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# Concurrent anti-vascular therapy and chemotherapy in solid tumors using drug-loaded acoustic nanodroplet vaporization

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ABSTRACT

Drug-loaded nanodroplets (NDs) can be converted into gas bubbles through ultrasound (US) stimulation, termed acoustic droplet vaporization (ADV), which provides a potential strategy to simultaneously induce vascular disruption and release drugs for combined physical anti-vascular therapy and chemotherapy. Doxorubicin-loaded NDs (DOX-NDs) with a mean size of 214 nm containing 2.48 mg DOX/mL were used in this study. High-speed images displayed bubble formation and cell debris, demonstrating the reduction in cell viability after ADV. Intravital imaging provided direct visualization of disrupted tumor vessels (vessel size <30 μm), the extravasation distance was 12 μm in the DOX-NDs group and increased over 100 μm in the DOX-NDs + US group. Solid tumor perfusion on US imaging was significantly reduced to 23% after DOX-NDs vaporization, but gradually recovered to 41%, especially at the tumor periphery after 24 h. Histological images of the DOX-NDs + US group revealed tissue necrosis, a large amount of drug extravasation, vascular disruption, and immune cell infiltration at the tumor center. Tumor sizes decreased 22%, 36%, and 68% for NDs + US, DOX-NDs, and DOX-NDs + US, respectively, to prolong the survival of tumor-bearing mice. Therefore, this study demonstrates that the combination of physical anti-vascular therapy and chemotherapy with DOX-NDs vaporization promotes uniform treatment to improve therapeutic efficacy.

**Statement of Significance**

Tumor vasculature plays an important role for tumor cell proliferation by transporting oxygen and nutrients. Previous studies combined anti-vascular therapy and drug release to inhibit tumor growth by ultrasound-stimulated microbubble destruction or acoustic droplet vaporization. Although the efficacy of combined therapy has been demonstrated; the relative spatial distribution of vascular disruption, drug delivery, and accompanied immune responses within solid tumors was not discussed clearly. Herein, our study used drug-loaded nanodroplets to combined physical anti-vascular and chemical therapy. The *in vitro* cytotoxicity, intravital imaging, and histological assessment were used to evaluate the temporal and spatial cooperation between physical and chemical effect. These results revealed some evidences for complementary action to explain the high efficacy of tumor inhibition by combined therapy.



## 1. Introduction

Tumor vasculature transports oxygen, nutrients, and drugs to the tumor tissue, which plays a crucial role in tumor therapy. In order to supply adequate oxygen and nutrients for rapid proliferation of tumor cells, angiogenic regulators such as vascular endothelial growth factor and angiopoietin are excessively secreted to induce tumor vascular growth by angiogenesis and vasculogenesis [1]. In addition, the imbalance in secretion of angiogenic regulators induces an abnormal morphology of tumor vessels with leaky, fragile, and tortuous structure, which affects drug delivery and therapeutic efficacy [2]. Due to the immature morphological characteristics of tumor vessels, nano-sized drug carriers can passively extravasate into tumor tissue through the gap junctions (380–780 nm) between vascular endothelial cells to improve intratumoral drug accumulation by the enhanced permeability and retention (EPR) effect [3–5]. However, the specific sizes of the gap junctions in different tumor models directly influence

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the efficiency of drug extravasation [6]. Interstitial fluid pressure is also increased because of the leaky tumor vessels and absence of lymphatic drainage to decrease the osmotic and hydrostatic pressure gradients (between intravascular and interstitial space), which impedes the delivery of oxygen, nutrients, and drugs especially at the tumor center [7,8]. The center of a tumor presents lower blood flow perfusion than the tumor periphery with absent drug extravasation since the low vascular density and small size of vessels are compressed by proliferating tumor cells and high interstitial fluid pressure [9–11]. Therefore, the heterogeneity of the vasculature within tumors contributes to uneven drug distribution, with drugs mainly extravasating into the peripheral region of tumors and increasing the probability of drug resistance after chemotherapy [12,13].

Anti-vascular therapy induces vascular disruption and blood flow shortage to suppress growth of the tumor by depriving it of oxygen and nutrients [14,15]. Vascular disrupting agents (VDAs) have been applied as chemical anti-vascular therapy to dissolve the cytoskeleton of immature vascular endothelial cells [16]. However, previous studies have reported that adverse side effects might be induced if VDAs affect some fragile or injured normal vessels since VDAs are intravenously injected throughout the systemic circulation [17]. Ultrasound-stimulated microbubble destruction (USMD) simultaneously provides US contrast imaging and locally disrupts tumor vessels by inertial cavitation, which is defined as a physical anti-vascular therapy [18–20]. The theranostic characteristic of USMD could prevent significant damage to the peritumor muscle or skin by precise external US guiding [21]. Although tissue necrosis is significantly shown at the tumor center to overcome the limitation of chemotherapy in both chemical and physical anti-vascular therapy, the inhibition of vascular disruption in the tumor periphery displays perfusion recovery and an intact vessel pattern might induce tumor recurrence after treatment [15,18,21,22]. The probable reasons for this limitation include: (1) the relative mature vessels covered with pericytes in the tumor periphery are difficult to disrupt [21]; (2) the adjacent normal vessels supply oxygen and nutrients to assist in the repair and proliferation of tumor peripheral cells [15].

Acoustic phase-change droplets have been applied to encapsulate chemotherapeutic drugs and then locally disrupt vessels and release drugs with bubble formation by US stimulation [5,23–26]. This phenomenon is called acoustic droplet vaporization (ADV) when droplets are converted into gaseous bubbles during US sonication [27,28]. Due to the smaller size distribution and more stable construction relative to microbubbles (MBs), drug-loaded nanodroplets (NDs) can passively extravasate into tumor tissue by the EPR effect to directly provide intertissue drug release, tumor cell damage, and tissue contrast imaging after ADV [5,23,24,29–35]. In the previous study, we demonstrated the feasibility of improving nanoparticles penetration via vascular disruption induced by ADV [5]. Moreover, the process of drug-loaded NDs vaporization might simultaneously produce physical anti-vascular therapy and chemotherapy to combine the spatial distribution between two treatment strategies. The continuous extravasation of drug-loaded NDs might complement the therapeutic efficacy even without vascular disruption in the tumor regions.

In this study, doxorubicin (DOX), one of the most commonly used chemotherapeutic drugs, was encapsulated into NDs (DOX-NDs) for chemotherapy. The physical anti-vascular effect induced by ADV was observed by real-time intravital and ultrasonic imaging, and then the alterations in drug extravasation and tumor perfusion were quantified. Histological assessment revealed the morphological changes in the tumors, which demonstrates the spatial distribution between vascular disruption and drug extravasation; moreover, the accompanied immune response was evaluated after various treatment strategies. Therefore, the aim of our study was to investigate the correlation between vascular disruption, drug extravasation, and therapeutic efficacy by DOX-NDs vaporization for combining physical anti-vascular therapy and chemotherapy (Fig. 1A).

## 2. Materials and methods

### 2.1. Characterization of DOX-NDs

#### 2.1.1. Preparation of DOX-NDs

The DOX-NDs structure consisted of a lipid shell, perfluoropentane core, and DOX, as shown in Fig. 1A. Preparation of the lipid film involved dissolving DPPC, DSPG, and DSPE-PEG5000 (see Supplementary Materials and Methods) with weight ratio of 10:4:4 in 1 mL chloroform and evaporating for 24 h at room temperature. The DOX solution (2–6 mg/mL) was mixed with the lipid film in 1 mL of degassed phosphate-buffered saline (DPBS) using a sonication bath (100 W; 2510, Branson, Danbury, CT, USA). To improve the conjugation of DOX and lipids, the hybrid solution was incubated in a water bath at 40 °C for 1 h and cooled to 4 °C. To fabricate DOX-NDs, 75 μL of perfluoropentane was added to the hybrid solution and homogenized for 20 min by high-intensity sonicator (200 W; UTR200, Dr. Hielscher Company, Teltow, Germany). To avoid bubble production due to the overheating for the duration of sonication, the hybrid solution in a 2 mL sealed glass vial was stored in an ice-bath for 5 min after sonication for 5 min, and this process was repeated four times. After that, DOX-NDs were isolated from free DOX, DOX-liposomes, and micro-sized DOX-NDs by differential centrifugation at 5000 rcf for 5 min and 1000 rcf for 1 min. The fabrication of NDs involved the same procedures as those for DOX-NDs, but without loaded DOX. Finally, the supernatant DOX-NDs and NDs emulsions were stored at 4 °C for stabilization.

#### 2.1.2. Size distribution, DOX payload, and stability of DOX-NDs

The size distributions of DOX-NDs and NDs were measured with a NanoSight LM10 device (Malvern Instruments, Worcestershire, UK) with a detection range of 10 nm to 2 μm. The cryotransmission electron microscope (Tecnai F20, Philips, San Francisco, CA, USA) was used to visualize the morphology of DOX-NDs. The drug payloads of DOX-NDs with various initial dosages of 2, 3, 4, 5, and 6 mg DOX were determined by a plate reader system (Tecan Infinite M200, Tecan Trading AG, Switzerland) at an absorbance wavelength of 490 nm. The original DOX-NDs emulsions before centrifugation with a full initial amount of DOX were used to obtain the standard calibration line. According to the results of the DOX payload in NDs, subsequent *in vitro* and *in vivo* experiments used DOX-NDs with initial dosages of 5 mg DOX.

The *in vitro* stability of DOX-NDs was established based on the leakage of DOX under the various time points at 37 °C. Samples were diluted 80 times with Dulbeco's modified Eagle's medium (DMEM) to simulate the *in vivo* concentration of DOX-NDs for tumor therapy. At time points of 0, 1, 2, 3, 4, and 24 h, samples were centrifuged at 5000 rcf for 5 min to separate free DOX and DOX-NDs. Free DOX leakage from various time points was quantified using the plate reader and normalized to the zero time point.

#### 2.1.3. Efficiency of drug release by DOX-NDs vaporization

To determine the efficiency of DOX release by ADV, DOX-NDs were diluted 80 times with DMEM and injected into a PE20 tube (BD Corp., Franklin Lakes, NJ, USA) with flow velocity of 20 mL/h. The tube was fixed and immersed in a water tank maintained at 37 °C, and the US sonication system with 2-MHz high-intensity focused ultrasound (HIFU) transducer was focused on the tube to generate acoustic pulses with various sonication parameters; 8,