an incubation method, and then anchored the protective coating CP05-TK-mPEG onto the exosomes through the interaction between the peptide CP05 and the exosome surface marker CD63, forming SmartExo (Fig. 3A) that are shielded from aggregation and phagocytosis. Due to the action of the hydrophilic polymer polyethylene glycol (PEG), SmartExo can avoid aggregation and escape phagocytosis by major organs, extending circulation time in the blood. Subsequently, therapeutic drugs were loaded onto SmartExo to form smart drug-loaded exosomes (Fig. 4). By irradiating the targeted site with ultrasound, the Ce6 in the smart drug-loaded exosomes generates reactive oxygen species (ROS) that act on the TK tendon between CP05 and mPEG (Fig. 3B), causing it

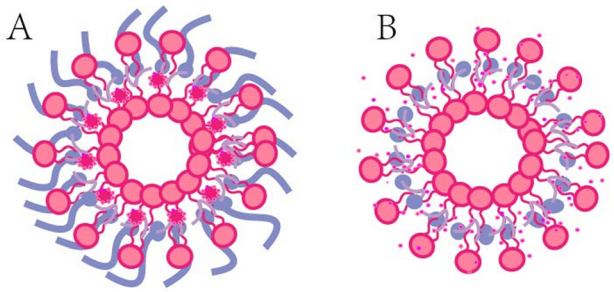

Fig. 3 (A) Schematic diagram of Smart exosomes (SmartExo). (B) Schematic diagram of drug-loaded exosomes after the removal of the invisible coating mPGE.

to break, thus enabling on-demand drug delivery at the targeted site. Yitong Guo *et al.* successfully delivered bone morphogenetic protein (Bmp7) mRNA in a controllable and targeted manner to the membrane tissue (OTA), inducing browning of OAT, which may assist in weight loss treatment. Their research results indicate that ultrasound irradiation significantly improved the delivery effect of SmartExo in adipose tissues.

Tang *et al.*[23] loaded interleukin-10, which has strong anti-inflammatory effects, into exosomes to create drug-loaded exosomes (AI-Exo) with anti-inflammatory properties. After intravenous injection, ultrasound was applied to the rheumatoid inflammatory ankle joint, and the results indicated that ultrasound can effectively enhance the targeted delivery of drug-loaded exosomes. Research by Yichen Liu *et al.* found that ultrasound can induce exosomes to target tumors, accumulate, and penetrate.[21]

Although exosomes can cross biological barriers such as the blood–brain barrier (BBB),[42] the brain-targeted delivery of exosomes may be hindered by the BBB, limiting their effective concentration in the brain.[43] It has been reported that the blood–brain barrier can be non-invasively opened using ultrasound. Therefore, when exosome-based drug formulations are combined with ultrasound, they can significantly penetrate the blood–brain barrier. It is known that focused ultrasound (FUS)-mediated blood–brain barrier opening is a temporary, safe, and reversible method for the brain-targeted delivery of exosomes. Low-intensity focused ultrasound (FUS) with microbubbles can non-invasively open the blood–brain barrier, thereby enhancing the brain-targeting capability of exosomes.[44] Research by Deng Zhiting *et al.* also indicates that FUS–BBB opening is beneficial for increasing the accumulation of exosomes in the brain, further enhancing their targeting efficiency.[15] Research by Yuanjiao Tang *et al.* shows that exosomes can target inflamed joints with the assistance of ultrasound, even in the absence of microbubbles[23] (Fig. 5).

Multiple studies have shown that ultrasound can enhance the permeability of exosomes to blood vessels and cell membranes, as well as the enhanced permeability and retention (EPR) effect, thereby improving the efficiency of targeted delivery of exosomes.[12,20,21,45,46] The combined use of ultrasound and microbubbles enhances the ability to deliver drugs to target tissues, which can be attributed to the effects of sonoporation and cavitation. The sonoporation effect caused by ultrasound contrast agent microbubbles is considered an important factor in the transient disruption of cell membrane permeability.[47] Fluid refers to the phenomenon of aligning

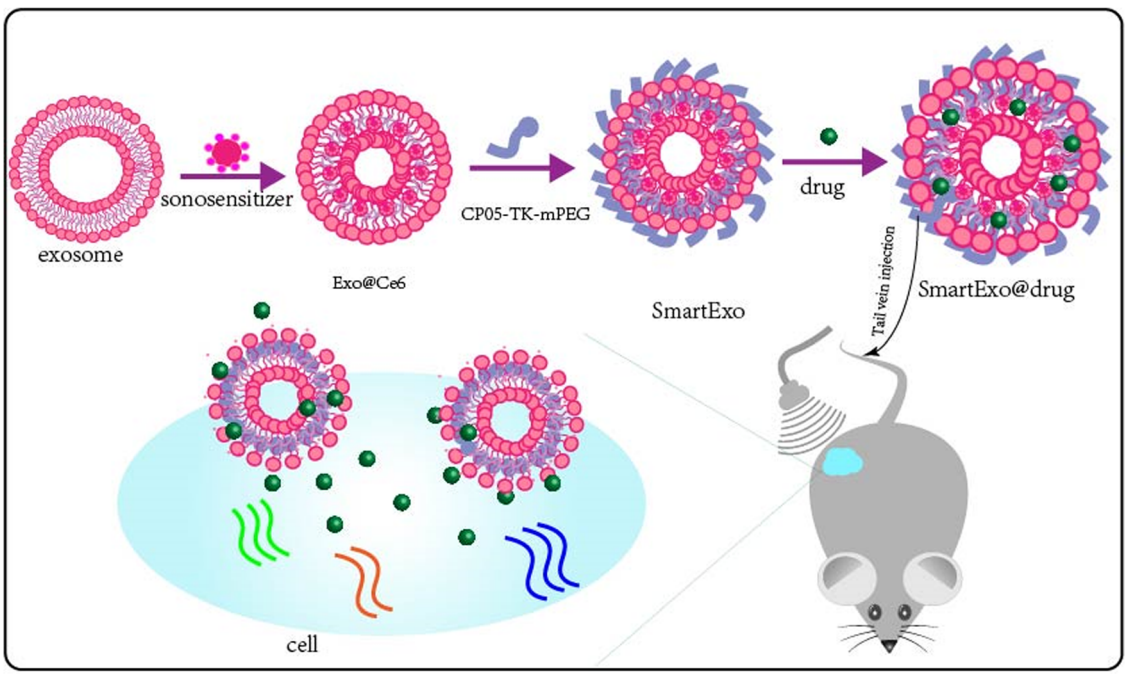

Fig. 4 The construction process of the smart drug delivery system (SmartExo); after the drug is loaded, it is injected into the systemic circulation *via* the tail vein, where it generates reactive oxygen species at the targeted site under ultrasound irradiation, sheds its invisible coating, and then releases the therapeutic drug at the target cells.

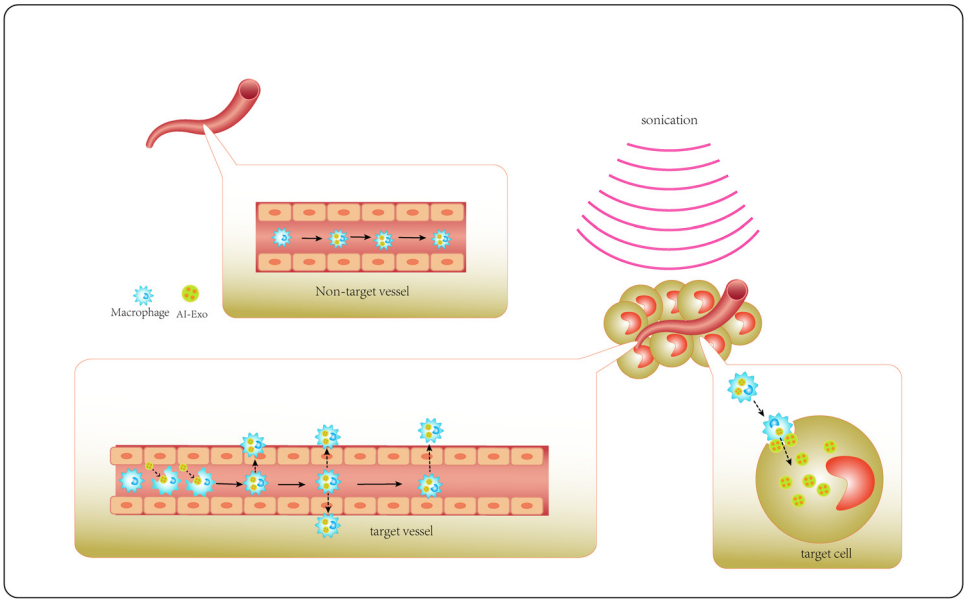

Fig. 5 Ultrasound promotes activated macrophages' phagocytosis of AI-Exo and increases the permeability of blood vessels and cell membranes through mechanical effects, thermal effects, cavitation effects, and others to promote AI-Exo targeted to cells.

reflective and scattering objects along the direction of ultrasound radiation force. The circulation of fluid around cavitation particles is known as the microstreaming effect. These mechanical flow effects can alter blood flow velocity and the movement of particles within the blood, which significantly aids in the delivery of drugs to target tissues.[17]

Ultrasound-assisted cellular uptake of exosomes or cargo

According to reports,[48,49] the possible mechanisms by which cells uptake exosomes include: (1) direct membrane fusion: exosomes fuse with the plasma membrane of the recipient cell; (2) receptor–ligand interactions or lipid interactions with target cells; (3) exosomes are internalized into recipient cells through endocytosis or pinocytosis. When ultrasound is applied, pores are formed and endocytosis occurs. It is generally believed that membrane pore formation (*i.e.*, sonoporation, endocytosis, and cavitation) is the main mechanism by which drugs enter cells during ultrasound application.[50] Research by Liu *et al.*[21] found that both exosome formulations and ultrasound stimulation can increase the cellular uptake of cargo, and when exosome formulations are combined with ultrasound, the uptake of cargo within cells is maximized. This may be due to the increased permeability of the cell membrane induced by the acoustic perforation effect of ultrasound. In contrast, Yuanjiao Tang *et al.*[23] suggested that ultrasound enhances the phagocytic activity of activated RAW264.7 cells, increasing their uptake of AI-Exo. Their findings indicate that ultrasound has a positive effect on promoting cellular uptake of exosome formulations. Additionally, research by Zhiting Deng *et al.*[15] shows that in conjunction with focused ultrasound (FUS)-mediated blood–brain barrier opening, exosomes can be rapidly taken up, thereby increasing their accumulation in the brain.

The ultrasonic cavitation effect refers to the formation or activity of bubbles in a medium under the action of ultrasound. The physical effects of cavitation can damage cell membranes and increase the permeability of cells and microvessels, leading to enhanced drug uptake.[51,52] The violent collapse of bubbles caused by high MI ultrasound, known as inertial cavitation, is associated with extreme local pressure and temperature, which can disrupt drug carriers and enhance drug uptake.[53] Sonoporation is a physical effect that may temporarily increase membrane permeability by creating transient membrane pores and stimulating endocytosis. Membrane pores may facilitate the intracellular delivery of small molecules (less than 4 kDa), while endocytosis may induce the uptake of large molecules (greater than 4 kDa).[54] Ine De Cock *et al.*[50] evaluated the mechanisms of cellular uptake within a range of acoustic pressures from 100 to 500 kPa. When other acoustic parameters (such as center frequency, pulse repetition frequency, *etc.*) were fixed, the results indicated that the drug uptake mechanism depends on the applied acoustic pressure. Low pressure primarily enhances uptake by stimulating cellular endocytosis, while high acoustic pressure mainly facilitates uptake through membrane pores.

Ultrasound augmented therapeutic effects of exosomes

Exosome carriers possess the advantages of both cell-based drug delivery and nanotechnology. Exosomes from different cell types contain various contents but share some common characteristic markers (such as CD9/CD63). miR-155 and miR-146a derived from dendritic cell (DC)-derived exosomes play

roles in promoting and inhibiting inflammation, respectively. Exosomes derived from bone marrow-derived dendritic cells (BMDCs) contain contents such as miR-16 and miR-21, which have anti-inflammatory effects. Research by Xuefeng Li *et al.*[18] shows that exosomes obtained from BMDCs after LIPUS irradiation contain approximately ten times more miR-16 and miR-21 than exosomes derived from untreated BMDCs. miR-16 can target IKKα and IKKβ,[55] enhancing their degradation[55] and increasing IκBα levels, which sequesters more NF-κB proteins in the cytoplasm, thereby reducing the activity of the NF-κB signaling pathway.[18] miR-21 can activate its own expression and enhance the suppression of pro-inflammatory factor expression, further limiting NF-κB activity.[18] As mentioned above, ultrasound irradiation may promote an increase in the expression of therapeutic contents within exosomes, thereby enhancing their therapeutic efficacy. Research by Nafar *et al.*[28] similarly found that after ultrasound treatment of exosomes containing hsp70 protein, the expression of the HSP70 protein in the exosomes increased. The HSP70 protein can prevent *in vitro* neurodegeneration by reducing misfolded proteins and lowering cytotoxicity, offering hope for the treatment of Alzheimer's disease by alleviating amyloid-β-induced neurotoxicity.

Exosomes can specifically act on target cells, regulate the external environment and inflammation, and promote the regeneration of damaged tissues.[56] Research has shown that exosomes derived from bone marrow mesenchymal cells can increase the expression of extracellular matrix proteins such as type II collagen (COL2) and aggrecan (AGG), thereby promoting cartilage regeneration in rats.[57] Therefore, ultrasound can enhance therapeutic effects by increasing the release of cell-derived exosomes. Xia *et al.*[14] found that low-intensity pulsed ultrasound (LIPUS) can enhance the efficacy of bone marrow mesenchymal stem cells (MSCs) in cartilage repair for osteoarthritis (OA) by increasing autophagy-mediated exosome release. However, the research by Liao *et al.*[26] suggests that LIPUS enhances the promotion of cartilage regeneration in osteoarthritis through the exosomes derived from bone marrow mesenchymal stem cells primarily by strengthening the inhibition of inflammation, which further promotes the proliferation of chondrocytes and the synthesis of a cartilage matrix. The potential mechanism may be related to the activation of the IL-1β-induced NF-κB pathway. Additionally, low-intensity ultrasound (LIUS) can enhance the biogenesis and docking of exosomes, thereby inducing their anti-inflammatory effects.[28] Research by Deng *et al.*[15] indicates that ultrasound significantly increases the release of exosomes derived from human astrocytes (US-HA-Exo). US-HA-Exo exhibits neuroprotective effects *in vitro* by reversing cell toxicity induced by oligomeric amyloid-β, and when combined with focused ultrasound (FUS) to induce blood–brain barrier (BBB) opening, it can clear amyloid-β plaques *in vivo*, thereby alleviating the neurotoxicity caused by amyloid-β, which may aid in the treatment of Alzheimer's disease.

Ultrasound can enhance the targeted delivery and cellular uptake of exosomes, thereby improving their therapeutic effects. For instance, ultrasound can promote the targeted accumulation of AI-Exo (anti-inflammatory exosomes) in inflammatory tissues and facilitate cellular phagocytosis, reducing the levels of inflammatory cytokines (including IL-6, TNF-α, and IL-1β) and promoting M2 macrophage polarization, thus targeting the treatment of inflammatory arthritis.[23] Research by Sun Wenqi *et al.*[12] found that UTMD significantly promotes the delivery of exosomal miR-21, providing substantial protection to the heart against doxorubicin-induced cardiotoxicity. In the study by Guo *et al.*,[22] ultrasound exposure was used to target the delivery of Smart-Exo@Bmp7 (a gene drug formulation based on exosomes) to the omental adipose tissue (OAT) to induce browning, demonstrating its weight loss therapeutic effect. The efficacy of SDT (sonodynamic therapy) depends on the ability of the sonosensitizer to generate ROS (reactive oxygen species) under ultrasound exposure. When ultrasound is used as an energy source, SDT exhibits stronger tissue penetration capabilities, making it suitable for the treatment of deep tissues. Studies indicate that the potential mechanisms of SDT may include ultrasonic cavitation effects, free radical production, apoptosis, or a combination of any of these mechanisms.[58,59] Research by Thuy Giang Nguyen Cao *et al.*,[24] Wang *et al.*,[25] and Liu *et al.*[21] also involves SDT, where ultrasound exposure targets specific sites, promoting the controllable release of sonosensitizer-loaded exosomes and increasing reactive oxygen species to enhance SDT for tumor treatment. In Liu *et al.*'s study, ultrasound was applied twice: the first was guiding ultrasound (US1), and the second was therapeutic ultrasound (US2). Initially, guiding ultrasound was used to promote the local accumulation of sonosensitizer-loaded exosome formulations (EXO-DVDMS) in the tumor region, followed by therapeutic ultrasound, under which EXO-DVDMS exhibited controlled ultrasound-responsive drug release and enhanced ROS generation, thereby improving the anticancer efficacy of SDT. Furthermore, their findings showed that the SDT of EXO-DVDMS effectively inhibited lung metastasis of breast cancer, potentially due to the high-level accumulation of EXO-DVDMS with tumor-derived exosomal coats in tumor tissues, downregulating the release of exosomes from the tumor, thus reducing the pro-metastatic and immunosuppressive effects of tumor-derived exosomes. Some limitations of SDT include the properties of sound waves, such as scattering and diffraction. Additionally, SDT cannot affect the lungs, which serve as air-carrying organs, and the exposure time for SDT is typically longer, which may lead to severe adverse reactions. However, compared to traditional treatments (such as chemotherapy or radiotherapy), SDT is appreciated for its non-invasive nature and selective targeting of cells.[60]

Conclusion and future perspective

In this review, we discuss the combined application of exosomes/exosome drug formulations with ultrasound to explore their potential applications. Currently, the combined use of exosomes/exosome drug formulations is primarily focused on exosome loading, delivery of exosome drug formulations,

cellular uptake, and enhancing the therapeutic effects of exosome drug formulations. As mentioned earlier, ultrasound irradiation can increase the expression of certain contents within exosomes, which may promote their efficacy to some extent. However, the underlying mechanism remains unclear. Future research could aim to elucidate the fundamental biophysical mechanisms by which ultrasound enhances specific contents in exosomes, which may help in the ultrasound-assisted preparation of exosomes containing more functionally specific contents for more effective treatment of related diseases.

There are still several issues regarding the application of ultrasound-assisted exosomes: (1) it may be difficult to maintain the structural and molecular integrity of exosomes under ultrasound irradiation; (2) although targeting peptides or proteins in exosomes can deliver molecules to specific cells, ultrasound can also assist in enhancing the targeted delivery of exosomes, yet they are still inevitably engulfed by non-target organs; (3) the limited penetration of ultrasound may prevent effective sonodynamic therapy (SDT) in deep tissues; (4) the free diffusion of drugs after ultrasound-mediated disruption may impair drug delivery efficiency. Future studies should explore how to prepare exosome formulations with good stability under ultrasound action, further improving targeting specificity and evading immune system-mediated destruction of exosome formulations.

Author contributions

Conception and design: YH; analysis and interpretation of the data: XW; writing – original draft: XW; revision – review: YH; final approval of the version: YH. All authors agree to be accountable for all aspects of the works.

Date availability

All data generated or analysed during this study are included in this published article.

Conflicts of interest

The authors declare that they have no competing interests.

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