Advances in Targeted Tumor Diagnosis and Therapy Based on Ultrasound-Responsive Nanodroplets

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Abstract: The ultrasound contrast agents currently used in clinics are microbubbles with a large particle size and short circulation time, and their approved clinical applications are limited to endovascular diagnosis and therapy only. The development of ultrasound-responsive nanodroplets (NDs) provides a new approach for extravascular diagnosis and therapy, especially for molecular imaging and targeted therapy of tumors. The NDs with a nano-scaled particle size and a liquid core can maintain their shape and initial diameter during injection, enhancing their EPR effects and facilitating the accumulation of NDs at the tumor site. When exposed to ultrasound, NDs can vaporize and exhibit contrast enhancement at the sites of interest. In addition, the destruction of microbubbles can provide a driving force to facilitate the release of drugs or genes from the microbubbles into target cells, allowing the NDs to act as drug carriers. The development of ultrasound-responsive NDs has shown rapid progress in recent years, while a variety of NDs with excellent properties have been fabricated for targeted diagnosis and drug delivery. In this article, the development of ultrasound-responsive NDs was reviewed in terms of their structure, phase transition properties, and applications in targeted tumor diagnosis and therapy.

Key words: Ultrasound contrast agents; Nanodroplets; Targeted diagnosis and therapy; Tumor

Introduction

Ultrasound has been widely used in clinical diagnosis and therapy due to its high sensitivity, portability, and safety, as well as relative low cost [1]. The application of ultrasound contrast agents (UCAs) enhances the ultrasound signal and further improves the accuracy of diagnosis. The UCAs used in clinics are typically gas-filled micron-sized bubbles (MBs) with a diameter of 1–8 µm and a limited *in vivo* circulation time, so these MBs cannot extravasate beyond the vasculature [2]. It has been reported that nano-scaled particles can extravasate beyond the vasculature and passively accumulate in the tumor microenvironment via enhanced permeation and retention (EPR). Unfortunately, the reduction in the size of MBs not only reduces their echogenicity under ultrasound but also reduces the stability of gas-filled bubbles in solutions [3], making it extremely difficult to develop small yet highly echogenic particles. Therefore, it is necessary to use other non-traditional strategies to develop nano-scaled UCAs.

The advancements in nanomedicine resulted in a rapid development of echogenic and biocompatible nano UCAS (nUCAs) (50–600 nm) with promising physical properties. These particles are composed of a

phospholipid-based shell and/or polymer-based shell and a solid, liquid, or gas core [2]. Among various nUCAs, nanodroplets (NDs) have attracted a lot of attention due to their unique phase transition properties. As shown in Figure. 1, the advantage of NDs is to maintain their shape and initial diameter during injection while undergoing the liquid-to-gas transition upon their exposure to ultrasound to produce micron-sized bubbles at the sites of interest, via a process called acoustic droplet vaporization (ADV)
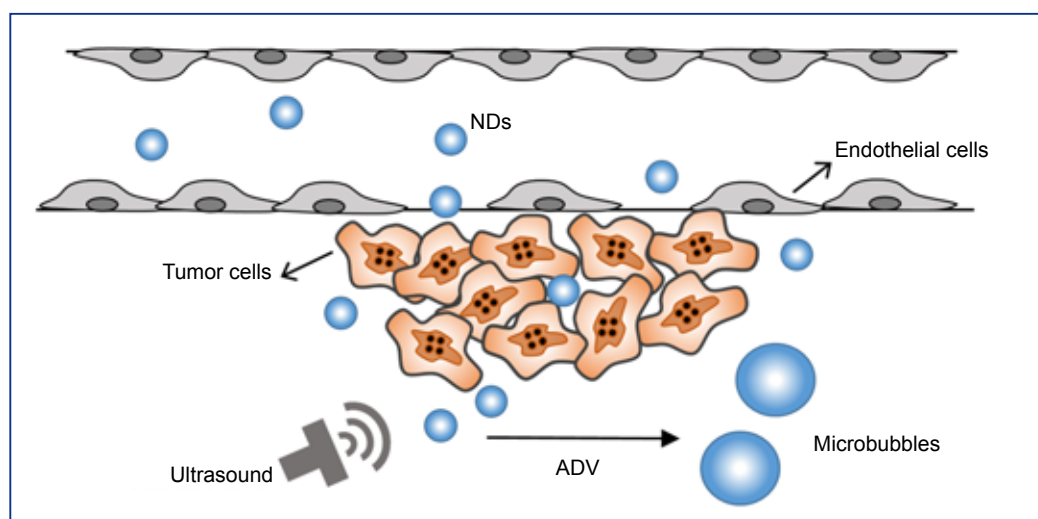
[4,5]. This allows NDs to extravasate and accumulate at the tumor site before transforming into bubbles under the effect of ultrasound [6]. NDs have attracted great attention in targeted tumor diagnosis and therapy, and have become a research hotspot in recent years as novel UCAs. Herein, the development of NDs and their potential applications in tumor diagnosis and treatment were reviewed.

Figure 1 Schematic representation of passive accumulation of ultrasound-responsive NDs in the tumor sites through EPR effect and ADV under ultrasound.

The Composition of NDs

The shells of NDs

NDs are comprised of perfluorocarbon (PFC), a liquid with a low boiling point, encapsulated within a shell (Fig. 2). Lipids and polymers are the most common materials used to make the shell of NDs. The materials used to prepare the lipid shell of NDs are similar to those used to prepare microbubble contrast agents such as SonoVue. Lipid NDs are more sensitive to ultrasound, and therapeutic effect of targeted drug release generated from microbubble rupture could be achieved at a lower acoustic output [4]. However, lipid NDs have poor stability *in vivo*, short circulation time and are vulnerable to phagocytosis by the reticuloendothelial system.

NDs with high molecular polymers shell showing strong pressure resistance, high *in vivo* stability and biocompatibility are expected to solve the problems of lipid NDs. Various NDs with polymer shells have been fabricated with the development of polymer materials. The most often used polymer materials include natural polymers such as chitosan [7,8] and alginate [9], as well as synthetic block polymer materials such as poly (ethylene glycol)-block-Poly (ε-caprolactone) (PEG-PCL) [10,11]. Due to the stability and stiffness of the shell, polymeric NDs usually have a higher cavitation threshold and longer circulation time than lipid NDs [12].
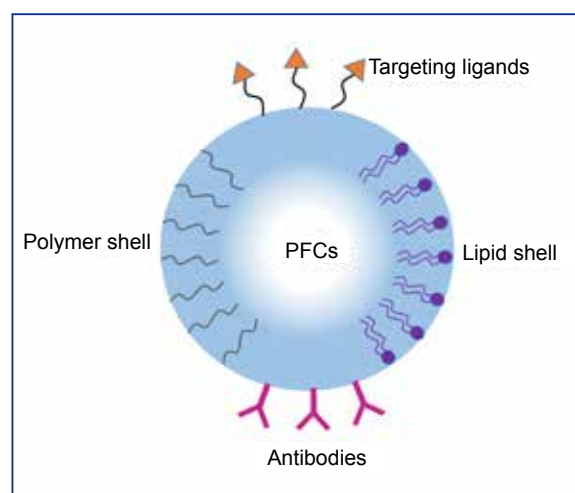

Figure 2 Schematic of the shell compositions, core of PFCs and targeting moiety of ultrasound-responsive NDs.

Baghbani et al. developed multifunctional smart curcumin-loaded chitosan/perfluorohexane (PFH) NDs for contrast-ultrasound imaging and on-demand drug delivery [7]. The optimal formulation with the size of

101.2 nm and 77.8% curcumin entrapment was chosen for release study and cytotoxicity evaluation. Sonication at the frequency of 1MHz, 2W/cm^2 for 4min triggered the release of 63.5% of curcumin from optimal formulation (Cur-NDs-2). B-mode ultrasound imaging confirmed strong ultrasound contrast of chitosan NDs even at low concentrations via droplet to bubble transition. The cytotoxicity evaluation in-vitro on 4T1 human breast cancer cells revealed that ultrasound exposure significantly increased cell growth inhibitory effects of curcumin-loaded NDs. According to these results, the ultrasound responsive curcumin-loaded chitosan/PFH NDs have a great potential for imaged-guided cancer therapy.

Gao et al. developed systems comprised of PFC NDs stabilized by the walls made of biodegradable block copolymers [10]. Upon heating to physiological temperatures, the nanodroplets convert into nano/ microbubbles. The phase state of the systems and bubble size may be controlled by the copolymer/PFC volume ratio. Upon intravenous injections, a long-lasting, strong and selective ultrasound contrast is observed in the tumor volume indicating nanobubble extravasation through the defective tumor microvasculature, suggesting their coalescence into larger, highly echogenic microbubbles in the tumor tissue.

A novel, acoustically-responsive, polydopamine (PDA) ND with particle size of about 400 nm was recently reported [13]. The biocompatible PDA NDs can be well dispersed in saline solutions with high colloidal stability and showed no harmful side effects to red blood cells or MCF7 cells. The small particle size and the hydrophilic segment prolongs the circulation time of NDs *in vivo* through avoiding the recognition by the reticuloendothelial system. It was demonstrated that PDA offers some important benefits: (1) facile fabrication, as dopamine monomers are directly polymerised on the nanodroplets, (2) high polymer biocompatibility, and (3) ease of functionalisation with other molecules such as drugs or targeting species. This suggests great potential of PDA NDs as an acoustically-active nanodevice, which is highly valuable for biomedical applications including drug delivery and treatment monitoring.

The cores of NDs

The core of NDs is usually made from liquid perfluorocarbons (PFCs). Since the radius of NDs is inversely proportional to the vaporisation threshold [14], a nano-scale droplet requires a PFC with a lower boiling point to achieve a vaporization threshold similar to that of micron-scale droplets. The vast majority of studies to date have selected perfluoropentane (PFP) [15-19], which has a boiling temperature of 29 °C. The NDs with a PFP core can be easily converted into microbubbles under the action of ultrasound, and both stable cavitation and inertial cavitation were observed under focused ultrasound [11,20]. However, NDs with a PFP core are unstable during storage, and their irreversible droplet-to-bubble transition is hard to control [21].
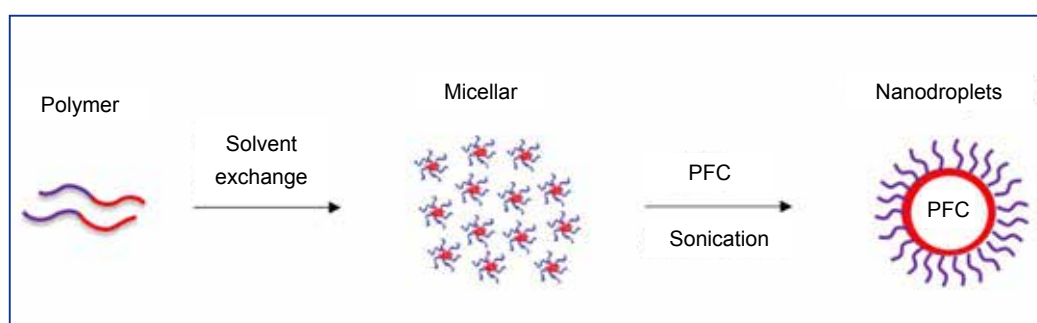

Figure 3 Schematic of preparation method for block copolymer stabilized NDs

In order to obtain nanodroplets with higher stability, PFH was chosen for the core of NDs because of its higher boiling point (58-60 °C) compared to other common PFCs including PFP [7,9,22]. In the study of Baghbani et al. [9], despite the relatively high boiling temperature of PFH, NDs converted into microbubbles under the action of ultrasound. It was also demonstrated that chitosan/PFH NDs can be detected even at low concentrations, which could serve as highly sensitive theranostic agents for simultaneous tumor imaging and therapy [7].

Rapoport et al. have screened various PFC compounds with a higher stability. Among those, perfluoro-15-crown-5-ether (PFCE, boiling temperature of 146 °C) has attracted special attention. PFCE NDs manifest both ultrasound and fluorine (^{19}F) MR contrast properties, which allows monitoring pharmacokinetics and biodistribution of NDs using multimodal imaging and 19F MR spectroscopy. The droplet-to-bubble transition of PFCE NDs with polymer shells could be triggered by

sonication with 1-MHz therapeutic ultrasound, and about 50% of the injected NDs remaining in circulation 2 h after the systemic injection [21].

Alternatively, combining PFCs have been used to increase stability of NDs. Phillips et al. [23] created a novel phase- shift nano agent by combining highly volatile perfluorobutane (DFB) with less volatile PFP. By using both in a 1:1 ratio, they found that vaporisation was achieved at relatively low high intensity focused ultrasound (HIFU) intensities compared with that of NDs with only PFP. and droplets were still stable enough to remain in the liquid phase for up to 48 hours.

The fabrication of NDs

The fabrication methods of NDs are dependent on the materials in their shell. The thin-film hydration method is mainly used to fabricate phospholipid-based NDs, and the double emulsion method is used to fabricate polymer-based NDs [12]. Rapoport et al. developed block copolymer stabilized NDs [11,20,21]. In their approach, the micellar solutions of PEG-PLLA and PEG-PCL block copolymers were first prepared by a solvent exchange technique. Then, the micellar solutions were mixed with liquid PFP and sonicated to obtain NDs (Fig. 3).

In addition, in several reports, sub-micron droplets were prepared using a newly developed condensation method [25, 27]. As described by Sheeran et al. [24], microbubbles of desired size were prepared followed by pressurization and condensation to generate the decafluorobutane (DFB, natural boiling points of –2 ℃)-condensed droplets. The volumetric change of each condensed bubble resulted in liquid DFB droplets mostly in the sub-micron range. This method can be very useful in preparing NDs from existing commercial UCAs, so as to avoid in-house synthesis, minimize resource requirements, and possibly improve the particle yield [2].

Tumor Targeted NDs

Nano-sized ultrasound contrast agents can take advantage of the EPR effect to produce passive accumulation in tumor site. However, it has been found that nanodrug delivery to tumors by the EPR effect can offer only a twofold improvement in nanodrug delivery compared with normal tissue, which results in an insufficient drug concentration for treating most cancers [28]. With respect to cancer, it has been found that some kinds of receptors are overexpressed on the surface cancer cells, but not in normal human tissue. According to this, ultrasound contrast agents with tumor targeting properties can be constructed by grafting targeting moiety (Fig. 2).
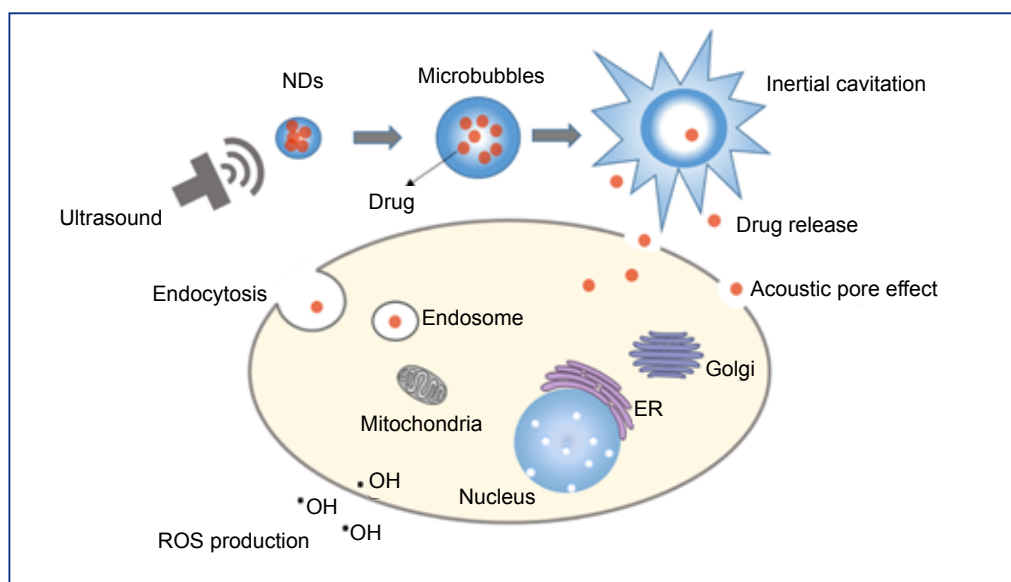

Figure 4 Schematic of drug delivery mediated by ultrasound-responsive NDs.

Folate receptor targeted NDs

Folate receptor (FR) is overexpressed on the surface of breast, ovarian, cervical and colorectal cancer cells [29, 30]. Recent work using FR-targeted NDs [31] indicates that targeted agents can also selectively promote intracellular delivery, which can be incorporated as a strategy for using NDs for even more precise cancer

cell imaging and therapy [25]. The study of Liu and colleagues described [32] the successful fabrication of phase changeable folate-targeted PFP NDs (termed FA-NDs) for tumor molecular imaging with ultrasound. Notably, these FA-NDs can be triggered by low-intensity focused ultrasound (LIFU) sonication, providing excellent enhancement in B-mode and contrast-enhanced

mode in vitro. After intravenous administration into nude mice bearing SKOV3 ovarian carcinomas, the labeled FA-NDs were found to accumulate in the tumor region.

FA-NDs injection followed by LIFU sonication exhibited remarkable ultrasound contrast enhancement in the tumor region.

Table 1 Properties of commonly used PFCs for fabrication of NDs.

Compound name	Acronym	Molecular formula	Boiling point (°C)	Droplet shell and reference
Perfluorobutane	DFB	C_4F_{10}	-2	Lipid [24,25]
Perfluoropentane	DDFP/PFP	C_5F_{12}	29	Lipid [12]
				PEG-PCL [10]
				PGA-g-Mpeg [18]
Perfluorohexane	PFH	C_6F_{14}	58	Chitosan [7]
				Alginate [9]
				Polypyrrole [22]
				Polydopamine [13]
Perfluoro-15-crown-5-ether	PFCE	$C_{10}F_{20}O_5$	146	PEG-PLLA [21]
Perfluorooctyl bromide	PFOB	C_8BrF_{17}	142	Egg lecithin, PEG [26]

Anti-Her2 NDs

The overexpressed Her2 on breast cancer was also regarded as a target for design of tumor targeted NDs. A tumor-targeting, ultrasound-triggered, phase-transition ND was developed by introducing an anti-Her2/neu peptide (AHNP) aiming to targeted treatment of Her2-overexpressing breast cancer [33]. The average diameter of AHNP-PFP-NDs was below 400 nm. The modification of AHNP improved the affinity between nanodroplets and Her2-overexpressing breast cells. Both intratumor and intravenous injection of NDs into nude mice bearing HGC-27 xenografts showed that the gene transfection efficiency and the ultrasound contrast effect were significantly enhanced after exposure to the insonation with optimized ultrasound parameters. This targeting NDs system could serve as a potential theranostic vector for tumor targeting, ultrasound diagnosis, and gene therapy.

$α_vβ_3$ integrins targeted NDs

RGD is a sequence of arginine, glycine, and aspartic acid, commonly known for targeting $α_vβ_3$ integrins overexpressed in tumor cells during angiogenesis. A pentapeptide, which includes the RGD sequence followed by a D-Phe-Lys moiety to close the cyclic structure, i.e. cyclo (Arg-Gly-Asp-D-Phe-Lys) or cRGD, has been chosen to tether onto the surface of the poly (10,12-pentacosadiynoic acid) NDs [34]. With cRGD conjugated to the surface via PEG spacer, the bioadhesion of NDs with HUVEC cells is significantly enhanced (0.6 ± 0.15 ND/cell) as compared to the plain NDs used as control (0.25 ± 0.06 ND/cell). The conjugation of cyclic RGD and PEG chains of the particles showed targeting ability toward endothelial cells.

Acoustic Droplet Vaporization (ADV)

Ultrasound can be used to induce the vaporization of NDs, a process known as ADV [16]. Surface tension plays a key role in determining the vaporization threshold of a droplet. Beyond ambient pressure, a PFC droplet will experience an additional Laplace pressure as the result of surface tension effects over a defined radius [35-37]:

$$\Delta P_{Laplace} = P_{Inside} - P_{Outside} = 2\sigma/r \qquad (1)$$

where P_{Inside} is the pressure inside the droplet, $P_{Outside}$ is the ambient pressure in the surrounding media, r is the radius of the droplet, and σ is the surface tension.

Due to the presence of Laplace pressure, PFP NDs will not spontaneously vaporize when they are injected *in vivo* at 37 °C until the droplets are exposed to ultrasound under a sufficiently high acoustic pressure. According to the Antoine vapor-pressure equation [38], the energy (in the form of temperature and/or ultrasound) required to induce phase transition increases over the increasing pressure on the core of the droplet. The Laplace pressure is an inverse function of radius, so this effect becomes more pronounced for droplets with a nano-scaled diameter.

The ultrasound pressure required to induce the

phase transition of the droplets is affected by a large number of factors, which can be divided into three categories: environmental factors, droplet design, and ultrasound parameters. Through encapsulation in a lipid or polymer shell, Kandadai and colleagues showed that the interfacial surface tension of PFP droplets may be modified by the choice of emulsifier to provide more favorable properties for dispersion in aqueous media [39]. The threshold of ADV is inversely proportional to the frequency and is proportional to the amplitude [17,40].

The mechanism that induces the ADV of the NDs has not been fully understood. The insight into the ADV mechanism allows for the optimization of the acoustic pressure required for activation, thereby minimizing the negative bio-effects of ultrasound [41]. In related studies, the mechanism has been discussed, and physical models have been developed to simulate droplet vaporization [42, 43]. It has been hypothesized that the vaporization of NDs occurs due to acoustic heating. However, most studies have shown that increasing pulse duration at clinically relevant ultrasound frequencies results in no significant decrease in the vaporization pressure needed to achieve ADV, unless the duration is on the order of 1 millisecond or greater [44-46]. In addition, Lo and colleagues found that by using shorter periodic pulses unlikely to cause heating, but with an equivalent total 'on-time' as the longer pulses, a similar decrease in required ultrasound pressure resulted, which indicated that heating may not be the primary mechanism at work [46]. Other studies have provided evidence that ADV may be initiated by mechanical effects such as acoustic cavitation [38]. The mechanical effects such as micro-streaming, liquid jet, and shock wave produced by cavitation may impinge on the surface of droplets and induce vaporization [45,47]. However, more studies have shown that phase transition can be induced independently of inertial acoustic cavitation [15,44]. A recent interpretation validated with experimental data captured with an ultrahigh-speed camera states that ADV was initiated by a combination of two phenomena: highly nonlinear distortion of the acoustic wave before it hits the droplet and focusing of the distorted wave by the droplet itself [41]. At high excitation pressures, nonlinear distortion causes significant superharmonics with wavelengths on the order of the droplet size. The superharmonic focusing of acoustic energy inside the droplet causes a spot of negative pressure that spreads throughout the liquid volume [4]. This mechanism could explain the observed pressure thresholding effect.

Once a droplet has vaporized into a microbubble it has the potential to undergo inertial cavitation (IC). The pressure required to achieve IC is distinct from the vaporization threshold, and depending on the therapeutic application is either strived for (i.e., in sonothrombolysis for the dissolution of clots) or avoided (as in drug delivery to prevent tissue damage).

Application of NDs in Diagnosis and Therapy of Tumor

Molecular imaging of tumor based on NDs

Contrast enhanced ultrasound (CEUS) uses highly echogenic contrast agents to amplify the ultrasound signal, allowing low ultrasound contrast imaging of organs (i.e., blood pool). In order to expand the utility of CEUS in the field of molecular imaging, effort has been spent in developing microbubbles targeting vascular endothelial markers by attaching biomolecules onto their surface [48,49]. Unfortunately, one major limitation in tumor targeted molecular ultrasound imaging is the lack of submicron-size UCAs that can extravasate beyond the vasculature to directly target the biomarkers located on tumor cells [2].Since many tumors possess blood vessels permeable to particles with a diameter of 200 nm, it is possible that submicron PFC droplets can be used as a novel extravascular UCA that can selectively enhance tumor images [50]. The relative stability of NDs in circulation facilitates their targeted accumulation in tumor sites prior to ultrasound-induced vaporization. Once vaporized, these NDs induce contrast enhancement similar to that provided by microbubbles.

Choi and co-workers developed functionalized chitosan-based PFP NDs (300-500 nm) for multimodal X-ray/CT and ultrasound imaging [51]. For in vivo ultrasound imaging, the strong echo signals began to be observed in tumor at 1 h post-injection and gradually increased for 12 h, indicating that NPs could be accumulated in tumor tissue. Moreover, the echogenicity of the NPs in tumor tissue was coincided with NIRF accumulation property, and the echogenicity of the NPs allowed feasible ultrasound imaging at the deep tissue region of the SCC7 tumor. These results indicated that the NPs could encapsulate and retard evaporation of PFP, resulting in prolonging echogenicity in the physiological condition.

To further enhance the imaging specificity of NDs in the tumor, Li and co-workers developed folate receptor (FR) targeted-nanoparticles (FRNPs) [26]. The nanoparticles were composed of a liquid core of perfluorooctyl bromide (PFOB) liposome and a targeted shell chemically conjugated with folic acid (FA) and polyethylene glycol (PEG). The FRNPs exhibited an average particle size of 301±10.8 nm, and a relatively high tumor-targeted distribution in FR-overexpressing tumors was observed compared with that in other non-

targeted sites. The *in vivo* ultrasound imaging of the FRNPs was compared with that of Sonovue. From 1 to 10 min post-injection, the imaging of the tumors was significantly enhanced in the Sonovue group, and the intensity value of tumor imaging in this group was considerably higher than that of the other groups (P<0.001). At 20 min post-injection, the intensity value in the Sonovue group decreased rapidly, and almost disappeared within 40 min post-injection. However, the intensity value in the FRNPs groups gradually increased after the injection and reached the peak levels at 10 and 20 min post-injection. Until the time point of 160 min post-injection, the enhancement of the FRNPs remained at a high level of intensity. This suggested that the FRNPs exhibited a longer duration of effective enhanced ultrasound imaging in tumor.

Application of NDs on targeted therapy of tumor

NDs have the same cavitation effects as microbubbles after undergoing phase transition. The ultrasonic cavitation effects refer to a series of dynamic processes such as shock, expansion, contraction, and implosion of microbubbles in liquid under the action of ultrasound. The ultrasonic cavitation effects are accompanied by various kinds of energy release behaviors, such as transient high temperature, high pressure, shock wave, discharge, and microjet. Ultrasonic cavitation at the histological interface can widen the gap between surrounding target cells (including vascular endothelial cells and tissue cells), facilitate cell endocytosis, form transient pores at cell surface (acoustic pore effect), and produce reactive oxygen species (ROS) (Fig. 3), which can serve as a driving force to facilitate the delivery of drugs or genes from the microbubbles into the target cells [47].

Dimcevski et al. showed that ultrasound combined with microbubbles and gemcitabine for pancreatic cancer treatment could improve the tolerance of patients to chemotherapy without increasing side effects compared with simple gemcitabine therapy, and the survival time of patients were prolonged significantly [52]. Microbubbles have been used to deliver therapeutic agents in tumor chemotherapy [53-55]. However, the short circulation time and large size of microbubbles hamper their versatile applications in drug delivery [12]. Ultrasound responsive NDs are a kind of non-invasive carriers that can carry particles through the endothelium to enter the target tissue. Through the ADV and cavitation effects of NDs at target sites, drugs and genes can be transferred and released to target tissues to achieve therapeutic effects.

ND-mediated enhanced tumor chemotherapy

A variety of chemotherapeutic agents have been loaded in NDs in study of targeted tumor therapy. The most commonly used chemotherapeutic drugs include doxorubicin (DOX) [12,18], paclitaxel (PTX) [21], docetaxel (DTX) [22] and camptothecin (CPT) [56]. The drug-loaded NDs can accumulate in tumor tissues by passive targeting due to their nano-scaled sizes. When exposed to targeted ultrasound insonation, these NDs are converted into microbubbles in tumor sites. Through the ultrasound-targeted microbubble destruction (UTMD) effect [47,57], the microbubbles cavitate and collapse to release the encapsulated drug and dramatically enhance intracellular drug uptake by the tumor cells [10]. ND-mediated enhanced tumor chemotherapy can reduce the side effects of drugs and improve treatment efficacy and patient tolerance.

In a recent study, an ultrasound-triggered delivery system using PFP/C_9F_{17}-PAsp(DET)/CAD/PGA-g-mPEG NDs was developed, which combined ultrasound responsive phase-change contrast agent, acid-cleavable DOX prodrug, and cationic amphiphilic fluorinated polymer carrier, aiming to achieve both high imaging contrast and preferable ultrasound-triggered anti-cancer therapeutic effects [18]. The optimized NDs were characterized as monodispersed particles with a diameter of about 400 nm, slightly positive surface charge, and high drug-loading efficiency. The functional augmenter PGA-g-mPEG provided the NDs with good sustainability, low cytotoxicity, and good serum compatibility. In the ultrasound imaging study, the NDs produced significant contrast with ultrasound inosation (3.5 MHz, MI=0.08) at 37℃. Cell uptake and cytotoxicity studies in HepG2 and CT-26 cells showed the enhanced drug uptake and therapeutic effect with the combination of NDs and insonation.

Cao et al. [12] constructed a phase-changeable drug-delivery nanosystem with programmable low-intensity focused ultrasound (LIFU, 1 MHz, acoustic intensity: 1.2 W/cm^2, duty cycle: 50%) that could trigger drug-release and significantly enhance anticancer drug delivery. Liquid-gas phase-changeable PFP and an anticancer drug (DOX) were simultaneously encapsulated in lipid-based NDs (LN) and PLGA-based NDs (PN). The average diameters of LN and PN were around 409.4 ± 24.7 nm and 357.1 ± 15 nm, respectively. Based on the acoustic property of shell materials, such as shell stiffness, these two types of NDs were activated by different acoustic pressure levels. Various ultrasound energies were introduced to induce the phase transition and microbubble collapse of NDs in vitro (3 W/3 min for LN; 8 W/3 min for PN). The intratumoral accumulation and distribution of the drug with LIFU exposure were significantly enhanced, and tumor proliferation was substantially inhibited. Co-delivery of two drug-loaded NDs could overcome the physical barriers of tumor

tissues during chemotherapy. This study provides a new strategy for the efficient ultrasound-triggered chemotherapy by nanocarriers with programmable LIFU capable of achieving the on-demand drug release.

In another study, the therapeutic properties of PTX loaded PFCE NDs was evaluated [21]. The nanodroplet size (200 - 350 nm) as well as long residence in circulation favored their passive accumulation in tumor tissue that was confirmed by ultrasonography. In breast and pancreatic cancer animal models, ultrasound-mediated therapy with paclitaxel-loaded PFCE nanoemulsions showed excellent therapeutic properties characterized by tumor regression and suppression of metastasis.

NDs-mediated gene therapy

Ultrasound-induced microbubble sonoporation has been shown to effectively improve drug/gene delivery efficiency by enhancing tissue and cell permeability. Recent studies have indicated that NDs can function as a carrier for cellular gene transfection, and the successful release of micro RNA and DNA upon ultrasound exposure has been demonstrated [58-60].

One kind of ultrasound-triggered phase-transitioning ND was developed to deliver microRNA-122 (miR-122) for hepatocellular carcinoma (HCC) treatment [60]. PFP served as an ultrasound-sensitive core for ultrasound-triggered phase transition and size change from the nanoscale to the microscale. Positively charged C_9F_{17}-PAsp (DET) ensured adequate miRNA loading. PGA-g-mPEG, which served as the shell of the ND, enhanced their stability in serum, and protected miR-122 from degradation *in vivo*. The PFP-TNDs/miR-122 has a diameter of 362 ± 15 nm, and remained stable for 24 h. After treatment with PFP-TNDs/miR-122 combined with ultrasound (frequency: 1 MHz; intensity: 1.2 W/cm2; duty cycle: 20%; 2 duration time: 1 min; twice at 30 min and 4 h), the miR-122 expression level was significantly increased by approximately 600-fold in HepG2 cells, 500-fold in SMMC-7721 cells, and 30-fold in human HCC xenografts. Moreover, PFP-TNDs/miR-122 combined with ultrasound effectively suppressed the growth, migration, and invasion of HCC cells, and inhibited tumor proliferation in mice. The biodegradable PFP-TNDs is a promising therapeutic gene carrier with functions of gene protection and effective gene delivery for clinical applications.

In the study of Dong et al., pre-miRNA plasmids were electrostatically adhered to positively-charged lipid-shelled NDs to obtain plasmid-loaded NDs (PLNDs). The PLND-generated microbubbles oscillated and even collapsed under ultrasound exposure to release the loaded pre-miRNA plasmids and enhance their cellular uptake. Fluorescence microscopy revealed that PLNDs could effectively deliver the aforementioned pre-miRNA plasmids into SMMC-7721 cells in vitro under ultrasound exposure (1.2-MHz focused transducer). The therapeutic efficacy in a subcutaneous tumor of the mouse xenograft HCC model revealed that the PLNDs loaded with pre-miR-139 and -378a plasmids could effectively suppress tumor growth after ultrasound treatment [19].

In another study, PFP/pDNA-loaded NDs were primarily formulated by an oil/water emulsification method, followed by surface modification with PGA-g-mPEG. The average diameter of the NDs ranged from 300 to 400 nm. With ultrasound exposure, the gene transfection efficiency was enhanced 14-fold on HepG2 cells, and ultrasound-triggered phase-transition cationic nanodroplets also displayed a good ultrasound contrast effect [59]. These results suggest that the phase-transition NDs can be utilized as efficient theranostic agents for targeting gene delivery.

Combination of NDs with other therapies

The NDs could provide ultrasound imaging or enhanced cancer inhibition when combined with other therapies. In the study of Zhao and colleagues [61], phase-shift and NIR photoabsorbing poly(lactic-co-glycolic acid) (PLGA) nanocapsules (PFH@PLGA / Fe_3O_4) were fabricated for MRI/US dual-modal imaging-guided photothermal therapy (PTT). The multifunctional nanocapsules can be used not only to increase the local tumor temperature by absorbing the NIR energy but also as bimodal contrast agents for both MRI and ultrasound imaging. Similarly, Yang et al. [22] constructed DTX-loaded, PFH NDs coated with a polypyrrole (PPy) shell and modified by FA on the surface. The NDs had an active targeting ability to tumor cells, and their photothermal-chemotherapy synergistically inhibited the proliferation of tumor cells. *In vivo* study indicated that the NDs not only effectively inhibited the growth of 4T1 breast cancer, but also inhibited lung metastasis. This multifunctional nanoparticle is expected to become a new nanoplatform for the visualized photothermal-chemotherapy of cancer.

Sonodynamic therapy (SDT), a promising alternative for cancer therapy, utilizes a sonosensitizer combined with ultrasound to damage tumor cells/tissues for therapeutic purposes. Inertial cavitation is crucial for the therapeutic effects of SDT. Therefore, approaches that can induce highly efficient inertial cavitation should be of benefit for sonodynamic effect. Several studies report the use of ultrasound-activated sonosensitizer-based NDs for SDT, which provide numerous benefits for killing cancer cells compared with traditional methods. For instance, NDs loaded with IR780 (a hydrophobic sonosensitizer)

showed effective surface-to-core diffusion both in vitro and *in vivo*. In the presence of ultrasound, the ADV effect significantly assisted the conveyance of IR780-NDs from the circulatory system to tumor regions, and the acoustic wave force also increased the penetration depth within tumor tissues. Furthermore, IR780-NDs possesses mitochondrial targeting capabilities, which improves the precision and accuracy of SDT delivery [62].

It was reported that highly efficient inertial cavitation activity can be achieved through the combinatorial use of a short-pulsed focused ultrasound (SPFU) sequence and PFH nanodroplets. IR780 iodine was loaded inside denatured bovine serum albumin-shelled PFH (PFH@BSA-IR780) NDs. The sonodynamic efficacy was validated by treating HeLa cervical cancer cells. Under SPFU exposure, PFH@BSA-IR780 NDs were highly effective in promoting reactive oxygen species (ROS) generation and inducing cancer cell death. A significant decrease in cell viability was achieved within just 10 s. Besides the cytotoxicity of ROS, the mechanical bioeffects of inertial cavitation also led to severe cell death resulting from higher acoustic power or the longer treatment time [63]. Therefore, with combined modalities, IR780-NDs can be a promising theranostic nanoplatform for cancer therapy.

Conclusion and Prospect

This review has outlined the advances in ultrasound-responsive NDs, including their structure, fabrication, ADV, tumor targeting, and applications in targeted tumor diagnosis and therapy. The literature has revealed the advantages of ultrasound-responsive NDs in tumor diagnosis and therapy due to their nano-scaled size and the property of phase transition. The NDs have provided a novel platform for molecular imaging and targeted therapy of extravascular diseases, and the treatment efficiency of traditional therapy could be enhanced through combination with NDs. However, factors about safety and bio-effects of NDs must be taken into account to enable clinical translation. The components of the construct should be biocompatible, the vaporization should be responsible only for intended bio-effects, and the formulation and package should be in a way that is easy for storage and medical use. In addition, it is necessary to establish safe and effective criteria of ultrasound parameters for NDs, and more clinical trials need to be conducted to evaluate whether they can used as a valuable tool in clinic.

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Conflict of Interests

The authors have declared that no competing interests exist.

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