

Perfluorooctylbromide nanoparticles for ultrasound imaging and drug delivery

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Abstract: Perfluorooctylbromide nanoparticles (PFOB NPs) are a type of multifunctional nanotechnology that has been studied for various medical applications. Commercial ultrasound contrast agents (UCAs) suffer from the following limitations: short half-lives in vivo, high background signal and restricted distribution in the vascular circulation due to their micrometer dimensions. PFOB NPs are new potential UCAs that persist for long periods in the circulatory system, possess a relatively stable echogenic response without increasing the background signal and exhibit lower acoustic attenuation than commercial UCAs. Furthermore, PFOB NPs may also serve as drug delivery vehicles in which drugs are dissolved in the outer lipid or polymer layer for subsequent delivery to target sites in site-targeted therapy. The use of PFOB NPs as carriers has the potential advantage of selectively delivering payloads to the target site while improving visualization of the site using ultrasound (US) imaging. Unfortunately, the application of PFOB NPs to the field of ultrasonography has been limited because of the low intensity of US reflection. Numerous researchers have realized the potential use of PFOB NPs as UCAs and thus have developed alternative approaches to apply PFOB NPs in ultrasonography. In this article, we review the latest approaches for using PFOB NPs to enhance US imaging in vivo. In addition, this article emphasizes the application of PFOB NPs as promising drug delivery carriers for cancer and atherosclerosis treatments, as PFOB NPs can transport different drug payloads for various applications with good efficacy. We also note the challenges and future study directions for the application of PFOB NPs as both a delivery system for therapeutic agents and a diagnostic agent for ultrasonography.

Keywords: perfluorooctylbromide nanoparticles, ultrasound enhancement, drug delivery, therapy coupled with diagnosis

Introduction

Perfluorooctylbromide ($C_8F_{17}Br$, PFOB; Figure 1), which is sometimes called as perflubron, is a linear perfluorocarbon (PFC) well known for its biocompatibility and good tolerance in human beings.¹ PFOB is a dense, biochemically inert liquid with a high spreading coefficient. Unlike most PFCs, it is highly hydrophobic and shows small but finite lipophilicity due to the covalently bound bromine atom.²⁻⁵ Following in vivo administration, PFOB is not metabolized and instead is eliminated as a vapor from the body through exhalation via the lungs with a 3-day biological half-life.⁶

Nanoparticles (NPs), particularly polymer-based NPs, offer several advantages for anticancer drug/gene delivery.^{7,8} The small volume enables their progressive accumulation in the interstitial space of tumors through the enhanced permeation and retention (EPR) effect and endows them with a long circulation half-life.⁹ For in vivo ultrasound (US) imaging and drug delivery, PFOB is typically prepared as perfluorooctylbromide nanoparticles (PFOB NPs), which consist of a PFOB core encapsulated within a monolayer

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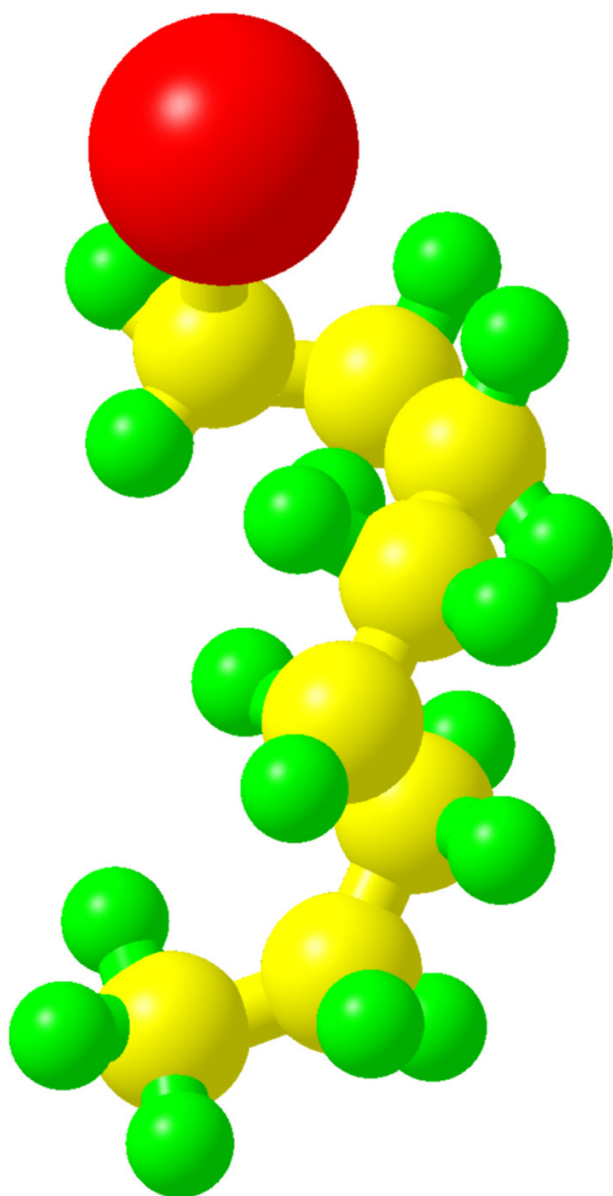


Figure 1 The structure of PFOB along with all atoms (red, bromine; yellow, carbon; green, fluorine).

Abbreviation: PFOB, perfluorooctylbromide.

of phospholipids or a polymeric shell. PFOB emulsions used in the clinic are marketed as LiquiVent (Alliance Pharmaceutical Corporation, San Diego, CA, USA),¹⁰ an oxygen-carrying liquid drug, and Oxygent™ (Alliance Pharmaceutical Corporation, San Diego, CA, USA),¹¹ a blood substitution agent. In addition, the use of PFOB NPs in various medical applications, including ¹⁹F or ¹H magnetic resonance imaging (MRI) tracers,^{12–19} cell-tracking agents,^{20–22} X-ray contrast agents^{21,23} and computed tomography (CT) tracers, has been explored.²⁴

US imaging is a clinical diagnostic technique that is frequently used because of its advantages, namely its real-time monitoring capability, low cost, high safety, convenience and

portability.²⁵ To improve the visualization in US imaging, US contrast agents (UCAs) have been developed. Mattrey et al²⁶ reported that PFOB NPs considerably increased the echogenicity of the liver relative to that of the kidney 48 hours after intravenous infusion and produced an echogenic rim around VX2 carcinoma, thereby enhancing tumor detection. Therefore, the authors concluded that PFOB represented a promising US contrast material. Lanza et al²⁷ reported that liquid PFC-filled NPs could potentially be used as new UCAs because of their long circulation half-life and acoustic stability. Since then, PFOB, one of the PFCs most frequently used as an NP core,²⁸ has attracted the interest of researchers. PFOB NPs present a low intensity of US reflection and require higher concentrations or more binding events, which are the physical bases of the ability of PFOB NPs to serve as UCAs, to produce a relatively high backscatter signal.^{29,30} Because of its poor solubility in water and very low speed of sound (approximately 600 m/s), PFOB persists in the circulatory system for long periods and displays a relatively stable echogenic response.³¹ Consequently, PFOB NPs are designed for therapeutic applications, such as drug delivery or gene transfer, and for potential use as UCAs. The use of PFOB NPs as carriers has the potential advantage of selectively delivering payloads of therapeutic agents to the site of interest while improving the visualization of the site by US imaging. Nevertheless, an important question remains: Does this process really work? This review will focus on the use of PFOB NPs for US imaging and drug delivery in diagnosing and treating various diseases to determine the challenges in and provide future perspectives of the applications of PFOB NPs in targeted therapeutic delivery coupled with targeted US imaging.

Structures of PFOB NPs

For in vivo US imaging and drug delivery, PFOB is typically prepared as PFOB NPs with a surfactant coating for stabilization and functionalization (Figure 2). Typical PFOB NPs contain a lipid–surfactant mixture encapsulating a liquid core of PFOB using an oil-in-water emulsion solvent evaporation process.^{32–34} The surfactant mixture consists of phospholipids typically derived from either egg or soybean lecithin, which display good biocompatibility. These phospholipid preparations have been used for many applications such as cosmetic, food and drug applications owing to their safety.³⁵ Thus, the final formulated PFOB NPs mainly comprise a PFOB core encapsulated within a lipid monolayer with a diameter <1,000 nm.³⁶ Other typical PFOB NPs consist of poly(lactide-*co*-glycolide) acid (PLGA),^{37,38} poly(lactic

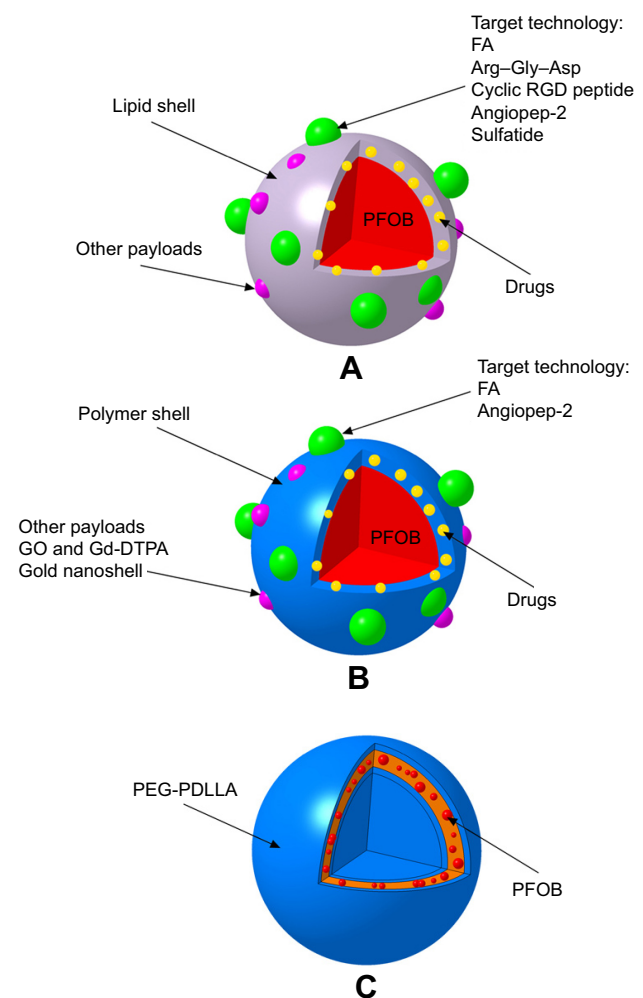


Figure 2 Schematic of the structures of PFOB NPs with different shells functionalized by different ligands.

Notes: (A) Representative images of typical PFOB NPs with a lipid shell for functionalization, representing an extremely versatile platform for targeted US/pool imaging and drug delivery applications. PFOB NPs carry drugs in the lipid layer of their shell. FA and Arg-Gly-Asp are used for ligand-directed, tissue-targeted US imaging. The targeting peptides Arg-Gly-Asp, a cyclic RGD peptide, angiopep-2 and sulfatide are used for ligand-directed tissue-targeted drug delivery. (B) Representative images of other typical PFOB NPs with a polymer shell for functionalization, representing an extremely versatile platform for US pool imaging and drug delivery applications. PFOB NPs carry drugs in the polymer layer of their shell or in the center of the PFOB NPs. FA and angiopep-2 are used for ligand-directed tissue-targeted drug delivery. (C) Representative images of novel self-assembled PFOB NPs with a low PFOB content in the outer lipophilic layer, representing a platform for US pool imaging.

Abbreviations: FA, folic acid; Gd-DTPA, gadolinium diethylene triamine pentacetate acid; GO, graphene oxide; PEG-PDLLA, poly(ethylene glycol)-*b*-poly(D,L-lactic acid); PFOB, perfluorooctylbromide; PFOB NPs, perfluorooctylbromide nanoparticles; US, ultrasound.

acid) (PLA)³⁹ or another polymer^{26,40} encapsulating a liquid PFOB core prepared using an oil-in-water emulsion solvent evaporation process.⁴¹ These polymers are biodegradable, are widely used in drug products and possess good general biocompatibility.^{42,43} An amphiphilic block copolymer, poly(ethylene glycol)-*b*-poly(D,L-lactic acid) (PEG-PDLLA), was developed to construct novel self-assembled PFOB NPs with a low content of PFOB in the outer lipophilic layer.⁴⁴

This self-assembled structure has mainly been studied as a UCA and has been established as a type of PFOB NP. The particle shell formulations and main applications, which are described in the text, are summarized in Table 1. Schematics showing the structures of PFOB NPs with different shells functionalized by different ligands are shown in Figure 2.

PFOB NPs for US imaging

Commercial UCAs (eg, Optison™ (Mallinckrodt Inc., St. Louis, MO, USA) and Definity® (Lantheus Medical Imaging, N. Billerica, MA, USA)⁴⁶ comprise encapsulated gas microbubbles with a diameter between approximately 2 and 10 μm. Upon intravenous injection, these UCAs are restricted to the vascular system of perfused organs or tissues. Despite the very sensitive detection offered by the strong nonlinear response of microbubbles, these agents present several limitations due to their short half-lives in vivo and high background signal. Because of their micrometer dimensions, their transportation in the vascular circulation is restricted to only a few minutes, and they cannot traverse outside highly permeable tumor vessels with pores ranging from 400 to 600 nm in diameter to achieve tumor imaging.⁴⁷ Compared with microbubbles, NPs offer several advantages in targeted US imaging. Their small volume contributes to a long circulation half-life and enables their progressive accumulation in the interstitial space of tumors through the enhanced EPR.⁹ These NPs have primarily been designed to address key issues, such as longevity and stability in the body.

PFOB was encapsulated within a Pluronic F-68 shell with a diameter of approximately 500 nm by Mattrey et al.²⁶ The resulting PFOB NPs considerably increased the echogenicity of the liver relative to that of the kidney 48 hours after intravenous infusion and produced an echogenic rim around VX2 carcinoma in New Zealand white rabbits, thereby enhancing tumor detection. Therefore, the authors concluded that PFOB NPs represent a promising UCA for selectively increasing detection in the liver and in tumors.²⁶ According to Lanza et al.,²⁷ targeted perfluorodichlorooctane (PFDCO)-filled NPs (PFDCO NPs) markedly enhance the acoustic reflectivity of thrombi in vivo in a canine model. Targeted PFDCO NPs persist in the blood for a long period, specifically bind to pre-targeted fibrin and generate a marked acoustical enhancement of acute vascular thrombi without increasing the background signal. In their follow-up study, the authors reported that both targeted PFOB NPs and targeted PFDCO NPs significantly increased the acoustic reflectivity of the nitrocellulose membrane samples and plasma thrombus substrate samples compared with their individual baseline values. Targeted

Table 1 Broad applications of PFOB NPs

| No. | Main component of the shell | Application | Target epitope | References |
|-----|---|---|---|--|
| 1 | Polymer (PLGA, PCL, PLA, PEG-PDLLA or PLGA-PEG and ingredients) | US pool imaging | | 9, 28, 39, 44, 52, 58 |
| 2 | Lipids (lecithin, cholesterol and ingredients) | US pool imaging Tumor-targeted US imaging | – | 30 63, 71, 72 |
| 3 | Lipids (lecithin, cholesterol and ingredients) | Loading different drugs for several therapies | VCAM-1, $\alpha_v\beta_3$ integrin, $\alpha_5\beta_1$ integrin, TN-C or LRP-1 | 33, 96, 99, 104, 105, 119–122, 127–129 |
| 4 | Polymer (PLGA-PEG, Bi ₂ Se ₃ or PLGA and ingredients) | Cancer therapy | LRP-1 or none | 17, 106, 107, 112 |

Abbreviations: FR, folate receptor; LRP-1, low-density lipoprotein receptor-related protein-1; PCL, polycaprolactone; PEG-PDLLA, poly(ethylene glycol)-*b*-poly(D,L-lactic acid); PFOB NPs, perfluoroctylbromide nanoparticles; PLA, poly(lactic acid); PLGA, poly(lactide-co-glycolide) acid; PLGA-PEG, poly(lactide-co-glycolide) acid-polyethylene glycol; TN-C, tenascin-C; US, ultrasound; VCAM-1, vascular cell adhesion molecule 1.

PFOB NPs and targeted PFDCO NPs enhanced reflectivity on both nitrocellulose membrane and plasma thrombus substrates to nearly identical levels.²⁹ Thus, US imaging with site-targeted PFOB NPs is expected to improve the diagnosis and detection of specific pathologies and tissue types, such as fibrin, as described earlier. PFOB NPs, which are resistant to pressure changes and mechanical stress, possess a relatively stable echogenic response without increasing the background signal, exhibit low acoustic attenuation and have received substantial attention with regard to fabricating a new UCA that can overcome the limitations of microbubble UCAs. Unfortunately, PFOB NPs are relatively incompressible and thus generally produce relatively poor echogenicity^{27,30,48} in circulation. Thus, the low intensity of US reflection has limited the application of PFOB NPs in the field of ultrasonography. Therefore, how to overcome the limitations of PFOB NPs in ultrasonography remains an open question. Accordingly, different researchers have developed alternative approaches to apply PFOB NPs in ultrasonography.

Adaptation of ultrasonography parameters for blood pool imaging

Some researchers have adapted ultrasonography parameters for blood pool imaging. High-frequency US (HFU), also known as US biomicroscopy,⁴⁹ is an established tool for imaging human beings and small animals *in vivo*.⁵⁰ Some researchers have reported the feasibility of detecting thick PLGA-shelled PFOB NPs at a high frequency and explored the ranges of concentrations, acoustic pressures and pulse durations capable of detecting the PLGA-shelled PFOB NPs in the 20–40 MHz frequency range.²⁸ Although the detectability of PFOB NPs would potentially be improved by HFU (>15 MHz), the acoustic responses remain relatively weak.²⁸ PFOB NPs exhibit a low inherent echogenicity and are poor blood pool contrast agents when employed under the conditions used for conventional two-dimensional (2D) echocardiography or

harmonic imaging or when imaged with color flow or spectral Doppler.³⁰ Thus, a new ultrasonic imaging modality, power Doppler harmonic imaging (PDHI), has been introduced.⁵¹ Wickline et al³⁰ have shown that PFOB NPs coated with a lipid shell with a mean diameter of approximately 400 nm provide excellent blood pool contrast when using PDHI at PFOB NP doses of 0.5 mL/kg. The contrast effect was unaffected by continuous US imaging at high transducer power outputs and persisted for more than 1 hour.³⁰ Li et al⁵² intravenously injected New Zealand white rabbits with PFOB NPs with a PLA shell for US imaging of their kidneys using both pulse inversion harmonic imaging (PIHI) mode and conventional B-mode. The rabbit kidney was more clearly observed using PIHI mode than using B-mode US imaging. These results were consistent with the abovementioned observations.

Adaptation of the formulations designed for blood pool imaging

The mechanical properties of PFOB NPs are very important for their use as UCAs because USs are mechanical waves.⁵³ In the classical mechanics of shells, the mechanical properties usually depend on the shell thickness-to-radius ratio. Pisani et al⁵³ concluded that the shell thickness-to-radius ratio depends only on the PFOB/PLGA ratio and that this versatile process could be adapted to other biodegradable polymers. In their follow-up study,⁴¹ the authors designed a process to obtain PFOB NPs within a PLGA shell of homogeneous thickness to develop more stable UCAs. Flegg et al⁴⁰ applied a novel modified theory based on Rayleigh scattering of US to a UCA consisting of PFOB encapsulated in polycaprolactone (PCL) and concluded that the shell of the PFOB NPs may play important roles in the scattering from each PFOB NP and the echogenicity of an agglomeration of PFOB NPs. Because the modulation of the thickness-to-radius ratio of PLGA and PFOB allows researchers to tune the compressibility and echogenicity of PFOB NPs *in vitro*, Jafari et al²⁸ compared the

US responses of PFOB NPs with a PLGA shell of different thicknesses. Diou et al⁵⁴ also utilized the very same strategy with PEGylated PFOB NPs with a PLGA shell to optimize their echogenicity. The results are consistent with a previous report by Pisani et al⁵³ showing that the thickness of the PFOB NP shell is an important parameter for US contrast enhancement. However, the acoustic responses remain relatively weak, and additional experiments are necessary to further modify PFOB NPs and obtain a better acoustic response.²⁸

Consequently, subsequent studies have adapted formulations designed for blood pool imaging but have met with limited success in vivo.^{55–57} Meanwhile, several researchers have successfully designed PFOB NPs for use in blood pool imaging.

According to Pisani et al,⁹ the thickness of the PFOB NP shell is an important parameter for US contrast enhancement: thinner shells produce a larger signal-to-noise ratio (SNR) and more compressible PFOB NPs. These authors have designed PFOB NPs with a thick PLGA shell as a blood pool contrast agent. The initial bolus passage enables significant US enhancement of the blood pool in mice during hepatic imaging (14 MHz, tissue harmonic imaging [THI] mode) after intravenous injection.⁹ Similar multifunctional UCAs featuring gold nanoshells coating the outside of PFOB NPs with a PLA shell have been designed by Ke et al⁵⁸ and integrated with superparamagnetic iron oxide (SPIO) dissolved in PFOB for combined dual-mode US/CT imaging-guided photothermal tumor ablation. Their platform is different from the PFOB NPs developed by our laboratory or by other researchers, which presented a spherical shape with

a smooth surface (Figure 3A), while their PFOB NPs with the gold nanoshell coating presented a rather rough surface morphology (Figure 3B). Several seconds after intravenous injection of their PFOB NPs, the rabbit kidneys showed clear enhancement under the PIHI contrast mode (mechanical index [MI] of 0.70). However, the authors believed that the US contrast enhancement was provided by the PFOB inside and that SPIO was used to obtain more accurate diagnostic information from contrast-enhanced MRI images. We are skeptical about this hypothesis. SPIO has also been utilized as a US imaging material in many studies.^{25,59,60} Therefore, the US enhancement may be caused by the SPIO, not the PFOB, in their formulation. In the same year, Ke et al³⁹ also designed another multifunctional UCA using a gold nanoshell coating the outside of PFOB NPs with a PLA shell for combined dual-mode US/CT imaging and photothermal cancer therapy. The rabbit kidney showed clear enhancement in PIHI contrast mode (at an MI of 0.59) after intravenous injection of their PFOB NPs.³⁹ A novel multifunctional theranostic UCA using PFOB NPs with a PLA shell surface functionalized with graphene oxide (GO) and a gadolinium diethylene triamine pentacetate acid (Gd-DTPA) was successfully fabricated by Li et al.⁵² Again, the rabbit kidney showed clear enhancement in PIHI contrast mode (at an MI of 0.42) after intravenous injection of their PFOB NPs.⁵² Other novel PFOB NPs with an extremely low PFOB content were prepared through the self-assembly of an amphiphilic block copolymer, PEG-PDLLA (Figure 2C). After intravenous injection of these PFOB NPs, all cavities of the heart, liver and kidney, particularly the

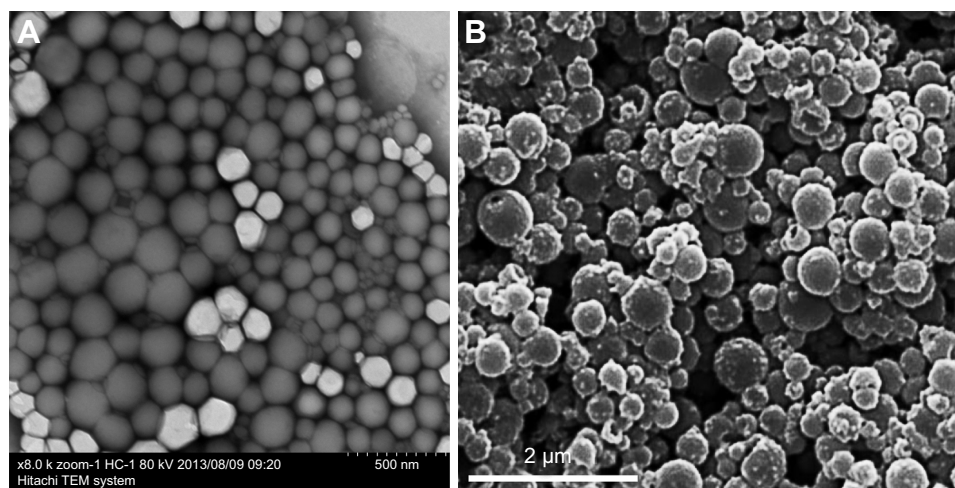


Figure 3 TEM images of normal PFOB NPs (A). TEM images of PFOB NPs coated with a gold nanoshell (B).

Notes: (A) Reprinted from Li X, Qin F, Yang L, Mo L, Li L, Hou L. Sulfatide-containing lipid perfluorooctylbromide nanoparticles as paclitaxel vehicles targeting breast carcinoma. *Int J Nanomedicine*. 2014;9:3971–3985.³³ (B) Reprinted from the study by Ke H, Wang J, Tong S, Jin Y, Wang S, Qu E, Bao G, Dai Z. Gold Nanoshelled Liquid Perfluorocarbon Magnetic Nanocapsules: a Nanotheranostic Platform for Bimodal Ultrasound/Magnetic Resonance Imaging Guided Photothermal Tumor Ablation. *Theranostics* 2014;4(1):12–23. doi:10.7150/thno.7275. Available from <http://www.thno.org/v04p0012.htm>.⁵⁸

Abbreviations: PFOB NPs, perfluorooctylbromide nanoparticles; TEM, transmission electron microscope.

horizontal axis of the right kidney, displayed a slight and homogeneous enhancement without any highlighted spots, which indicated a good contrast enhancement effect for blood pool imaging. The authors believed that both the compressibility and shell density of their PFOB NPs serving as US scatterers were enhanced, resulting in a much higher echo intensity than that of other PFOB formulations.⁴⁴

Fabrication of targeted PFOB NPs for tissue-targeted imaging

PFOB NPs may be less liable to nonspecific signal enhancement events since larger concentrations (more binding events) are required to produce a relatively high echogenicity.^{29,61} Thus, a high contrast between specific and nonspecific agents may be obtained by adding a targeted ligand to PFOB NPs. Although nanosized UCAs achieve passive imaging of tumors via the EPR effect, the specific capability of nanosized UCAs to bind to tumor tissues to achieve US enhancement is still limited.⁶² Some investigators have recognized the future clinical importance of PFOB NPs in tumor-targeted US imaging and developed targeted PFOB NPs to enhance US imaging; however, this objective was realized only *in vitro*.⁶¹ Subsequently, PFOB NPs targeted to $\alpha_v\beta_3$ integrins expressed on new vessels in tumors were formulated by adding an “Arg–Gly–Asp” mimetic binding ligand to the lipid layer.⁶³ Tumor-specific enhanced imaging was accomplished by either I_f (1.99) or H_f receivers (different receivers of ultrasonography) in transgenic K14-HPV16 mice whose ears typically carried squamous metaplasia after intravenous injection of these targeted PFOB NPs.⁶³ Another laboratory modified the surface of PFOB NPs with PEGylated phospholipids to allow them to escape recognition and clearance from the mononuclear phagocyte system and achieve passive tumor targeting. In this study, tumors were observed by ultrasonography only after intratumoral injection. To explain the absence of an echogenic signal in the tumor after intravenous injection of these PEGylated PFOB NPs, a histological analysis was conducted, revealing their limited accumulation within the tumor tissue at a level that was not sufficient to achieve US imaging enhancement.⁶⁴ The folate receptor (FR) binds to folic acid (FA) with high affinity and mediates its intracellular transport via receptor-mediated endocytosis.⁶⁵ The FR is overexpressed on the surface of various types of tumors, including pancreatic, prostate, lung, head and neck, breast, ovarian and mesothelioma tumors, whereas it shows limited expression and restricted distribution in normal tissue.^{66–70} Hu et al⁷¹ bound an FA–polyethylene glycol (PEG)–chitosan (CS) conjugate to PFOB NPs through electrostatic interactions, promoting the formation of new FR-mediated PFOB NPs using a layer-by-layer assembly technique. The US

signals of the NPs *in vitro* increased as the concentration of the FR-mediated PFOB NPs increased. Based on the experimental results, the authors predicted that the FR-mediated PFOB NPs are more promising agents for tumor-targeted US imaging.⁷¹ In a subsequent study,⁷² the researchers injected their FR-mediated PFOB NPs along with an immobilized probe into mice via the tail vein and set the ultrasonography parameters to an MI of 0.7 and a gain of 80%. Between 20 and 160 minutes after injection, the images from FR-overexpressing tumors displayed a higher intensity value than those from tumors expressing low levels of FR. Thus, the FR-mediated PFOB NPs achieved specific enhanced imaging of FR-overexpressing tumors and a longer duration of effective enhanced US imaging.⁷² The abovementioned alternative approaches to applying PFOB NPs in ultrasonography are summarized in Table 2.

PFOB NPs for drug delivery

PFOB NPs have been formulated to adhere to the surface of a target and produce highly concentrated zones.⁶¹ These targeted imaging effects may persist in the body from several minutes to possibly several days.⁷³ Therefore, a new generation of PFOB NPs as stable UCAs designed for therapeutic applications such as drug delivery or gene transfer has become available.⁵³ With the attachment of targeting ligands to the PFOB NP surface, PFOB NPs have been specifically directed to bind biomarkers of cancer, angiogenesis, thrombosis and other diseases.⁷⁴ Furthermore, targeting PFOB NPs to the tissue of interest also localizes drug release to the target site, resulting in a much higher effective drug concentration at that site. In addition, PFOB NPs may function as an efficacious drug delivery vehicle because their safety has been approved by the Food and Drug Administration (FDA).⁷⁵ PFOB NPs may serve as drug delivery vehicles in which drugs are dissolved in the outer lipid or polymer layer for subsequent delivery to target cells for site-targeted therapy. Therefore, many researchers have designed PFOB NPs with a drug or gene⁷⁶ payload as effective systems for delivery to a targeted site. In contrast to liposomal drug delivery, which generally requires endocytosis, the mechanism by which targeted PFOB NPs with a lipid-coating transport drugs involves lipid exchange or lipid mixing between the lipid monolayer of the delivery system and the targeted cell membrane, termed “contact-facilitated drug delivery.”^{77–80} PFOB NPs have been loaded with different drugs for various applications, such as anti-inflammatory arthritis therapy,^{32,81,82} bone fracture healing,^{83,84} dust mite-triggered asthma therapy,⁸⁵ cytotoxicity reduction in the loaded drug,⁸⁶ analyses of complement activation,⁸⁷ thrombolytic therapy,^{88,89} prevention of restenosis,^{77,90} atherosclerosis therapy and cancer therapy. The capacity

Table 2 Alternative approaches for applying PFOB NPs to ultrasonography in vivo

| Methods to achieve US imaging | Component of the shell | Target epitope | Main points | References |
|--|--------------------------|----------------------------|---|------------|
| Adaptation of ultrasonography parameters | PLGA | – | The detectability of PFOB NPs may potentially be improved using HFU | 28 |
| | Lecithin and cholesterol | – | PFOB NPs provided excellent blood pool contrast images with PDHI for >1 hour | 30 |
| | PLA | – | Novel multifunctional PFOB NPs were fabricated by loading PFOB into a PLA shell followed by surface functionalization with GO and Gd-DTPA The rabbit kidney was more clearly observed in PIHI mode than in B-mode US imaging | 52 |
| Adaptation of formulations | PLGA | – | A thinner shell produces a larger SNR and more compressible PFOB NPs PFOB NPs with a thick PLGA shell present a significant US enhancement of the blood pool during hepatic imaging | 9 |
| | PLA | – | Novel multifunctional PFOB NPs were fabricated by loading PFOB and SPIO into a PLA shell followed by surface functionalization with a PEGylated gold nanoshell The kidney of the rabbit showed obvious enhancement in PIHI contrast mode | 58 |
| | PLA | – | Multifunctional PFOB NPs underwent surface functionalization with a PEGylated gold nanoshell The kidney of the rabbit showed obvious enhancement in PIHI contrast mode | 39 |
| | PLA | – | Novel multifunctional PFOB NPs were fabricated by loading PFOB into a PLA shell followed by surface functionalization with GO and Gd-DTPA The rabbit kidney showed obvious enhancement in PIHI contrast mode | 52 |
| | PEG-PDLLA | – | All cavities of the heart, liver and kidney, particularly the horizontal axis of the right kidney, showed slight and homogeneous enhancement without any highlighted spots | 44 |
| Fabrication of targeted UCAs | Lecithin and cholesterol | $\alpha_v\beta_3$ integrin | Specific enhanced US imaging was achieved in transgenic mice whose ears exhibited squamous metaplasia | 63 |
| | Lecithin and cholesterol | FR | Images from the FR-overexpressing tumors displayed a higher intensity value than images from tumors expressing low levels of FR | 72 |

Abbreviations: FR, folate receptor; Gd-DTPA, gadolinium chelate; GO, graphene oxide; HFU, high-frequency US; PDHI, power Doppler harmonic imaging; PEG-PDLLA, poly(ethylene glycol)-*b*-poly(D,L-lactic acid); PFOB, perfluorooctylbromide; PFOB NPs, perfluorooctylbromide nanoparticles; PIHI, pulse inversion harmonic imaging; PLA, poly(lactic acid); PLGA, poly(lactide-co-glycolide) acid; SNR, signal-to-noise ratio; SPIO, superparamagnetic iron oxide; UCAs, US contrast agents; US, ultrasound.

to deliver a payload to the targeted site could be of great benefit, leading to the development of targeted anticancer and anti-atherosclerosis therapeutics based on this platform.

Drug delivery for cancer therapy

Although a number of anticancer drugs have been tested clinically, either their low uptake efficacy at the target or systemic toxicity has limited their therapeutic value.^{91–93} A satisfactory delivery vehicle can facilitate improved pharmacokinetics and biodistribution, decreased toxicities, improved solubility and stability and controlled release.^{94,95} PFOB NPs represent a potential suitable delivery vehicle, and surface functionalization of these NPs may improve the tolerability, site-specific delivery and controlled release of therapeutic agents.³³

Soman et al⁹⁶ incorporated melittin into the outer lipid monolayer of PFOB NPs. The favorable pharmacokinetics of PFOB NPs allow melittin to accumulate in murine tumors in vivo and produce an obvious reduction in tumor growth without any apparent toxicity compared with melittin alone.⁹⁶ Vascular cell adhesion molecule 1 (VCAM-1), which is expressed in tumor and atherosclerotic vessels, plays a pivotal role in the course of many inflammatory diseases.^{97–101} VCAM-1-targeted PFOB NPs were generated by Pan et al⁹⁹ for in vivo targeting in breast cancer (STAT-1-deficient) models. The authors observed a 4.9-fold increase in the number of targeted PFOB NPs in the tumor vasculature compared with nontargeted PFOB NPs, indicating that VCAM-1-targeted PFOB NPs may function as an ideal breast cancer-targeted delivery system for certain anticancer drugs.⁹⁹ Sulfatide is a

ligand of several extracellular matrix (ECM) glycoproteins, particularly tenascin-C (TN-C), which is highly upregulated in many different cancers, including breast, ovarian and prostate cancer.^{102,103} Sulfatide, a type of lipid, actively incorporates with other lipids in PFOB NPs and functions as part of the outer lipid monolayer component of the targeted ligand.³³ We added sulfatide to PFOB NPs as a targeted delivery system

for loading paclitaxel (PTX) to treat breast carcinoma. These sulfatide-targeted PFOB NPs were observed in breast cancer tissues, facilitated the delivery of the drug to the targeted breast cancer tumor (Figure 4) and improved the therapeutic outcomes of PTX in a mouse model (Figure 5).³³ Pan et al¹⁰⁴ incorporated a c-Myc inhibitor prodrug into $\alpha\beta_3$ -targeted PFOB NPs and reported that the formulation displayed good

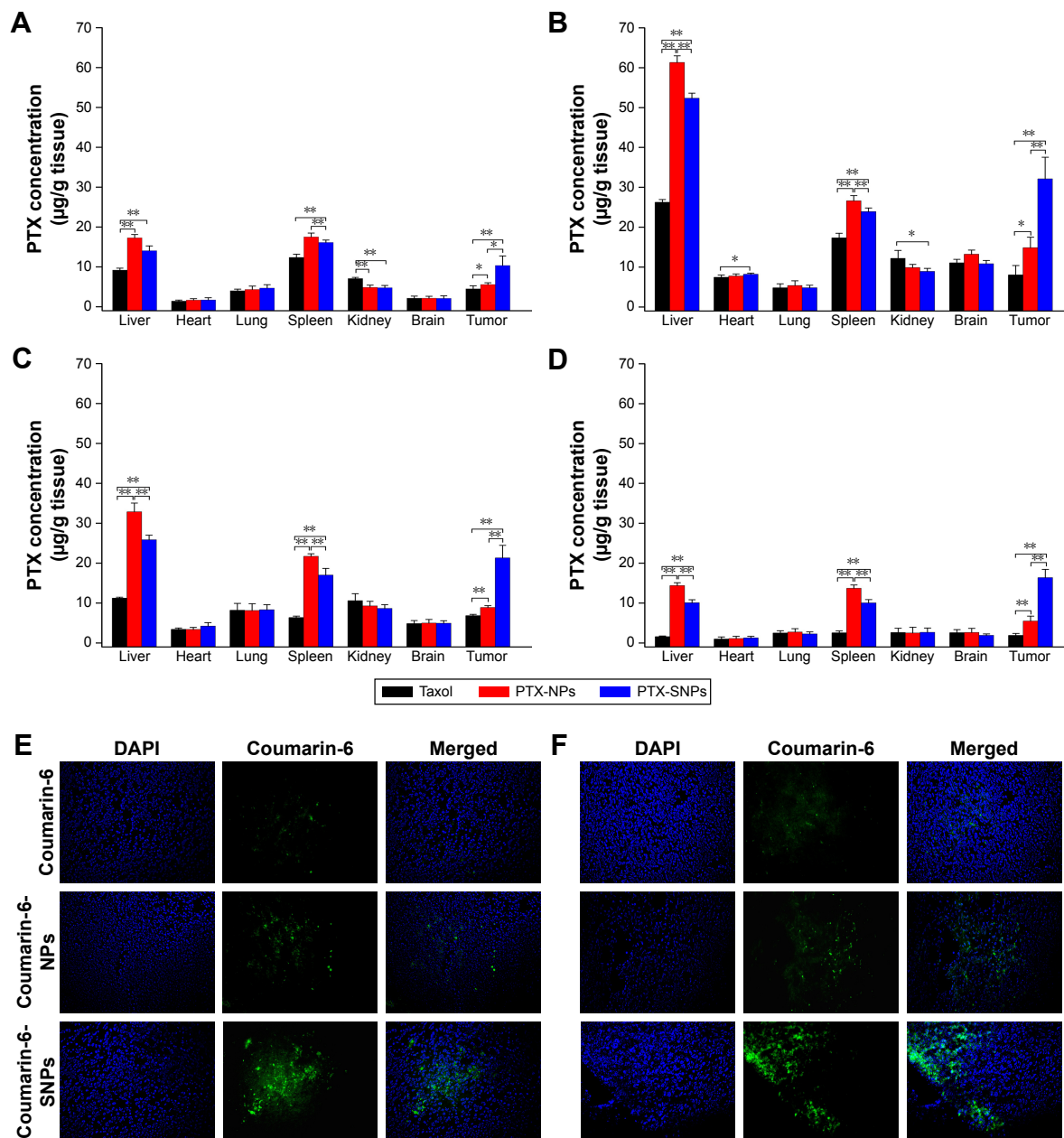


Figure 4 Images of the tissue distribution of PTX in EMT6 tumor-bearing mice obtained at 1 hour (A), 8 hours (B), 12 hours (C) and 24 hours (D) after intravenous administration of different PTX formulations. Values are presented as mean \pm SD (n=3). * P <0.05, ** P <0.01. (E) Representative images of the fluorescence of different coumarin-6 formulations in frozen slices of breast tumor tissues 1 hour after administration. (F) Representative images of the fluorescence of different coumarin-6 formulations in frozen slices of breast tumor tissue 8 hours after administration. Representative images of tumor sections were obtained at 200 \times magnification. PTX-NPs, PFOB NPs loaded with PTX; PTX-SNPs, sulfatide-targeted PFOB NPs loaded with PTX; coumarin-6-NPs, PFOB NPs loaded with coumarin-6; coumarin-6-SNPs, sulfatide-targeted PFOB NPs loaded with coumarin-6.

Note: Reprinted from Li X, Qin F, Yang L, Mo L, Li L, Hou L. Sulfatide-containing lipid perfluorooctylbromide nanoparticles as paclitaxel vehicles targeting breast carcinoma. *Int J Nanomedicine*. 2014;9:3971–3985.³³

Abbreviations: PFOB NPs, perfluorooctylbromide nanoparticles; PTX, paclitaxel; SNPs, sulfatide-targeted perfluorooctylbromide nanoparticles.

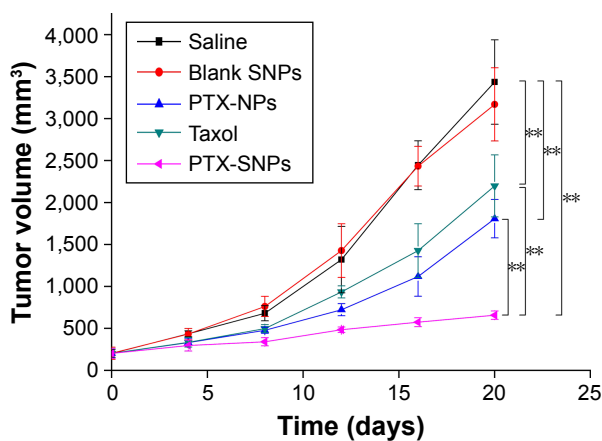


Figure 5 Tumor growth curves of EMT6 tumor-bearing mice after the administration of different PTX formulations.

Notes: Values are presented as mean \pm SD (n=6). PTX-NPs, PFOB NPs loaded with PTX; PTX-SNPs, sulfatide-targeted PFOB NPs loaded with PTX; blank SNPs, sulfatide-targeted PFOB NPs. $**p < 0.01$. Reprinted from Li X, Qin F, Yang L, Mo L, Li L, Hou L. Sulfatide-containing lipid perfluorooctylbromide nanoparticles as paclitaxel vehicles targeting breast carcinoma. *Int J Nanomedicine*. 2014;9:3971–3985.³³

Abbreviations: PFOB NPs, perfluorooctylbromide nanoparticles; PTX, paclitaxel; SNPs, sulfatide-targeted perfluorooctylbromide nanoparticles.

efficacy in preventing melanoma. Their laboratory subsequently showed that their $\alpha_v\beta_3$ -targeted PFOB NPs loaded with a c-Myc inhibitor prodrug extended the survival time in a mouse model of disseminated multiple myeloma.¹⁰⁵ Song et al¹⁰⁶ designed PFOB NPs loaded with hollow Bi₂Se₃ for the timely supply of oxygen under near-infrared irradiation to enhance cancer radiotherapy with good effectiveness. Vu-Quang et al¹⁷ designed multifunctional FA-targeted PFOB NPs loaded with doxorubicin in a PLGA shell and showed their good anticancer efficacy in vitro. PEGylated PFOB NPs loaded with PTX, which was dispersed in the PLGA shell of the PFOB NPs, were designed by Boissenot et al¹⁰⁷ and produced a promising and statistically significant twofold reduction in CT-26 tumor growth compared with generic Taxol® (Bristol-Myers Squibb Company, Princeton, NY, USA). Angiopep-2, which is known to bind to low-density lipoprotein receptor-related protein-1 (LRP-1), is an ideal dual-targeting moiety for a brain tumor tissue-targeted delivery system that also targets glioblastoma cells.^{108–111} A novel high-intensity focused US (HIFU)-responsive angiopep-2-targeted PFOB NP drug delivery system with a PLGA shell containing doxorubicin was developed by Luo et al¹¹² to enhance the targeted therapy of glioblastoma. The rate of drug delivery was greatly increased by the application of HIFU irradiation, which enabled the PFOB NP drug delivery system to achieve the strongest efficacy against glioblastoma.¹¹² Interestingly, the locations of the drugs (PTX or doxorubicin) in the PFOB NPs with a polymer shell for cancer therapy developed by Vu-Quang et al,¹⁷ Boissenot et al¹⁰⁷ and Luo et al¹¹² were ambiguous (Figure 6). Vu-Quang et al¹⁷ and Boissenot et al¹⁰⁷ reported

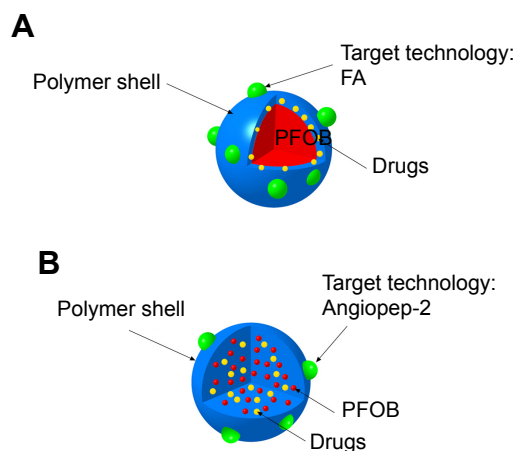


Figure 6 Schematic illustration of the location of doxorubicin or PTX in the PFOB NPs designed by Vu-Quang et al¹⁷ and Boissenot et al¹⁰⁷ (A). Schematic illustration of the location of doxorubicin in the PFOB NPs designed by Luo et al¹¹² (B).

Abbreviations: FA, folic acid; PFOB, perfluorooctylbromide; PFOB NPs, perfluorooctylbromide nanoparticles; PTX, paclitaxel.

that the loaded drug was located in the polymer shell (Figure 6A), whereas Luo et al¹¹² concluded that the doxorubicin used in their study was located in the center of the NPs along with PFOB (Figure 6B). For drug delivery, more studies have focused on PFOB NPs with a lipid coating than on PFOB NPs with a polymer coating. Thus, the location of the drugs in PFOB NPs with a polymer shell should be verified in further studies.

Angiogenesis is a normal physiological process required for the formation of new blood vessels from existing vessels.¹¹³ The maintenance, growth and metastasis of solid tumors also require angiogenesis, making it an attractive therapeutic target in the treatment of cancer.^{114,115} Biomarkers for angiogenesis, such as $\alpha_v\beta_3$ integrin, vascular endothelial growth factor (VEGF), VEGF receptor-2 (VEGFR2) and $\alpha_5\beta_1$ integrin, can serve as tumor targets.^{116–119} In the study by Schmieder et al,¹¹⁹ $\alpha_5\beta_1$ -targeted PFOB NPs containing fumagillin further decreased the extent of neovessel formation but did not affect the tumor volume in an MDA-MB-435 xenograft mouse model. The MDA-MB-435 tumor model exhibited a very sparse neovasculature with relatively slow growth, which may have caused the negative results.¹¹⁹ One molecular biosignature, $\alpha_v\beta_3$ integrin, has attracted prominent attention for angiogenesis-targeted applications, because it is involved in endothelial cell recruitment and proliferation, which are important steps in the formation of new blood vessels.⁷⁴ The utility of $\alpha_v\beta_3$ integrin as a biomarker of angiogenesis in cancer was studied in the VX-2 xenograft rabbit model. The $\alpha_v\beta_3$ -targeted fumagillin-loaded PFOB NPs designed by Winter et al¹²⁰ suppress neovasculature formation and inhibit the growth

of VX-2 adenocarcinoma xenografts in a targeted manner. The authors subsequently combined the antiangiogenic effect of $\alpha_v\beta_3$ -targeted fumagillin PFOB NPs with zoledronic acid to treat VX-2 adenocarcinoma xenografts. The dual antiangiogenic therapy decreased angiogenesis, stabilized tumor progression and enhanced the anticancer efficacy of chemotherapy.¹²¹ Pan et al¹²² utilized $\alpha_v\beta_3$ -targeted taxane prodrug-loaded PFOB NPs to suppress neovasculature formation and inhibit the growth of VX-2 adenocarcinoma in rabbits in a targeted manner with good efficacy.

Drug delivery for atherosclerosis therapy

Atherosclerotic plaques, which progress from an early atheromatous lesion to a vulnerable plaque through aggressive inflammatory and immune responses, comprise macrophage infiltrates with necrotic core enlargement, neovascular expansion of the vasa vasorum and intraplaque hemorrhage.^{123,124} Angiogenesis of the vasa vasorum is required for the progression of atherosclerosis;^{125,126} therefore, $\alpha_v\beta_3$ -targeted PFOB NPs, which were mentioned earlier as an antiangiogenesis tumor therapy, also act as a vehicle for atherosclerosis therapy. Winter et al¹²⁷ utilized the $\alpha_v\beta_3$ -targeted PFOB NPs for molecular imaging of angiogenesis in subjects with early-stage atherosclerosis and concluded that $\alpha_v\beta_3$ -targeted PFOB NPs can carry several drugs in their lipid monolayers, making them an attractive candidate for drug delivery to early atherosclerotic lesions. The $\alpha_v\beta_3$ -targeted PFOB NPs loaded with fumagillin prepared by Winter et al¹²⁸ delivered fumagillin and elicited a marked antiangiogenic response with minimal drug dosage. Rabbits with early atherosclerotic lesions administered a single

dose of $\alpha_v\beta_3$ -targeted fumagillin PFOB NPs displayed markedly reduced macroscopic adventitial angiogenic expansion of the vasa vasorum compared with the rabbits in the control group.¹²⁸ In a subsequent study, the authors developed a prolonged antiangiogenesis therapy for atherosclerotic rabbits using atorvastatin and $\alpha_v\beta_3$ -targeted PFOB NPs loaded with fumagillin. The combination of atorvastatin and $\alpha_v\beta_3$ -targeted fumagillin-loaded PFOB NPs synergistically sustained a prolonged antiangiogenic effect in atherosclerotic rabbits.¹²⁹ VCAM-1-targeted PFOB NPs were generated by Hua et al⁹⁹ for targeted atherosclerosis therapy in a (ApoE-deficient) mouse model in vivo. Their results prompted subsequent studies showing that VCAM-1-targeted PFOB NPs loaded with a suitable drug are an effective targeted atherosclerosis therapy.⁹⁹ The shell materials, loaded drugs and their locations and target epitopes of the PFOB NPs as a drug delivery system, which are described in the text, are summarized in Table 3.

Future perspectives

PFOB NPs are now a multifunctional nanotechnology with potential as both a drug delivery system and a diagnostic platform, and the promising results reported thus far have encouraged us to focus our attention on the following possibilities.

PFOB NPs for delivering drugs while serving as diagnostic agents

PFOB NPs offer great potential for coupling therapeutic functions with diagnostic functions, which indicates that PFOB NPs can serve as theranostic agents.^{19F}, which is highly enriched in PFOB NPs, functions as an excellent

Table 3 Topical delivery of certain drugs using PFOB NPs as potential carriers for cancer and atherosclerosis therapies

| Therapy | Shell material/ loaded drug | Target epitope | Drug localization | Drug transport mechanism | References |
|--|------------------------------------|----------------------------|----------------------|-----------------------------|------------|
| Loading of antitumor drugs for cancer therapy | Lipid/melittin | – | Lipid monolayer | Lipid exchange | 96 |
| | Lipid/PTX | TN-C | Lipid monolayer | Not determined | 33 |
| | Lipid/c-Myc inhibitor prodrug | $\alpha_v\beta_3$ integrin | Lipid monolayer | Lipid exchange | 104, 105 |
| | Bi ₂ Se ₃ /– | – | – | – | 106 |
| | PLGA/doxorubicin | FR | Polymer shell | No exposure | 17 |
| | PLGA-PEG/PTX | – | Polymer shell | No exposure | 107 |
| | PLGA/doxorubicin | LRP-1 | Center of the NPs | No exposure | 112 |
| Antiangiogenesis drugs for cancer therapy | Lipid/fumagillin | $\alpha_5\beta_1$ integrin | Lipid monolayer | Lipid exchange | 119 |
| | Lipid/fumagillin | $\alpha_v\beta_3$ integrin | Lipid monolayer | Lipid exchange | 120, 121 |
| | Lipid/taxane prodrug | $\alpha_v\beta_3$ integrin | Lipid monolayer | Lipid exchange | 122 |
| Antiangiogenesis drugs for atherosclerosis therapy | Lipid/fumagillin | $\alpha_v\beta_3$ integrin | Lipid monolayer | Lipid exchange | 128, 129 |

Abbreviations: FR, folate receptor; LRP-1, low-density lipoprotein receptor-related protein-1; NPs, nanoparticles; PFOB NPs, perfluorooctylbromide nanoparticles; PLGA, poly(lactide-co-glycolide) acid; PLGA-PEG, poly(lactide-co-glycolide) acid-polyethylene glycol; PTX, paclitaxel; TN-C, tenascin-C.

probe for quantitative MRI. PFOB NPs have been used to deliver drugs to a targeted site while concurrently providing direct confirmation of drug delivery and therapeutic efficacy via ^{19}F MRI.¹³⁰ A limitation for ^{19}F MRI is the low signal from targeted/localized PFOB NPs. Thus, PFOB NPs have been modified with payloads of paramagnetic chelates (eg, Gd-DTPA) for high-resolution diagnostic imaging and have been integrated with therapeutic agents for antiangiogenesis therapy.^{77,120,128,129,131,132}

In the clinic, two or more biomedical imaging techniques are usually applied together to achieve more reliable diagnostic results, as each imaging technique has its specific advantages and limitations.⁵⁸ Hyaluronic acid, a biocompatible and biodegradable material utilized in the development of various drug delivery systems, has also been widely used in the design and fabrication of drug conjugates and NPs for therapy.¹³³ Near-infrared dye-conjugated hyaluronic acid-encapsulating PFOB NPs represent another approach to realize the theranostics: the unique properties of PFOB NPs as contrast agents were enhanced by the coating agents to which they had been conjugated, endowing them with fluorescence in near-infrared imaging and cancer cell-tracking capabilities due to their interaction with hyaluronidases.^{134,135} Multifunctional UCAs designed by coating the outside of PFOB NPs with hyaluronic acid certainly have potential as theranostic agents for applications in which two or more biomedical imaging techniques are applied together.

PFOB NPs for delivering drugs while serving as diagnostic agents via ultrasonography

As a multifunctional nanotechnology, PFOB NPs have been evaluated for use in various medical applications. In contrast to CT/MRI, US imaging is a more cost-effective and accessible imaging technique and does not use ionizing radiation.¹³⁶ Nevertheless, the question remains: how do we design PFOB NPs to achieve therapeutic functions coupled with diagnostic functions via ultrasonography?

Importantly, in recent years, two laboratories^{63,72} have designed targeted PFOB NPs that successfully enhanced tumor US imaging *in vivo*. The most recently reported success in the application of targeted PFOB NPs with a lipid coating to targeted US imaging has inspired a substantial number of subsequent studies on achieving therapeutic functions coupled with diagnostic functions via ultrasonography. The capacity to enhance US imaging will be tremendously improved by developing targeted PFOB NPs to enhance US imaging of the site of interest,^{63,72} however, there is still

no study on the use of PFOB NPs to deliver drugs while serving as diagnostic agents for ultrasonography *in vivo*. The accumulation of targeted PFOB NPs within the target tissue at a level that is not sufficient to achieve high-quality US imaging enhancement remains a challenge for subsequent studies in this area. Although a single ligand might improve the tumor-targeted US imaging to some extent, the Arg–Gly–Asp-⁶³ or FA-modified⁷² PFOB NPs may not be sufficient to target tumor tissue for higher-quality tumor US imaging. Therefore, additional targeting strategies should be applied to improve the targeting ability of PFOB NPs with a lipid coating. We predict that to address this need, researchers will introduce various biological ligands into PFOB NPs as part of a novel dual-targeting concept, which may provide them with an opportunity to obtain higher-quality tumor US images. This novel dual-targeting concept has already improved the selective delivery of drugs to tumor tissues¹³⁷ and is anticipated to facilitate the use of PFOB NPs to further improve tumor US imaging and to achieve the goal of delivering drugs while serving as diagnostic agents for ultrasonography *in vivo* in the future.

In addition, to extend their applications, PFOB NPs must exhibit minimal toxicity. Recent studies have shown that cell fate can be dictated by the stiffness and topographical characteristics of the ECM.¹³⁸ Thus, the cellular uptake of NPs can also be regulated, which would influence the efficacy and toxicity of these nanomaterials. Through optimization of the shape and dimensions, particularly the height of nanotopography, the nuclear volume can be modulated through focal adhesion rearrangement to regulate cell function for the end application.^{139–141} Thus, delineation of the relationships between cell adhesion and nucleus and cell function may provide insight into the rational design of PFOB NPs as novel theranostic agents.

Conclusion

Our laboratory and many other groups have reported that PFOB NPs are a useful system for the delivery of therapeutic agents to target tissues with good efficacy. For drug delivery, more studies have focused on PFOB NPs with a lipid coating than on PFOB NPs with a polymer coating. In summary, we conclude that PFOB NPs, particularly PFOB NPs with a lipid coating, can serve as an effective drug delivery carrier for cancer and atherosclerosis therapies.

Although PFOB NPs are capable of increasing the visualization of US imaging and have been extensively studied, the pace of their development has been relatively slow. Sensitive US imaging will benefit from the optimization

of ultrasonography parameters, formulations and targeting abilities, as this nascent application still requires additional efforts. Polymer-coated PFOB NPs have been studied more frequently to optimize ultrasonography parameters and develop formulations for pool imaging in vivo, whereas lipid-coated PFOB NPs can be functionalized with specific ligands and have been studied for targeted tumor ultrasound imaging in vivo.

With a diverse portfolio of therapeutic and potential diagnostic functions via ultrasonography, PFOB NPs may play a promising role in many of the cutting-edge technologies that are currently being developed to fight the most pressing challenges facing the field of medicine today.

Disclosure

The authors report no conflicts of interest in this work.

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