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Phase-shift Nanodroplets as an emerging sonoresponsive nanomaterial for imaging and drug delivery applications

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Abstract:

Nanodroplets – the emerging phase-changing sonoresponsive materials – has drawn substantial attentions in biomedical applications for both tumour imaging and therapeutic purposes due to their unique response to ultrasound. As ultrasound is applied at different frequencies and powers, nanodroplets have shown to cavitate by the process of acoustic droplets vapourisation (ADV), causing the development of mechanical forces which promotes sonoporations through cellular membranes. This allows drugs to be imaged and delivered efficiently into deeper tissues where tumours are located. Recent reviews on nanodroplets are mostly focused on the mechanism of cavitation and their applications in biomedical fields. However, the chemistry of the nanodroplets components has not been discussed or reviewed yet. In this review, the commonly used materials and preparation methods of nanodroplets are summarized. More importantly, this review provides examples of variable chemistry components in nanodroplets which links them to their efficiency as ultrasound-multimodal imaging agents to image and monitor drug delivery. Finally, the drawbacks of current research, future development, and future direction of nanodroplets are discussed.

1. Introduction

During the last few years, ultrasound technologies have evolved in biomedical applications through imaging and therapeutic areas. These technologies are non-invasive, free of ionising radiation, and could operate by theranostic exposures. Ultrasound is used in both preclinical and clinical research for anatomical imaging, as well as exerting mechanical forces that lead to either the increase of temperature or cavitation that result in changes in cell membranes while allowing penetration into deep tissue regions. The recent combination of MRI and High intensity focused ultrasound (HIFU) have led to specific site cell ablation in tumour tissues from numerous studies[1]. However, reports of toxicities related to high temperatures exposures have hindered the clinical transition. HIFU can induce local hyperthermia that can help the drugs to reach tumour regions[2]. However, remarkably, amplified by ultrasound contrast agents (UCAs), also known as microbubbles and recently phase change nanodroplets (PCN), ultrasound-mediated drug delivery through cavitation has drawn substantial attention for clinical applications, where physiologic and structural tissue barriers significantly limit delivery of therapeutic agents to disease sites.

Ultrasound has been a valuable tool for diagnostic and therapeutic applications for more than 60 years[3]. As a mechanical wave, ultrasound will generate cycles of alternating acoustic pressure when propagates through body tissues and lead to the change of pressure *in situ* [4]. In order to meet clinical requirements, ultrasound contrast agents (UCAs) were developed to enhance ultrasound signals and diagonstics. UCAs possess great clinical values, as they can produce various distinctive imaging and drug delivery characteristics. UCAs can oscillate under the low amplitude of ultrasound and produce acoustic signal, whereas strong oscillation creates shear forces that may lead to poration of nearby cell membranes, *i.e.,* sonoporation. Apart from diagnostic, ultrasound also shows promising potential in therapeutic applications*, e.g.* sonodynamic treatment (SDT). SDT requires two essential components: ultrasound and sonosensitizer molecules. The mechanism of SDT will be introduced later in this review. Sonosensitizing nanoparticles could act as cavitation nuclei, including liposomes, micro-nanobubbles, nanodroplets, metal and metal oxide nanoparticles [5]. Metallic and inorganic nanoconstructs such as gold[6], titanium dioxide (TiO_2)[7], magnetite (Fe_3O_4)[8] and porous silicon nanoparticles[9] are believed to be promising sonosensitizers for future anticancer therapy. Their enormous surface area can be modified with functional groups for various therapeutic applications and their submicrometer size allows them to penetrate deeply into tissues and be taken up efficiently by cells[6]. SDT might be useful for nanodroplets applications. However, this review will focus into the development of phase changing nanodroplets. Other nanoparticles used in SDT can be further referred from a review produced by Canavese *et al*[5].

Traditional UCAs are gas-filled micro-particles with acoustic properties, *i.e.,* the ability to produce echogenicity from acoustic exposures, widely known as microbubbles[10]. The first generation of microbubble was Albunex®, an echo-enhancer with an air core and shell constructed with protein albumin. Highly echogenic contrast agents can majorly amplify the ultrasound signals even in low contrast medium, such as blood[11]. However, microbubbles have several disadvantages which restrict their application in the clinic. The size of microbubbles is around 1~10 μm, which confines their distribution in the vascular space, and limits *in vivo* circulation time to a few minutes as they are rapidly cleared *via* liver[12]. To overcome these problems, nanodroplets have be developed sice the last two decades[12]. When compared to microbubbles, nanodroplets have advantages which make them more desirable for clinical application[13].

Nanodroplets are composed of a stabilising shell and perfluorocarbon core. The core remains liquid at body temperature but vaporises into microbubbles under ultrasound. The PCNs can enhance the extravasation of therapeutic agents into a target tissue site under ultrasound-induced ADV and subsequent acoustic cavitation[14]. To further understand the mechanism of ADV, the vapour pressure equilibrium between the liquid and gas phases in the core region of PCNs are aspects to be observed and characterized. Vapourisation takes place when the vapour pressure in the liquid phase of volatile liquids such as perfluorocarbons elevates above the surrounding gas phase pressure. What ultrasound does is that it can reduce the pressure surrounding PCNs below the vapour pressure of the liquid perfluorocarbons encapsulated in the core region of PCNs. This results in liquid perfluorocarbon vapourisations, leading to the generation of microbubbles[15]. Nanodroplets are 10-fold smaller than microbubbles and able to pass through endothelial gaps and accumulate into the tumour site or lesions. The *in vivo* dwell time of nanodroplets is also prolonged for up to 4-5 hours, which offers the potential to better target cancers[7]. Besides, the selection of perfluorocarbon core offers nanodroplets

ultrasound-responsive tuneable properties and high precision[13,16,17].

The pioneering work of nanodroplets was initiated by Apfel through the design of perfluorocarbon droplets that can vaporise into microbubbles under ultrasound irradiations[18]. Nanodroplets started to gain more attention and the number of publications increases each year (Figure 1). PCNs can be combined with ultrasound technologies to produce local cavitations that can be used for contrast enhancement, tumour ablation, antivascular therapy and release of therapeutic agents loaded in nanodroplets[19]. Different applications of PCNs are achieved by adjusting ultrasound parameters[20]. Stable or inertial cavitation can be achieved depending on the intensity, amplitude and frequency of the ultrasound wave[3]. At low frequencies and low intensities, stable cavitations could produce strong echoes for imaging, but PCNs need different ultrasound frequency for vaporisation and imaging[21]. Microstreaming produced during stable cavitation could also temporarily enhance permeability of physiological barriers such as BBB (Blood brain barrier) and endothelium[22]. Higher amplitude and lower frequency can be used for therapy application encompass sonoporation and sonodynamic therapy[22,23]. At high intensity, including HIFU (High intensity focused ultrasound), PCNs can also be used for histotripsy and tumour ablation[21]. The choice of ultrasound frequencies and intensities settings used in previous studies for both diagnose and therapy are summarised in Loskutova's review[21].

Although there are no clinically approved nanodroplets in the market, there are several clinically approved microbubbles used in a wide variety of biomedical applications[26]. PCNs clinical prospect looks promising as PCNs have the similar ability of contrast enhancement as microbubbles do but are superior to microbubbles. However, the drawbacks of nanodroplets are that they cannot be imaged before being acoustically activated by ultrasound[27]. To solve this problem, multimodal imaging nanodroplets can be developed by incorporating different imaging probes, for example, fluorescence imagining, magnetic resonance imaging (MRI) and positron emission tomography (PET)[13]. In summary, these formulations are gaining attention as the amount of research increases which will push clinical and commercial translation of this novel transformable nanoparticle.

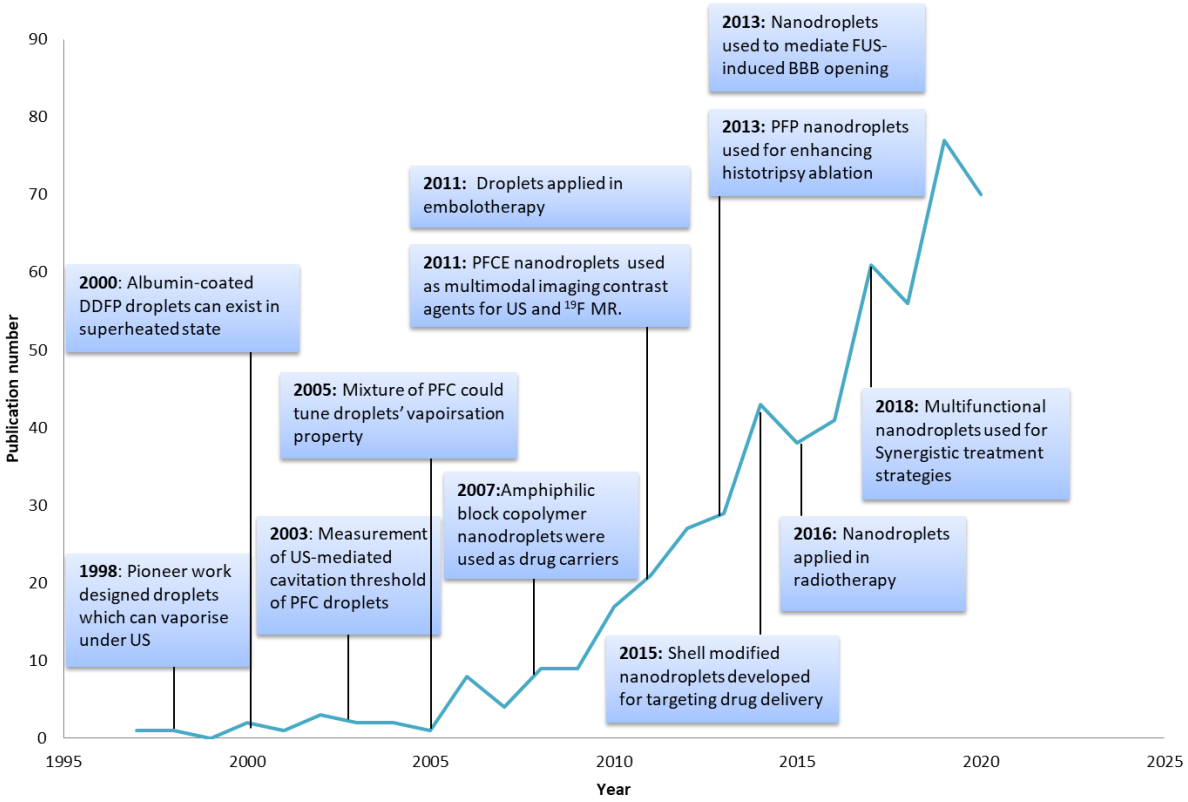


Figure 1. Milestones in the development history of nanodroplets [18,28–36] and number of publications related per year (Search from the web of science Clarivate Analytics using keywords: perfluorocarbon nanodroplets, perfluorocarbon droplets, phase change nanodroplets and phase-change contrast agents).

This review aims to present the development of PCN for imaging and therapy purposes, focusing on chemical compositions and characteristics. Although as an important topic, the chemistry of these nanodroplets' components has not been discussed or reviewed yet. Therefore, we provided examples of various chemical characteristics of nanodroplets while linking them with their efficiency as ultrasound/multimodal imaging agents as well as cavitation mediators. Cavitation can promote drug delivery through sonoporation (stable cavitation) or through jetting (inertial cavitation). The nanodroplets composition could be a strong attribute to this effect.

2. Chemical composition of nanodroplets:

Nanodroplets are composed of two parts: encapsulation shell and core filled with liquid perfluorocarbon (PFC). The formulation is critical as it will influence the properties of the nanodroplets. The shell must be designed to maintain droplets' shape and original diameter after intravenous injections, as well as being able to expand into bubble upon ADV[10]. The actual surface tension of nanodroplets is largely dependent on the state of the shell. The low-boiling point perfluorocarbons can remain liquid as a superheated state at physiological temperatures

due to Laplace pressure provided by the shell[3]. Laplace pressure is a force generated by the surface tension at the interface of the shell and PFC core. Therefore, nanodroplets remain stable *in vivo* until sufficient acoustic energy is induced to promote vaporisation through ADV[13] (Figure 2).

Different type of perfluorocarbons could also influence the vaporisation property and thermal stability of droplets[37]. Optimising the perfluorocarbon core could develop more precisely tuneable droplets with maximum performance in each application[29]. The property of the shell and core which influence the nanodroplets characteristics will be discussed later. Apart from the shell and core, drug encapsulation or/and droplets decoration (*e.g.,* imaging probe, targeting ligand) might be located inside the liquid perfluorocarbon core, embedded in the shell or attached to the shell surface depending on their molecular weight and lipophilicity. These substances could also influence the acoustic property of nanodroplets.

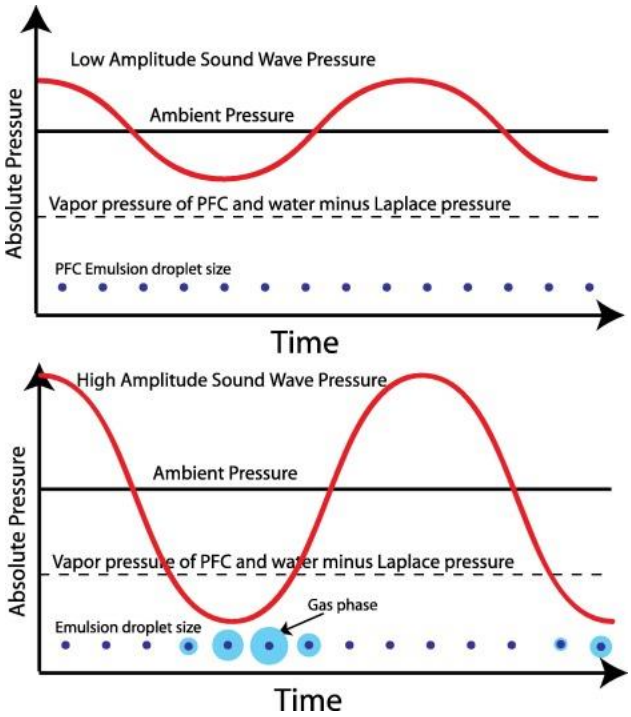


Figure 2. Schematic of an ultrasonic acoustic wave in a perfluorocarbon emulsion. The graphs show emulsion droplets (not to scale) that only vaporise into the gas phase when acoustic pressure is below Laplace pressure. (Copyright from Pitt *et al.*[38])

2.1 Phase change nanodroplet shell composition

The choice of nanodroplets' shell is based on the criterion to find a balance between mechanical resistance to provide enough Laplace pressure and compliance to enable large deformation during ADV (Figure 2)[13]. Lacour *et al.* built a mathematical model to study the influence of hyperelastic shells on droplets acoustic propert[39]. They concluded that the most favourable droplets shell elastic properties correspond to soft materials, with a low value of shear modulus (G) and high nonlinearity (β). G is rheological property related to a material's response to shear stress; nonlinearity is significant as it corresponds to the large deformation of the shell[39]. Currently, commonly used shell materials include surfactants, albumin, lipids, and synthetic polymers[13] (Table 1). Among all the studies, lipids and polymers are the most popular material to

form the droplets shell. Lipids and fluorinated surfactants are considered as soft-shell materials whereas polymers and proteins are considered as hard-shell materials[40]. The advantages and disadvantages of each material are listed in Table 2.

Albumin is a pioneer shell used in both microbubble and nanodroplets formulation. It has been used extensively in fabricating droplets due to its ability to stabilise the surface of the droplets[28]. This material could thicken at the droplet state and thin to form ideal bubble shells upon vaporisation[41]. Most albumin-coated nanodroplets were prepared using sonication[26]. Among these studies, the albumin used is the commonly bovine serum albumin (BSA)[42,43] and denatured BSA[25]. The albumin shell can also be modified. For example, Chang *et al.* loaded sonosensitizer IR780 iodine through hydrophobic interactions with albumin[25]; the surface of the shell could also be functionalised for molecular targeting of specific biological targets[44].

Surfactants have been explored to form stable microbubbles, but for nanodroplets, fluorosurfactants appear to be more favourable[13,40]. Although perfluorocarbon and alkanes emulsifiers are both hydrophobic, surfactants still exhibit a very low affinity for perfluorocarbons[45]. Therefore, droplets fabricated with normal surfactants are not very stable[46]. To solve this stability issue, the approach is to replace the lipophilic hydrocarbon part of the emulsifier with a fluoro-philic perfluorocarbon part to make fluorinated surfactant[45]. For example, a commercially available fluorosurfactant- Zonyl® (surface tension at 20℃ 16-23 mN/m) has been used in a few studies[47,48]. Low surface tension fluorosurfactant could provide appropriate stabilisation for droplets against coalescence phenomena[40].

Lipids are frequently used in biodegradable and biocompatible nanoparticle formulations. Lipid-based nanoparticles such as liposomes are one of the most useful carriers used for biomedical imaging and drug delivery because different mixtures can be easily formulated and modified in lipid-coated particles[26,49]. Lipids are successfully adopted in the fabrication of ultrasound-responsive droplets due to their elasticity[29,50]. One major advantage of the lipid shell is it has good mechanical flexibility which contributes to its ability to expand and collapse repeatedly[51]. Such properties ensure that the PCN is stabilised against dissolution and coalescence[13]. Most of the studies considered lipids to form a monolayer shell on nanodroplets. Chattaraj *et al*. used Transmission Electron Microscopy (TEM) to examine the shell property of nanodroplets, and the image shows a uniform texture of lipid monolayer (Figure 3a)[52]. However, Mountford *et al*. showed microscopy evidence that the nanodroplets prepared using microbubble condensation may have one or more bilayer lamella structures (Figure 3b)[53]. They propose that the lipid monolayer could fold into bilayer folds upon compression or self-healing, and bilayer folds can then deform into the monolayer upon expansion (Figure 3c)[54]. The state of lipids on nanodroplets still needs more studies to explore.

Lipids composition could play a tuning role in the acoustic property of nanodroplets. Although numerous studies used lipid formulated nanodroplets, only a few studies have investigated the effect of lipid shell composition on nanodroplets' size distribution and acoustic property [52,55]. Mountford *et al*. used a series of lipids with acyl chain lengths ranging from C14 to C20 to form nanodroplets. Results showed that energy needed to reach vaporisation threshold increases linearly with the acyl chain length of lipids, which indicated that lipid intermolecular cohesion force plays an important role for slowing the vaporisation process of nanodroplets [54]. A recent

study by Chattaraj *el al*. suggested nanodroplets with a mixture of 40% DOPC, 40% DPPC with 20% Cholesterol as shell have ten times higher ultrasound contrast to DPPC-only formulation[52]. Besides, lipid-shelled nanodroplets are commonly coated with hydrophilic block polymers, usually poly (ethylene oxide) (PEG) chains. PEGylation is a typical approach to reduce non-specific cellular uptake and prolong blood half-life for nanocarriers[56]. For nanodroplets, PEGylation can not only influence biological behaviour, but also size and acoustic response. Increasing the molar percentage of PEGylated lipid reduces the average size and size variation of nanodroplets, and facilitates ultrasound imaging contrast in a murine model of breast cancer[55]. The influence of PEG chain length was also addressed by Melich *et al*. They prepared 90% DPPC nanodroplets with 10% DSPE-PEG5000 or DSPE-PEG2000 using the microfluidics method. They concluded that there was no significant impact on the nanodroplet formulation quality under their operating parameters [40].

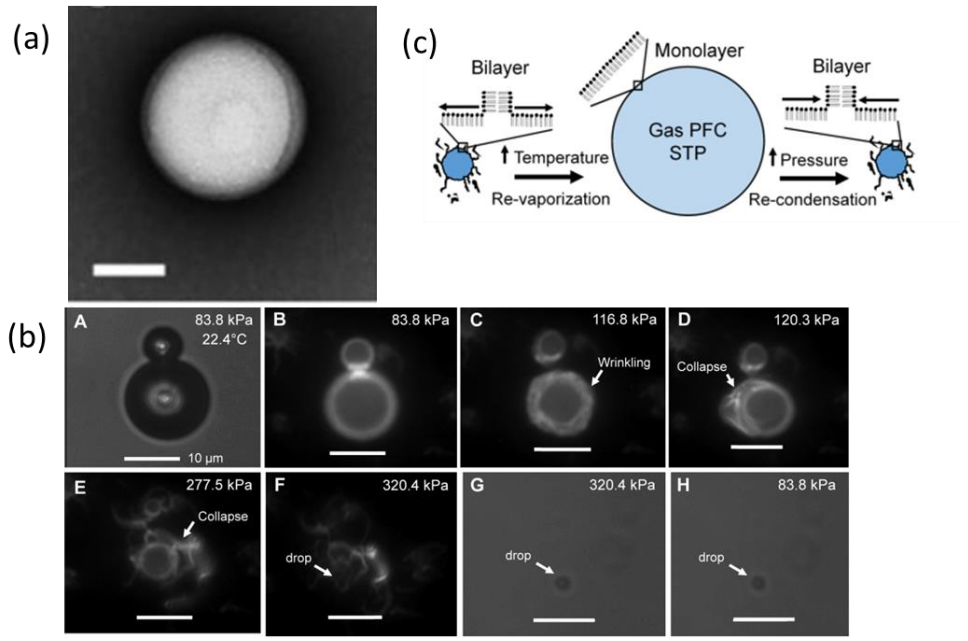


Figure 3. (a) TEM images of 4 v/v % PFH droplets, scale bar=100nm. (shell: DPPC/PEG 1.3 mM/40 Mm in TBS) (Copyright from Chattaraj *et al*.[52])

(b) Microscopy images of DiI, DSPC: DSPE-PEG2000 (9:1)-coated microbubbles undergoing condensation, the image shows sinuous lipid collapse structures (Copyright from Mountford *et al*.[53])

(c) Schematic of microbubble-condensed nanodroplets with the lipid shell during vaporisation and condensation (Copyright from Mountford *et al*.[54])

Amphiphilic block copolymers as shell material for nanodroplets could facilitate the loading of lipophilic drugs with high loading efficiency[13], combining tumour-targeting, enhancing intracellular drug delivery as well as enhancing the ultrasound contrast properties[57]. Different types of polymers are used in the formulation of nanodroplets, including PLGA (Poly (lactic-co-glycolic acid), PCL (Polycaprolactone), chitosan, PLA (Poly (lactic acid) and PDA (polydopamine)[58,59]. PEG is mostly used in combination with other polymers for size reduction and targeted drug delivery. Gao *et al*. had shown that count rate value (corresponding to

concentration) of nanodroplets without PEG loads decreases significantly in solution containing serum due to protein aggregation with nanodroplets. However, nanodroplets that are modified with PEG could remain stable in same condition[60]. PLGA is a widely used biocompatible polymeric carrier with extended release property for loaded drug [58]. Cao et al. has loaded doxorubicin (DOX) into PLGA-coated nanodroplets and the drug-releasing profile shows that drug was continuously released from nanodroplets without LIFU (low-intensity focused ultrasound) but burst drug release was observed after LIFU exposure[61]. Chitosan is a cationic linear polysaccharide extracted from marine animals. Its physicochemical properties such as nontoxicity, hydrophilicity, biocompatibility, biodegradability and high resistance to heat make it suitable for biomedical application [58]. Baghbani *et al.* has prepared curcumin-loaded chitosan-stabilised nanodroplets for curcumin smart delivery and this formulation shows good curcumin entrapment efficiency (77.8%) due to high affinity between chitosan and curcumin[62].

Most polymer-shelled nanodroplets load drugs in the polymer shell and the drug loading capacity is dependent on the concentration of polymer[58]. Perfluorocarbon/copolymer ratio is also important for nanodroplets formulation. As, when perfluorocarbon /copolymer is low, perfluorocarbon dissolved in the core of the micelle and no nanodroplets exist; with increasing ratio of perfluorocarbon to polymer more nanodroplets are formed leading to droplet stabilisation and micelles disappearing[63]. For microbubbles, decreasing the initial thickness of copolymer shell could facilitate encapsulated drug transferring from bubble to the neighbouring cells [64]. The influence of polymer shell thickness of nanodroplets has not been investigated but we could hypothesis this will influence the drug releasing profile of nanodroplets. And it was hypothesised that gaps between polymer molecules become larger on nanodroplets shell after they changing phase and this could facilitate drug release from polymer shell[61].

Several studies have reported that polymer-coated nanodroplets have higher stability and vaporisation threshold compared with lipid-coated nanodroplets. Melich *et al*. prepared nanodroplets with different shell materials, and polymer shell (PLGA) showed better stability than lipid shell (DPPC:DSPE-PEG^{2000}) nanodroplets. Results indicated PLGA coated nanodroplet exhibit good storage stability in the fridge (5℃) over 1 month without any impact on size and polydispersity, whereas lipid shell nanodroplets lack stability after storage probably due to vesicles aggregation [40]. The study by Cao *et al*. indicated that polymer-based (PLGA shell) nanodroplets need higher ultrasound energy to be activated into microbubble compared with lipid-based (DPPC, DPPG, DPPE, and cholesterol) nanodroplets formulation due to stiffness of polymer material[61].

Table 1. Commonly used shell material in nanodroplets

Shell type	Material	References
Protein	Albumin	Zhang *et al*. [65] Kripfgans *et al*. [28] Giesecke *et al*. [41]
Polymer	PLGA: Poly (lactic-co-glycolic acid)	Pisani *et al*. [66] Astafyeva *et al*. [67]
	PCL: Polycaprolactone	Rapoport *et al*. [68] Ji *et al*. [69]
	Chitosan	Magnetto *et al*. [70]

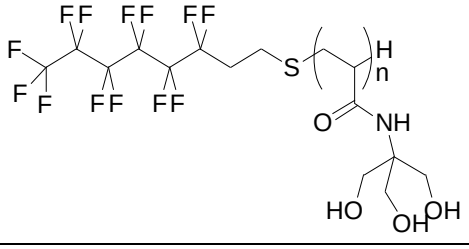
	PLA: Poly (lactic acid)	Wei *et al*. [71]
	PDA: Polydopamine	Mannaris *et al*. [59]
Lipids	DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine)	Zhang *et al*. [65]
	DPPA (1,2-dipalmitoyl-*sn*-glycero-3-phosphate monosodium salt)	Zhang *et al*. [65]
	DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine)	Sheeran *et al*. [72] Yarmoska *et al*. [55]
	DSPE-PEG^{2000} (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy(polyethyleneglycol)-2000	Sheeran *et al*. [72] Yarmoska *et al*. [55]
	Cholesterol	Schad *et al*. [50]
	Lecithin	Schad *et al*. [50]
Surfactant	Zonyl® (Linear Formula $(C_2H_4O)_x(CF_2)_yC_2H_5FO$)	Williams *et al*. [48]
	FTAC (a series of fluorinated surfactants F_iTAC_n, e.g., F_6TAC_n) 	Astafyeva *et al*. [45]

Table 2. Advantages and disadvantages of different shell material [51,58]

Material	Advantages	Disadvantages
Albumin	Easy preparation method.	More rigid than other materials.
Surfactants	Fluoro-surfactants could provide appropriate stabilisation for droplets against coalescence phenomena.	Alkane surfactants show a low affinity for perfluorocarbons. May required considerable peak negative pressures to induce vaporisation
Lipids	Different mixtures can be easily formulated and modified in lipid shells.	A hydrophobic mismatch between the lipids may inevitably lead to lateral phase separation
Polymers	The polymer could facilitate the loading of drugs with high loading efficiency in the shell. Polymer nanodroplets possess a high surface to volume ratio so the rate of adsorption is enhanced, and the kinetics of the reaction is accelerated. Polymer shell could act as an effective tool for targeting infections and wounds.	Upon vaporisation, bubbles formed are highly unstable due to the presence of gas molecules within the polymer shell. Estimation of long-term shelf-life is difficult. Defects in the outer shell may result in the leaking of perfluorocarbon before reaching the target site. Acoustic threshold is higher than lipids or surfactants formulated nanodroplets.

2.2 The Core composition

The liquid core chosen for nanodroplets is preferably hydrophobic, bioinert and able to circulate

safely in the body before being vaporised into a gas, i.e., have an appropriate boiling point. Thus, unlike microbubbles which are normally formed using air, nitrogen or sulphur hexafluoride as the core, nanodroplets use perfluorocarbon to meet these criterias[3]. The perfluorocarbon family differs in chain length, giving rise to unique boiling points (Table 3)[13]. After injection, perfluorocarbon released in blood fluid would be expected to be eliminated through the lungs. Perfluorocarbons have low partition coefficients in blood, so perfluorocarbons binding to blood proteins would be expected to be minimal[68]. Physicochemical properties of perfluorocarbons make them an attractive candidate for ADV, and Laplace pressure provided by shell could allow them to remain stable within nanodroplets at body temperature until vaporised by sufficient acoustic energy[39]. PFC concentration could also influence the size of nanodroplets. Ferri et al. indicated increasing the volumetric concentration of PFP from 5% to 15% v/v will lead to double size larger nanodroplets[73].

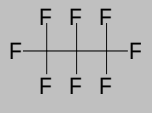
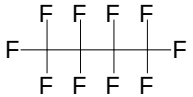
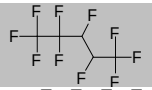
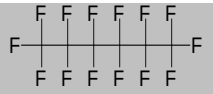
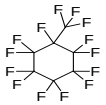
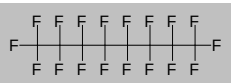
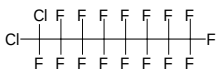
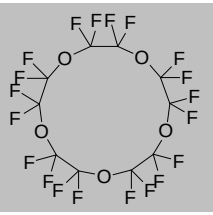
The dodecafluoropentane DDFP (C_5F_{12}) was the first-ever studied droplet core from the early 2000s[28]. Even though the boiling point of DDFP (C_5F_{12}) is 29℃, which is lower than body temperature, the superheated DDFP droplets could remain stable and circulate freely *in vivo* until activated by ultrasound due to the existence of Laplace pressure[31]. However, studies also showed that decreasing the size could increase vaporisation thresholds of perfluorocarbon droplets[74], so nanodroplets with highly volatile perfluorocarbon, e.g., decafluorobutane DFB (C_4F_{10}) and octafluoropropane OFP (C_3F_8), were developed. This allows droplets with a size below 200nm to be formed. These small size PCN can passively accumulate in the targeted tumour tissue[75]. In addition, nanodroplets with volatile perfluorocarbons play an important role in the diagnostic applications (Ultrasound imaging) because a low boiling point core induces the droplets to have an earlier vaporisation[21]. Other perfluorocarbons also offer different advantages. For example, the boiling temperature of perfluorocarbon ether (PFCE) ($C_{10}F_{20}O_5$) is 146℃. Compared with DDFP, PFCE have a higher boiling point, therefore, has greater storage ability than DDFP. At the same time, activating phase transition in PFCE nanodroplets requires only slightly higher acoustic energy than those for DDFP confirmed in experiment[16]. PFCE is also a fluorine-19 MR (magnetic resonance) imaging contrast agent with high sensitivity which could be used in image tracking[76] because PFCE contains 20 equivalent ^{19}F nuclei that generate a single resonance peak in ^{19}F MRI[16].

Early studies focused on developing nanodroplets used single perfluorocarbons as the core. Kawabata *et al*. first used a mixture of DDFP and DFP as the droplet core to reduce the vaporisation threshold[29]. A perfluorocarbon mixing is a valuable tool to manipulate the thermal stability and the vaporisation threshold of droplets simultaneously to maximise the performance for specific applications[37]. Melich et al. then discovered that the ADV threshold of nanodroplets elevated with the increase of PFH percentage in a liquid core made up of PFH and PFP mixture [40]. Perfluorocarbon mixing is not limited to perfluorocarbons that exist in the same state (gas or liquid), but on the feasibility to mix across different states to produce tuneable droplets which are "flexible" for clinical applications[29].

Apart from perfluorocarbons, adding other particles into nanodroplets could also influence vaporisation properties. For example, quantum dots are used as cavitation seeds in nanodroplets by mixing them into the perfluorocarbon solution[77]. Quantum dots could be visualised in

fluorescence imaging and lower the vaporisation threshold[78]. Loading iron oxide nanoparticles within nanodroplets' inner surface of the shell could also reduce the vaporisation threshold[79].
Currently, most of the studies have only fabricated phase-shift nanodroplets with a core composed of a single kind of perfluorocarbon and the number of studies using the mixture of perfluorocarbon as the core is limited. However, the perfluorocarbon mixture tends to offer more ideal properties to phase-shift nanodroplets such as a reduced vaporisation threshold and changeable composition for clinical use. Hence, in the future, perfluorocarbon mixture is supposed to be developed as an essential part of phase-shift nanodroplets.

There are numerous studies indicated that nanodroplets composition exhibit effects on their behaviour, but most of studies focused on evaluating nanodroplets properties in aqueous buffer or water settings. It is essential to highlight that nanodroplets possess different characteristics either the *in vitro* or *in vivo* ambiences. To date, only several studies have evaluated nanodroplets characteristics in serum/blood-mimicking fluid. Serum could slightly influence the stability of nanodroplets. Meng *et al*. has incubated polymer shell nanodroplets in buffer solution containing 10% Fetal Bovine Serum (FBS) at 37℃ where particle size only increased slightly within 24h [80]. Other studies showed that nanodroplets size remains stable in serum, which indicates that nanodroplets could possess good stability in physiological condition, even for nanodroplets with low-boiling point PFC such as PFP[59–61]. Besides, fluid viscosity and environmental parameters also affect nanodroplets' acoustic property. Rojas *et al*. has compared the vaporisation threshold of nanodroplets in phosphate-buffered saline (PBS, viscosity = 1 cP) and a blood-mimicking fluid (viscosity = 5.4 cP). Result indicated that increasing viscosity have a significant effect on nanodroplets' vaporisation threshold in 30-mm tube[81]. They also suggested that the increase of vaporisation threshold in *in vivo* rather than *in vitro* experiments was due to boundary constraints and hydrostatic pressure derived from the tissue and capillary walls, as well as the high blood fluid viscosity [81]. Helfield *et al*. has shown that fluid viscosity may influence the fragmentation and acoustic emission of lipid-shell microbubbles although not a similar research has been conducted on nanodroplets[82]. Unfortunately, with limited numbers of studies relating to nanodroplets characterisations in biological fluids, existing studies have suggested that the *in vitro* studies should evaluate nanodroplets in different meida before transitioning into *in vivo* studies.
Table 3. Summary of perfluorocarbons used in various phase change nanodroplets.

Compound name	Molecular Formula*	IUPAC Name*	Chemical Structure*	Molecular Weight (g/mol)*	Boiling Point (℃)*	References
Octafluoropropane (OFP)	C_3F_8	1,1,1,2,2,3,3,3-octafluoropropane		188.02	-39	Sheeran *et al.*[51] Doinikov *et al.* [83]
Perflubutane (PFB)/Decafluorobutane (DFB)	C_4F_{10}	1,1,1,2,2,3,3,4,4,4-decafluorobutane		238.03	-36.7	Chen *et al.* [33] Matsunaga *et al.*[75] Doinikov *et al.* [83]
2H,3H-Decafluoropentane (DFP)	$C_5H_2F_{10}$	1,1,1,2,2,3,4,5,5,5-decafluoropentane		252.05	55	Kawabata *et al.* [29]
Perfluoropentane (PFP)/Dodecafluoropentane (DDFP)	C_5F_{12}	1,1,1,2,2,3,3,4,4,5,5,5-dodecafluoropentane		288.03	29	Vlaisavljevich *et al.* [84] Li *et al.*[85]; Miles *et al.*[86]; Kripfgans *et al.* [28] Giesecke *et al.*[41]
Perfluorohexane (PFH)	C_6F_{14}	1,1,1,2,2,3,3,4,4,5,5,6,6,6-tetradecafluorohexane		338.04	58	Vlaisavljevich *et al.* [84] Strohm *et al.*[87]
Perfluoromethylcyclohexane (PFM)	C_7F_{14}	1,1,2,2,3,3,4,4,5,5,6-undecafluoro-6-(trifluoromethyl)cyclohexane		350.05	76	Giesecke *et al.* [41]
Perfluorooctane (PFO)	C_8F_{18}	1,1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-octadecafluorooctane		438.06	105.9	Fabiilli *et al.* [43]
Perfluorodichlorooctane (PFD)	$C_8Cl_2F_{16}$	1,1-dichloro-1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-hexadecafluorooctane		470.97	176	Lanza *et al.* [88]
Perfluoro-15-crown-5-ether (perfluorocarbonE)	$C_{10}F_{20}O_5$	2,2,3,3,5,5,6,6,8,8,9,9,11,11,12,12,14,14,15,15-icosafluoro-1,4,7,10,13-pentaoxacyclopentadecane		580.07	146	Rapoport *et al.*[57] Rapoport *et al.*[76]

3. Phase change nanodroplets preparation techniques:

A variety of techniques has been developed to manufacture nanodroplets, including sonication, homogenisation, extrusion, microfluidics, and microbubble condensation. Different preparation methods could influence the property of nanodroplets, especially their size and size uniformity. As mentioned earlier, nanodroplets have smaller size than conventional microbubbles, which allows them have advantages like prolonged *in vivo* circulation, deep penetration into the tissues via the extravascular space. Their nano-scale size also allows them to passively accumulate in tumour tissue due to EPR (Enhanced Permeability and Retention) effect. Nanodroplets size also influences their acoustic properties. For micron-sized droplets, the acoustic activation threshold depends on the initial diameter (Figure 4)[74]. It was reported vaporisation thresholds are reduced with increasing droplets size [89]. Although data for nanosized droplets do not exist yet, it appears that the size is a critical parameter for the activation pressure threshold. Size distribution also influences the acoustic performance of nanodroplets [51]. Polydisperse droplets will not respond the same under ultrasound energy [90]. To improve the uniformity of activation, the most important thing is to use techniques that can create nanodroplets with low polydispersity[37]. It is important to choose an appropriate method for preparing nanodroplets according to their desired properties for future application, either for imaging or drug delivery.

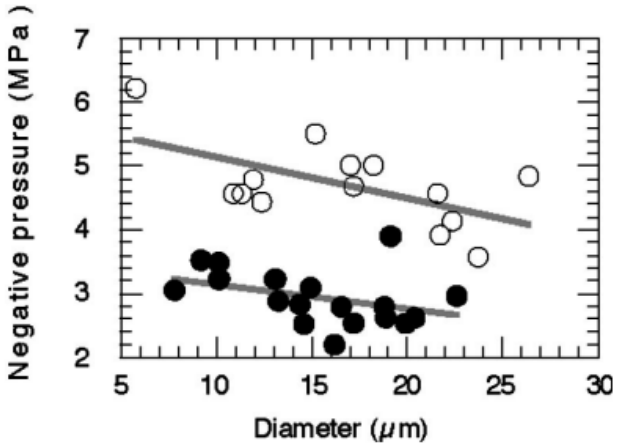


Figure 4 Relationship between pressure threshold for ADV and diameter of. Decreasing droplets diameter will increase pressures for vaporisation (Copyright from Kripfgans *et al*.[74]).

3.1 Method of Agitation/Homogenisation

Some of the earliest reports related to PCN mention production by agitation. The methods vary from simply shaking by hand to commercial homogenisation systems[18,91], with protocols varying significantly. In general, these methods produce droplets first by mixing the shell components with an aqueous solution and then adding perfluorocarbon and homogenising into emulsions. Since the entire droplet solution is within a single container, agitation techniques could avoid material losses. However, these methods often produce droplets with wide size distribution and low reproducibility[13].

3.2 Preparation by Sonication

Sonication is a common and simple method to produce nanoparticles including nanodroplets. In previous studies, both sonication bath[63] and probe sonicator[48,78,92] were used. In this method, the component of droplets (shell material and perfluorocarbon) is emulsified by ultrasound in a continuous aqueous phase/buffer solution (Figure 5). Usually, the vial must be kept in an ice bath during sonication to prevent excess heating[51]. The parameters of sonication also influence the property of nanodroplets. Ferri et al. have studied the influence of power and duration of sonication on nanodroplets. The result shows that longer sonication time and sonication intensity leads to lower size and size dispersity[73].

The main advantage of this method is the ease of use and low cost. Besides, this method could avoid material loss compared with some flow techniques because the emulsion system is closed. This technique is also applicable to incorporate other agents (e.g., solid nanoparticles) into nanodroplets. However, the disadvantage of this method is relatively low nanodroplet uniformity. Gao *et al.* used a sonication bath, and the final product is a mix of nanodroplets with micelles[63]. Sheeran *et al.* also showed an example of the increased polydisperse size distribution of probe-sonicated nanodroplets[51]. In addition, erosion of the probe tip also has the potential to contaminate nanodroplets solution with metals during preparation. That's why it is very important to inspect the tip to avoid any defect on its surface[51].

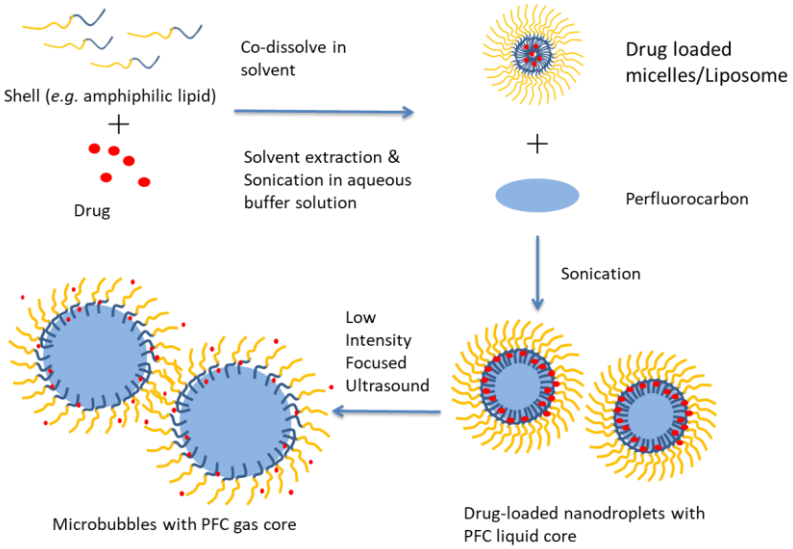


Figure 5. Schematic representation of preparation of PCN and their phase change with focused ultrasound.

3.3 The method of Extrusion

Extrusion was used for the preparation of liposomes and may now also be adopted to fabricate nanodroplets[87,90]. Extrusion is commonly used in combination with other approaches (e.g., sonication, condensation) inside of using alone. Sheeran *et al*. fabricated nanodroplets using a 1μm porous membrane filter. The shell material was first dried in the vial to form a thin film and hydrated with a buffer solution. DFB (C_4F_{10}) was added to lipid suspension mixed with glycerol in a cold room. Then the solution was extruded at -5℃ to avoid freezing of the aqueous solution and maintain the liquid state of the DFB[90].

Compared with sonication, extrusion has higher monodispersity. However, extrusion did not appear to be capable of manufacturing submicron droplets regardless of the membrane size used, maybe due to the low perfluorocarbon surface tension and the very high viscosity of the phospholipid solution at -5℃[17]. Besides, extrusion is more complex than sonication. For phospholipids formulated nanodroplets, extrusion may preferentially form liposomes instead of droplets[51].

3.4 The Microbubble condensation method

Preparing nanodroplets by microbubbles condensation first appeared in works of literature due to the challenge of producing liquid nanoscale droplets from highly volatile perfluorocarbon (e.g., DFB, OFP) which exist as a gas at room temperature[89]. In the method, microbubbles with volatile perfluorocarbon core ideal for ultrasound interaction are generated and then condensing the gaseous precursors into liquid state droplets by cooled and pressurised[93]. Once the liquid core is formed, the reduction in size results in a submicron distribution of droplets, and the Laplace pressure could stabilise the droplets against re-expansion at room temperature until the nanodroplets are activated by ultrasound or increased temperature[37]. Microbubble condensation will generate liquid core nanodroplets instead of gas core nanobubbles.

This method has several advantages[37,51]. First, it offers a method to generate a high concentration of nanodroplets of volatile compounds simply[51]. Second, microbubbles condensation could manipulate functionalised droplets at microscale before microbubbles were condensed. It is relatively simple to incorporate particles, dyes and targeting ligands into the droplet shell[72,94]. Third, since this method begins from a population of microbubbles ideal for imaging, if condensation and vaporisation proceed optimally, after vaporisation, nanodroplets could become bubbles with ideal size[37]. Finally, this technique provides an opportunity to produce nanodroplets directly from well-developed microbubbles. Research related to microbubbles is earlier than nanodroplets. There are many publications related to novel microbubbles applied in molecular imaging and drug/gene delivery, and these modifications can be applied directly into droplets with microbubble condensation[51].

However, there are some drawbacks. First, as most microbubbles tend to be polydisperse[95], preparing droplets with narrow size distribution is difficult[51]. Second, although it is simple to incorporate components into the droplet shell, it is difficult to encapsulate components into the perfluorocarbon core due to phospholipid shedding during condensation and condensation of microbubble can be impeded by the low purity of perfluorocarbon[53].

3.5 The method of microfluidics

Microfluidic technologies offer a promising route to produce uniform emulsions. Microfluidics for forming droplets can be either passive or active (Figure 6). In a passive microfluidic device, an aqueous phase (continuous phase) was injected into the first inlet cartridge, whereas the organic phase (usually ethanol or acetonitrile solvent) containing dissolved perfluorocarbon and coating material (dispersed phase) is injected into the second inlet via a pressure-driven flow[96]. The two phases meet at a junction, at which the perfluorocarbon liquid extends to form a ‘figure’ or ‘jet’

and eventually pinched off to form droplet[97]. The speed of the organic phase and aqueous phase pumped through the two separate microfluidic cartridges are different[40]. The particles size can be controlled by altering the flow rate ratio of the two phases[37]. In most studies, nanodroplets are manufactured using the passive method. Compared with passive techniques, active techniques modulate droplet formation with the aid of additional energy input. Droplets generation can be manipulated by two strategies: first by introducing additional forces from electrical, magnetic, and centrifugal controls; second by modifying viscous, inertial, and capillary force by varying intrinsic parameters like flow velocity and material properties[96].

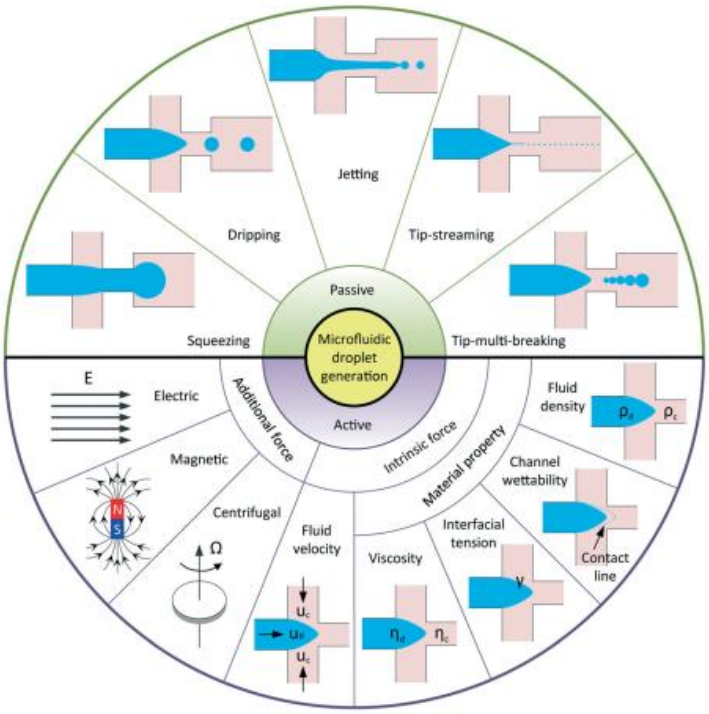


Figure 6. Schematic of droplet generation in passive and active methods (Copyright from Zhu *et al*. [96])

Microfluidics presents a powerful approach to optimising current formulations of nanodroplets. The advantage of this method is it allows for monodisperse size distributions; therefore, activation thresholds are highly uniform and vaporisation efficiency is increased[51]. It is also worth mentioning that this advantage also offers an opportunity to characterise the physical aspects of emulsions. An experimental relationship between particle size and vaporisation temperature could be tested to estimate Laplace pressure as well as surface tension[37]. However, there are some disadvantages. The ease and speed of manufacturing droplets are limited[13]. Microfluidics requires specialised equipment which is relatively expensive and not easy for novice users. It is even more challenging to produce nanoscale droplets. It either needs nanofluidic devices[98], or combines microfluidics with condensation[99].

3.6 Preparation of PCN by spontaneous nucleation

A novel method for producing nanodroplets using spontaneous nucleation, also called the OUZO method, was demonstrated by David *et al.*[99]. Lipid surfactants, or any other stabiliser, are first

dissolved in an organic solvent. They first prepared an initial lipid-ethanol stock solution. Perfluorocarbons were dissolved in the stock solution until it was fully saturated and adjusted with a stock solution to achieve the desired percentage. Finally, the aqueous solvent was added to the solution to reduce the solubility of lipid and perfluorocarbon, forcing droplets to spontaneously nucleate[99]. A stabiliser can be added to increase the stability of nanodroplets. This method is easy to operate but not commonly used in literature.

4. Phase change nanodroplets for bioimaging

Nanodroplets allow simultaneously therapeutic and diagnostic application. Unlike microbubbles, which are unable to enhance image contrast outside blood vessels, nanodroplets can migrate through hyperpermeable vessel walls in tumours and accumulate in the interstitial tissue[100]. Another advantage of droplets is that they can retain their nano-scale size in the bloodstream, enabling them to circulate for longer. Nanodroplets with liquid cores can be converted to gas bubbles, which make good contrast agents for ultrasound imaging[21].

Ultrasound imaging is broadly used imaging techniques with real-time, non-ionising, high frame-rate imaging as well as low cost[101]. Ultrasound contrast agents are a good tool to investigate sites of inflammation and solid tumour due to highly permeable vascular networks in these tissues[75] (Figure 7). The vaporisation of nanodroplets results in acoustic emissions, which are usually observed by B-mode (Brightness) ultrasound probe[28,62,63]. However, in the beginning, nanodroplets that use perfluorocarbon compounds with a boiling point above room temperature [62] (*e.g.,* DDFP, PFH) need a significant amount of acoustic energy to vaporise, making diagnosis and molecular imaging with nanodroplets especially difficult[75]. Therefore, nanodroplets using highly volatile perfluorocarbons were developed later, which are inherently more sensitive to acoustic energy[89]. Apart from this, acoustic imaging could also monitor the size of bubbles through harmonic emissions produced by vaporised nanodroplets[100].

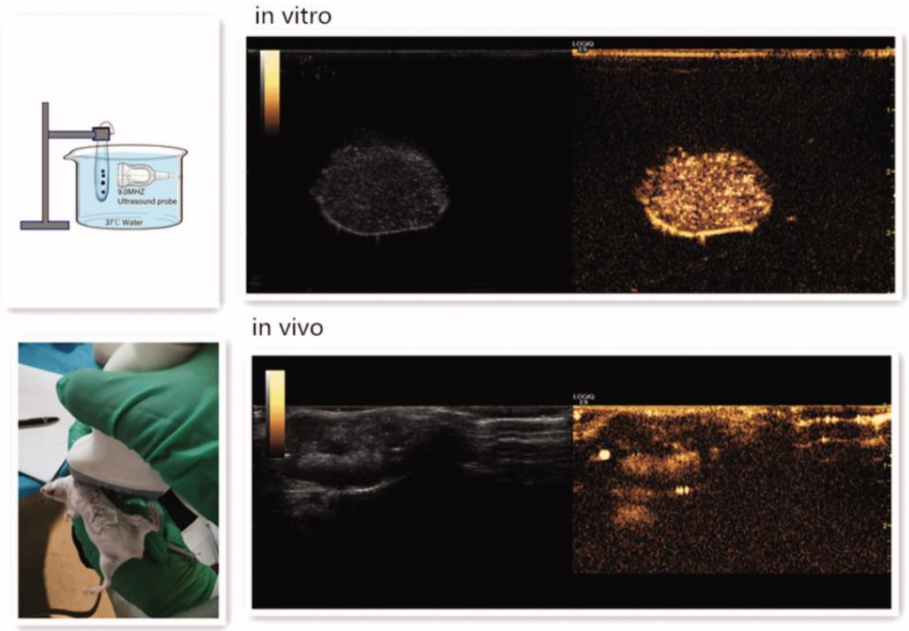


Figure 7. Ultrasound enhancement images of nanodroplets in vitro and in vivo (copyright from Zhou *et al*.[102])

However, unlike microbubbles, nanodroplets remain inert and virtually undetectable by conventional ultrasound imaging before vaporisation[12]. Besides, the ultrasound as an imaging tool has poor tissue discrimination ability compared with MRI and largely depends on the analysis of operator[103]. Thus, another imaging system can be used to assist in guiding the focused ultrasound[13]. Different imaging probes are added to nanodroplets to make them into multimodal imaging contrast agents. Multimodal imaging nanodroplets will become a future developing direction because other imaging tools could offset the weakness of nanodroplets and work in correlation with ultrasound. The core and shell of nanodroplets can be adopted to make particles detectable under other kinds of imaging *e.g.*, fluorescence/MRI/PET/X-ray [78,104]. Since each type of imaging has its advantages and disadvantages, different imaging modalities are generally considered complementary rather than competitive.

Photoacoustic (PA)/Ultrasound (US) imaging is a hybrid biomedical imaging technique. It combined the contrast superiority of optical imaging with the resolution superiority and deep tissue penetration of ultrasound imaging[105]. Nanodroplets used for PA/US imaging are prepared by adding photo-absorber in nanodroplets formulation[106]. This dual-modality agent can undergo vaporisation induced by ultrasound energy or optical energy by laser activation[107] and produce high US/PA contrast on demand. After absorbing optical energy, photo-absorber in nanodroplets produces heat and photoacoustic pressures, which lead to liquid-to-gas phase transition of perfluorocarbon core. The activation process of nanodroplets by optical energy is called ODV (optical droplet vaporisation) instead of ADV[105].

One unique property of laser-activated nanodroplets is they can vaporise and recondense with different perfluorocarbon core compositions. When nanodroplets are formed with perfluorocarbon core with low-boiling point which is lower than body temperature (37℃), they remain gas phase after vaporisation and not able to condense back to liquid droplets. However, if nanodroplets are formulated with high-boiling point perfluorocarbon like perfluorohexane, they can recondense back to liquid droplets from gaseous bubble, which allows repeat activation and deactivation (Figure 8)[108]. Worth mentioning that repeat activation and deactivation of nanodroplets is not only controlled by perfluorocarbon core, but a combination of several parameters including particle size, laser fluence, amount of dye and imaging conditions[107]. Therefore, a thoughtful choice of parameters is necessary to design the recondensation of nanodroplets.

Apart from perfluorocarbon core, the photo absorber is also an important parameter for PA contrast agent. The laser activation and photo absorber vary based on clinical needs. Laser activation in the near-infrared region was used in several studies by adding ICG (indocyanine green) and plasmonic nanoparticles in nanodroplets, because in this region the optical penetration is effective[106]. ICG is an FDA approved commonly used intravenous dye to measure cardiac output, hepatic function and for ophthalmic angiography in clinics with rare side effects. The study of Hannah *et al.* prepared PA/US nanodroplets by loading ICG in albumin shell and sample can be irradiated at 780nm wavelength laser pulse[109]. Hannah *et al*. encapsulated gold nanorods in nanodroplets consisting of BSA shell and PFP core. Upon pulsed laser irradiation at

780nm, liquid perfluorocarbon undergoes phase transition yielding giant photoacoustic transients and the gaseous phase provides ultrasound contrast enhancement. After vaporisation, PA signal decayed but still exist. At this stage, PA signal is originated from expelled nanorods and endogenous thermal expansion [44]. Laser pulse with 1064nm wavelength is also a commonly used optical source for PA/US dual-modality imaging. The advantages of using 1064nm light for biomedical imaging are it can improve contrast due to minimal absorption by blood-perfused tissue, and this laser source is inexpensive[106]. Santiesteban *et al*. loaded coated copper sulfide nanoparticles (CuS NPs) in PFP core and nanodroplets have good PA/US contrast as well as good biocompatibility over other metallic particles[110]. Photo absorber can also be encapsulated on nanodroplets shells. Santiesteban *et al.* has prepared two nanodroplets with different photoabsorber dye on lipid shell, and these nanodroplets can be activated by 680nm and 1064nm laser pulse separately[111].

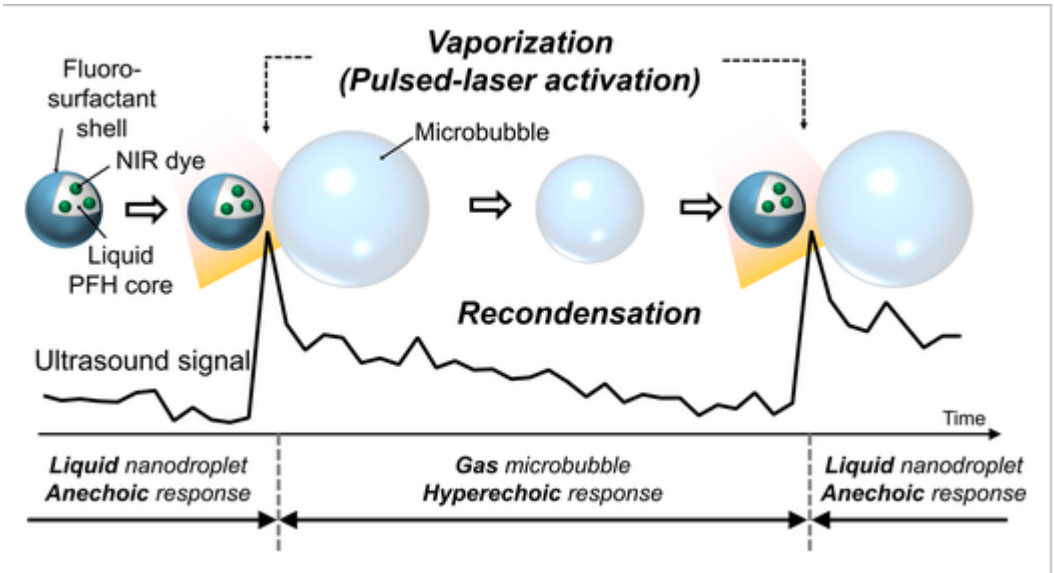


Figure 8. Vaporisation and recondensation of laser-activated nanodroplets. When activated, nanodroplets immediately produce strong ultrasound signals (Copyright from Yoon *et al*.[108])

Fluorescence imaging is imaging with high sensitivity but is only semi-quantitative and has poor tissue penetrating ability. Currently, most fluorescence labelled droplets are used for *in vitro* and preclinical studies, especially for optimising droplets for further human injection.[78] Gorelikov *et al*. suspended CdSe/ZnS core/shell quantum dots (QDs) in the perfluorocarbon core of droplets for rapid, preclinical optical assessment [78]. To understand the condensation process of microbubble to nanodroplets, Mountford *et al*. added fluorescent lipid DiI into the membrane of microbubbles to visualise their deformation during pressurisation under fluorescence microscopy[53].

MRI is an accurate imaging tool for soft tissue anatomy with high spatial resolution but low sensitivity. Conversely, ultrasound is an imaging technique with high sensitivity but low spatial resolution. Besides, unlike ultrasound which can provide real-time monitoring, MRI needs a relatively long imaging time for high-resolution imaging[104]. Therefore, ultrasound and MRI are combined as a compliment in many clinical applications[103]. Previous studies adopted nanodroplets into T2-weighted contrast agents by loading Superparamagnetic iron oxide nanoparticles (SPIO NPs) or Fe_3O_4 [112–114]. SPIO NPs have been loaded into the shell of

nanodroplets by the interaction between aliphatic terminated SPIO NPs and lipid to form a stable monolayer shell[113]. SPIO could greatly improve nanodroplets' liquid-to-gas phase change efficiency upon ultrasound exposure and make the nanodroplets magnetically responsive. SPIO loaded droplets have the potential to be manipulated via an external magnetic field which is highly advantageous for drug delivery in the previous work[79]. Recently, some studies focus on adopting nanodroplets into T1-weighted contrast agents[115]. Other nanoparticles, like liposomes and micelles, have been adopted into MRI contrast agents by incorporating ligand onto the macromolecular membrane to increase gadolinium ion payload. The same method can be used in nanodroplets. Maghsoudinia *et al*. has embedded a small molecular contrast agent Gadovist into the alginate polymer shell of nanodroplets. Nanodroplets show a higher T1-weighted MRI signal than free molecule Gadovist[115].

As fluorinated compounds can be monitored by ^{19}F (Fluorine) MR spectroscopy, nanodroplets loaded with PFCE or PFOB (perfluorooctyl bromide) in the core was used as multimodal contrast agents directly for ultrasound and ^{19}F MRI [76]. Lanza and Wickline's team has conducted a series of studies on PFCE and PFOB nanoemulsions as ^{19}F MR contrast agents[116–118]. Apart from adding an MRI probe into nanodroplets, changes in proton resonance frequency could be used to monitor temperature changes under MR thermometry. Crake *et al*. used MR thermometry to measure the thermal effects induced by vaporising DDFP lipid-coated nanodroplets[119].

PET is one of the most effective techniques to quantify the agents in preclinical models and patients[120]. Adapting nanodroplets to be detectable by PET could allow understanding of pharmacokinetics and biodistribution of nanodroplets, which is very important for developing safe and efficient drug carrier[121]. Amir *et al*. made PET contrast nanodroplets by dissolving $[^{18}F]CF_3(CF_2)_7(CH_2)_3F$ into PFOB core [122]. Contrast-enhanced digital mammography (CEDM), as one of the techniques of X-ray mammography, can provide good sensitivity and specificity in breast cancer detection and characterisation. Hill *et al*. used PFOB nanodroplets as CEDM contrast agents because the bromine atom in the molecule has good X-ray attenuation characterisation[123].

Imaging of nanodroplets is important not only because it can be used as imaging contrast agents, but also allow us to understand the in vivo stability, biodistribution and pharmacokinetics of nanodroplets. However, few studies have investigated these aspects of nanodroplets. Rapoport *et al.* have tested the pharmacokinetics of polymer-coated nanodroplets by measuring the PFCE core using ^{19}F MRI. The result indicated that 40 to 50% were still circulating 2 h after the injection and after 24 h most signals were found from liver [30]. Pre-clinical biodistribution and pharmacokinetics are essential for the future development of nanodroplet. PET, MR and fluorescence imaging have great potential to be used in these studies. Fluorescence imaging can be used for short-term real-time biodistribution imaging in small animals [124] whereas PET imaging has clinical translatability for large animals and human. Besides, PET allows treatment monitoring and planning [122].

5. Development of sonoresponsive nanodroplets for drug delivery

The combination of therapeutic ultrasound and microbubbles is broadly used in the medical area

while the large size has become an inevitable restriction of microbubbles to extravasate beyond the blood vessels and significantly constrained their therapeutic efficacy[125]. Compared to microbubbles, nano-sized droplets with a superior *in vivo* stability tend to have a broader therapeutic application such as in ablation, embolotherapy and drug delivery which are listed in Table3 [65,84,126].

Histotripsy, a novel ablation method, can fractionate the tumour tissues in a non-invasive manner by taking the advantage of the cavitation generated by the high-pressure ultrasound[32]. The ultrasound frequencies used in currently approved clinical application are normally below 1MHz [21]. However, this approach is unable to handle tumours with micrometastases and small nodules as they are challenging to be visualized before operation [84]. Nanodroplets as cavitation nuclei can reduce the threshold of cavitation and the introduction of nanodroplets into histotripsy can realize the selective and targeted tumor ablation as the nanodroplets are capable of penetrating tumor vasculature and accumulating into tumors [127]. Embolotherapy is another method, suppressing the tumor outgrowth through ischaemic damage [65]. The microbubbles converted from nanodroplets under the stimulation of ultrasound have a diameter larger than blood vessels, resulting in the occlusion of blood vessels and blocking the blood flow in tumor site to induce the shrink of tumors [31]. Currently, the development of this therapeutic approach is still in the pre-clinical stage, and in order to get into clinical use, controlling the migration of these gas emboli to avoid arterial occlusion in healthy tissues and optimizing the ultrasound parameter to trigger the ADV efficiently are two challenges standing ahead.

Up to now, the preclinical research using sonoresponsive nanodroplets in drug delivery has been comparatively more diverse, covering chemotherapy, gene therapy, sonodynamic therapy, photo-thermal/dynamic therapy and the combination of them. The sonoreponsive nanodroplets will turn into microbubbles through the ADV process and the cavitation of these microbubbles in the blood can generate mechanical force such as shock waves and microfluids, causing the disruption of biological barriers through the perforation on cell membranes , thus enhancing the drug delivery where the mechanism is described in Figure 9 [3].

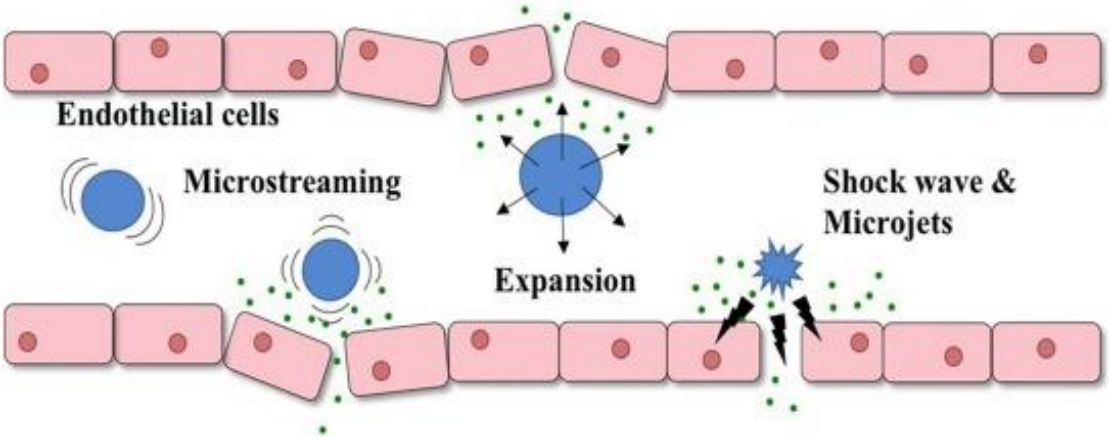


Figure 9. Schematic illustration of how UCAs respond to ultrasound which results in the increase of endothelial cells permeability. UCAs will oscillate and collapse in ultrasonic exposure, generating mechanical forces such as shock wave, microjets and microstreaming which

temporarily disrupts endothelial cells and cause sonoporation. (Copyright from Kee *et al*.[3])

For chemotherapy, most the recent studies loaded chemotherapy drugs on the shell of nanodroplets, and ultrasound could facilitate phase change of nanodroplets as well as drug release. Baghbani *et al*.[128], fabricated the DOX-loaded nanodroplet using alginates as the outer shell and perfluorohexane (PFH) as the liquid core. Surfactant Tween 20 in this formulation could prevent the recognition of nanodroplets from the reticulo-endothelial system and prolong their half-life[128] . An increased biodistribution of DOX was discovered, from approximately 2μg/g in its free state to around 12μg/g in nanodroplets under sonication. Using biocompatible material, for example, chitosan can ensure the safety of DOX-loaded nanodroplets[102]. No significant cellular structure impairment was found in functional organs, indicating that loading DOX inside this drug carrier could considerably mitigate its cardiotoxicity and nephrotoxicity, while the anti-tumour rate increase from 8.35% in the DOX control group to 39.50% in the DOX-loaded nanodroplet group, which revealed that DOX-loaded nanodroplets could offer a great potent in suppressing the tumour outgrowth[102]. Cao et al. discovered that nanodroplets composed of different outer shells required different ultrasound intensities to activate acoustic cavitation[61]. Hence, it is estimated that the co-delivery of nanodroplets composed of lipid and polymer could enhance the delivery of chemotherapeutic agents to a large extent as the cavitation of lipid nanodroplets could facilitate the accumulation of polymer nanodroplets in tumors [61].

The outer shell of nanodroplets can be modified by attaching various ligands to optimise their therapeutic performance. Zhao *et al.* managed to use this property to prepare a drug-loading nanodroplet with cell-penetrating and targeting capability[129]. The primary outer shell of this nanodroplet was composed of DPPC, DSPE-CPP and cholesterol, while the cargo loaded inside the nanodroplet was another anti-cancer drug named 10-hydroxycamptothecin (HCPT). Modifying the surface of the nanodroplets with transactivating transcriptional activator (TAT) protein, which facilitates the translocation of large molecules across the cellular membrane, could exploit the cell-penetrating capability of HCPT into the cytoplasm or nuclei[130] . And the addition of hyaluronic acid (HA) in this formulation could enhance the targeting capability of nanodroplets by binding to the overexpressed cluster of differentiation (CD-44) in human hepatoma[131]. These elaborate nanodroplets under the irradiation of low-intensity focused ultrasound showed a three-fold increase in mean tumour suppression rate compared to the free drug-treated group, from 29.17% to 94.97%, and displayed great potential in treating hepatoma. Following the same design route, a novel tumour homing-penetrating peptide tLyP-1 as an alternative to TAT was attached to the same lipid-based nanodroplets for deeper tumour penetration, and the enhanced accumulation of HCPT-loaded nanodroplets was discovered as well[132]. In another study, the folic acid (FA) was attached to the surface of HCPT-loaded nanodroplets made up of DSPE-PEG2000, DPPG and cholesterol[115]. FA could bind to the overexpressed FA receptor in SKOV3 tumour cells and is broadly used as a ligand to enhance the targeting capability of nanocarriers, increase the accumulation of therapeutic agents in tumours and mitigate their off-target possibility[133]. A significant increase in nanodroplets distribution and HCPT concentration was observed in the tumour. The adequate accumulation of therapeutic agents in tumours remarkably improved the tumour inhibitory rate to 73.6% compared to the control group[114].

The cargo loaded in nanodroplets can not only be chemotherapeutic agents but also be therapeutic genes. Gao *et al.* prepared a gene loaded nanodroplets to improve the gene transfection rate through the cavitation and sonoporation triggered by ultrasound[60]. The outer shell of the nanodroplets was fabricated by PGA-g-PEG-AHNP synthesised from γ-glutamic acid (γ-PGA) and PEG-AHNP. The core of this nanodroplet was made up of PFP and $C_{11}F_{17}$-PAsp-DET, (synthesized from $C_{11}F_{17,}$ aspartate and diethylenetriamine). To optimise the targeting capability of this gene-loaded nanodroplet to breast cancer, anti-Her2/*neu* peptide (AHNP), which could bind to the overexpressed Her2/*neu* receptors in breast cancer, was modified to the surface of the nanodroplets. A dramatically improved gene expression was discovered in the mice intravenously and intratumoral administrated with gene-loaded nanodroplets. Recently, four pre-microRNA plasmids (miR139, miR203a, miR378a and miR422a) downregulating the PIK3 CA mutation in hepatocellular carcinoma were loaded onto the positively charged surface of nanodroplets to investigate their anti-tumour potential[134]. The nanodroplets in this study were constructed using DPPC and DSPE-PEG2000-NH_2. After directly intratumoral injection of these four plasmid-loaded nanodroplets, inhibited tumour growth rate and prolonged survival time were discovered in these four groups after sonication, among which the pre-microRNA-139 group displayed the most outstanding therapeutic efficacy, with a four-times increase in the anti-tumour rate compared to the group treated with bare nanodroplets.

The mechanism involved in cancer treatment varies, so as the therapeutic agents. Aside from chemotherapy, photothermal or photodynamic therapy (PDT) can also treat a wide range of tumours. The mechanism of photothermal therapy is to induce the apoptosis of tumour cells via temperature elevation, while for PDT, the death of tumour cells attributes to the generation of reactive oxygen species (ROS) via the interaction between the photosensitizer and laser light[135,136]. Photosensitizers irridated by light can transfer energy to oxygen, which can overcome tumor hypoxia and cause cell death[137]. Moreover, oxygen (O_2) has a superior solubility in liquid perfluorocarbon, turning the nanodroplet into an O_2 reservoir and enhancing the generation of cytotoxic ROS [138]. Liu *et al*., prepared the Au nanoparticles (AuNP) loaded nanodroplets to amplify the anti-tumour efficacy of photothermal therapy[139]. The outer shell of the nanodroplets was made up of human serum albumin (HSA), and dodecafluococarbon (DDFC) was used as the liquid core. When treated with AuNP loaded nanodroplets, sonication, and laser radiation, the tumour temperature could reach 50^{o}C, considerably higher than the other control groups. In terms of PDT, the therapeutic efficacy is hampered by hypoxia in tumours. As previously stated, nanodroplets can ameliorate this condition through the production of ROS[138]. A study compared the anti-tumour efficacy of IR780 loaded lipid nanodroplets with or without PFH, and a remarkably inhibited tumour outgrowth was discovered in the mice treated with IR780 and PFH encapsulated nanodroplets which indicated that the hypoxia of tumour microenvironment could be adjusted by using appropriate drug delivery system[140].

Sonodynamic therapy (SDT) was developed recently as a novel treatment method for tumour. It damages cancer cells by ultrasound stimulation of sonosensitizer[141]. The mechanism underlying the effects of SDT is not fully understood, but it is thought that acoustic cavitation induced by the interaction between ultrasound waves and the aqueous environment can activate sensitisers to

transfer energy to nearby oxygen molecules, subsequently resulting in the formation of ROS. Compared with photodynamic therapy (PDT), SDT has higher tissue penetration because sonosensitizer can be activated by low-intensity ultrasound whereas PDT uses light as a stimulator [5]. IR780 iodide has been used as a sonosensitizing agent to be encapsulated in ultrasound-responsive nanodroplets for SDT. This is a lipophilic, near-infrared fluorescence (NIRF) dye that does not only perform NIRF imaging but can effectively target organic-anion transporting polypeptides (OATPs) – commonly overexpressed in cancer cells[142–144]. Zhang *et al*. have fabricated this IR780-loaded nanodroplet [145]. The outcome of *in vivo* biodistribution test revealed that encapsulating IR780 into the nanodroplets shell could enhance the accumulation of IR780 to the tumours because of the EPR effect of nanodroplets and the mitochondria-targeting capability of IR780. When the mice were treated with pristine nanodroplets, a comparatively large amount of nanodroplets was discovered in the liver and spleen rather than the tumour. Moreover, the administration of IR780-loaded nanodroplets with ultrasound could significantly slow down the growth of tumours, indicating a promising potential of this treatment strategy. Another agent that has been evaluated is hematoporphyrin monomethyl ether (HMME) – an effective sonosensitizer with lower toxicity and higher singlet oxygen yield to cause cellular apoptosis through the mitochondrial apoptotic pathway[146]. In order to treat ovarian cancer, Yang *et al*. encapsulated HMME into the lipid shell of nanodroplets with perfluoropentane as core[147]. FA was conjugated to the surface of nanodroplets, targeting the overexpressed FA receptor in 90% of ovarian cancers[133]. The nanodroplets together with ultrasound also induced ROS formation, resulting in tumour necrosis and apoptosis. The tumour inhibitory rate was 87.68% higher than the control group, which firmly supports the potential utilisation of lipid-based nanodroplets to improve SDT efficacy in clinical studies[147].

Synergistic treatment strategies have been introduced to strengthen the therapeutic outcomes by combining two or more single treatment strategies where nanodroplets can also display their ability to enhance therapeutic agents' delivery. For instance, liposomes with cisplatin prodrug encapsulated (cisPT-Lip) in chemoradiotherapy could further use PFCE as a liquid core to inhibit tumour outgrowth and significantly prolong the median survival time of treated mice by 6-8 days compared to the other control groups[148]. The PFCE with great O_2 loading capacity could increase the oxygenation in tumours, and this sufficient O_2 accumulation was essential for radiotherapy to cause DNA damage of tumour cells[149]. Chemotherapy has been integrated with anti-vascular therapy to inhibit tumour growth, where the ADV of DOX-loaded nanodroplets can disrupt the tumour vasculature, reduce cell proliferation, increase the distribution of DOX, and restrain the growth of tumours eventually[150]. Apart from these treatment strategies, many other preclinical studies have also taken advantage of nanodroplets in chemo/photothermal therapy, photo-dynamic/thermal therapy, and radio/photodynamic therapy to optimise drug distribution a better anti-tumour efficacy [35,151,152].

The application of sonoresponsive nanodroplets in drug delivery is in its developing state, and there is still a long way to go before getting into clinical use. Firstly, standard ultrasound protocols haven’t been set to effectively enhance drug delivery and cause minimal damage to peripheral tissues according to different diseases in patients. Secondly, immunotherapy as a rising star in the medical field has attracted great attention. Using sonoresponsive nanodroplets to deliver

therapeutic agents and trigger immune response can be a new trend. Apart from these, exploring a new administration route for sonoresponsive nanodroplet, for example, intranasal delivery, may widen its application in the future.

Table 3. Recent preclinical studies using nanodroplets to deliver therapeutic agents

Therapy	Therapeutic agent	Outer shell	Liquid core	Delivery mechanism	Reference
Chemotherapy	DOX	Alginate, Tween 20	PFH	Nanodroplets can form microbubbles through the ADV process, and encapsulated DOX can be released through the cavitation triggered by ultrasound. The addition of Tween 20 prolongs the circulation time of nanodroplets via the avoidance of RES.	Baghbani *et al.*[128]
	DOX	DPPC, DPPG, DPPE and cholesterol	PFP	Both lipid and polymer nanodroplets can turn into microbubbles under the sonication of ultrasound, and the DOX can be released locally through the collapse of microbubbles. However, the polymer nanodroplets with a hard shell require a higher intensity of ultrasound to stimulate the ADV process.	Cao *et al.*[61]
		mPEG-PLGA			
	DOX	Chitosan	PFH	The chitosan nanodroplets can develop into microbubbles through the ADV process and release the DOX after the disruption of microbubbles. The use of biocompatible chitosan can improve the biosafety of this drug delivery system.	Zhou *et al.*[102]
	HCPT	DPPC, DSPE-CPPs, cholesterol, HA	PFP	The nanodroplets modified with CPP can actively deliver the HCPT across the cellular membrane. The addition of HA can increase the targeting capability of nanodroplets through the binding of overexpressed CD44 in human hepatoma.	Zhao *et al.*[129]
	HCPT	DPPC, DSPE-PEG_{3400}-tLyP-1, DPPG, cholesterol	PFP	tLyP-1 is a homing-penetrating peptide binding to the neuropilin-1 receptor overexpressed in human tumour cells. The addition of tLyP-1 to the nanodroplets could enhance their penetration and accumulation into tumours	Zhu *et al.*[132]
	HCPT	DSPE-PEG_{2000}-FA, DPPG cholesterol	PFP, Fe_3O_4	The addition of FA binding to the overexpressed FA receptor in SKOV3 ovarian cancer cells can increase the targeting capability of nanodroplets. The Fe_3O_4 acting as a contrast agent can improve the PAI of nanodroplets and realize the theranostic of	Liu *et al.*[114]

				cancers.	
Gene therapy	Luciferase gene	PGA-g-PEG-AHNP	PFP, $C_{11}F_{17}$-PAsp-DET	The use of AHNP on the nanodroplets binding to the overexpressed Her2/*neu* receptor in breast cancer can improve the targeting capability of nanodroplets, and the peptide itself can have an anti-tumour efficacy. The amphiphilic core can help to condense the negatively charged genes into the nanodroplet efficiently.	Gao *et al*.[60]
	miRNA-139, miRNA-203a, miRNA-378a, miRNA-422a	DPPC, DSPE-PEG_{2000}-NH_2	PFP	These nanodroplets loaded with four different genes were intratumorally administrated. The released gene via the ADV and cavitation process can have anti-tumour efficacy by inhibiting the PIK3 CA mutation in hepatoma cells.	Dong *et al.*[134]
Sonodynamic therapy	IR780	DPPC, DSPE-$mPEG_{2000}$, cholesterol	PFP	The ADV process involved in the release of sonosensitizer IR780 could facilitate the leakage and accumulation of nanodroplets into tumours. Moreover, the loaded IR780 itself displayed a significant tumour penetration and mitochondria-targeting ability.	Zhang *et al*.[145]
	HMME	DPPC, DSPE-$mPEG_{2000}$-FA, cholesterol	PFP	The sonosensitizer HMME was released through the ADV and cavitation process, which could disrupt the vasculature and enhance the penetration of HMME deep into the tumour. The addition of FA binding to the overexpressed FA receptor in the ovarian cells could enhance the targeting ability of nanodroplets.	Yang *et al*.[147]
Photothermal therapy	AuNP	Human serum albumin	DDFC	The sonoporation caused by the cavitation of nanodroplets could enhance the delivery of AuNP into the tumour cells. The interaction between light and AuNP could increase the temperature of tumours and induce the apoptosis of tumour cells.	Liu *et al*.[139]
Photodynamic therapy	IR780	Lecithin, cholesterol, DSPE-PEG_{2000}	PFH	The dissolved O_2 in the liquid core (PFH) could ameliorate the hypoxia tumour environment, ensure the generation of cytotoxic ROS and enhance the therapeutic efficacy of photodynamic therapy.	Tang *et al*.[140]
Chemo-radiotherapy	Cisplatin prodrug	DPPC, DSPE-PEG_{5000}, cholesterol	DFCE	The therapeutic outcome was caused by chemotherapy and radiotherapy. The use of nanodroplets in this study enhanced the delivery of chemotherapeutic agents, reduced its systematic toxicity, and the O_2 dissolved in the DFCE could help tackle the hypoxia in the tumours, thus amplify the impact of radiotherapy.	Yao *et al*.[148]

Chemo-antivascular therapy	DOX	DPPC,DSPG,DSPE-PEG$_{5000}$	PFP	The mechanical waves generated through the ADV of nanodroplets could disrupt the tumour vasculature and inhibit cell proliferation. The vascular disruption could also facilitate the diffusion of DOX into tumours and obtain an enhanced anti-tumour effect.	Ho *et al.* [150]
Chemo-photothermal therapy	Melanin, DOX	PVA	PFP	The loaded melanin was a photosensitizer which could trigger the vaporisation of PFP under laser irradiation and generate a photothermal therapeutic effect. The cavitation of nanodroplets could facilitate the penetration of DOX in tumours to have an enhanced chemotherapeutic effect.	Hu *et al*.[151]
Photothermal/ photodynamic therapy	$ZnF_{16}Pc$ molecules	PEG-based perylene diimide(PDI)	PFP	The outer shell of the nanodroplets was a photoabsorber which could increase the temperature of tumours and trigger the vaporisation of PFP under the laser irradiation, while the O_2 dissolved in the liquid core could improve the photodynamic therapeutic outcome of $ZnF_{16}Pc$ molecule which was a photosensitizer in this study.	Tang *et al.*[35]
Radiotherapy/ photodynamic therapy	TaOx nanoparticles	C18PMH-PEG	PFH	The TaOx nanoparticles decorated on the nanodroplets could enhance the therapeutic outcomes of radiotherapy and lead to the damage of DNA. The O_2 dissolved in the PFH was beneficial for radiotherapy and photodynamic therapy to generate abundant cytotoxic ROS and kill cancers.	Song *et al.* [152]

5. Conclusion

Nanodroplets are novel nanoparticles for both diagnostic and therapeutic applications. In this review, the chemical composition and preparation methods were introduced and compared. Then applications of imaging and therapeutic application of nanodroplets were summarised and how they were chemical adopted for different applications was highlighted. This review gives us an overview of how to design nanodroplets according to their desired application.

Nanodroplets have great potential for future development. Although there are no commercially available nanodroplets by now, recent research and the fact that nanodroplets are composed of safe and previously tested materials indicate that they have a great opportunity for application in the clinic. The therapeutic potential of nanodroplets is currently explored and is demonstrating the extraordinary ability of tumour drug penetration and improved treatments in the mouse model. However, the biodistribution and pharmacokinetic studies related to nanodroplets are limited and need more investigation. Besides, more preclinical studies are needed before nanodroplets are clinically approved. From a translation point of view, nanodroplets with good size uniformity and stability are needed.

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