Research Advances in Ultrasound Imaging for Tumor *in Situ*

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Abstract: Known for being highly sensitive and noninvasive, ultrasound imaging using microbubble contrast agents is widely used in the clinic. To use ultrasound to image tissue beyond the vasculature, researchers have developed strategies that include nanobubbles, ultrasound contrast agents generated *in situ*, and gene expression of ultrasound contrast agents *in situ*. All of these strategies offer the capability of targeting tumor cells, intratumoral imaging tumor cells and require just a small incision or no incision. In this review, we will first describe the application of nanobubbles acting as ultrasound contrast agents. Then, we will briefly introduce the stimuli-responsive formulations to generate ultrasound contrast agents *in situ*. Finally, we will provide an overview of the use of state-of-the-art of gene expression of ultrasound contrast agents *in situ* to monitor cellular location and function inside living organisms.

Key words: Ultrasound contrast agents; Nanobubbles; Gene expression; Ultrasound

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Introduction

“A cloud of echoes between the undulating margins of the aortic root” was observed after injection of agitated saline in 1968, proving for the first time that bubbles in the agitated saline could be used as ultrasound contrast agent for echocardiography [1]. The United States approved the first human ultrasound contrast agent, Albunex, in 1994 [2]. Since then, ultrasound contrast agents have been used to improve the sensitivity and accuracy of conventional ultrasound imaging [3]. All commercially available ultrasound contrast agents are currently microbubbles (MBs), which are typically designed with a diameter of 1-8 μm to serve as blood pool agents [4,5]. These micro-sized bubbles are, however, too large to pass through the vasculature [6]. To penetrate deep into tissue, visualize cellular functions, and load therapeutic agents, tumor *in situ* ultrasound imaging is necessary [7].

Tumor *in situ* ultrasound imaging is a new strategy for visualizing cells or structures by using a new

generation contrast agent. Among the promising solutions used for this imaging approach are nanobubbles (NBs) [8,9], ultrasound contrast agents generated *in situ* [10], and gene expression of ultrasound contrast agents *in situ* [11,12]. These new generation contrast agents exhibit several advantages over MBs: (1) imaging tumor cells beyond the vasculature; (2) targeting tumor by the enhanced permeability and retention (EPR) effect or by conjugating targeted molecules on the surface; (3) stimuli-responsive to generate ultrasound contrast agents *in situ*; and (4) gene expression of ultrasound contrast agents *in situ* to directly monitor tumor location.

Advanced nanotechnology has enabled the development of many new types of MBs for ultrasound imaging, but the exploitation of tumor *in situ* ultrasound imaging is still in its infancy (Table 1). We believe that with the rise of sophisticated advancements in nanomedicine, *in situ* ultrasound imaging agents would receive more attention in future imaging research and clinic practice. This review aims to provide an overview of the state-of-the-art of the tumor *in situ* ultrasound

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imaging strategies.

Contrast Nanobubbles

NBs acting as ultrasound contrast agents have increasingly received attention in molecular ultrasound

imaging for tumors *in situ*. NBs usually exhibit a uniform size of 380–780 nm, which is compatible with the pore sizes of leaky tumor blood vessels [16]. Different from MBs, NBs are able to passively leak through tumor blood vessels by EPR effect [17].

Table 1 Tumor in situ ultrasound imaging strategies

Contrasts	Classification	Advantages	Disadvantages	Ref.
Nanobubbles	Imaging	Passing through vasculature	Poor echogenic ability	[4,17]
	Loading drugs	Imaging and therapy	Poor drug loading ability	[13]
	Targeting	Accumulating more in tumor tissue	Some tumor cells may negatively express receptor ligand	[14,19]
	Multifunction	Imaging and treating tumor	Difficult to fabricate multifunctional nanobubbles	[20]
Ultrasound contrast agents generated in situ	Heat responsive	Treating tumor and release contrast agents	Harmful to normal tissue	[27]
	pH responsive	Noninvasive	pH value may not low enough to trigger the generation of contrast agents in some tumors	[26]
	Near-infrared (NIR)/Ultraviolet responsive	Noninvasive	Short tissue penetrating depth	[15]
	Enzymatic responsive	Noninvasive	Enzyme specifically dependent	[28]
Gene expression of ultrasound contrast agents in situ	Gas vesicles	Imaging living organism	The production process of genetic encoding gas vesicles is complex	[30,31]

At present, researchers are searching the potential of NBs for contrast enhancement, loading drugs, and tumor-targeting. For example, Yin and coworkers fabricated a lipid NBs, constructing them with phospholipid shells to enclose the C_3F_8 gas core, for enhanced ultrasound imaging of tumors [18]. The constructed NBs, with uniform nano-size 436.8 ± 5.7 nm, were found to provide ultrasonically similar contrast enhancement to MBs in *in vitro* study. In *in vivo* tumor imaging study, researchers found that the MBs rarely showed contrast enhancement after 15 minutes, while the NBs offered contrast enhancement lasting more than 15 minutes, with this persistence perhaps due to the accumulation of NBs in the tumor site (Fig.1).

Although NBs can accumulate in tumor passively by the EPR effect, they appear to have poor tumor selectivity. To make NBs more selective, they can be modified with targeting molecules that improve their anti-tumor efficacy. Targeting molecules may include RGD (Arg-Gly-Asp) peptide and antibody. Yang et al. investigated the possibility of increasing the accumulation of affibody-conjugated NBs in breast cancer BT-474 site [19]. They observed a large number of affibody-conjugated NBs on the surface of BT-474 cells by inverted fluorescence microscopy, and the *in*

vitro contrast study proved that the affibody-conjugated NBs exhibited ultrasonic contrast enhancement that was similar to SonoVue MBs. In *in vivo* study, the affibody-conjugated NBs had better ultrasonic contrast enhancement than the SonoVue MBs and unconjugated NBs.

Scientists are interested in developing NBs not only for the purpose of ultrasound contrast imaging, but also for the purpose of loading drugs to improve tumor treatment. Zhang et al. constructed multifunctional targeted poly (lactic-co-glycolic acid) (PLGA) NBs (denoted mAb_{HLA-G}/MTX/PLGA) [20]. By filling them with perfluorocarbon gas, the multifunctional nanobubbles served as ultrasound contrast agent for tumor imaging and enhanced high intensity focused ultrasound (HIFU) ablation efficiency. Loaded with methotrexate, the multifunctional NBs can serve as nanocarriers for treating tumors. Tumor targeting ability can also be improved by conjugating the multifunctional NBs with monoclonal anti-HLA-G antibodies. Both *in vitro* and *in vivo* study have demonstrated that the multifunctional NBs have excellent ultrasound contrast ability, tumor-targeted accumulation, and drug-loading and non-invasive HIFU therapy potential (Fig. 2).

Figure 1 The NBs for ultrasound imaging. (A) Structure of the NBs; (B) Surface morphology of the NBs as visualized using scanning electron microscopy; (C) Representative *in vitro* ultrasonic images of NBs and MBs at concentration of 8.0×10^5 bubbles/ml, which shows that NBs exhibit better ultrasound contrast ability than MBs; (D) Representative subcutaneous tumor images before (blue dotted line) and after the injection of NBs compared with MBs at various time points (0, 1, and 15 minutes). The NBs exhibit longer contrast-enhanced time than MBs. (Reproduced with permission from [18])

Ultrasound Contrast Agents Generated *in Situ*

Clinically available ultrasound contrast gas-filled MBs have undesirable intrinsic drawbacks that include short half-life and low stability in blood [21]. Researchers are actively working to prolong the half-life and improve the stability of MBs for *in vivo* ultrasound imaging [22-25]. Researchers are also exploring stimuli-responsive formulations to generate ultrasound contrast agents *in situ* (pH, heat, enzyme, light). Min et al. reported a pH-controlled CO_2 -generating theranostic nanoparticle system consisting of doxorubicin-loaded CaCO_3 for ultrasound imaging and treating tumors [26]. The acidic tumor micro-environment can hydrolyze CaCO_3 to generate CO_2 bubbles, which could trigger the release of doxorubicin and act as ultrasound contrast agents for tumor imaging. *Ex vivo* research showed that the amount of CO_2 generated at pH = 6.8 is 1.59 times higher than the amount of CO_2 generated at pH = 7.4.

And the nanoparticle system produced significantly better ultrasound contrast images at pH = 6.8. An intratumoral injection method was used to demonstrate the potential of the nanoparticle system for ultrasound imaging of the tumor site. Thirty minutes after injection, enhancement in ultrasound images was observed, and it lasted up to 120 minutes. The researchers also confirmed that the echogenic signals were pH-decreasing dependent by measuring pH values of the subcutaneous tissues, the SCC-7 tumor xenograft model, and the liver (Fig.3).

In other research, Zhang and coworkers constructed folic acid-conjugated nano-lipid carriers for *in situ* ultrasound/computed tomography (CT) imaging and tumor photothermal therapy (PTT) [27]. The bubble-generating nano-lipid carriers were designed by loading gold nanorods, doxorubicin, and ammonium bicarbonate into folic acid-conjugated liposomes. Both *in vitro* and *in vivo* experiments revealed that folic acid-conjugated nano-lipid carriers can selectively target more tumor cells

than doxorubicin and liposomes. Excellent ultrasound contrast enhancement was also observed after near-infrared (NIR) laser irradiation.

Traditionally, gas cores of the ultrasound contrast agents were used in clinic solely for the purpose of ultrasound imaging. Nowadays, scientists are trying to explore additional functions of gas cores, such as tumor ablation. NBs can pass through tumor blood vessels via the EPR effect, but they may be too small to scatter clinical ultrasonic waves, leading to insufficient ultrasound contrast. Thus, the goal is to develop a nanostructure that can escape from blood vessels to tumor sites, transform intratumorally into MBs for ultrasound imaging, and then collapse to release gas cores to kill tumor cells. Liu et al. developed nanostructures-to-microstructures microbubble bombers for ultrasound imaging and tumor therapy [28]. The

nanoplateform consisted of anethole dithiolethione (ADT) (hydrogen sulfide (H_2S) pro-drug) and magnetic nanoliposome (AML), which could be guided by external magnetic field to the interstitial matrix. Spatiotemporally, ADT was catalyzed by cystathionine- β synthase (CBS) and cystathionine- γ -lyase (CSE) *in situ* to produce H_2S bubbles within the tumor microenvironment [29]. When the nanoplateforms were endocytosed by tumor cells, optical microscopy was used to map the real-time H_2S bubbles generated by ADT *in vitro*. After 6 h of incubation, the cells were disrupted by H_2S bubble bombers. In *in vivo* study, 4 h after intravenous injection, the researchers found that the echo intensity value of ultrasound with the anethole dithiolethione-loaded group reached almost 3.4-times higher than the blank group. In addition, the nanoplateform showed acceptable safety to normal organs and significantly inhibited tumor growth.

Figure 2 (A) Schematic illustration of the structure of $mAb_{HLA-G}/MTX/PLGA$ NBs and how $mAb_{HLA-G}/MTX/PLGA$ NBs act as a synergistic agent improving the treatment efficacy of HIFU ablation for tumor and residual tumor cells; (B) SEM image of $mAb_{HLA-G}/MTX/PLGA$ NBs with diameter of 477.6 ± 119.7 nm; (C) *In vitro* ultrasound images of the different samples: (1) air (control); (2) 200 mg/mL $mAb_{HLA-G}/MTX/PLGA$ NBs; (3) 200 mg/mL pure PLGA NBs; (4) 200 mg/mL MTX/PLGA NBs; (5) de-gassed and deionized water (control); (D) 0.5 mL of DiI-labeled $mAb_{HLA-G}/MTX/PLGA$ NBs are injected via the tail vein, then *in vivo* imaging of JEG-3 cells in the back of choriocarcinoma subcutaneous transplant sarcoma model by DiI-labeled $mAb_{HLA-G}/MTX/PLGA$ NBs at 3 h and 48 h time point after injection. The results display the target ability of $mAb_{HLA-G}/MTX/PLGA$ NBs; (E) The diagnostic sensor of HIFU shows the echoes of targeted tissue in the subcutaneous transplant tumor before and after HIFU ablation: (E1) HIFU+saline; (E2) HIFU+MTX/PLGA NBs; (E3) HIFU+ $mAb_{HLA-G}/MTX/PLGA$ NBs. The results indicate that the acoustic signal of tumor tissues in mAb_{HLA-G} -targeted NBs group was significantly higher than those in the saline control group and the non-targeted groups. (Reproduced with permission from [20])

Figure 3 (A) TEM image of DOX-CaCO₃-MNPs after mineralization for 12 h; (B) Optical micrographs of CO₂-generating profiles of sub-micron-size aggregates of DOX-CaCO₃-MNPs incubated in pH = 6.8 (B1) and pH = 7.4 (B2) for 30 and 90 minutes; *In vitro* ultrasound images of DOX-CaCO₃-MNPs incubated in pH = 6.8 (C1) and pH = 7.4 (C2) for 30 and 90 minutes; (D1) *In vivo* ultrasound imaging of the SCC-7 tumor mice by intratumoral injection of DOX-CaCO₃-MNPs for 30 and 120 minutes; (D2) *In vivo* ultrasound imaging of the SCC-7 tumor-free mice by subcutaneous injection of DOX-CaCO₃-MNPs for 30 and 120 minutes. (Reproduced with permission from [26])

Gene Expression of Ultrasound Contrast Agents *in Situ*

Ultrasound also provides a promising method for mapping living organisms within the body. Recently, Shapiro et al. found that gas vesicles genetically encoded by bacteria and archaea can be used as ultrasound contrast [30]. Those gas vesicles, with lengths of 100–600 nm and width of 45–250 nm, consist of protein shell and gas cores. The gas vesicles show good echogenic ability both *in vitro* and *in vivo*. However, the gas vesicles are produced by prokaryotic cells *in vitro*. And they must be injected through subcutaneous or intravenous to work as ultrasound contrast agents *in vivo*.

In their another work, Shapiro et al. imaged the gene expression of tumors in mammalian cells by using the gas vesicles as ultrasound contrast agents [31]. They first screened the gas vesicle-edited genes by synthesizing them from microbial species and then cotransfected them into human embryonic kidney (HEK) 293T cells

to identify whether detectable gas vesicles could be expressed or not. After identifying the genes of interest, they linked these genes with the viral cotranslational self-cleavage peptide P2A to construct a poly-cistronic mammalian operon. Then, the as-prepared polycistronic operons were incorporated into the cellular genome for consistent gas vesicles expression induced by doxycycline. The expressed gas vesicles with diameters of 64 ± 12 nm and lengths of 274 ± 212 nm showed characteristic ultrasound contrast ability *in vitro*. At last, the researchers established the mARG-HEK tumor xenografts in immuno-compromised mice in left flanks and mCherry-HEK control cells in the right flanks. They observed that gas vesicles were expressed by the as-prepared polycistronic operons and could be visualized with ultrasound in left flanks after systemic injection of inducer. On the contrary, nothing could be seen from the control side. Interestingly, the gas vesicles could constantly re-express themselves after acoustic collapse. The results demonstrated that the engineered genetic

construct enabled gene expression imaging *in vivo* and highlighted the potential for ultrasound to visualize intricate patterns of gene expression noninvasively to

monitor cellular location and function inside living organisms (Fig.4).

Figure 4 (A) Schematic of the transient cotransfection assay used to identify combinations of genes capable of producing gas vesicles in mammalian cells, and representative TEM image of purified gas vesicles expressed in HEK293T cells; (B1) Representative ultrasound images and contrast measurements in mARG-HEK cells as a function of time; (B2) Schematic and representative ultrasound images of gas vesicles (GV) expression from mARG-HEK cells in Matrigel; (B3) Re-expression of gas vesicles (GV) after acoustic collapse; (C1) Diagram of a mouse implanted with a subcutaneous tumor model, and the expected spatial pattern of vascularization and doxycycline-induced reporter gene expression; (C2) Representative ultrasound image of tumors containing mARG-HEK cells after 4 days of doxycycline administration. mARG-specific contrast shown in the hot color map is overlaid on an anatomical B-mode image showing the background anatomy; (C3) Representative ultrasound image of tumors containing mCherry-HEK cells after 4 days of doxycycline administration. (Reproduced with permission from [31])

Conclusion

In this article, we first reviewed the application of NBs acting as ultrasound contrast agents for tumor *in situ* imaging. Second, we summarized the stimuli-responsive formulations to generate ultrasound contrast agents *in situ*. Subsequently, we demonstrated the application of gene expression of ultrasound contrast agents *in situ* to monitor cellular location and function inside living organisms.

In conclusion, in recent decades we have witnessed significant progress in the development of ultrasound contrast agents. It is worthwhile to note that the use of ultrasound contrast agents for imaging tumor *in situ* has also achieved magnificent outcomes, especially when nanotechnology has been employed. Nevertheless, the following scientific questions currently restrict tumor *in situ* ultrasound imaging:

(1) The sizes of NBs applied for ultrasound imaging of tumor should be smaller than the size distribution of the tumor vasculature. Creation of small yet highly echogenic NBs still remains to be solved.

(2) There is a need for the ultrasound imaging NBs and nanostructures to be more tissue biocompatible and contain lower toxicity components.

(3) To date, gene expression of gas vessels for ultrasound *in situ* imaging of tumor is a proof-of-concept method. Efforts should be made to further optimize the genetic constructs so that gene expression of gas vessels for ultrasound *in situ* imaging are more sensitive and practical.

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Conflict of Interest

There is no conflict of interest and no disclosure associated with the manuscript.

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