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Therapeutic oxygen delivery by perfluorocarbon-based colloids

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ABSTRACT

After the protocol-related indecisive clinical trial of *Oxygent*, a perfluorooctylbromide/phospholipid nanoemulsion, in cardiac surgery, that often unduly assigned the observed untoward effects to the product, the development of perfluorocarbon (PFC)-based O₂ nanoemulsions ("blood substitutes") has come to a low. Yet, significant further demonstrations of PFC O₂-delivery efficacy have continuously been reported, such as relief of hypoxia after myocardial infarction or stroke; protection of vital organs during surgery; potentiation of O₂-dependent cancer therapies, including radio-, photodynamic-, chemo- and immunotherapies; regeneration of damaged nerve, bone or cartilage; preservation of organ grafts destined for transplantation; and control of gas supply in tissue engineering and biotechnological productions. PFC colloids capable of augmenting O₂ delivery include primarily injectable PFC nanoemulsions, microbubbles and phase-shift nanoemulsions. Careful selection of PFC and other colloid components is critical. The basics of O₂ delivery by PFC nanoemulsions will be briefly reminded. Improved knowledge of O₂ delivery mechanisms has been acquired. Advanced, size-adjustable O₂-delivering nanoemulsions have been designed that have extended room-temperature shelf-stability. Alternate O₂ delivery options are being investigated that rely on injectable PFC-stabilized microbubbles or phase-shift PFC nanoemulsions. The latter combine prolonged circulation in the vasculature, capacity for penetrating tumor tissues, and acute responsiveness to ultrasound and other external stimuli. Progress in microbubble and phase-shift emulsion engineering, control of phase-shift activation (vaporization), understanding and control of bubble/ultrasound/tissue interactions is discussed. Control of the phase-shift event and of microbubble size require utmost attention. Further PFC-based colloidal systems, including polymeric micelles, PFC-loaded organic or inorganic nanoparticles and scaffolds, have been devised that also carry substantial amounts of O₂. Local, on-demand O₂ delivery can be triggered by external stimuli, including focused ultrasound irradiation or tumor microenvironment. PFC colloid functionalization and targeting can help adjust their properties for specific indications, augment their efficacy, improve safety profiles, and expand the range of their indications. Many new medical and biotechnological applications involving fluorinated colloids are being assessed, including in the clinic. Further uses of PFC-based colloidal nanotherapeutics will be briefly mentioned that concern contrast diagnostic imaging, including molecular imaging and immune cell tracking; controlled delivery of therapeutic energy, as for noninvasive surgical ablation and sonothrombolysis; and delivery of drugs and genes, including across the blood-brain barrier. Even when the fluorinated colloids investigated are designed for other purposes than O₂ supply, they will inevitably also carry and deliver a certain amount of O₂, and may thus be considered for O₂ delivery or co-delivery applications. Conversely, O₂-carrying PFC nanoemulsions possess by nature a unique aptitude for ¹⁹F MR imaging, and hence, cell tracking, while PFC-stabilized microbubbles are ideal resonators for ultrasound contrast imaging and can undergo precise manipulation and on-demand destruction by ultrasound waves, thereby opening multiple theranostic opportunities.

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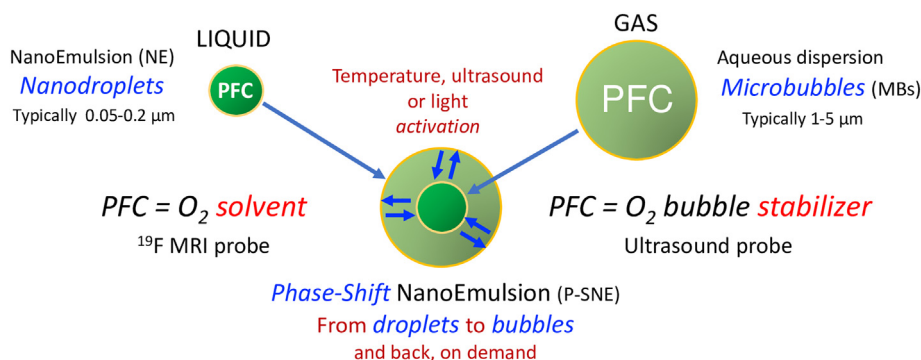
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1. Introduction

Once the flagship of medically-dedicated perfluorocarbon (PFC) research, and catching most of the limelight, oxygen-carrying PFC nanoemulsions, the so-called “blood substitutes”, have so far not met expectations. No satisfactory injectable O₂-delivering product has yet reached the marketplace. By contrast, another primary endeavor in medically-oriented PFC research, that is, the development of contrast-enhancing agents for diagnostic imaging, has been more successful. Several products have been licensed for use as contrast agents for ultrasound (US) imaging, as well as for ophthalmology and for in vivo cell tracking. The search for O₂-delivering therapeutics

has, however, not been forsaken. Further colloidal PFC products, including nanoemulsions (NEs), microbubbles (MBs) and phase-shift nanoemulsions (P-SNEs) (Scheme 1), are undergoing clinical investigations for O₂ delivery, in particular for O₂-dependent cancer radiotherapy, chemotherapy and photodynamic therapy. Concurrently, further PFC-based products are undergoing clinical trials for molecular imaging diagnosis of liver, breast, ovarian, prostate and colon cancers; guidance for surgical procedures; enhancement of immunotherapy; ablation of uterine fibroids; treatment of allergic asthma; treatment of smoke inhalation injury; delivery of drugs to the brain, as for management of brain tumors and neurodegenerative diseases, and assessment of treatment efficacy.



Scheme 1. Injectable PFC colloids most investigated in medicine: nanoemulsion (NE) droplets, phase-shift emulsion (P-SNE) droplets, and microbubbles (MBs). While for emulsions, the liquid PFC operates as an O₂ solvent, in MBs the primary role of the PFC gas is to stabilize the MB, which represents a drastic shift in paradigm and provides an alternative option for O₂ delivery: the direct administration, instead of O₂-loaded PFC nanodroplets, of PFC-stabilized O₂ MBs. P-SNEs, when activated by heat, ultrasound, laser light or other forms of energy, turn into PFC-stabilized MBs. Re-condensation into NE droplets can be achieved by pressurizing and/or cooling.

Starting with a critical assessment of a problematic Phase III trial of *Oxygent* (Alliance Pharmaceutical Corp., San Diego, CA), an O₂-carrying PFC NE product, in cardiac surgery, this short review will present PFC-based colloids that are, or may be, used for O₂ delivery. It will insist on the critical question of PFC selection and NE design and fabrication, highlight recent advances in the field and progress in our understanding of PFC behavior at fluid interfaces. After a brief reminder of the basics of PFC NE-based O₂ transport and delivery, it will expose further demonstration of their capacity to deliver O₂ to tissues in various circumstances. We will then discuss alternative O₂-delivery options that rely on the administration of PFC-stabilized injectable MBs or on MBs generated in vivo by vaporization of P-SNEs, as well as on other colloids and scaffolds that involve PFCs or other highly fluorinated components. We will also allude to other emerging PFC-based procedures and indications that are being investigated for diagnostic, therapeutic and theranostic purposes, embracing contrast imaging, cell tracking, controlled delivery of energy and of therapeutics, including across the blood-brain barrier. Even when developed for other purposes, these products possess a definite O₂ carrying capacity and the knowledge acquired in their investigation could benefit to O₂ carrier development. Finally, the Prospects section will highlight some critical issues, desirable developments, and offer some opinions about the development of PFC-based O₂ delivery products. The paper's organization is further detailed in the Table of Content.

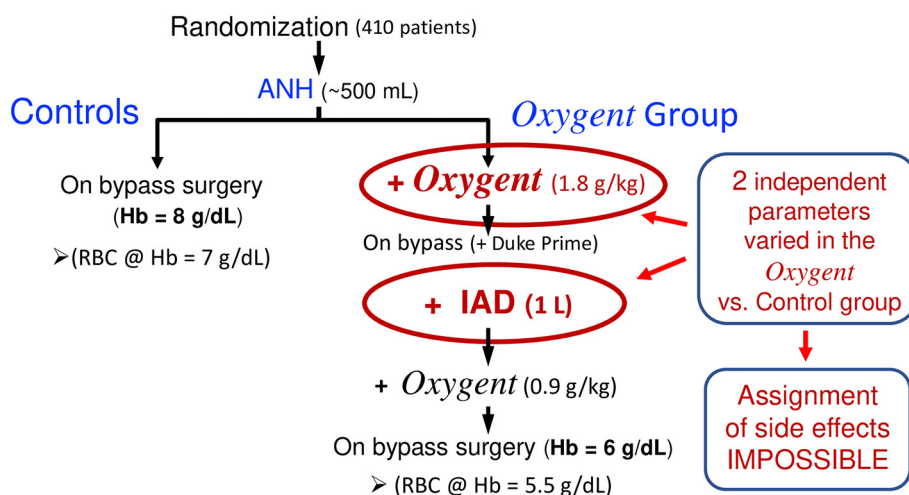
Citations are leaning towards recent reviews from which numerous earlier papers can be retrieved. The italicized prefix *F*- conventionally signifies perfluoro. *F*-colloids will refer to colloids comprising highly fluorinated components. Recourse to acronyms will be minimized. Commercial PFC-based product names are in *italics*.

2. An inconclusive clinical trial

PFC-based oxygen carriers have suffered a serious drawback when Alliance Pharmaceutical Corp. closed its operations subsequent to a failed clinical study. The development of the most advanced PFC nanoemulsion of that time, *Oxygent* AF0144, a phospholipid-shelled 60% w/v *F*-octylbromide (C₈F₁₇Br **3**) emulsion comprising a small fraction of *F*-decylbromide (C₁₀F₂₁Br **4**, 2%) for stabilization against Ostwald ripening [1,2], was interrupted in 2001 when a Phase III study in coronary artery bypass graft surgery patients showed a higher incidence of adverse neurologic and bleeding events in the test group versus control patients [3].

A previous Phase III clinical study in general surgery had established that *Oxygent* effectually reversed physiological transfusion triggers and diminished the need for transfusion of allogeneic red blood cells (RBCs) [4,5]. The *Oxygent*-treated patients remained stable and were adequately oxygenated at substantially lower hemoglobin (Hb) levels than the control patients; no major safety issues were reported. In cardiac surgery, an earlier Phase II study had indicated that *Oxygent*-treated patients could tolerate a greater amount of preoperative autologous blood harvesting and that the procedure tended to reduce or avoid allogeneic blood transfusion [5,6].

The failure of the subsequent cardiac surgery Phase III study was essentially due to a flawed clinical protocol that, by design, could not be conclusive. Indecisiveness was indeed inscribed in the protocol design (Scheme 2) because two independent parameters that affected the test patients and control patients differently were varied simultaneously [3,7]. Both patient groups first underwent autologous normovolemic hemodilution (ANH), but the *Oxygent* patients i) then received a first dose of the test material, while the controls did not, and ii) also underwent a rapid additional one-liter blood withdrawal (or intraoperative autologous donation, IAD), which the control group did not incur, thus creating a double imbalance between the two groups. Consequently, this protocol design made it impossible to allocate any observed higher incidence of untoward effects to the test material or to the additional large and rapid blood harvesting procedure solely incurred by the *Oxygent* patient group. Although the overall rates of adverse events were within expectations or lower than those reported in the literature, they were higher in the *Oxygent*-treated patients than in the control patients, leading to voluntary suspension of the trial [3]. These results should draw attention on the protocol that may have involved too rapid and too aggressive autologous blood harvesting, resulting in hypotension and hypoxia in critical organs, including the brain. At the time of the studies, PFC-augmented ANH was considered as rather safe [6,8]. However, the efficacy of mild ANH has been questioned [9], whilst deep ANH exposes the patient to low Hb levels that may not be well tolerated by some patients [10]. Hemodilution during cardiopulmonary bypass was since reported to cause neurologic injury in rats [11]. An independent direct association was also found between degree of hemodilution during cardiopulmonary bypass surgery and increased risk of perioperative stroke [12]. Greater decline in cognitive function was observed with elderly patients, for whom extreme hemodilution is thus not advisable [13]. The severe hemodilution



Scheme 2. Schematic representation of the study protocol (intraoperative phase) of a Phase III clinical trial of *Oxygent* in coronary artery bypass graft surgery patients. Both *Oxygent* and Control patients first underwent acute normovolemic hemodilution (ANH). Then, in contrast with the control patients, the test patients received both a dose of *Oxygent*, and underwent an additional blood withdrawal (intraoperative autologous donation, IAD), which reduced their hemoglobin (Hb) level to only 6 g/dL. Two independent variables (circled and squared area) were thus changed simultaneously in the control vs. test group, therefore forbidding assignment of any untoward effect to either product or protocol. Hence, no causal link can be drawn between the observed bleeding and neurologic effects and PFC emulsion. Adapted from [7].

Table 1
PFC properties useful for diagnostics and therapeutics.

<ul style="list-style-type: none"> o Thermal stability and chemical inertness o High gas solubilities and transport capacity o Extreme hydrophobicity, and hence, low water solubility, associated with strong lipophobicity o High propensity for self-segregation o High volatility/low Bp relative to MW o High density and compressibility o Low surface tension o Low dielectric constant and refractive index o Low viscosity 	<ul style="list-style-type: none"> o High bioacceptance o No metabolism, pulmonary excretion o Heat transfer capacities, cooling agents o Functional fluids for mechanical and acoustic pressure transmission o Can have a positive spreading coefficient o Magnetic susceptibility close to tissues' o ^{19}F MRI, most sensitive nucleus after ^1H o Absence of ^1H o Radiopaque, e.g. if brominated
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Outstanding properties and combinations of properties provide a unique toolbox for delivery of O_2 , energy, contrast, drugs, genes and other therapeutics.

used in the *Oxygent* trial may likely have constituted an independent risk of adverse neurologic outcome [14].

Subsequent analysis of all available clinical data concluded that excessive and too rapid hemodilution, rather than *Oxygent* administration, was the likely cause of, or primary contributor to the observed imbalance in adverse effects [3,15]. In other words, the clinical testing protocol, rather than the O_2 carrier, is probably responsible for these effects. It is noteworthy that the rate of untoward effects was also associated with a lower familiarity of surgical teams with the ANH procedure and with an inadequate management of blood pressure during rapid blood harvesting. The clinical data signpost the combined ANH and excessive and rapid IAD as an inappropriate procedure. The procedure was subsequently discouraged for patients with coronary or pulmonary disease [10]. No evidence was found that linked the imbalance in side effects to the emulsion. No perturbation of hemostasis, viscosity or tendency for coagulation was induced by the colloidal product that could be relevant to bleeding and neurologic events [7]. Altogether, this means that the too often repeated statement that the neurologic side effects seen during that trial were due to the product is, as of now, unfounded. Although the regulatory institutions (FDA and EMEA) did not object to resuming the *Oxygent* trials, the Company's financial situation did not allow it.

This setback did not preclude research and development of PFC-assisted diagnostic, therapeutic and theranostic procedures and nanomedicines from taking momentum in multiple areas. An effective, safe oxygen delivery system is still needed, including as a bridge to blood transfusion in case of emergency, trauma or shock; for treatment of ischemia related to myocardial infarction and stroke; for perioperative organ protection; potentiation of O_2 -dependent cancer therapies (e.g. radio-, chemo-, immunotherapies); regeneration of damaged tissues; preservation of isolated organs; and beyond. Many more PFC-related opportunities have emerged that do not aim primarily at O_2 delivery but could also serve for O_2 -delivery or co-delivery applications.

3. The perfluorocarbon colloids at work

Intravascular administration of PFCs requires their dispersion as injectable aqueous formulations. The colloids used in nanomedicines for O_2 delivery (Sections 3–6) and other purposes (Section 7) consist primarily of NEs, MBs, and P-SNEs (Scheme 1). The latter involve liquid NE droplets that can be converted in vivo into gas MBs upon activation (vaporization) by heat, sound or light. Further colloids intended for biomedical use that can carry and deliver O_2 include reverse water-in-PFC emulsions, multiple emulsions, highly fluorinated polymeric micelles, and various types of PFC-loaded nanoparticles (NPs) and scaffolds (Section 6).

3.1. The key components—their selection is a critical step

Selection of suitable PFCs and other colloid components, of their chemical structure, molecular weight (MW), purity, physical and biological characteristics, is a critical step in any investigation or development endeavor involving PFC colloids and depends decisively

on their intended use (Tables 1–3). PFC selection largely determines colloid stability, effectiveness, safety, practical developability, regulatory approval, and commercial success. Product consistency, current good manufacturing practices (cGMP) and quality control need to comply with regulations and will condition the acceptability of the drug product. Thorough pharmacokinetics (adsorption, distribution, metabolism and excretion) and pharmacodynamics (mechanism of action, biochemical and physiological dose-responses to drug, beneficial and adverse) studies are mandatory. Neglecting these rungs may nurture difficulties further along, from experimental data reproducibility and interpretation to sub-optimal patient care. As a result, clinical translation may be compromised and regulatory hurdles anticipated.

3.1.1. Selecting the perfluorocarbon

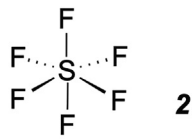
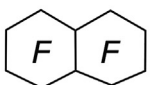
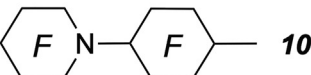
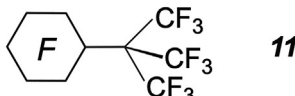
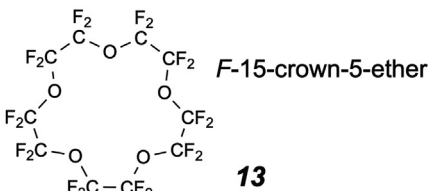
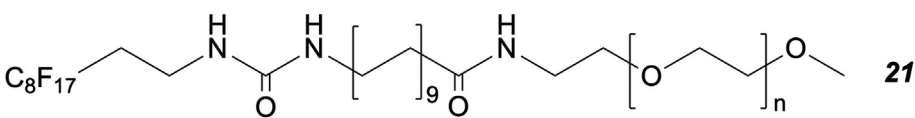
Perfluorocarbons form a vast family of man-made molecules that offer unique combinations of physical, chemical and biological properties, including high gas-dissolving capacity, volatility, density, compressibility and heat-transfer capacity; extreme hydrophobicity, distinctively coupled with marked lipophobicity; low surface tension, magnetic susceptibilities that can match those of tissues; high ^{19}F nuclei concentration and absence of protons (Table 1). These features are associated with excellent thermal, chemical and biological inertness, including absence of metabolism [2,16–20]. The chemistry of PFCs is much simpler than that of hemoglobin and can be precisely controlled, especially for linear PFCs [1,21]. The PFCs used for medical purposes should be clearly distinguished from *F*-alkylated surfactants (next sub-section).

Our basic understanding of PFC behavior at interfaces and in dispersed self-assemblies has progressed significantly [2,7,22–25]. PFCs were found, quite unexpectedly, to exercise co-surfactant activity in phospholipid films [26–28]. PFCs are partly soluble in phospholipid monolayers, especially when light or lipophilic, and can modify their properties [29–31]. The amount of *F*-hexane present in phospholipid monolayers has been quantified by neutron reflectometry [32]. A trans-membrane fluorine/fluorine recognition interaction has been discovered that has implications for drug loading and delivery [23,33–35]. More basically, it has been recognized that a PFC present within a colloid can no longer be viewed as forming an entirely separate inert phase, but needs now to be considered as an active component that can exercise its influence well beyond and even across interfacial boundaries (Fig. 1). The notion that, PFCs being inert, any interactions with cells and tissues are solely with the colloid's shell, is no longer valid.

The earliest commercial PFC NE-based injectable O_2 -carrying product, *Fluosol* (based on a mixture of *F*-decalin **9** and *F*-tripropylamine **6**; Green Cross Corp., Japan) was approved by the FDA in 1989 for reducing tissue ischemia in high-risk transluminal coronary balloon angioplasty, but could not achieve commercial success, largely because, prior to use, it required a complicated and cumbersome, multi-step thawing and reconstitution procedure involving three separate packages, one of which, the stem emulsion, had to be shipped and stored in the frozen state.

The criteria for PFC selection for NE products have since been periodically reassessed and refined [1,2,36]. Relevant properties can indeed

Table 2
Perfluorochemicals and fluorosurfactants frequently used in medical fluorinated colloid design.

<i>F</i> - <i>n</i> -alkanes C_nF_{2n+2} 1	$n = 3$: <i>F</i> -propane $n = 4$: <i>F</i> -butane $n = 5$: <i>F</i> -pentane $n = 6$: <i>F</i> -hexane $n = 8$: <i>F</i> -octane	 Sulfur hexafluoride 2
$C_8F_{17}Br$ <i>F</i> -octylbromide 3		 <i>F</i> -decalin 9
$C_{10}F_{21}Br$ <i>F</i> -decylbromide 4		 10
$ClC_8F_{16}Cl$ <i>F</i> - α,ω -dichlorooctane 5		<i>F</i> -methylcyclohexylpiperidine
$N(C_3F_7)_3$ <i>F</i> -tripropylamine 6		 11
$N(C_4F_9)_3$ <i>F</i> -tributylamine 7		<i>F</i> - <i>t</i> -butylcyclohexane
$C_4F_9CH = CHC_4F_9$ 8		
$R[CF_2CF_2O]_nR'$ <i>F</i> -poly(ethylether) 12		 <i>F</i> -15-crown-5-ether 13
$C_nF_{2n+1}C_mH_{2m+1}$ 14 <i>F</i> -(alkyl)alkane (<i>F</i> <i>nH</i> <i>m</i> diblock)		
$C_nF_{2n+1}(CH_2)_mOP(O)[N(CH_2CH_2O)]_2$ (<i>F</i> -alkyl)alkyl dimorpholinophosphate 15		
$C_7F_{15}C(O)NH(CH_2)_3N(O)(CH_3)_2$ <i>F</i> -alkylated nitric oxide 16		
$F-(CF_2CF_2)_{3-8}-[CH_2CH_2O]_y-H$ (<i>Zonyl</i> FSO) 17		
$F-[CF(CF_3)CF_2O]_n-CF(CF_3)COOH$ (<i>Krytox</i> 157FS) 18		
$C_3F_7O-[CF(CF_3)CF_2O]_n-CF(CF_3)C(O)NH-[CH_2CH_2O]_m-CH_2CH_2NHC(O)CF(CF_3)-[OCF_2CF(CF_3)]_n-OC_3F_7$ 19		
$C_3F_7O-[CF(CF_3)CF_2O]_n-CF(CF_3)C(O)N(CH_2CH_2OCH_2CH(OH)CH_2OH)_2$ 20		
 21		

differ substantially among PFCs, including within a homologous series, as they are often highly sensitive to MW. For any in vivo use, PFCs should be pure and well defined in terms of molecular composition, which depends greatly on synthetic procedure. When used parenterally, their organ retention half-life should be limited to a few days. Industrial production feasibility in better than 99% purity on a large scale at a reasonable cost is needed. The PFC must also be amenable to production of stable, highly reproducible small-sized narrowly dispersed heat-sterilizable and easily scalable NEs. Frozen storage or need for any reconstitution procedure in the operating theater is hardly acceptable.

The tetrafluoroethene telomerization route can achieve the required degree of purity for linear PFCs and also benefits from easy scalability. Linear PFCs have a non-negligible advantage over cyclic ones of comparable MW in term of O_2 solubility (e.g. 50 vol% for *F*-octylbromide **3** vs. 40 vol% for *F*-decalin **9** at 25 °C) [1]. Notably, CO_2 solubility is always much larger than O_2 solubility. The organ retention half-life of PFCs, a critical selection parameter, is primarily correlated, and increases exponentially with their MW (e.g., for the same dose, *F*-decalin **9**, MW 462: ~7 days; *F*-tripropylamine **6**, MW 521: ~65 days; *F*-tributylamine **7**, MW 671: over 2 years). Organ retention can be effectually lessened by

Table 3
Selected physical properties of frequently used perfluorochemicals in medical colloids.

Compound	F-pentane	F-hexane	F-octane	F-octylbromide	F-decalin (cis + trans)	F-tripropylamine	F-tributylamine	F-15-crown-5-ether
Formula	C ₅ F ₁₂	C ₆ F ₁₄	C ₈ F ₁₈	C ₈ F ₁₇ Br	C ₁₀ F ₁₈	N(C ₃ F ₇) ₃	N(C ₄ F ₉) ₃	(CF ₂ CF ₂ O) ₅
MW (g mol ⁻¹)	288	338	438	499	462	521	671	580
Molar volume (cm ³ mol ⁻¹)	177	201	253	261	238	285	381	–
Boiling point (°C)	28–30	58–60	99–106	143	140–143	131	178	145
Density (g cm ³ , 25 °C)	1.59	1.68	1.73	1.92	1.94	1.74	1.88	1.78
Saturated vapor pressure (10 ³ Pa, 25 °C)	85 ^a	27–29 ^a	5 ^a	1.4 ^b	1.9 ^b	1.4	0.2	–
Water solubility (mol L ⁻¹)	4.0 10 ⁻⁶ ^a	2.7 10 ⁻⁷ ^a (25 °C)	3.8 10 ⁻⁹ ^a (25 °C)	5 × 10 ⁻⁹ ^b	10 × 10 ⁻⁹ ^b	2.8 × 10 ⁻¹⁰ ^a	–	–
O ₂ solub. (vol%, 25 °C)	54 (est.)	70.0	52.1 ^c	52.7 ^c	40.3 ^c	45.3 (37 °C) ^d	33.2–38 ^d	–
CO ₂ solub. (vol%, 25 °C)	–	156	–	210 ^c	142 ^c	166 (37 °C) ^d	127–152 ^d	–
CST _{hex} (°C) ^e	–	5	20	–20	22	32	60	–
Organ T _{1/2} (days) ^{e,f}	1.8–2.5 min ^g	~8 h (est.)	~3 (est.)	~4	6–7	65	900	–

^a [39].

^b [1].

^c [40].

^d [41].

^e Estimated using [38].

^f [36].

^g [42].

conferring some lipophilic character to the PFC (as reflected by a reduction of its critical solution temperature in hexane or increased solubility in olive oil). This was achieved by introducing a polarizable bromine atom as in *F*-octylbromide (MW 499), thus reducing its organ half-life to 3–4 days in humans (for a 2.7 g/kg dose). Similar lipophilic character is obtained with *F*-octyl ethane, C₈F₁₇C₂H₅ **14** ($n = 8, m = 16$) or by introducing two chlorine atoms, as in *F*- α,ω -dichlorooctane **5**. The PFCs most frequently used for medical purposes are collected in Table 2 and some of their physical characteristics provided in Tables 3 and 4. Eventually, only a very small number of PFCs were found eligible for intravascular O₂ delivery [1,2,37,38].

F-octylbromide (PFOB, perflubron, **3**) stands out among PFCs for medical uses. It provides a good compromise in terms of excretion rate, O₂ and CO₂ solubilities, capacity for forming stable NEs and cost-effective high-purity industrial feasibility. The very low equilibrium interfacial tension with water that can be reached in the presence of egg phospholipids, near 1 mN m⁻¹ [47], heightens phospholipid-based NE stability. Additionally, its radiopaque bromine atom provides contrast for diagnostic radiography, and its positive spreading coefficient (2.7 mN m⁻¹) is advantageous in pulmonary applications.

The perfluorochemicals selected for preparing MBs and P-SNEs are generally the short volatile *F*-alkanes (C_{*n*}F_{2*n*+2}, $n = 3$ to 6, **1**), several

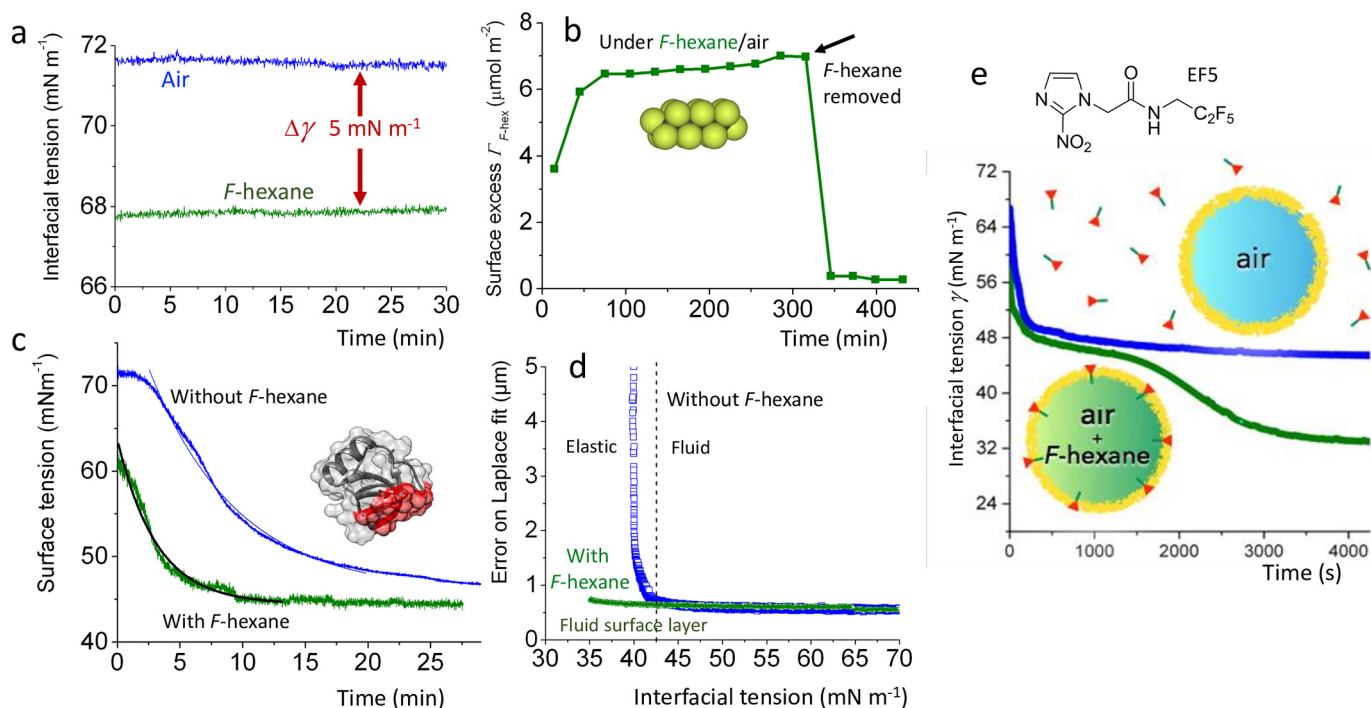


Fig. 1. Exposure to a PFC gas can impact colloid shell structure and properties, as demonstrated on interfacial film studies. a) *F*-hexane is surface active, reduces air/water interfacial tension (bubble shape analysis tensiometry); b) its surface excess is ~6 μmol m⁻², as measured by neutron reflectometry; c) it accelerates the diffusion of film-forming protein hydrophobin HFB II at the air/water interface; d) it can erase the fluid/elastic phase transition, thereby fluidizing the interfacial protein film; e) *F*-hexane can also help recognize, recruit and immobilize hydrophilic species, here a hypoxia biomarker (EF5), across an interfacial phospholipid monolayer. Adapted from a) [27], b) [32,35], c-d) [33] and e) [23].

Table 4

Selected physical properties of frequently perfluorochemicals in their gas phase.

Compound	Sulfur hexafluoride	F-propane	F-butane	F-pentane	F-hexane
Formula	SF ₆	C ₃ F ₈	C ₄ F ₁₀	C ₅ F ₁₂	C ₆ F ₁₄
MW (g mol ⁻¹)	146	188	238	288	338
Molar volume (cm ³ mol ⁻¹)	64	145	153	177	201
Boiling point (°C, 1 atm)	-68 (subl.)	-36 to -39	-1.7	28-30	58-60
Density (g cm ⁻³)	1.37 ^a	1.35 ^b	1.52	1.59	1.69 ^c
Saturated vapor pressure (10 ³ Pa, 25 °C)	2.24 × 10 ³ (20 °C) ^a	0.98 × 10 ³ (29 °C) ^b	2.6 × 10 ^{2d}	85 ^d	27-29 ^d
Saturated vapor concentration (25 °C, mol m ⁻³)	-	535 (30 °C) ^b	105.6 ^d	34.14 ^d	11.66 ^d
Water solubility ^e (mol m ⁻³)	-	-	0.021 ^d	4.0 × 10 ^{-3 d}	2.7 × 10 ^{-4 d}
Ostwald coefficient ^e (C _{water} /C _{gas})	5.4 × 10 ⁻³	5.2 × 10 ⁻⁴	2.02 × 10 ⁻⁴	1.17 × 10 ⁻⁴	2.3 × 10 ⁻⁵
Diffusivity coefficient in water ^e (m ² s ⁻¹) [*]	1.2 × 10 ⁻⁹	7.45 × 10 ⁻¹⁰	6.9 × 10 ⁻¹⁰	6.3 × 10 ⁻¹⁰	5.8 × 10 ⁻¹⁰
Permeability through a shell (m s ⁻¹) ^{e**}	8.7 × 10 ⁻⁶	1.2 × 10 ⁻⁶	2.57 × 10 ⁻⁷	9.04 × 10 ⁻⁸	4.44 × 10 ⁻⁸

^a [43].^b [44].^c [45].^d [39].^e [46].^{*} Diffusivity of air in water: 2.05 × 10⁻⁹ m² s⁻¹.^{**} Permeability of air through the shell: 2.857 × 10⁻⁵ m s⁻¹.

of which are gaseous at room or body temperature, or sulfur hexafluoride, SF₆ **2**, which has a comparably dense fluorine covering and properties similar to those of C₂F₆ and C₃F₈. *F*-pentane is commonly used because its boiling point (29 °C) is close to body temperature. Partially hydrogenated PFCs (e.g. 2H,3H-decafluoropentane) have been proposed to help adjust the boiling point [48]. PFC mixtures, e.g. *F*-butane with *F*-pentane, should be used precociously since the lighter PFC will tend to diffuse out of a nanodroplet or microbubble faster than the heavier one, causing composition and properties to vary over time.

The rather heavy *F*-15-crown-5-ether **13** and *F*-poly(ethylethers) (R(CF₂CF₂O)_{*n*}R' **12** (*n* ~ 10), with their numerous magnetically equivalent fluorine nuclei, are often selected when ¹⁹F MRI is a primary objective and the injected doses are small. Bis(*F*-butyl)ethene **8**, which is easily produced in pure form, has an organ retention half-life comparable to that of *F*-decalin and a superior O₂ solubility [1], may also provide a valuable candidate for O₂ delivery. Various functional *F*-alkylated or *F*-arylated components that are being used in micelles and nanoparticles generally intended for drug delivery, also display O₂-transport abilities (Sections 3.5.4 and 6).

3.1.2. Emulsifiers and other shell components

The most commonly used emulsifiers and shell components of medical *F*-colloids are pharmaceutical grade phospholipids. Many injectable NEs rely on egg yolk phospholipids, a natural mixture of phospholipids that has a long history of use in lipid emulsions for parenteral nutrition. Complementation of phospholipids with molecular fluorocarbon-hydrocarbon diblock amphiphiles (C_{*n*}F_{2*n*+1}C_{*m*}H_{2*m*+1}, *F**n*H*m* **14**) provides outstanding NE stability [49,50]. Other frequently used shell constituents include proteins such as albumin (generally

heat-denaturized), red blood cell or macrophage envelope fragments or components [51–55], and polysaccharides such as chitosan [56], dextran [57] or hyaluronic acid [58]. Synthetic polymers include poly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene) triblock copolymers (poloxamers, such as *Pluronics*) and poly(lactic-co-glycolic acid) (PLGA), which is biodegradable and FDA-approved, and silica. The latter usually provide more rigid shells or capsules. Multiple superposed coatings are also utilized [59,60]. The capacity to emulsify PFCs, stabilize PFC colloids, and other properties have been investigated for a large panoply of molecular and polymeric surfactants [17,61]. A study of the safety of poly(*n*-butyl cyanoacrylate)-encapsulated *F*-decalin uncovered several side effects and the accumulation of polymeric material in various organs [62]. Product formulation may also include electrolytes, oncotic agents and other active components. In any case, PFC formulations need to be compatible with any fluid that may be administered concurrently during treatment [63–66].

MBs, which are generally dispensed in much smaller doses than O₂-carrying NEs, are commonly shelled with synthetic mixtures of phospholipids such as dipalmitoyl- and distearoylphosphatidylcholine (DPPC and DSPC), often supplemented with poly(ethylene glycol)-fitted (PEGylated) phospholipids (e.g. DSPC-PEG2000) that provide added stability and a hydrophilic coating that helps evade phagocytosis. Addition of dipalmitoylphosphatidylethanolamine (DPPE), egg phosphatidylserine, or phosphatidic acids confers negative charges to the shell. An additional emulsifier (e.g. lipopolymers such as DSPE-PEG2000) appears indispensable for MB formation [67]. An amount of ~5% of DSPE-PEG2000 was deemed optimal for DPPC MB abundance, stability and mechanical properties [68]. Span 60 (sorbitan monostearate) was also used to stabilize O₂ MBs [69], as well as Tween 80 (poly(oxyethylene) sorbitan monooleate) [70]. The shell

Table 5

Properties and issues of PFC nanoemulsions related to PFC versus colloid shell and versus colloidal form.

Related to the PFC	Related to the shell	Related to the colloidal form
<ul style="list-style-type: none"> Linear O₂ vs. pO₂ dissolution – no bonding no saturation passive delivery, instant availability high extraction ratio O₂ solubility preserved at lower temp. CO₂ dissolution and removal ensured No NO scavenging and vasoconstriction Emulsion stability increases with MW Organ retention increases with MW, decreases with lipophilic character Diagnostic imaging (multiple ¹⁹F, no ¹H) Can dissolve highly fluorinated agents 	<ul style="list-style-type: none"> Contributes to stability Can affect droplet size Can affect rheology, mechanical prop. Reduces diffusion of dissolved gases Affects PFC's vaporization rate Can affect in vivo recognition, intravascular persistence organ distribution side effects Can be functionalized Can be targeted to disease Can host therapeutics for delivery Can serve for binding other colloids 	<ul style="list-style-type: none"> Small sizes vs. RBC (0.1-0.3 vs. 7 μm) no extravasation, blood pool agent Normally restricted to blood pool Many particles - facilitates O₂ diffusion Viscosity similar to water, plasma Mechanical resistance (pumps, filters) Foreign particles - RES clearance, short intravascular persistence, may activate macrophages (flu-like symptoms) High yield additive production process Easy large-scale manufacturing Terminal heat sterilization possible

formulation of P-SNEs that serve as precursors of MBs is often similar to that of MBs.

Fluorosurfactants (*F*-surfactants), which associate a highly fluorophilic and lipo- and hydrophobic *F*-alkyl tail with a hydrophilic polar head, develop the ultimate in terms of surface activity, fostering numerous industrial applications, as well as valuable “fluorous” synthesis and separation technologies [16,18,71,72]. *F*-surfactants hold a distinctive place when it comes to PFC colloid preparation [17,19,73–75]. Both molecular and polymeric *F*-surfactants (Table 2) allow production of stable NEs, MBs, reverse water-in-PFC emulsions, multiple emulsions or high internal phase ratio gel-emulsions. While use of *F*-surfactants is no longer needed for stable NE production, they are often used for preparing P-SNEs from volatile PFCs [76–78], and have so far been systematically used for reverse PFC emulsion preparation. *F*-surfactants are also relevant to colloid preparation through microfluidic procedures. Highly effective, single-molecule *F*-surfactants (e.g. **15**, **16**) have been derived from polar heads such as phosphatidyl groups, sugars, proteins and more [17,79]. Such *F*-surfactants have an outstanding propensity to segregate, self-assemble into compartmentalized colloids [80]. Polymeric *F*-surfactants fulfill numerous industrial purposes [71]. Those most frequently used for medical *F*-colloid preparation comprise a *F*-alkyl chain grafted on a hydrophilic PEG chain (e.g. *Zonyl* FSO **17**, now replaced by the *Capstone* series, e.g. *Capstone* FS-63, that features an environmentally more acceptable shorter *F*-hexyl hydrophobic chain) or a poly(*F*-propylene oxide) chain with a terminal carboxylic function (e.g. *Krytox* 157 FS **18**, Chemours, Wilmington, DE). Amphiphilic triblock PEGylated fluorinated amphiphiles **19** were used to stabilize phospholipid-shelled *F*-butane MBs [75]. *F*-surfactants **20** with poly(*F*-propylene oxide) chains and dendritic glycerol polar head groups were shown to minimize inter-droplet exchange of small encapsulated molecules [81]. *F*-surfactant **21**, with a *F*-octyl chain, a hydrocarbon spacer and a PEG chain was synthesized to stabilize cationic *F*-butane/lipid MBs [82]. Stable but large-sized Pickering-type (i.e. surface NP-stabilized) NEs of *F*-pentane, *F*-hexane, *F*-octylbromide were obtained with (*F*-octyl)ethyl grafted silica NPs [83]. When comparable in effectiveness, short molecular *F*-surfactants should likely be preferred over the less well defined, polydisperse polymeric ones. The use of long chain functional *F*-alkylated substances, which are eventually degraded into persistent *F*-alkyl acids, is now strictly regulated due to environmental concerns [84]. However, exemptions should be obtainable for medical uses when no satisfactory alternative is available, mainly because such uses only involve very small tonnages and deliver a very high benefit/risk ratio.

3.2. Nanoemulsions of gas-dissolving perfluorocarbons

Nanoemulsion design, formulation, stability and property control, and understanding of in vivo behavior have progressed. The core PFC, surrounding shell, and colloidal form play different roles in the colloid's overall behavior that need to be clearly differentiated (Table 5). NE droplet size and size distribution are here central parameters. Average droplet size in the currently investigated concentrated (20–60% w/v) injectable NEs is in the 0.1–0.3 μm range, well below the $\sim 7 \mu\text{m}$ of RBCs. Intravascular persistence, and hence O_2 -carrying duration, augments when droplet size is reduced (Fig. 2). Concurrently, side effects such as the flu-like symptoms related to particle clearance from the circulation by the reticuloendothelial system diminish when droplet size diminishes [85]. A tail of large particles in the droplet size histogram should therefore be avoided [1,2].

Particle size increase over time in PFC NEs is primarily due to molecular diffusion (Ostwald ripening). The latter can easily be counteracted by adding a higher MW PFC, which, however, does not go without a sharp, soon unacceptable increase in organ dwelling time. Resolution of this catch-22 situation can again be achieved by selection of a somewhat lipophilic higher MW PFC additive. Addition of 2 w/vol% of *F*-decylbromide (organ half-life around 23 days) in a 58 w/vol%

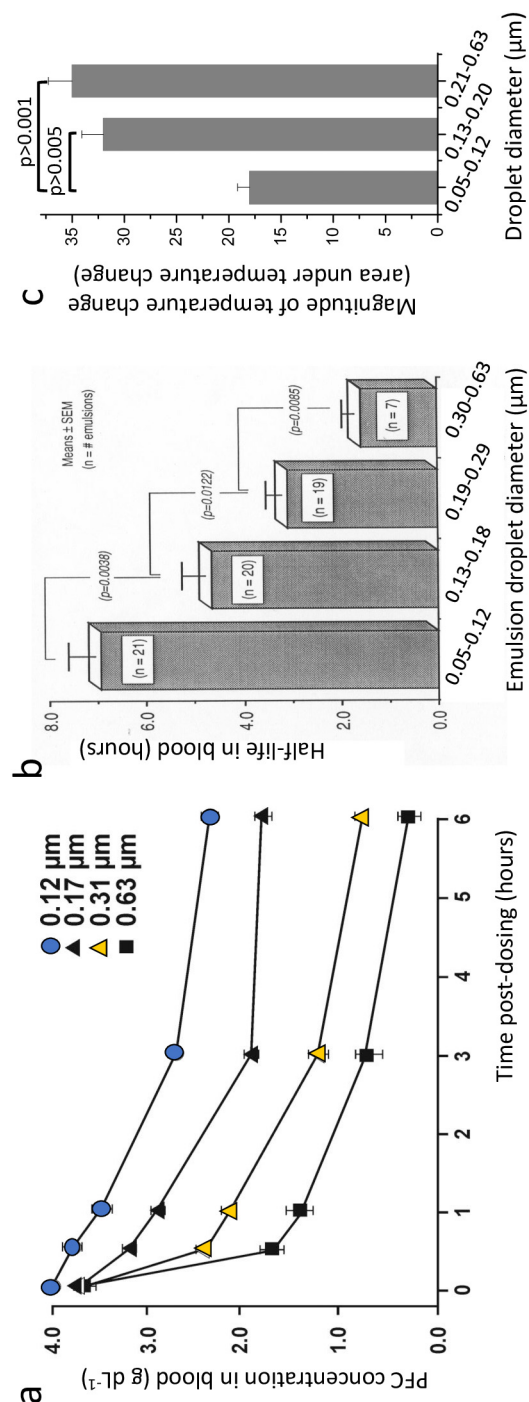


Fig. 2. Effects in rats of mean nanoemulsion droplet size on a) intravascular persistence over time; b) blood half-life; c) febrile response to NE administration (the area under the change in temperature over time curve integrates both intensity and duration). Adapted from [85].

emulsion of *F*-octylbromide solved the problem in the case of *Oxygent* [38,86].

A different approach consists in increasing the cohesion of the PFC/phospholipid interface by complementing the phospholipid emulsifier with an equimolar amount of a semi-fluorinated alkane (*F_nH_m*) diblock that can be envisioned as providing “molecular dowels” at the interface [22,49,50]. This approach produced NEs that withstand standard heat sterilization and forced coalescence through shaking, and proved stable for over two years at room temperature (Fig. 3). Precise control of NE droplet size could be achieved over an extended range, typically from 0.012 to 16 μm post-sterilization. Further stabilization schemes use more rigid polymeric droplet shells or capsules, which have their own advantages and limitations.

Diverse technologies can be implemented for PFC NE production. The most effective in terms of droplet size control, reproducibility and throughput rely on high-pressure, high-shear homogenizers commonly used in industry. Impinging jet devices (microfluidization) also produce quality PFC NE batches. Uniform commercial-scale terminal heat sterilization can be achieved in a rotating autoclave at 121 °C in standard conditions when the NE is stable enough to withstand these conditions. Sterilization of metastable colloidal formulations requires, however, specific knowhow, multiparameter optimization and thorough validation. Lab-scale samples are most often prepared by tip sonication, which is easy to implement but yields widespread droplet size distributions, has poor reproducibility and defies lab-to-lab comparisons. Small-capacity high-shear emulsifiers (e.g. *Emulsiflex*, Avestin, Ottawa, Canada) are available that secure much better droplet size control and reproducibility, and should be preferred for lab-size NE production.

3.3. Perfluorocarbon-stabilized injectable micro- and nanobubbles that respond to ultrasound

We will see (Section 5) that O_2 MBs can offer an alternate approach to parenteral NE-mediated O_2 administration. MB design, preparation and characterization have been discussed in numerous articles [67,87–108].

Naked MBs are extremely short-lived (Fig. 4). Gaseous PFCs (or SF_6) were chosen as the core of MB contrast agents for US imaging because of their much lower solubility in water as compared to O_2 or N_2 (e.g. $4.0 \cdot 10^{-6} \text{ mol L}^{-1}$ for *F*-pentane vs. $1.2 \cdot 10^{-3} \text{ mol L}^{-1}$ for O_2), thus extending the MBs circulatory half-life sufficiently to enable practical diagnostic examination.

When MBs containing only a PFC gas are introduced in the circulation they will absorb gases present in the blood and swell until a diffusion equilibrium is reached and the partial pressure of air in the MB equals the ambient concentration and the partial pressure of the PFC equals the joint Laplace and arterial blood pressures (Fig. 4b). To prevent in vivo swelling, MBs can be designed in which the partial pressure of the PFC exactly counterbalances the Laplace and blood pressures (Fig. 4c) thereby ensuring safe, sustained circulation [39,87,110]. This osmotic stabilization principle was applied to the development of *Imagent*, a product that was provided as spray-dried microspheres made of water-soluble hydroxyethylstarch, DMPC and a poloxamer under an atmosphere of *F*-hexane and N_2 in such proportions ($\sim 1/5$) that the MBs formed after dissolution of the microspheres would stabilize around 3 μm in vivo and could not grow. The same principle was used to produce DSPC/DSPE-PEG2000-shelled injectable O_2 MBs osmotically stabilized by 5% of *F*-butane [111]. In some cases MBs of SF_6 or *F*-

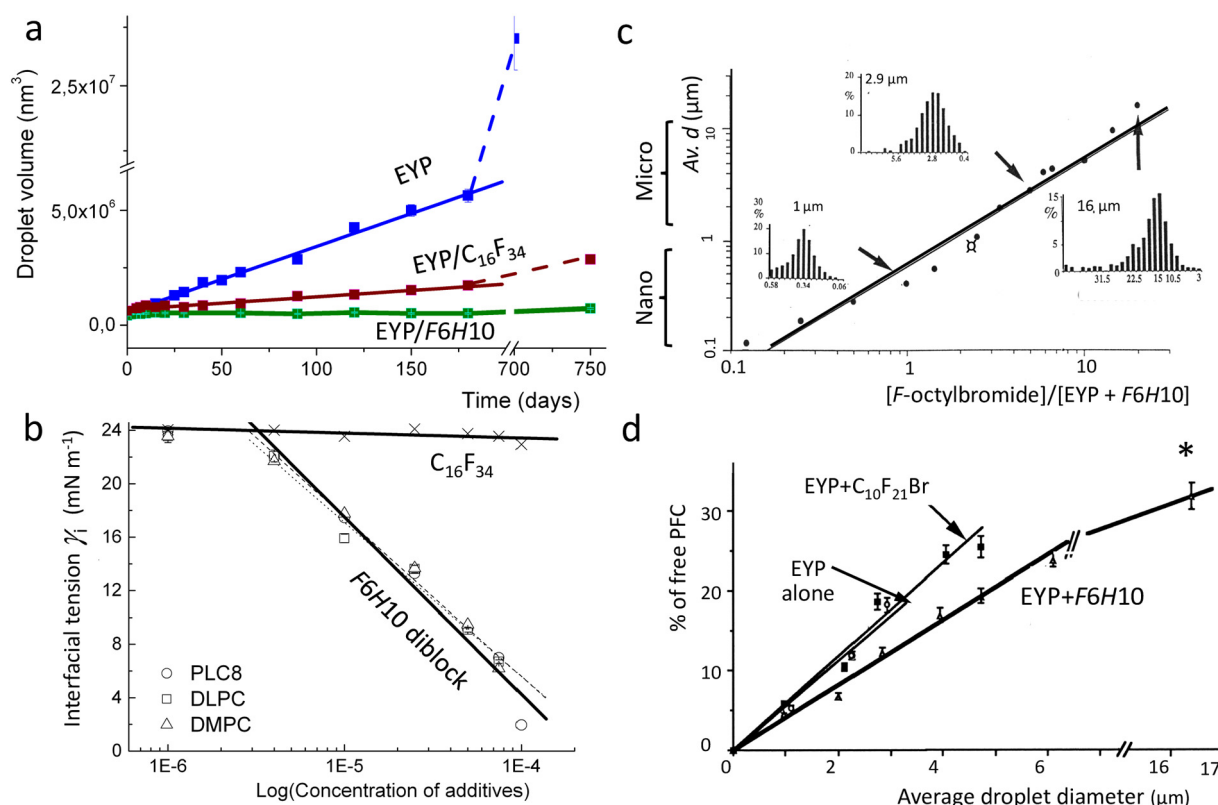


Fig. 3. F-octylbromide nanoemulsion stabilization by (F-alkyl)alkyl diblocks ($\text{C}_n\text{F}_{2n+1}\text{C}_m\text{H}_{2m+1}$, *F_nH_m*). a) Addition of F_6H_{10} diblock to egg yolk phospholipids (EYP) in equimolar amounts provides nanoemulsions that are stable for several years at room temperature (green); this stabilization approach is different and significantly more effective than the heavy PFC ($\text{C}_{16}\text{F}_{34}$, bordeaux) approach, and with EYP alone (blue); note the linear volume increase over time, characteristic of Ostwald ripening; b) F-octylbromide/water interfacial tension variation over concentration of added F_6H_{10} diblock for various phospholipids, as compared to addition of the heavy PFC $\text{C}_{16}\text{F}_{34}$; c) exquisite droplet size control is achievable over a large range of sizes (F_6H_{10} /EYP molar ratio is always 1); d) resistance to NE droplet coalescence through shaking is also remarkably superior with F_6H_{10} addition as compared to those achieved with EYP alone or with $\text{C}_{10}\text{F}_{21}\text{Br}$ adjunction. Adapted from [22,50]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

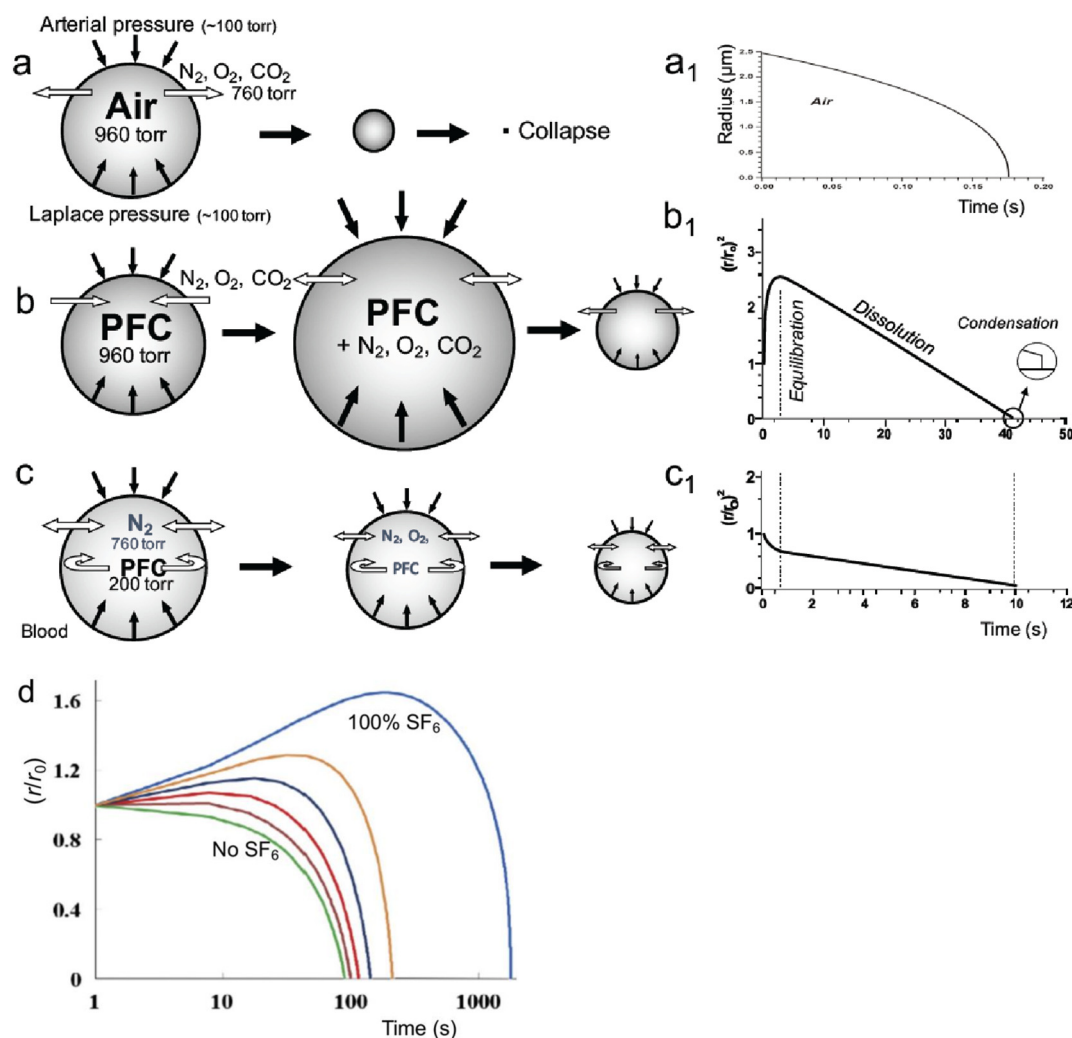


Fig. 4. In vivo fate in water of microbubbles of a) air; b) a PFC gas, and c) nitrogen osmotically stabilized by a PFC gas; b₁–c₁) calculated size change $(r/r_0)^2$ of the MBs over time. d) Calculated variation of relative radius, r/r_0 , as a function of partial pressure of SF₆ during dissolution of a MB coated with distearoylphosphatidylcholine in air-saturated water (surface tension 24.7 mN m^{−1}, shell resistance to gas transfer 3100 s m^{−1}, shell elasticity 8 mN m^{−1}); SF₆ fractions are 0 (green), 0.2 (brown), 0.4 (red), 0.6 (black), 0.8 (yellow) and 1 (blue). a–c) Adapted from [39,87]; d) from [109]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

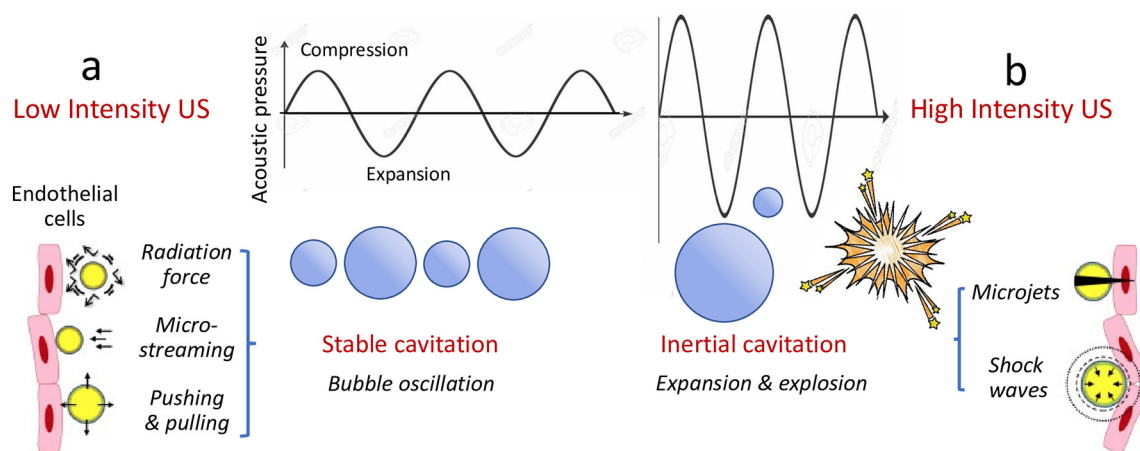
butane were formed that were subsequently sparged with O₂ [112,113]. Bubble dynamics in complex aqueous environments has since been modeled considering shell properties and their variations during dissolution and mass transfers between MBs [67,109,114,115], as exemplified for phospholipid shelled SF₆ MBs in Fig. 4d.

The shell of the MB, whether soft and resilient (e.g. phospholipids) or more rigid (polymers), also contributes greatly to MB stability and influences shell viscoelasticity, and hence, response to ultrasound. Phospholipids provide soft shells, ideal for sound reflection. Other shells involve albumin, dextran or hyaluronic acid. Acoustically active lipospheres were prepared for drug and gene delivery that are MBs, the gas core of which is surrounded by a layer of viscous hydrophobic liquid oil destined to contain a cargo and is enclosed in a phospholipid film [116]. MBs can be manipulated by US waves and emit energy in various forms, allowing various imaging modes, as well as control over O₂ release, thermal and mechanical energy delivery for surgical procedures, and delivery of therapeutics (Scheme 3) [117].

MBs can be obtained by sonication, extrusion, mechanical vibration/amalgamation, and other procedures [67,106,107]. Essentially all these techniques yield highly polydisperse MB populations. Subsequent population size narrowing can be achieved by flotation [93], differential centrifugation [118], pore filtration, acoustic sorting, which relies on the

size-related response to US [119], or microfluidics techniques such as pinched flow fractionation [120]. Direct preparation of narrowly sized MB populations can be attained with microfluidic procedures, including T-junction, and axisymmetric and asymmetric flow focusing [121,122], but the throughput of these techniques, although it has considerably improved [107,123], remains difficult to consider for industrial development.

All the commercial MB products approved for contrast US imaging rely on perfluorochemicals for stability. They include *SonoVue/Lumason* (Bracco Imaging, Milan, Italy), a SF₆/phospholipids formulation that is now approved in 36 countries for assessment of cardiac regional wall motion, characterization of focal liver and breast lesions, evaluation of cerebral arteries, carotid and peripheral arteries, and assessment of vesico-ureteral reflux in children; *Optison* (F-propane/albumin; GE Healthcare, Princeton, NJ); *Definity/Luminity* (F-propane/phospholipids; Lantheus, North Billerica, MA), and *Sonazoid* (F-butane/phospholipids; GE Healthcare, Amersham, UK), which is notably used in Japan for liver mass characterization. An osmotically stabilized MB product, *Imagent* (F-hexane/phospholipid; Alliance Pharm. Co.) was approved by the FDA but never marketed, a collateral victim of Alliance's debacle. These agents, which were all developed some 20 years ago, typically display an average MB size of 1–3 μm in number, with over 99% of them below



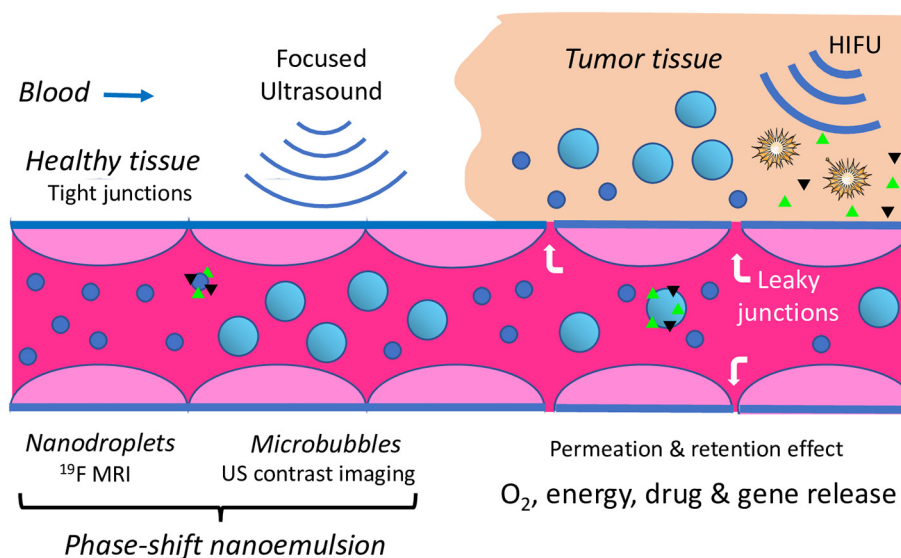
Scheme 3. Microbubbles in an ultrasound wave field. MBs can be manipulated by US waves, undergo a) stable or b) inertial cavitation on-demand, and emit energy in various forms, allowing different imaging modes, as well as control over O₂ release, thermal and mechanical energy delivery for surgical procedures, and delivery of therapeutics. Adapted in part from [117].

10 μm , and concentrations of $\sim 10^8$ – 10^{10} MB per mL [124]. Their main limitations, besides their being confined in the vascular space, include broad size distributions, short vascular half-life, usually below 5 min, absence of specificity and limited functionality. In a polydisperse MB sample, only a fraction of the bubbles is effective at a given US frequency.

A first molecular imaging agent, the vascular endothelial growth factor receptor 2 (VEGFR2)-targeted *F*-butane MB agent BR55 (Bracco, Geneva Switzerland) has been FDA-approved in 2016 for liver mass characterization and for voiding cystourethrography in children. VEGFR2 is a key regulator of the formation of blood vessels in cancer (neovascularization). Further clinical studies involve detection and monitoring of treatment of prostate [125], breast and ovarian [126], colon [127] or kidney [128] cancers. More targeted MB products are available for research, such as *Visistar* (Targeson, San Diego), a VEGFR2 or vascular cell adhesion molecule 1 (VCAM-1) or integrin-targeted *F*-butane MB, and *Vevo MicroMarker*TM (VisualSonics, Toronto), a *F*-butane MB that can be streptavidin-pre-targeted, as well as various *F*-propane/lipid MBs, e.g. *USphere Labeler* (Trust Bio-sonics, Zhubei, Hsinchu, Taiwan).

These MBs have all the capacity to absorb O₂ dissolved in blood and carry it to the tissues. Facile manipulation by sound waves, a distinctive attribute of MBs, can produce stable or inertial cavitation, eventually leading to focused, on-demand destruction and release of content. O₂ release from MBs can thus be activated by exposure to US (Schemes 3 and 4) [111–113]. The characteristics of MBs oscillating in an US wave field, in particular the knowledge about MB size, gas composition, MB shell structure and rheology, stabilization mechanisms, carrier capacity, response to US wave form, frequencies, intensities and pulse sequences, development of new MB-based imaging techniques have been thoroughly discussed [90,108,129–135]. Understanding of MB interactions with cells, especially endothelial cells, and tissues has progressed considerably [87,107,108,115,136]. Specific MB tailoring for MB-assisted US therapies and the difficulties that may be encountered in clinical translation have also been considered [137].

Among the currently tackled challenges, one can cite the development of nanometer-sized bubbles (NBs), in spite of the increasing Laplace pressure that should drive them towards dissolution [60,138–140]. Advantages resulting from the sub-micrometer size



Scheme 4. Phase-shift nanoemulsions in vivo. P-SNEs can be turned locally into MBs under US irradiation. They can extravasate through leaky capillaries and accumulate into tumor tissue, where they can be phase-shifted into MBs. High-intensity focused US (HIFU) can then cause inertial cavitation and eventual MB disruption for focused local delivery of O₂, energy and drugs (black and green triangles).

include longer half-life, easier extravasation to tumor tissue through EPR effect, and facilitated cellular uptake. NBs can be separated from MBs by centrifugation or filtration. *Pluronics* and PEGylated lipids were found effective to reduce bubble size [103,141]. Iron oxide NP-containing NBs have been prepared [142], in particular O₂-loaded superparamagnetic NBs to enhance tumor oxygenation during hyperthermia [143]. Further investigation of this topic is warranted both on a theoretical basis and through experimental NB characterization. A Special Issue on “Nanodroplets and Nanobubbles” is presently being published [144].

3.4. Perfluorocarbon nanoemulsions that phase-change into microbubbles

Phase-shift NEs that can reversibly switch from liquid NE droplets to gaseous MBs (Scheme 1) when activated by heat, sound or light after having been positioned in target tissues currently draw considerable attention [102,130,145–160]. P-SNEs benefit from the small initial size and relatively long intravascular circulation half-life (up to a few days) of NE droplets, allowing extravasation and retention in tumor tissue (the still debated EPR effect) [161,162], and, after vaporization, acquire the responsiveness of MBs to US (resonance, inertial cavitation and destruction) needed for on-demand, site-selected delivery of O₂ and other therapeutics (Table 6, Scheme 4). A specific research topic is the use of P-SNEs for crossing the blood-brain barrier (BBB) [163,164].

As far as we know, all the P-SNEs investigated for medical uses rely on a phase-changing PFC core. The P-SNE shell is similar to those used for MBs, but for a more frequent use of fluorosurfactants. It can be soft and resilient, as with phospholipids, or harder as with polymers or silica. *F*-pentane P-SNEs stabilized with a dextran/poly(vinylpyrrolidone) [165] or chitosan shell [56] have been reported, as well as polydopamine-shelled *F*-hexane P-SNEs [166]. Shift from the liquid to the gas state produces a marked increase in size (typically 5-fold or more) and a huge rise of acoustic impedance, and hence, in echogenicity. Soft (e.g. lipid)- and hard (e.g. polymer)-shelled NEs can require markedly different US intensities for phase-shift, inertial cavitation and drug release [167,168]. While liquid PFC droplets have an obvious aptitude for ¹⁹F MRI, their gaseous MB form is ideally suited for US imaging and manipulation by US waves. P-SNEs, upon vaporization, thus switch from ¹⁹F-MRI to US contrast agents, and vice versa upon condensation. Rapid repeated phase shifts (vaporization and recondensation) led to blinking nanodevices [169,170].

P-SNE preparation usually relies on sonication, extrusion, high shear homogenization, microfluidics, as well as on condensation of microbubbles [151,156,171]. Size distributions can be adjusted as for MBs. Reversal from MB to NE can be achieved by cooling or compression, meaning that P-SNEs can be obtained from commercially available MBs that were approved for use as contrast agents [160,172–179].

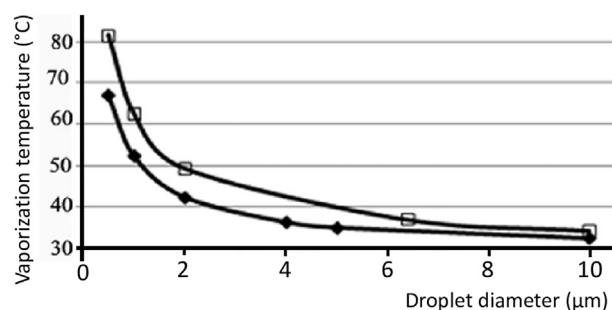


Fig. 5. Calculated effect of droplet size on *F*-pentane vaporization temperature for surface tensions of 30 and 50 mN m⁻¹ (black diamonds and open squares, respectively). Adapted from [146].

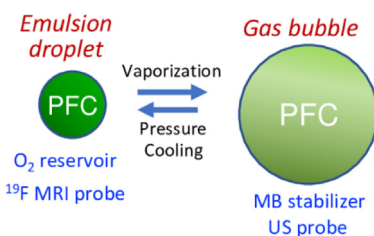
However, the much-investigated US-induced acoustic droplet vaporization (ADV) of the core PFC is a complex, multiparameter process that requires MB nucleation, depends on multiple MB, sound wave and physiologic parameters, can involve different mechanisms, and is difficult to control. [180–184] The persistence of superheated nanodroplets that remain in the liquid state at temperatures and pressures above the saturation curve cannot be explained by thermodynamics alone [151]. This suppression of nanodroplet thermal vaporization was first ascribed to Laplace pressure, which shifts the equilibrium saturation curve (Antoine's equation) to higher temperatures and lower pressures [185]. Suppressed vaporization was later assigned to the energy barrier that opposes nucleation of a vapor seed of critical size [186]. The homogenous nucleation theory accurately predicts the elevated pressures needed to condense MBs. The vaporization pressure increases as droplet size is reduced and also depends on nanodroplet shell and fluorocarbon [151,187,188]. A super-harmonic focusing effect was reported that combines two phenomena: a highly non-linear distortion of the acoustic wave and its focusing by the droplet; the larger the droplet, the stronger the focusing, which locally increases peak negative pressure and initiates vaporization [189].

Simply due to Laplace pressure the vaporization temperature of *F*-pentane NEs is expected to increase significantly when droplet size diminishes below ~4 μm (Fig. 5), causing 1 μm-size naked droplets to vaporize at over 60 °C only, versus 29 °C for bulk *F*-pentane, thus remaining in a superheated liquid state at body temperature [146]. Phase change can also be induced by ultrasound, laser light or use of small diameter injection needles [146]. Indocyanine green-loaded *F*-pentane/albumin MBs rapidly re-condensed into NE droplets when laser light activation was interrupted [150]. Even lipid-coated *F*-propane and *F*-butane NEs did not vaporize spontaneously at body temperature [190]. *F*-propane (boiling point minus 36.7 °C) droplets (condensed *Definity*) did not return spontaneously to MBs after

Table 6

Pros (left) and cons (right) of phase-shift PFC nanoemulsions.

- Small *sub-micron size*
- Long intravascular *half-life*
- Can *accumulate* in tumors (permeation & retention effect)
- *Multiple activation modes* (heat, sound, light, microwave..)
- Can be reversed
- Convert to MBs at *US- or laser-light focus*
- Ease *sonoporation* & *BBB crossing*
- Can release O₂, drug cargo *on-demand*
- Low response to US becomes *high*



- Vaporization (phase shift) is a *complex, multiparameter event*
- *Requires nucleation*
- *Vaporization energy threshold needs to be minimized*
- Inertial cavitation requires considerable attention
- *Control and reproducibility* of in vivo MB size is mandatory
- *Transfer* of PFC from NDs to MBs must be minimized to avoid inflation
- Above points are safety issues

intravenous injections in pigs, but required application of acoustic power to induce vaporization [175]. On the other hand, even heavy, high boiling point PFCs such as *F*-15-crown-5-ether and *F*-octylbromide can generate MBs under US activation due to mechanisms that involve recruitment of dissolved gases, including O_2 , from the surroundings [146,191].

The importance of a thorough command of the phase-shift event, including equilibrium with the environment, and proper control of acoustic cavitation, cannot be emphasized enough [78,135,155,160,192]. PFCs with a relatively higher MW (e.g. *F*-hexane rather than *F*-pentane) demonstrate advantages when repeated therapeutic activation is pursued, providing higher contrast, increased drug payload and higher in vivo stability, especially for theranostics applications [193,194]. A serious safety issue with P-SNEs concerns indeed the accurate control of vaporization and the size of the resulting MBs. Prolonged myocardial opacification with a *F*-pentane emulsion was, for example, related to the formation of very large (30–50 μm in diameter) MBs, with a negative impact on hemodynamic parameters [195]. A similar *F*-pentane emulsion when used as an O_2 therapeutic also showed a transient increase in mean pulmonary artery pressure [196]. Size increase results not only from PFC expansion through phase change, but also involves inward osmotic recruitment of gases dissolved in the surrounding aqueous medium, as well as transfer of PFC from co-existing, not yet vaporized PFC nanodroplets. In the case of *F*-butane/phospholipid MBs such transfer from droplet to bubble was shown to cause a MB volume

inflation by several orders of magnitude, with a risk of vessel occlusion (Fig. 6) [160]. Resolution of this issue was, however, deemed possible through suitable P-SNE design, including use of less volatile PFCs and of a polymeric heat-denatured albumin shell.

No phase-shift PFC emulsion is on the market yet. An early *F*-pentane (2% w/v)/*F*-surfactant- and *Pluronic*-based P-SNE, *Echogen* (Sonos, Bothell, WA), was approved for use as a contrast agent in Europe in 1998, but was never marketed, likely because of inadequate control of phase shift in vivo. A buffered version of *Echogen*, *NVX-108* (NuvOx Pharma, Tucson AZ) is presently being investigated, including for O_2 delivery during treatment of cancer and stroke [197]. The *F*-surfactant utilized is a purified *Zonyl-FSO*-type fluoropolymer [198].

3.5. Further medically-oriented, PFC-based oxygen-carrying colloids

Further PFC-based colloids and constructs investigated for O_2 delivery and other biomedical purposes include reverse and multiple emulsions and gel-emulsions, porous PFC-loaded NPs and scaffolds, nested and connected colloids, in particular liposomes, and polymeric micelles that comprise *F*-alkylated or *F*-arylated moieties.

3.5.1. Reverse and multiple emulsions

Reverse, water-in-PFC nanoemulsions (Scheme 5a) are mainly aimed at delivering water-soluble drugs, in particular through the pulmonary route, as with metered-dose inhalers [17,20,199–205].

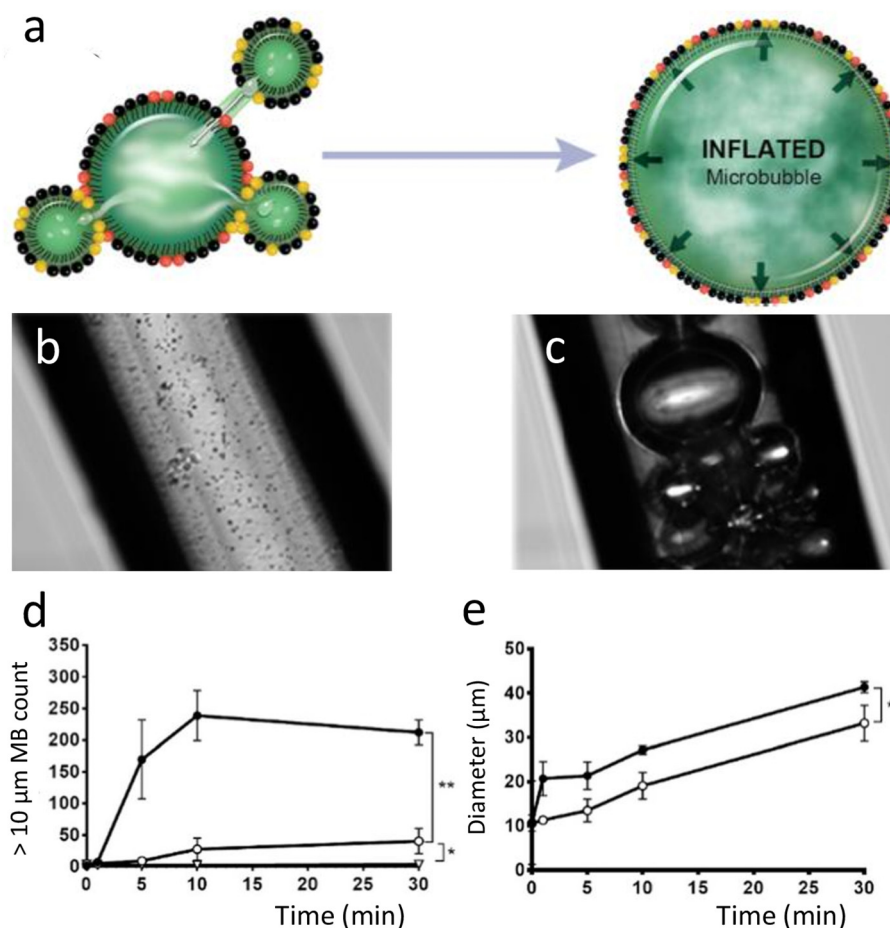
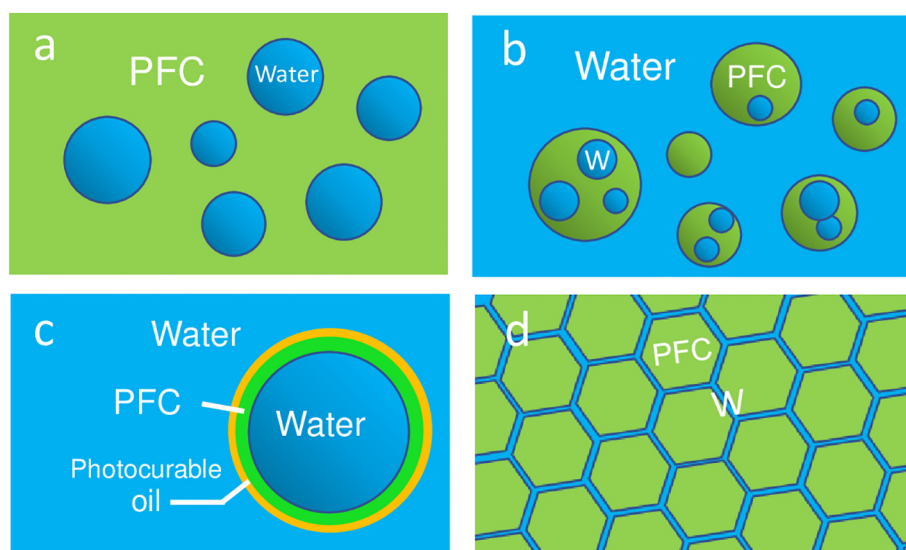


Fig. 6. a) Transfer of *F*-butane from co-existing not yet vaporized soft-shell lipid-coated NE droplets to MBs can cause a considerable increase in MB size (by up to 6 orders of magnitude), with a risk of vessel occlusion; b–c) bright-field micrographs of MBs before and after infusion of PFC nanodroplets (the inner tubing diameter is 200 μm); d) the same phenomenon as with *F*-butane (black circles) was observed with *F*-pentane (open circles), but to a lesser extent, and is no longer significant with *F*-hexane (open triangles); e) average size of inflated MBs over time after addition of *F*-butane (black circles) or *F*-pentane (open circles) emulsion nanodroplets to a MB suspension. Adapted from [160].



Scheme 5. Examples of further soft-shell PFC colloids. a) Reverse water-in-PFC emulsion; b) water-in-PFC-in-water double emulsion; c) triple water-in-PFC-in-oil-in-water emulsion droplet; d) high internal phase ratio (up to 99%) PFC-in-water gel emulsion. Adapted from [17,217,218].

A non-ionic, molecular *F*-alkyldimorpholinophosphate **15** (Table 2) produced, for example, stable, heat-sterilizable reverse emulsions of water-in-*F*-octylbromide in which a variety of drugs could be loaded [199,206]. Thermodynamically stable, transparent emulsions (called “microemulsions” [207]) were also obtained (average diameter 12 nm) [203]. Reverse emulsions can as well serve in microfluidics techniques for a range of applications, including single-molecule polymerase chain reactions (PCR), proteome analysis, clinical diagnosis on human physiological fluids, protein crystallization, and titration of anticoagulants [208–210]. They have also been used to encapsulate prokaryotic and eukaryotic cells [211].

Emulsions used in microfluidics usually involve a polymeric fluorosurfactant (Table 2) such as the poly(*F*-propylether)-PEG triblock copolymer **19** [75,211]. The PFCs used as continuous phase in the latter studies include a mix of tertiary *F*-alkylamines. Reverse water-in-PFC emulsions have also been produced using *F*-alkylated silica NPs in microfluidics channels, yielding NP-stabilized (Pickering-type) emulsions that effectively alleviated undesirable inter-drop exchange of contents [212]. Dendronized fluorosurfactant with mono- or polyglycerol polar head groups (e.g. **20**) can also minimize interdroplet transfer of small molecules [81].

Water-in-PFC-in-water double emulsions (Scheme 5b), chiefly destined for delivery of fragile hydrophilic drugs [17,171,213–215], again relied on a *F*-surfactant for the water-in-PFC emulsification step. For example, water-in-*F*-pentane NEs were prepared with Krytox [215] and water-in-*F*-hexane NEs with Krytox-derived *F*-surfactant **19** [171], which were re-emulsified in water using phospholipids or Pluronic F-68, respectively. A water-in-*F*-hexane-in-water phase-shift double emulsion was embedded in an acoustically responsive fibrin matrix for controlled drug release [216]. A poly(ethylene imine)-coated water-in-*F*-octylbromide-in-water double emulsion was loaded with indocyanine green and doxorubicin, and targeted to HER2-overexpressing cancer cells for photo- and chemotherapeutic treatments [214].

Triple emulsions, in which a thin layer of a heavy PFC separates the inner aqueous or organic encapsulant phase from an external photocurable oily shell within the continuous aqueous phase (Scheme 5c), have been prepared using microfluidics [217]. Highly stable, heat-sterilizable high internal phase ratio gel-emulsions (up to 99% of diverse PFCs) with a polyaphron structure (Scheme 5d), obtained with neutral molecular amine oxide *F*-surfactant **16**, could find topical uses [218].

3.5.2. PFC-loaded nanoparticles and matrices

PFCs have been loaded, along with various drugs, within diverse mesoporous nanomaterials [219], such as Prussian blue (ferric hexacyanoferrate) nanocubes [220], surface-modified hollow molybdenum sulfide NPs [221], porous paramagnetic Fe₃O₄ NPs [222,223], hollow Bi₂Se₃ NPs [224], gold nanoshells [225], silica NPs [19,226–228]. *F*-hexane-loaded fullerene NPs also helped generate reactive oxygen species [229]. *F*-15-crown-5-ether-loaded, poly(vinyl alcohol)-stabilized PLGA NPs with an atypical multicore structure, that is, ~100 nm NPs consisting of agglomerated smaller PLGA-coated *F*-15-crown-5-ether droplets, have been reported [24].

Rather complex “nanomotors” consisting of gold nanowires coated with *F*-octylbromide NE droplets cloaked with RBC membranes were built that could be propelled under US activation into hypoxic macrophages to provide intracellular O₂ delivery (Fig. 7) [55]. In the same vein, focused US-triggered phase-shift of *F*-nonane/*F*-surfactant droplets was used to fire silica or polymer “nanobullets” from “microcannons” fabricated through electrodeposition techniques [230,231].

PFCs have also been incorporated in implantable hydrogel scaffolds to deliver O₂ and bioactive agents for tissue engineering. For example, an O₂-carrying *F*-tributylamine-loaded fibrin hydrogel scaffold accelerated sciatic nerve regeneration and functional recovery in rats [232]. Further hydrogels that embedded PFC MBs or P-SNEs and provided acoustically responsive scaffolds for delivery of regenerative growth factors and drugs, can potentially transport O₂ as well [48,216,233–237].

3.5.3. Interconnected and nested colloids

Further PFC-based colloids that will inevitably transport some O₂ although they were not designed for this purpose include “acoustic clusters” that associate negatively charged *F*-butane MBs (*Sonazoid*) and positively charged, phase-shiftable *F*-methylcyclopentane/phospholipid/stearylamine microdroplets intended for US-mediated chemotherapeutic drug delivery [238,239]. Controlled aggregation of *F*-butane MBs was also obtained through avidin/biotin linkage [240].

PFC NE droplets have been associated with liposomes in order to combine their respective assets, i.e. increase the loading capacity of the NE for hydrophilic drugs and confer sensitivity to US to the liposomes (Fig. 8). Biotinylated cationic liposomes complexed with mRNA (lipoplexes) were thus attached on the surface of avidinylated *F*-butane/lipid MBs for mRNA delivery [241]. Paclitaxel-loaded liposomes have been tethered covalently to the periphery of SF₆ NBs, or through biotin/avidin linkage to *F*-propane MBs, for combined US imaging and

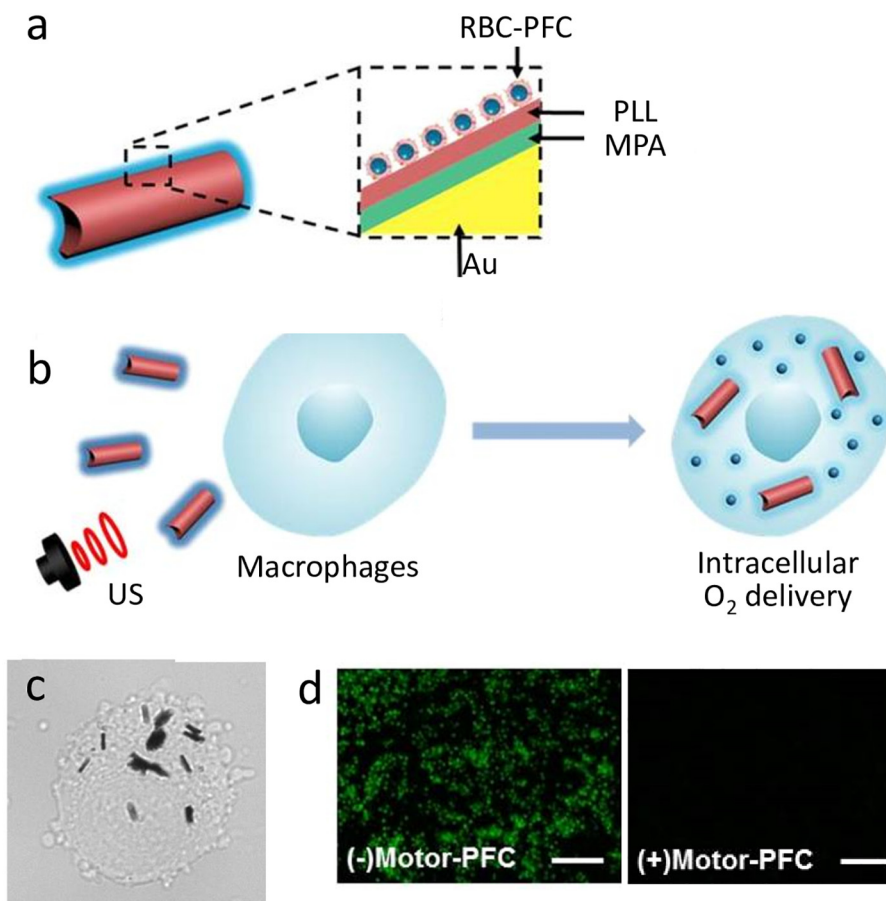


Fig. 7. A sophisticated “nanomotor”-based O_2 delivery system. a) A gold nanorod coated with mercaptopropionic acid (MPA), then poly-L-lysine (PLL), then with RBC fragments-shelled *F*-octylbromide droplets; b) these devices were propelled by US into hypoxic macrophages for acoustically-assisted O_2 delivery; c) they were internalized in macrophages under US exposure (optical microscopy), and d) they alleviated hypoxic stress as indicated by the suppression of the green fluorescence of a hypoxia marker. Adapted from [55].

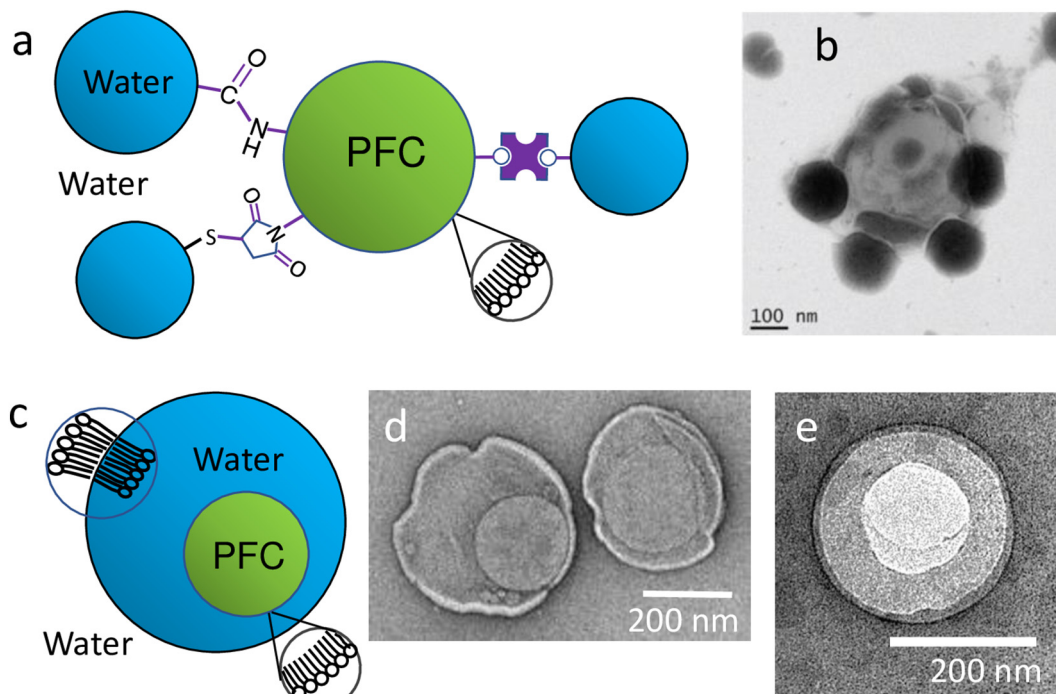


Fig. 8. a) Liposomes tethered to PFC MBs (schematic), also illustrating popular carbodiimide, maleimide and avidin-biotin coupling methods; b) transmission electron micrograph of a SF_6 nanobubble/paclitaxel liposome conjugate; c) a lipid-coated PFC MB nested in a liposome (schematic); TEM images of d) nested *F*-butane MBs, and e) a nested *F*-pentane phase-shift NE. Adapted from b) [244], d) [246] and e) [248].

US-triggered drug delivery [242–244]. Magnetically targeted doxorubicin-loaded liposome-*F*-propane MB conjugates allowed US-enhanced delivery [245].

F-butane NBs [246] and *F*-hexane or *F*-pentane P-SNEs [247,248] nested within liposomes allowed US-triggered drug release (Fig. 8b). Tumor-targeted fusogenic liposomes were loaded with a *F*-pentane P-SNE [249]. *F*-propane and recombinant tissue plasminogen activator protein (rtPA)-loaded liposomes destined for treatment of ischemic stroke by sonothrombolysis actually comprised a variety of structures including liposomes (with and without gas) and lipid-shelled MBs

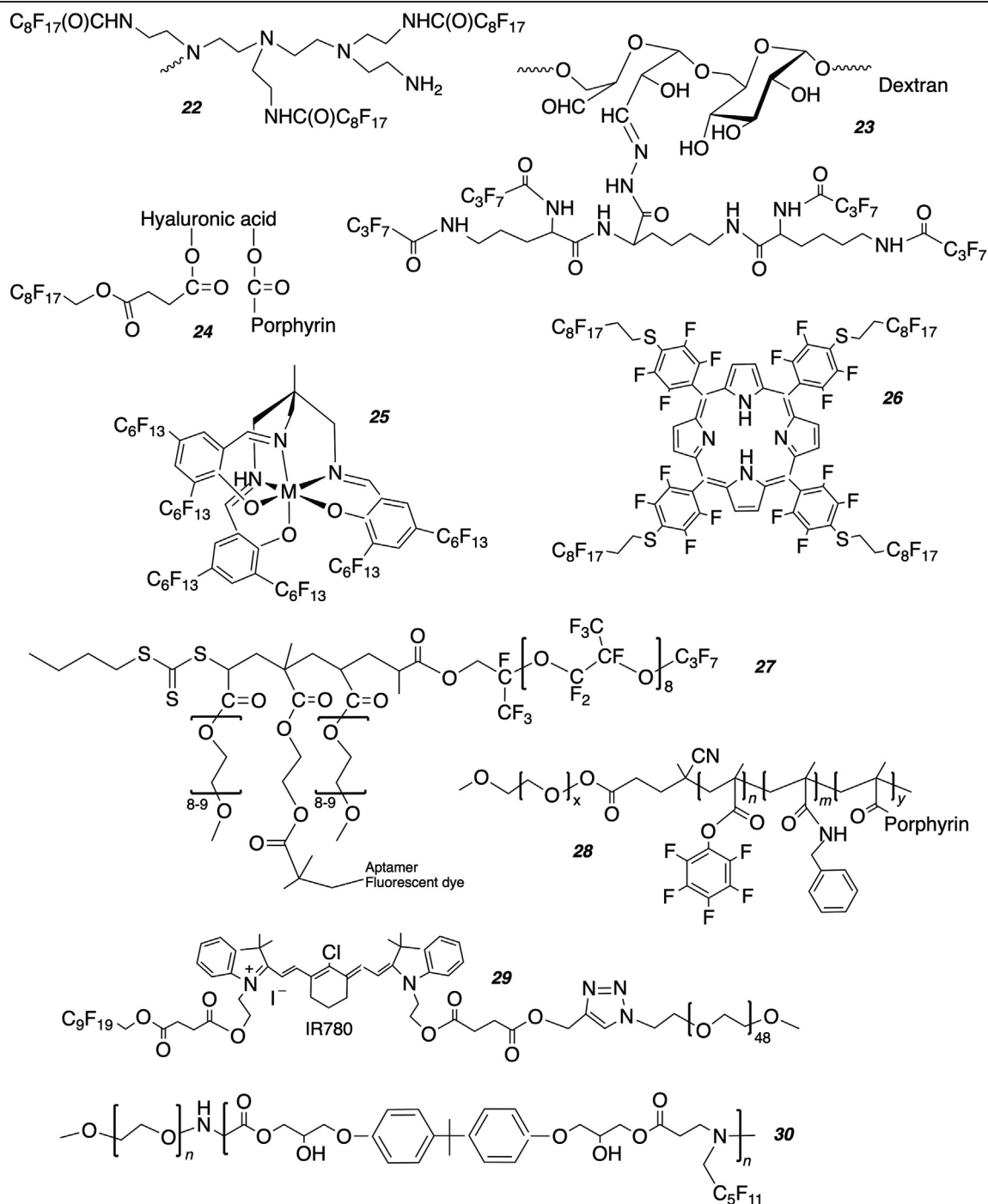
[250]. *F*-propane “bubble liposomes” enabled US-activated antisense oligonucleotide therapy of muscular dystrophy in mice [251].

3.5.4. Polymer micelles and nanoparticles with perfluoroalkylated or arylated components

Further colloids have been investigated that, without incorporating PFCs per se, contain components that contain sufficient *F*-alkyl or *F*-aryl moieties (Table 7) for them to acquire some of the properties and functions of PFCs such as gas dissolving capacity, solubility in PFCs, ^{19}F MRI probing ability, or control of cargo release. The capacity

Table 7

Examples of functional *F*-alkylated or *F*-arylated colloid components that can also contribute to oxygen delivery for cancer therapies.



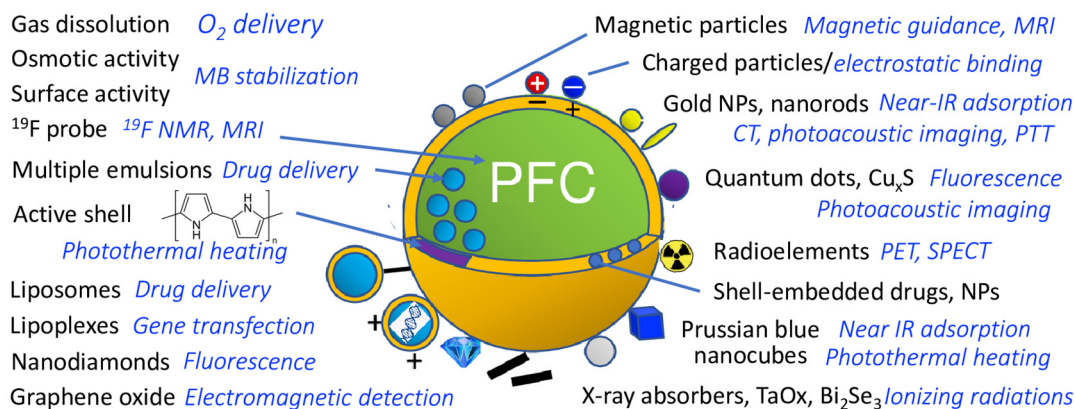
for such systems to facilitate O₂ delivery, when investigated, actually appears rather remarkable. For example, O₂-carrying micelles of *F*-octanoic acid-grafted poly(ethylene imine) **22**, also loaded with a photosensitizer, improved photodynamic glioma tumor growth inhibition [252]. A tumor-targeted, pH- and light-sensitive poly(lysine) dendron fitted with several *F*-propyl groups **23** provided the O₂-carrying NP component needed for photodynamic therapy (PDT) enhancement [57]. Micelles made of *F*-nonanoic acid- and porphyrin photosensitizer-conjugated hyaluronic acid **24**, designed for targeted O₂-enhanced PDT, increased singlet oxygen (¹O₂) production [58]. *F*-octyl chain grafting imparted US-triggered O₂ delivery capacity to a sonosensitized porous silica shell [253]. *F*-alkylated siloxane/polystyrene NPs incorporating a *F*-arylated zinc phthalocyanine photosensitizer were also produced [254].

F-alkylation has often been used to increase the solubility in PFCs of dyes for fluorescence imaging [255], sensitizers for PDT, or paramagnetic metal chelates for MRI as in **25** [256,257]. Four *F*-octyl chains bound via tetrafluorophenyl-based linkers onto a porphyrin **26** ensured its solubility in PFC NEs for concurrent O₂ delivery and photosensitizing [258]. The photosensitizer could thus be dissolved in a *F*-decalin/*F*-tributylamine NE, in which its undesirable aggregation was reduced, leading to superior ¹O₂ production and melanoma cell killing. Targeted *F*-polyether-based hyperbranched polymer NPs **27**, conjugated with a fluorescent dye, provided fluorescence imaging and sensitive ¹⁹F MRI detection of breast cancer in a mouse model [259]. Further examples of *F*-alkylated or *F*-arylated NPs can be found in Section 6.

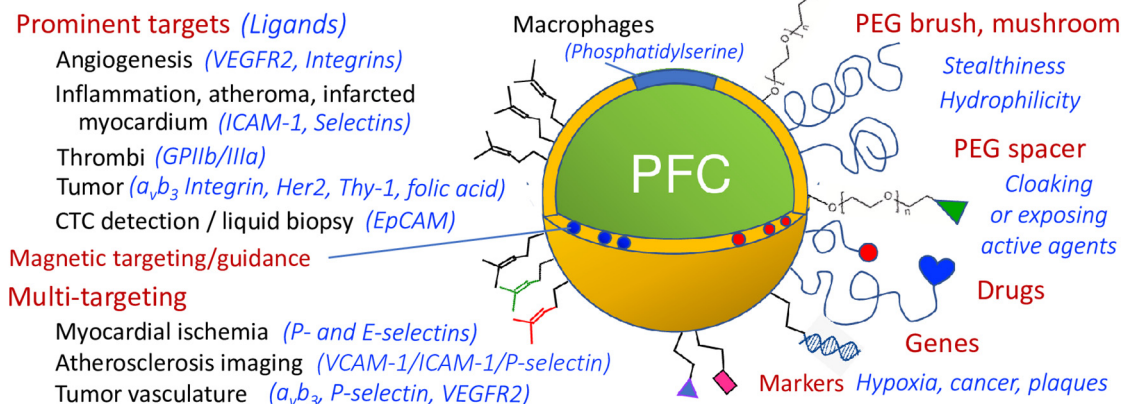
3.6. Functionalization and targeting of perfluorocarbon colloids

Surface modification, functionalization and targeting (Scheme 6) can expand the range of indications for PFC colloids well beyond O₂ delivery and help augment their efficacy and safety. Functionalization involves attachment (as through carbodiimide, maleimide, pyridyl disulfide or avidin/biotin coupling, see Fig. 8) [260,261], or incorporation, adsorption or electrostatic binding of fluorescent dyes, photo- and sonosensitizers, radiopaque elements, magnetic NPs, radiotracers, etc. New binding techniques based on biorthogonal click chemistry are emerging [262,263]. Poly(2-oxazoline) amphiphiles were used to control PFC nanoemulsion surface chemistry [264]. Such additions authorize fluorescence, photoacoustic and sono-photoacoustic imaging, computed tomography (CT), X-ray phase contrast imaging, positron emission tomography (PET) or single-photon emission computed tomography (SPECT), or MR imaging or guidance, as well as labeling and tracking of circulating cells. Incorporation of photo- and sonosensitizers also enables energy conversion (e.g. light or sound into thermal or mechanical energy) therapies, as well as reactive oxygen species (ROS)-based therapies. Still further functions can be conferred to PFC nanocarriers, such as: –responsiveness to environmental stimuli, including pH, temperature, O₂ level, redox potential, presence of specific enzymes, markers or ROS; –control of vaporization energy threshold for P-SNEs; –capacity for evading opsonization and phagocytosis by coating with hydrophilic surface brushes or RBC membrane components; –increase of drug cargo space, as by binding or incorporating drug-loaded liposomes and carrier NPs; –competency for condensing and

a PFC colloid functionalization



b PFC colloid targeting and decoration



Scheme 6. Schematic illustration of multiple a) functionalization and b) targeting possibilities of PFC colloids.

preserving genetic material; —control of in vivo drug cargo protection or presentation; —capacity for on-demand, externally triggered or stimuli-dependent focused cargo release or even projection into tissues [100,107,108,265–267]. Fluorination of the functionalizing agent can enable its dissolution in the PFC core [255–258].

PFC colloids have also been fitted with a range of targeting implements (Scheme 6b). Targeting is critical for such applications as molecular imaging or precisely disease-focused O₂, energy or drug delivery. PFC-based nanocarriers loaded with magnetic NPs can be directed to their target by applying an external magnetic field [245,268–270]. Different strategies for magnetic functionalization have been examined [271]. Dendronized iron oxide loaded-*F*-hexane MBs could serve as versatile, magnetically targetable multimodal imaging and drug delivery systems [34]. Detection of inflammation sites can be obtained by inclusion of phosphatidylserine in the nanocarrier shell, which promotes uptake by activated macrophages [100,272,273]. *Sonazoid*, with a phosphatidylserine-containing shell can thus act as a marker of phagocytosis [274]. Dual P/E-selectin-targeted MBs allowed US monitoring of anti-inflammatory treatment response in acute ileitis [275]. Active targeting to specific diseased tissues can be achieved by surface conjugation of appropriate monoclonal antibodies, peptides, peptidomimetics, oligosaccharides or folic acid. Privileged targets include infarcted myocardium, atheroma plaques, thrombi and activated platelets, sites of inflammation, or sites of angiogenesis related to malignant tumor growth [100,105,107,108,261,267,276–279]. A folate-targeted *F*-pentane P-SNE loaded with a chemotherapeutic drug and Fe₃O₄ provided multimodal imaging and treatment of ovarian cancer in mice [280]. Hyaluronic acid has also been used as a targeting moiety [58]. *F*-alkylated lipopeptides that are precursors of targeting lipopeptide conjugates have been synthesized and their incorporation in the phospholipid shell of *F*-hexane-stabilized MBs investigated [281]. Targeting can be assisted by US-generated radiation forces [282]. The development of targeted PFC-based devices for detection of circulating tumor cells (liquid biopsy) is also focusing considerable interest [283]. Multiple functionalization and targeting foster multimodal imaging, enhanced therapeutic efficacy, increased safety, and opens an endless range of theranostic possibilities.

4. Oxygen delivery by PFC nanoemulsions

The early objective of developing PFC-based O₂-delivering “blood substitutes” has not been forsaken. Further evidence of O₂ delivery efficacy by means of PFC NEs has been secured. New, highly stable size-controlled NEs have been developed. The alternative option of using MBs or P-SNE-derived MBs for O₂ delivery is being actively investigated. Additional indications for O₂-delivering therapeutics have emerged. Potentiation of the foremost O₂-dependent cancer treatment strategies, including radiotherapy, chemotherapy, photo- and sonodynamic therapy, and now immunotherapy, is also an important goal.

4.1. Basics of oxygen transport and delivery by perfluorocarbon nanoemulsions

The basics of O₂ transport and delivery by PFC NEs have been reviewed and compared to those that support hemoglobin-based carriers [1,7]. Briefly, PFCs dissolve O₂ physically, typically 20 times more than water or blood serum. Such solubility relies on weak non-specific van der Waals-type interactions. Contrary to hemoglobin, the O₂ content of PFCs is a linear function of O₂ partial pressure and there is no saturation. Full benefit for patients will thus be gained when the fraction of inspired oxygen (FiO₂) is 1 or close to 1. PFCs do not bind O₂ through strong directional chemical bonds as hemoglobin does, and O₂ release requires no allosteric factor. Oxygen is therefore immediately and close-to-totally available to tissues, with extraction ratios typically reaching 90% of the dissolved O₂. Administration of a PFC NE is comparable to increasing the O₂ content of the plasma compartment of blood.

High FiO₂ produces a large O₂ tension gradient, and hence, a potent driving force for O₂ diffusion into tissues. Additionally, the much more numerous, much smaller-sized PFC droplets relative to RBCs offer a huge interface for gas exchange and circulate more easily through capillary beds, thus further facilitating O₂ diffusion. Even when blood cell circulation is hindered, perfusion of tissues by the PFC nanodroplets is expected to persist along with residual plasma flow. When both PFC and hemoglobin are present, the PFC will release its dissolved O₂ first, while the hemoglobin-bound O₂ remains in reserve. As transport and delivery rely on physical dissolution, the same principles apply to other gases, including N₂, CO₂, NO, CO, Xe, and volatile anesthetics. The large solubility of CO₂ in PFCs should be noted. Also notable is that, contrary to Hb, PFCs do not scavenge nitric oxide and therefore do not cause vasoconstriction.

A multiparameter mathematical model allows calculation of the “hemoglobin equivalency” of a PFC NE. In typical clinical conditions, the patient’s hemoglobin level being about 8 g/dL, a 2.7 g PFC/kg body weight dose of *Oxygent* (110 mL of NE) was found equivalent to about 4 g/dL of hemoglobin [284]. This value was validated by clinical data from a Phase III study in general surgery.

Further, much needed insight into the mechanism of O₂ delivery in the microcirculation has been gained [285]. In a case of extreme hemodilution in a hamster model, infusion of 4.2 g/kg body weight of a PFC NE (*Oxycyte*) increased systemic and microvascular O₂ delivery by 25% when the animals breathed air and by 400% when they breathed pure O₂, as compared to a hydroxyethylstarch solution [286]. The PFC NE produced a significant increase of the amount of O₂ transported and consumed. Acid-base balance, mean arterial pressure and cardiac output were restored. No evidence for vasoconstriction or microvascular perfusion impairment was noted. Further studies of the physiology of microcirculation and its regulation mechanisms, the role of the vascular endothelium, and the effects of O₂ carriers on them, were encouraged [285].

A meaningful advance in the utilization of O₂-carrying *F*-colloids is the realization that release of O₂ from NEs could be triggered or accelerated and focused using external stimuli. On-demand local release of O₂ from PFC NEs to specific tissues has, in particular, been triggered by focused US (Fig. 9) [111,214,287,288].

4.2. Development status of O₂-carrying PFC nanoemulsion products

No satisfactory PFC NE has so far gained approval and substantial acceptance. As indicated previously, the early commercial product *Fluosol* could not be successful in view of its poor user-friendliness. *Oxygent* development did not survive Alliance’s financial debacle. The emulsion *Perforan* (Perforan, Pushchino, Russia), despite requiring frozen transportation and storage, and notwithstanding a long organ-dwelling component (*F*-methylcyclohexylpiperidine **10**, 30% of total PFC; MW 595; organ half-life of at least 3 months), is available in former Soviet Union Countries and has been used in Mexico [289–291]. Introduction in North America under the brand name *Vidaphor* (FluorO2 Therapeutics, Inc. Boca Raton, FL) is being sought. Development of *Oxyfluor* (HemaGen Corp., CA), a *F*- α,ω -dichlorooctane NE [292], with a misconstrued stabilization principle involving safflower oil supplementation that actually form separate droplets rather than the expected stabilizing oily sub-shell [293], was discontinued following funding problems. *Oxycyte* (Tenax Therapeutics, Morisville, NC), an emulsion of “*F*-*t*-butylcyclohexane” **11** with poorly documented purity, organ retention (estimated at about 2 weeks) and emulsion characteristics, has experienced a futile clinical trial in 2014 [14]. On the other hand, several PFC NEs are commercially available for research purposes, including a *F*-poly(ethylene) NE (CS-1000, Celsense, Pittsburgh, PA) that is used in several FDA-approved clinical trials involving ¹⁹F MRI tracking of circulating cells.

Yet, there is at this point no factual reason to discard the PFC NE-based O₂-delivery approach. Previous endeavors have suffered from

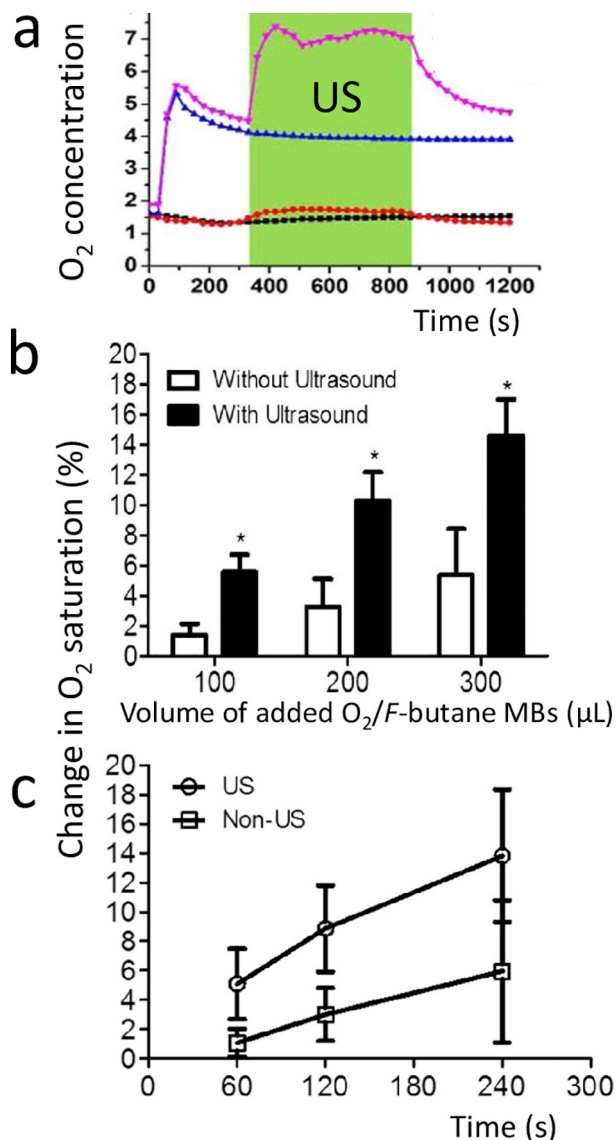


Fig. 9. Oxygen release from PFC colloids can be stimulated by exposure to US. a) Changes in dissolved O₂ concentration over time in deoxygenated water without (black) or with (blue) addition of an O₂-loaded F-15-crown-5-ether NE; US treatment was applied during the period highlighted in green (water: red; pink: with PFC). b) Change in O₂ saturation using O₂/F-butane-filled MBs with (black bars) and without (white bars) exposure to US after addition of 100, 200, and 300 μL of MB dispersion (10¹⁰ MBs per mL, * *p* < 0.05); c) effect of US exposure duration on O₂ release from MBs: change in O₂ concentration following US treated (circles) or untreated (squares) injection of 300 μL of MBs dispersion. Adapted from a) [288], b) and c) [111]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ill-conceived component selection, sub-optimal emulsion characteristics, clinical evaluation blunders, and/or funding issues. The capacity for well-designed, properly engineered PFC emulsions to deliver O₂ has been proven. Large-scale, industrial production of high purity, readily excretable PFCs, and of reproducible small and narrowly-dispersed sterile NEs with long-term shelf stability has been established. Side effects related to PFC NEs appear limited to short-lived flu-like symptoms and mild transient thrombocytopenia, which could likely be further reduced.

Moreover, advanced fluorocarbon-hydrocarbon diblock-stabilized, size-controlled NEs have been obtained that have a shelf-life of over two years at room temperature (Fig. 3) [1,22,49,50]. These emulsions effectively delivered O₂ to cells [294], isolated organs [66], animal models [295,296], and also proved advantageous for ¹⁹F MRI imaging [297,298].

4.3. Further demonstration of oxygen delivery efficacy by PFC nanoemulsions

Additional demonstrations of effective O₂ delivery by PFC NEs are continuously being reported. They support the ability of such O₂ therapeutics to protect organs and salvage tissues at risk of hypoxia, enhance O₂-dependent cancer therapies, assist regeneration of damaged tissues, preserve organs for transplantation, help regulate gas supply in biotechnology, and more. The early goal of a “blood substitute” to replace blood or RBC transfusion has all but vanished, primary due to rather short intravascular persistence. The potential of PFC-based O₂-carriers in emergency medicine and more generally while waiting for blood transfusion still holds. Some of the examples provided below also illustrate the concurrent (theranostic) capacity of PFC NEs for both diagnostic imaging and therapeutic performance.

4.3.1. Protecting and salvaging tissues from hypoxia

Administration of PFC NEs can help counteract hypoxia, a deficit in O₂ supply versus consumption. Notably, gut perfusion and oxygenation were significantly improved in a group of patients instrumented for gut tonometry during the cardiac surgery phase III trial of *Oxygent*, indicating protection of the gastrointestinal tract against hypoxia (Fig. 10) [299]. This could constitute a valuable indication for PFC NE products and an acceptable endpoint for clinical trials. Administration of *Perftoran* along with moderate ANH reduced the need for allogeneic RBCs in cardiac surgical valvuloplasty patients [300]. *Oxycyte* delayed the need for blood transfusion in acutely anemic hamsters [301]. Systemic and microvascular O₂ delivery was considerably augmented in hemodiluted, O₂-breathing hamsters by administration of this product; acid-base balance, mean arterial pressure and cardiac output went back to normal; no vasoconstriction was observed [285]. A F-octylbromide NE improved myocardial oxygenation and cardiac function in the isolated working rabbit heart [302]. *Oxygent* enhanced cerebral blood flow and tissue oxygenation in hemodiluted rats [303]. A FmHm-stabilized F-octylbromide NE allowed survival of “bloodless” rats after near-total exchange-transfusion, enabling high-resolution imaging of brain astrocytes and neurons by two-photon laser-scanning microscopy [296]. The same NE resuscitated rabbits exposed to acute hemorrhagic shock [295]. An intravenously administered *Oxygent*-type formulation alleviated acute lung injury in rats [304]. A (F-hexyl)octane NE lessened brain tissue hypoxia, preventing irreversible brain damage in an ischemic rat model [305]. *Oxycyte* improved cognitive function and reduced neuronal cell loss in rats subjected to percussion brain injury [306], and

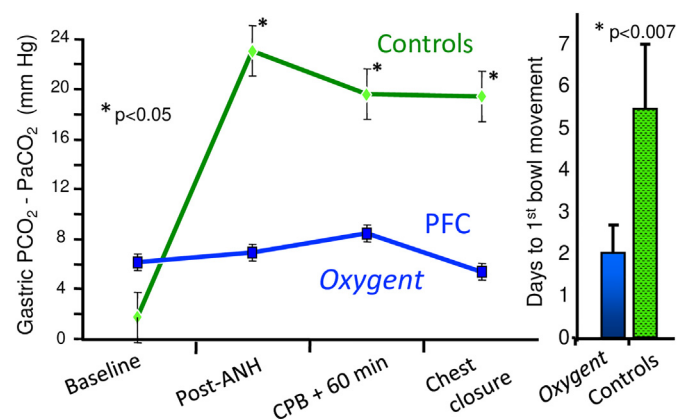


Fig. 10. Prevention of gastro-intestinal tract hypoxia and organ protection by a F-octylbromide NE during surgery. Gut tonometry data collected during a Phase III clinical trial of *Oxygent* in coronary artery bypass graft surgery patients (ANH: acute normovolemic hemodilution; CPB: cardiopulmonary bypass). The gastric pCO₂ gap (PCO₂ - PaCO₂), a marker of hypoxia, was preserved in the *Oxygent* group, while it increased dramatically when no emulsion was administered. Adapted from [299].

markedly reduced lesion area and cell death after traumatic spinal cord injury in rats [307]. The NE also reduced infarct size and improved functional recovery after acute ischemic stroke in the rat [308]. Resuscitation of mice from hemorrhagic shock using *F*-octylbromide emulsified with RBC membrane proteins was deemed comparable to that obtained with whole blood [51,54]. Reduction of tissue hypoxia with *Perftoran* in cats with post-hemorrhagic anemia was reported [309]. Blood from sickle cell disease patients, when mixed with a PFC emulsion in vitro, showed increased O_2 extraction, indicating facilitated O_2 transport [310].

Pulmonary administration of an aerosolized *F*-octylbromide NE achieved effective O_2 delivery in rats [311] and in a rabbit model of acute lung injury [312]. Topically applied 2H,3H-decafluoropentane NE droplets enhanced transdermal O_2 release under US-exposure for hypoxic wound healing [48,234,235].

4.3.2. Enabling oxygen-dependent cancer therapies

Hypoxia within solid tumors is a major impediment to most current cancer therapies, including radio-, photo(sono)dynamic-, chemo- and immunotherapies, most of which require a supply of O_2 for production of singlet oxygen and other ROS that are the actual cancer cell-killing agents [313–316]. These therapies themselves consume O_2 , which aggravates hypoxia and further impedes tumor response. The damage caused to the microvasculature of the tumor can also hamper O_2 delivery. In addition, hypoxia confers immunosuppressive attributes to the tumor microenvironment that allow tumors to escape detection by the immune system [317,318]. PFC-based O_2 carriers can help overcome tumor hypoxia and deliver the O_2 required for potentiating O_2 -dependent cancer therapies [288,315,316]. Of note, radicals such as 1O_2 benefit from considerably prolonged lifetimes in PFCs [319]. Moreover, small O_2 -loaded NE (or P-SNE) droplets can extravasate from leaky tumor vessels and accumulate in tumor tissue; O_2 release can then be actuated on demand, for example with focused US.

Radiosensitization was, for example, achieved using an *Oxygent*-type emulsion that suppressed hypoxia-associated radio-resistance in brain tumors in mice [320]. Hypoxic xenograft prostate tumors were re-oxygenated, even without augmented O_2 breathing, due to accumulation of a *F*-octylbromide NE in the tumor in mice [321]. Decoration of *F*-hexane NE droplets with TaO_x NPs improved tumor oxygenation, augmented the X-ray absorbing capacity and increased local radiation energy density in tumor tissue [322]. *F*-15-crown-5-ether NE droplets encapsulated in a polymer shell, then coated with RBC membrane proteins, accumulated in tumor tissue, reduced tumor hypoxia and enhanced radiotherapy in mice [51,54]. NEs of *F*-tributylamine and of a *F*-tripropylamine/*F*-decalin mixture delivered O_2 for radiotherapy enhancement and also augmented RBC infiltration in murine colon and breast tumors, which was deemed to provide a second-stage O_2 delivery [323]. However, clinical use of *F*-tributylamine and even tripropylamine can hardly be envisaged due to overlong organ retention; the former may also raise environmental issues [324]. Use of PFC-based O_2 -carrying systems and other nanomaterials to enhance radiotherapy has been reviewed [325].

There is also renewed interest in PFC-assisted photodynamic therapy (PDT) and sonodynamic therapy (SDT). PDT uses a photosensitizer such as porphyrins, cyanines, plasmonic gold NPs or quantum dots that, upon excitation by light, usually near-infrared laser light, assists generation of the ROS that destroy tumor cells. Numerous photosensitizer-loaded, O_2 -carrying PFC NEs have demonstrated their capacity to significantly reduce tumor hypoxia, enhance 1O_2 production upon irradiation and inhibit tumor growth in animal models (Fig. 11) [258,287,326–333]. A *F*-15-crown-5-ether/albumin emulsion achieved focused ultrasound (FUS)-activated local O_2 delivery for enhanced radio- and photodynamic therapy (Fig. 12) [288]. A NE comprising *F*-tributylamine and indocyanine green, and coated with RBC membrane fragments, accumulated in tumors and enhanced PDT efficacy in mice [52]. Dissolution of a fluorinated photosensitizer in PFC NEs led

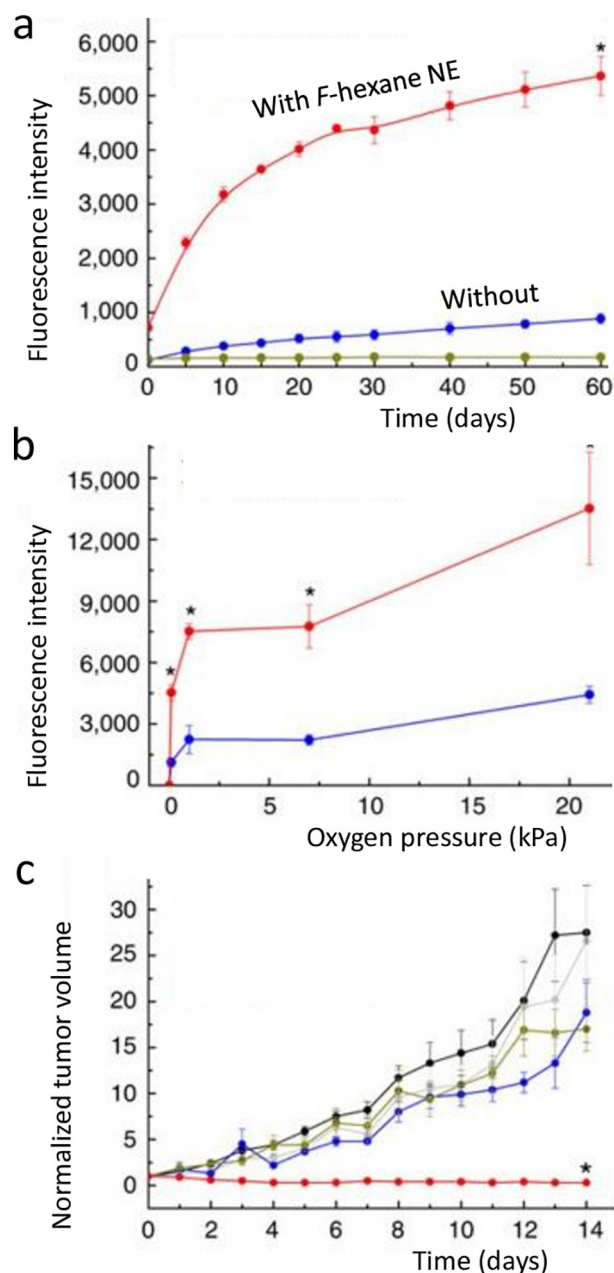


Fig. 11. Singlet oxygen (1O_2) production under near-infrared irradiation, a) as measured by singlet oxygen sensor green fluorescence intensity over time for water (green), heptamethine (IR780) dye-loaded liposomes (blue) and a dye-loaded, O_2 -carrying *F*-hexane NE (red); b) fluorescence intensity as a function of O_2 pressure for dye-loaded liposomes (blue) and dye-loaded *F*-hexane emulsion (red), and c) changes in tumor volume during photodynamic treatment of tumor-bearing mice receiving saline (controls, black), dye-loaded liposomes (light green), the same under near-infrared irradiation (blue), the *F*-hexane emulsion with the liposomal dye (green), and the latter under irradiation (red) [287]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to superior PDT production of 1O_2 and caused more melanoma cell death in vitro upon irradiation [258]. A *F*-octylbromide emulsion doped with a heptamethine photosensitizer and functionalized with a small tumor-penetrating peptide enhanced PDT efficacy in mice [331]. A *F*-hexane NE loaded with a redox-responsive tetrapyrrole photosensitizer provided fluorescence/photoacoustic imaging-guided enhanced PDT in tumor-bearing mice [332]. O_2 -carrying *F*-hexane NE droplets loaded with an inorganic photosensitizer consisting of hybrid gold/ SiO_2 /Cu $_2$ O NPs for enhanced plasmon-induced resonance energy

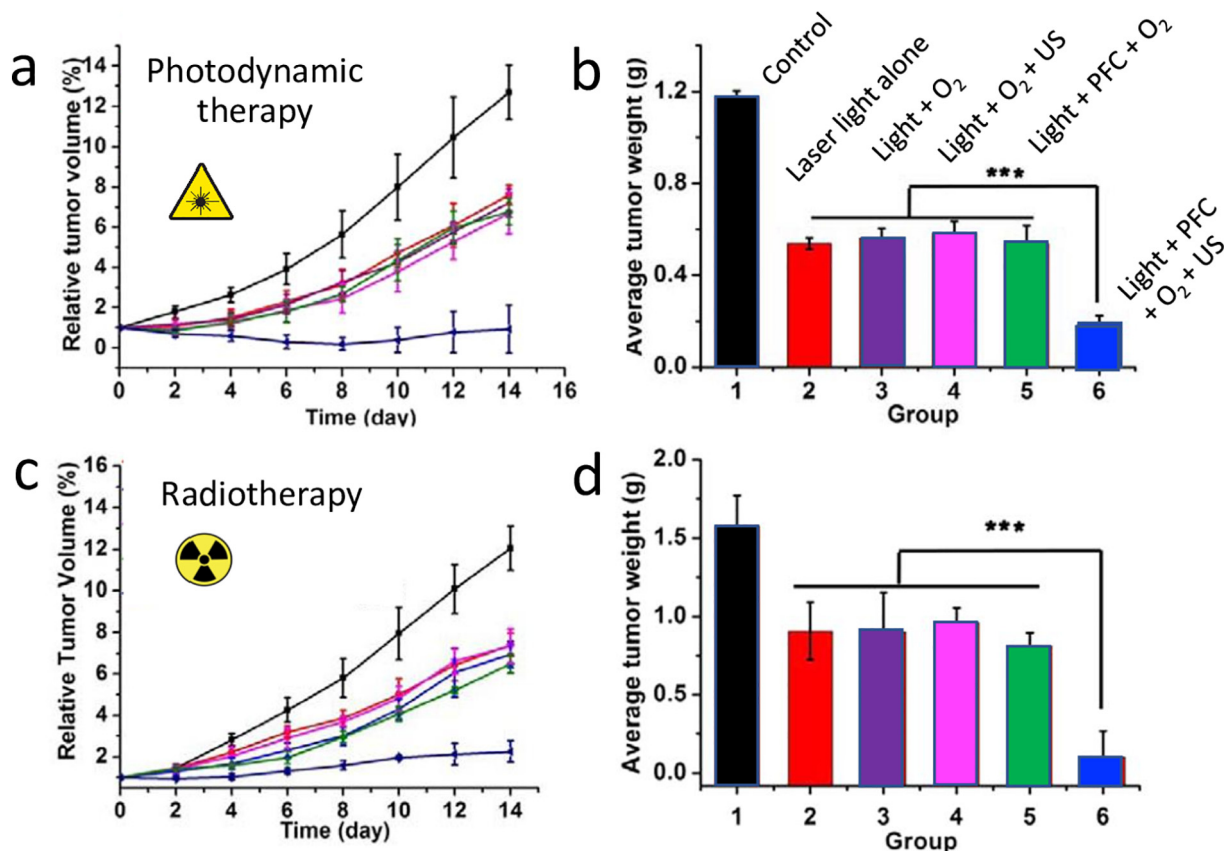


Fig. 12. US-triggered tumor oxygenation-enhanced photodynamic therapy (a and b) and radiotherapy (c and d) using a photosensitized F-15-crown-5-ether NE; the color code is provided in frame b). a) Tumor growth over time in mice after various photodynamic treatments; b) average tumor weight after these treatments; c) tumor growth, and d) average tumor weight after radiotherapy treatments. Adapted from [288].

transfer, led to deeper light penetration in tissues and improved PDT efficacy against tumor growth [334]. A tumor pH-responsive, photosensitive *F*-hexane NE permitted O₂-potentiated PDT and US/fluorescence imaging [330]. A *F*-polyether NE loaded with a photosensitizer also improved PDT in tumor-bearing mice [335]. Indocyanine green-loaded *F*-octylbromide “nanoliposomes”, more likely a PFC-in-water NE, delivered O₂ for synergistic photodynamic and photothermal tumor growth inhibition in mice while also providing fluorescence, photoacoustic and CT contrast imaging [336]. A *F*-hexane NE with a perylene diimide polymer shell provided O₂ for PDT promotion, as well as enhanced PTT, and US and photoacoustic imaging [329].

Sonodynamic therapy, an emerging modality that relies on US activation of sonosensitizers for converting O₂ into ROS, and operates via US-induced MB cavitation to produce the free radicals, can treat tumors at much greater depth than PDT and may elicit lesser side-effects (photo-toxicity) [337–339]. SDT, like PDT, is hampered by tumor hypoxia and can thus benefit from O₂-carrying PFCs. The mechanisms of PDT, SDT and combined PDT and SDT have recently been reviewed [340]. Diverse photosensitizer-loaded NEs demonstrated significantly reduced hypoxia, enhanced ¹O₂ generation by SDT, and inhibited tumor growth in animals. A *F*-tributylamine NE with a PLGA shell that incorporated an heptamethine dye and doxorubicin, improved combined SDT/chemotherapy treatment of hypoxic breast tumor in mice [341].

Response to many major chemotherapeutics (e.g. cisplatin, doxorubicin, paclitaxel, docetaxel) also relies on ROS generation, and hence, on O₂ supply. Several tumor hypoxia-induced chemoresistance mechanisms could be counteracted with PFC-based oxygenating nanocarriers. Hypoxia-induced resistance to cisplatin was overcome by administration of a *F*-octylbromide NE (Fig. 13) [342]. High-intensity focused

US-responsive tumor-targeted NEs of various PFCs (*F*-pentane, *F*-hexane, *F*-octylbromide and *F*-poly(ether)), stabilized by poly (organosiloxane)-derivatized phospholipids, were investigated for O₂ and doxorubicin co-delivery [343]. The *F*-hexane product produced the best results in terms of tumor hypoxia relief, multidrug resistance alleviation and tumor metastasis inhibition. A *F*-tributylamine/albumin NE displayed platelet inhibiting abilities, resulting in PFC accumulation in the tumor and decreased tumor hypoxia [344].

Immunotherapy, which boosts the body's natural defenses to fight disease, emerges as a highly promising novel cancer therapy. However, a main obstacle to its effectiveness is again that hypoxia can depress certain functions of immune cells. In particular, hypoxic tumor cells over-produce adenosine, which is a potent suppressor of T cells and natural killer (NK) cells [318]. Oxygenation of the tumor microenvironment can thus greatly help stimulate immunotherapy [315–317]. It was also noted that PFC NE-mediated platelet inhibition stimulated intratumoral infiltration of T cells, resulting in enhanced immunotherapy [345]. After exposure to focused US, bone marrow-derived monocytes loaded with a *F*-pentane NE and doxorubicin-containing poly(acrylic acid-co-distearin acrylate) NPs were used as a cellular Trojan horse that accumulated in the tumor and induced apoptosis of cancer cells in tumor-bearing mice [346].

4.3.3. Regenerating bones, nerves, and engineering tissues

O₂-delivering PFC products also appear promising for repairing damaged nerves, tendons and bones, as well as for culture of cells and tissues and diverse biotechnological productions. For example, a *F*-tributylamine-based O₂-carrying fibrin hydrogel injected into collagen-chitosan conduits seeded with cells of the peripheral nervous system provided O₂ for sciatic nerve regeneration and functional

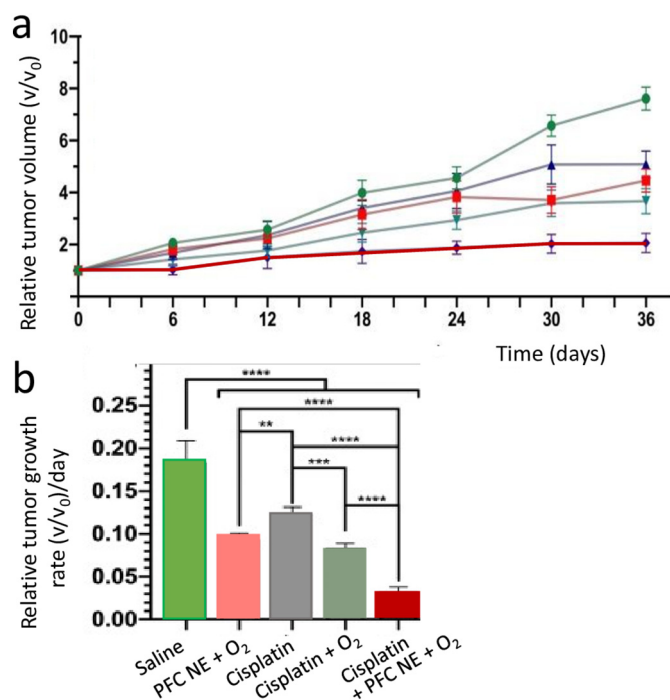


Fig. 13. An O₂-carrying *F*-octylbromide NE helps overcome hypoxia-induced resistance to cisplatin therapy. a) Tumor growth in mice treated with saline (green circles), the PFC NE + O₂ (red squares), cisplatin (grey triangles), cisplatin + O₂ (green triangles) or cisplatin + PFC NE + O₂ (diamonds); b) relative tumor growth rate for the same treatment. Adapted from [342].

recovery in rats [232]. *F*-octane NE droplets loaded in hollow polymeric microparticles delivered O₂, thereby extending the survival and preserving the osteogenic differentiation potency of hypoxic human periosteal-derived cells; new bone formation was accelerated and bone density was increased in cultured cells and in minipigs with mandibular osteomyelitis [347,348]. Degenerative intervertebral disc repair was realized using a simvastatin-loaded *F*-pentane phase-shift double emulsion, along with US, in isolated rabbit spinal discs [215]. Diverse biotechnological culture systems for enzyme, hormone, anticancer drug, monoclonal antibody, genetic material, or tissue graft production have investigated use of neat or emulsified PFCs, or PFC-enriched gels or scaffolds for transfer of O₂, CO₂ and other gases for improving

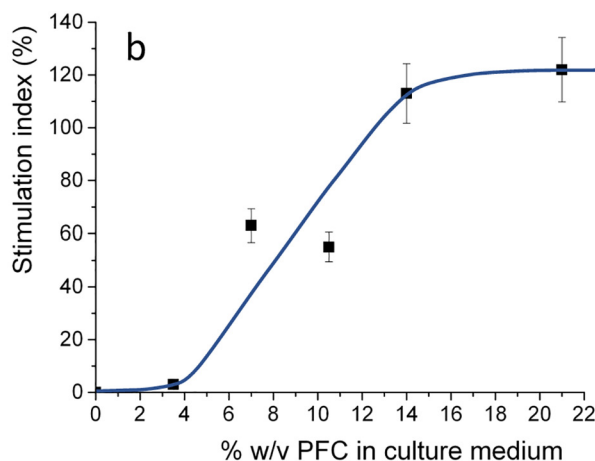
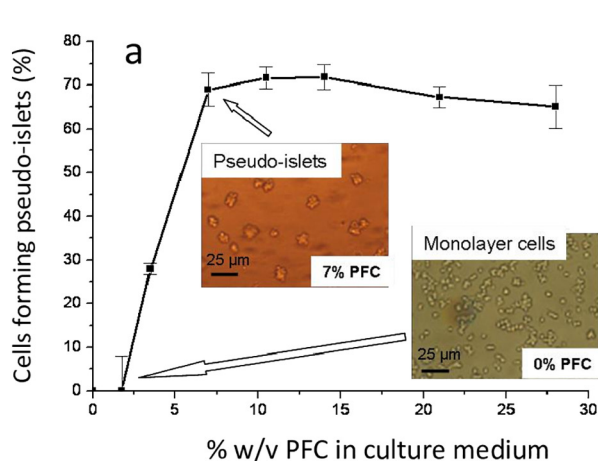


Fig. 14. Contact with an O₂-delivering *F**nm*-stabilized PFC NE increased cell viability and a) prevented adhesion of β -cells to the Petri dish and promoted formation of insulin-secreting pseudo-islets; b) stimulation of insulin production showing the beneficial effect on pseudo-islet functionality (the stimulation index is the ratio of insulin concentration at high versus low glucose concentration). Adapted from [352].

biomass yields [349]. Incorporation of *F*-octylbromide in an alginate gel resulted in a substantial increase in O₂ permeability and transport for tissue engineering [350].

4.3.4. Preserving isolated organs and tissues

Oxygen is indispensable for the preservation of organ grafts destined for transplantation. Advances in organ and tissue preservation could substantially increase the number of receivers. *Oxygent*-type NEs improved liver-transplantation survival in rats [351]. A *F**nm* diblock-stabilized *F*-octylbromide NE prevented hypoxia of pancreatic rat Langerhans islets and preserved their insulin production capacity (Fig. 14) [294,352,353]. Ex vivo lung reconditioning and transplantation with this NE was deemed safe and improved tissue preservation and graft function in a pig model [66]. Such a reconditioning strategy could help mitigate graft shortages. The pharmaceutical design and development of PFC nanocolloids for O₂ delivery in regenerative medicine has been reviewed [354].

5. The O₂ microbubble delivery option—a change in paradigm

An alternate option for O₂ delivery relies on the direct administration of PFC-stabilized O₂ micro- or nanobubbles rather than emulsion droplets. This represents a definite change in paradigm since in the former case the PFC does no longer act as a solvent for physical dissolution of O₂, but as a gaseous stabilizer of an O₂ microbubble. Such MBs can also be generated through vaporization of NE droplets. US exposure can prompt on-demand O₂ delivery from MBs located at the disease site.

5.1. Injectable PFC-stabilized micro- and nanobubbles

O₂-loaded PFC-stabilized MBs can be fabricated *de novo* and administered as such. In addition, they offer the possibility of US image-guided and triggered O₂ delivery. Thus, focused US-triggered destruction of lipid-shelled O₂ MBs osmotically stabilized by a small amount of *F*-butane significantly enhanced image-guided local O₂ delivery (Fig. 9b,c) [111]. In some instances perfluorochemical MBs have served as templates for the preparation of O₂ MBs: PFC-stabilized MBs are prepared first, after which the PFC is exchanged for O₂ [112,113,355]. US-targeted destruction of *F*-butane MBs sparged with O₂ and coupled to a sonosensitizer (Rose Bengal) and chemotherapeutics potentiated simultaneous chemo- and sonodynamic treatment of pancreatic [113,356] or breast [355] cancer in xenografted tumor-bearing mice. Similar MBs with superparamagnetic iron oxide NPs loaded in their shell allowed magnetic targeting [357]. *F*-propane-stabilized

O₂-loaded MBs substantially elevated pO₂ in a rabbit breast tumor model upon US-triggered burst [358]. *F*-propane enabled the formation of Span-60-shelled MBs that, after freeze-drying, were laden with O₂, allowing US-assisted sensitization of hypoxic breast tumors to radiotherapy in mice [359,360]. US-mediated local O₂ release from *F*-propane/O₂ MBs inhibited hypoxia and accomplished vascular normalization for enhanced tumor perfusion and doxorubicin delivery and penetration [361]. O₂ delivery by MBs and NBs for effective reversal of hypoxia and photo-triggered cancer theranostics has been thoroughly reviewed [99,140]. SF₆ MBs (*SonoVue*) enhanced the antitumor effects of sinoporphyrin sodium-mediated SDT in vitro and in mice [362]. Intraperitoneal administration of O₂/paclitaxel-loaded, folate-targeted *F*-propane MBs allowed US-mediated MB destruction for delivery of O₂ and paclitaxel to ovarian cancer cells and tumor-associated macrophages in a murine model [363,364].

5.2. Microbubbles generated through acoustic nanoemulsion droplet vaporization

A still further possibility is to generate O₂-delivering MBs by in vivo vaporization of previously infused P-SNEs of volatile PFCs. Acoustic droplet vaporization is most commonly used. P-SNEs of *F*-pentane, with its boiling point at 28–30 °C, i.e. somewhat below human body temperature, are the most thoroughly investigated. Dextran-shelled *F*-pentane nanobubbles showed US-enhanced O₂ delivery in vitro [165]. Small doses of the *Echogen F*-pentane P-SNE effectively protected O₂-breathing rats from severe anemia [365], and significantly reduced hypoxia caused by right-to-left shunt in the lung of pigs [366].

These early demonstrations were consolidated by studies using the *F*-pentane/*F*-surfactant P-SNE NVX-108 for overcoming the tumor hypoxia barrier to cancer radio-, chemo- & immunotherapy [198]. O₂ delivery by this P-SNE protected the ischemic brain in a rabbit stroke model, reducing stroke volume and improving neurological outcome [367]. It extended the “golden hour” time window for recombinant tissue plasminogen activator (rtPA) thrombolysis treatment (Fig. 15) [368]. The product also reduced the extent of infarction in a murine stroke model [369], and of myocardial ischemia-reperfusion injury in acutely infarcted rat hearts [370]. Its administration increased cerebral O₂ tension and lessened post-traumatic brain injury hypoxia in rats [371], and substantially reduced infarct size in a murine model of cardiac ischemia [372]. Use of NVX-108 as a resuscitation fluid for management of hemorrhagic shock and traumatic brain injury has been reviewed [373]. Its O₂ offloading behavior was studied in vitro [374]. O₂ delivery decreased ischemic stroke infarct volume in a rabbit model [375]. The emulsion markedly enhanced tumor pO₂ and radiotherapy efficacy in pancreatic tumor xenografts in mice [376]. The O₂ carried by another multifunctional *F*-pentane P-SNE contributed to enhanced

photodynamic treatment, leading to glioblastoma tumor eradication in mice (Fig. 16) [329]. A similar emulsion reduced brain damage after traumatic brain injury-related hypoxia in swine [196].

Clinical Phase Ib/II studies of the safety, tolerability and effectiveness of NVX-108 in overcoming tumor hypoxia have been conducted in primary glioblastoma multiforme patients, in combination with radiation and temozolomide chemotherapy [197,198]. No significant adverse effects were noted in this initial dose exploration phase. The product has received Orphan Drug Designation as an O₂ providing radiosensitizer for chemoradiation treatment of glioblastoma. A randomized prospective phase II trial is on-going. A small dose-escalation study in acute ischemic stroke patients concluded that the product was safe at the dose tested and may improve clinical outcomes [377].

A paclitaxel and indocyanine green-loaded *F*-pentane P-SNE provided combined photo(sono)dynamic therapy and chemotherapy as well as dual US and photoacoustic imaging [378]. A similar P-SNE loaded with oxaliplatin, when subjected to near-infrared and ultrasound, augmented the anti-tumor effect, enhanced immunological potency and provided dual-mode imaging in mice [379]. Other examples of uses of *F*-pentane and heavier PFCs for O₂-dependant cancer therapy enabling are provided in Section 4.3.2; it is sometimes difficult to appreciate whether phase shift is implicated.

6. O₂ delivery by further PFC-based colloids

A few recent studies that investigate O₂ transport by fluorinated colloids other than PFC NEs, MBs or P-SNEs, in which O₂ delivery is either a

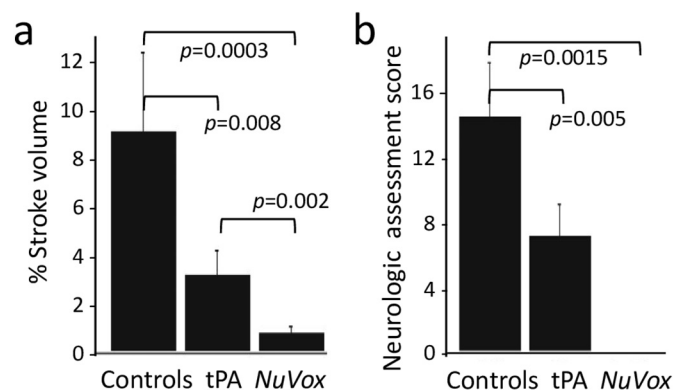


Fig. 15. Administration of an O₂-delivering *F*-pentane P-SNE (NuVox) extended the window for tissue plasminogen activator (tPA) therapy in a rabbit stroke model: a) % stroke volumes at 24 h for animals treated with the PFC emulsion and tPA vs. tPA alone, and vs. controls; b) neurologic assessment scores at 24 h. Adapted from [368].

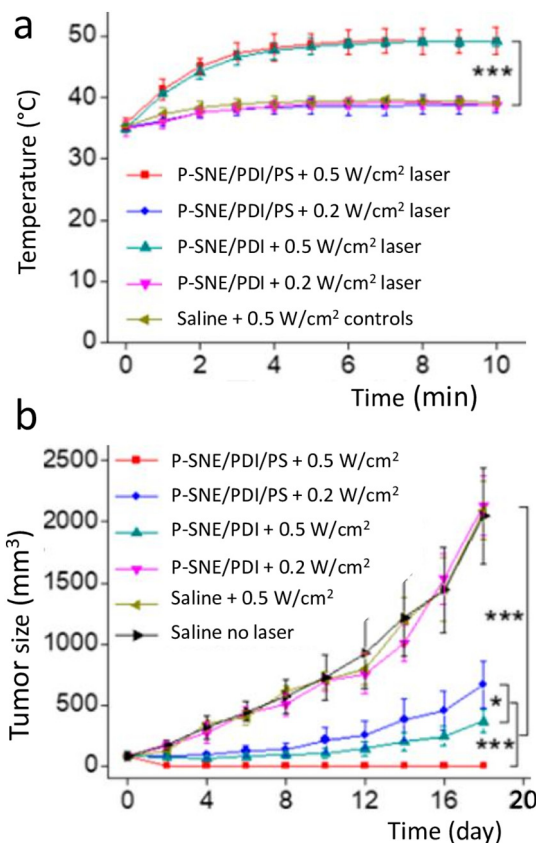


Fig. 16. Photoacoustic and US imaging-guided combined photothermal (PTT) and O₂-enhanced photodynamic (PDT) cancer therapy in mice using a *F*-pentane P-SNE with a perylene diimide photoabsorber shell (P-SNE/PDI) that can include a fluorinated zinc phthalocyanine photosensitizer (PS), under 0.5 or 0.2 W/cm² laser light irradiation. a) Temperature curves at tumor region; b) tumor growth curves; complete tumor eradication was observed in the O₂-supplying *F*-pentane P-SNE/PTT + PDT group subjected to 0.5 W/cm² laser irradiation. Adapted from [329].

deliberate part of a theranostic strategy, or that pursue other goals, but inevitably also provide an O₂ reservoir, are illustrated here. Examples of *F*-alkylated or *F*-arylated components of fluorinated polymeric micelles formed for this purpose are provided in Table 7. ¹O₂ production and PDT efficacy of micelles made of PEGylated copolymers bearing pending pentafluorophenyl groups and photosensitizing porphyrins **28** increased when the *F*-phenyl/porphyrin ratio increased [380]. Augmented ROS production for PDT therapy was also obtained with O₂-carrying, photosensitizer-doped micelles made from *F*-octyl chain-bearing branched poly(ethylene imine) **22** [252]. Doxorubicin-loaded micelles made of a polymer with PEG chains outside, a central photosensitive heptamethine dye, and C₉F₁₉ chains inside **29**, were reported to enhance O₂ delivery, resulting in synergistic PDT and chemotherapy in mice [381]. O₂-carrying photosensitive NPs of pegylated poly(β-amino ester) polymer with pending *F*-pentyl chains **30**, co-loaded with a specific oxydoreductase inhibitor (NLG919) involved in tumor escape from immuno-surveillance, relieved immune suppression and enhanced PDT

efficacy through alleviation of tumor hypoxia (Fig. 17) [382]. Fluorinated, hyaluronic acid-based polymeric micelles made of compound **24** showed O₂-enhanced PDT efficacy against choroidal melanoma [58].

F-hexane was encaged in targeted porous Fe₃O₄ NPs loaded with etoposide for MRI-monitored O₂-enhanced chemotherapy in mice [223]. *F*-hexane loaded in hollow MoS NPs surface-modified with a phthalocyanine and with human serum albumin, served as O₂ carriers for PDT as well as for fluorescence, photoacoustic and CT imaging in mice [221]. *F*-hexane-loaded PEGylated hollow Bi₂Se₃ NPs provided augmented near IR and X-ray absorbing capacity, and achieved enhanced light-induced O₂ delivery to fight hypoxia-associated radio-resistance in tumors in mice [224]. O₂ diffusion, hypoxia alleviation and enhanced radiotherapy were also obtained with *F*-pentane-loaded, ⁶⁴Cu-labeled mesoporous organosilica NPs, as well as US/photoacoustic and PET imaging (Fig. 18) [228].

F-octyl chains were grafted onto sonosensitizer-carrying hollow silica NPs for US-promoted O₂ delivery for enhanced SDT efficacy in

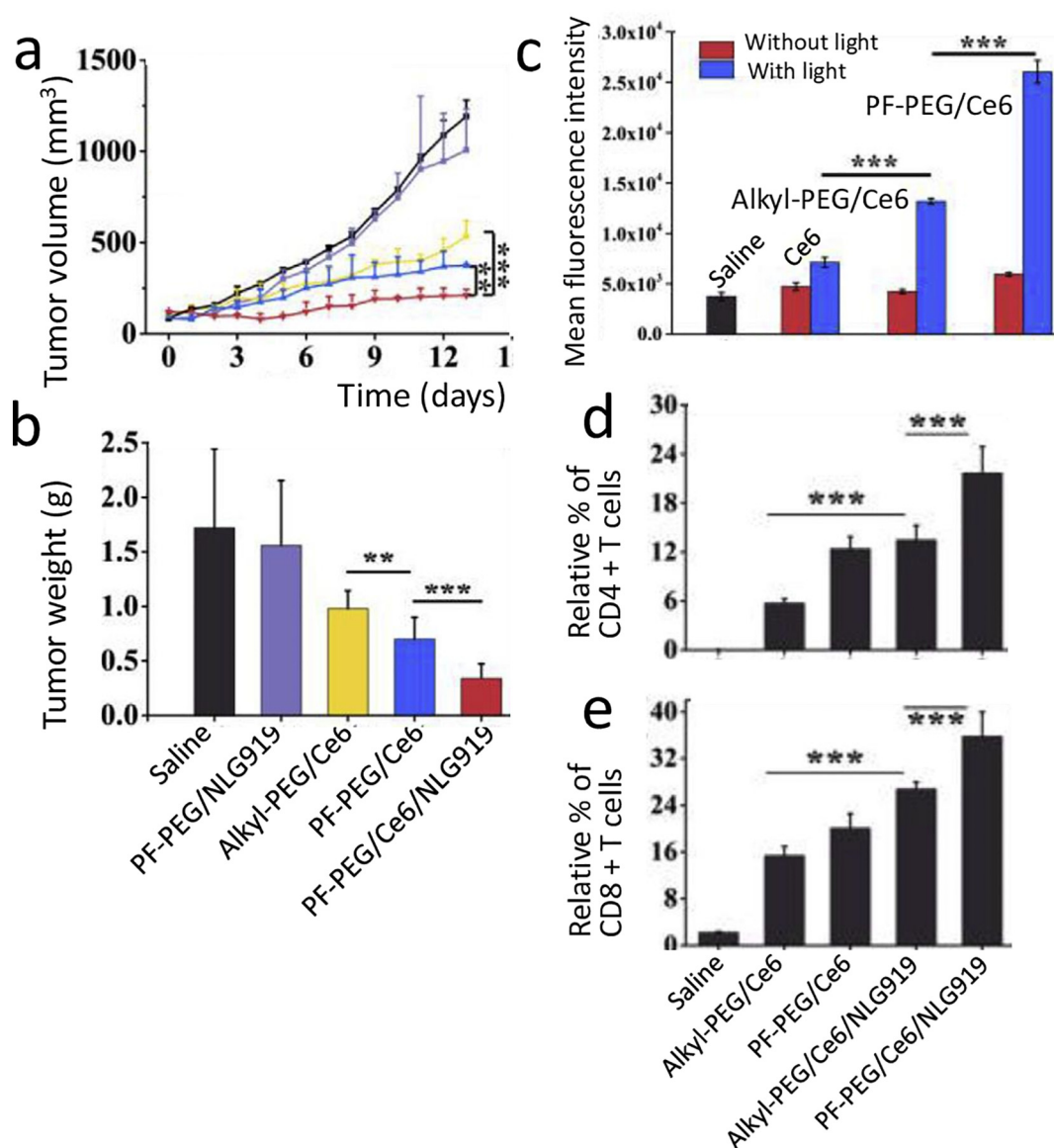


Fig. 17. Simultaneous O₂-enhanced photodynamic therapy and boosted antitumor immune response obtained in mice with O₂-carrying micelles of a pending *F*-pentyl chain-fitted PEG-polymer (PF-PEG) loaded with a chlorin (Ce6) photosensitizer and an inhibitor (NLG919) of indoleamine-2,3-dioxygenase (IDO), a tumor-expressed enzyme that is involved in tumor immune escape; a non-fluorinated analog (alkyl-PEG) polymer was also investigated. a) Inhibition of tumor growth (tumor volume over time) by various treatments (laser light was 55 W/cm²; same color code as in b); b) tumor weight at the last day of treatment; c) mean fluorescence intensity, denoting ROS generation capacity using singlet oxygen sensor green for various formulations, with and without laser light; d) percentage of tumor infiltrating CD4+ T cells, and e) CD8+ T cells. Adapted from [382].

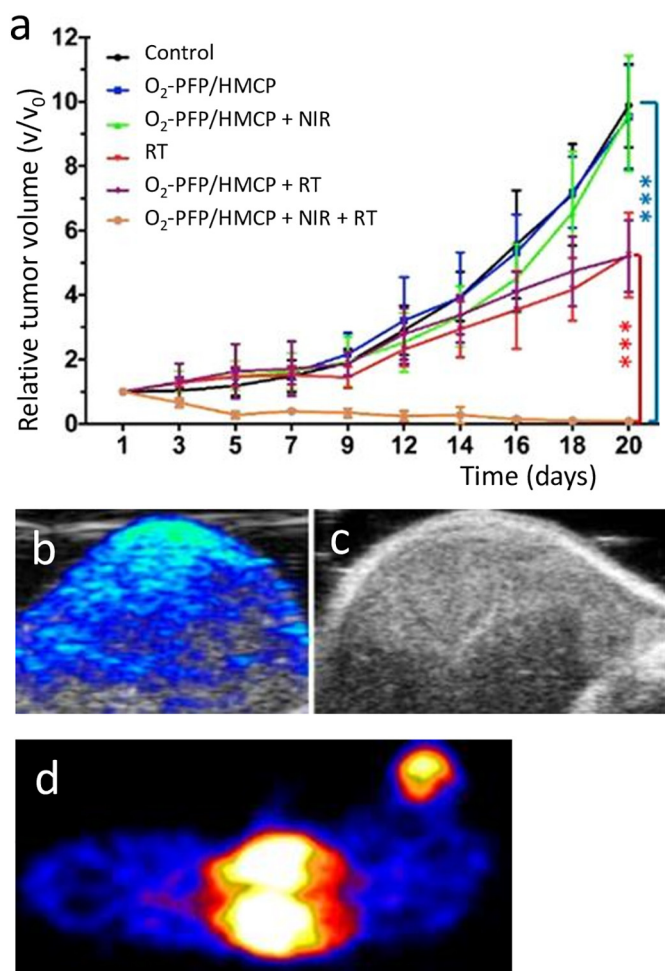


Fig. 18. F-pentane (PFP)-loaded pegylated biodegradable hollow mesoporous organosilica NPs with surface-bound CuS (designated as HMCP) for laser light-induced MB-enhanced, O₂-sensitized radiotherapy. a) Relative tumor volume over time in mice for various treatments (NIR: near-infrared, RT: radiotherapy); b-d) photoacoustic, US and PET images, respectively; the latter was obtained by ⁶⁴Cu labeling of the NPs. Adapted from [228].

pancreatic tumor cells and tumor xenografts in mice (Fig. 19) [253]. F-sebacic acid-bridged tetra(hydroxyphenyl)porphyrin-cross-linked porous PEGylated polymeric NPs filled with F-15-crown-5-ether provided O₂ for hypoxia relief and enhanced PDT in mice; ^{99m}Tc radioisotope-chelating enabled SPECT imaging [383]. Tumor-targeted, pH-sensitive dextran NPs, grafted with F-propyl poly(L-lysine) dendrons and doped with a cyanine photosensitizer, was used for PDT and inhibited breast tumor growth and reduced lung and liver metastasis in mice [57]. Enhanced ¹O₂ production and PDT were also reported with O₂-carrying hybrid F-alkylated siloxane-polystyrene NPs doped with a F-arylated zinc phthalocyanine photosensitizer [254].

7. Perfluorocarbon colloids can also deliver other gases, contrast, energy, drugs, genes, and have a clear vocation for theranostics

The recent years have witnessed an outburst of prospective PFC-based nanomedicines for an increasing number of indications other than O₂ delivery. Objectives include increasingly sophisticated diagnostic imaging modalities, focused delivery of energy in various forms, controlled as and when required delivery of therapeutics, ... and even control of urinary stress. Even when the primary goal is different, the F-colloids put to work predictably do carry, and can release a certain amount of O₂. Multiple functionalization can lead to theranostic

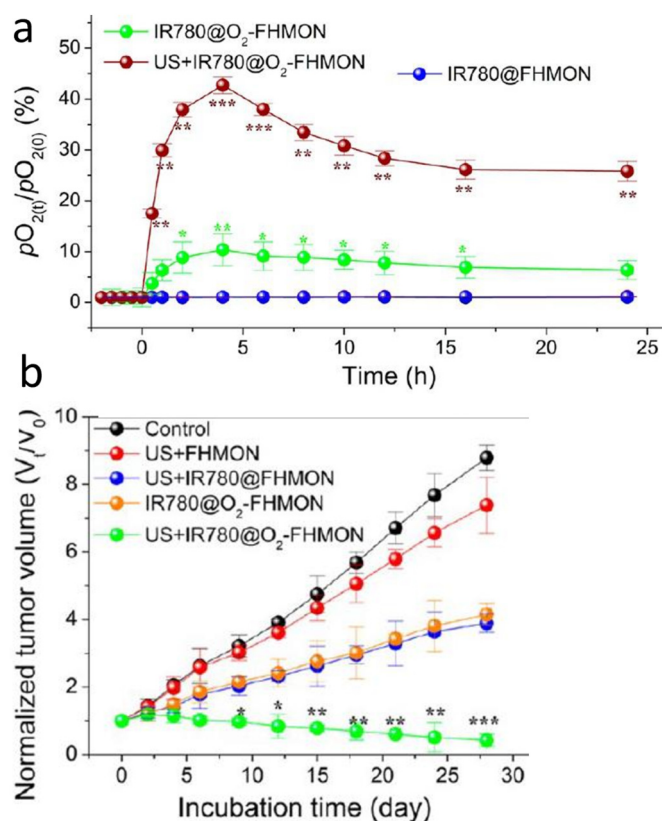


Fig. 19. F-octyl chain-functionalized hollow mesoporous organosilica NPs (named FHMON) loaded with a sonosensitizer (IR780) for alleviating hypoxia-induced resistance to sonodynamic pancreatic cancer therapy. a) Hypoxia reversion in solid pancreatic tumor implanted in mice, as indicated by relative pO₂ variation over time, for diverse treatments; b) reduction of hypoxia-induced resistance to treatment, as reflected by tumor volume changes over time. Adapted from [253].

procedures and synergistic effects. Safely crossing the blood-brain barrier for delivery of O₂, energy and other therapeutics is a major challenge that could be met by appropriate MB or P-SNE/US combinations. The capacity of PFC colloids for simultaneous diagnostic imaging/monitoring/assessing functions and O₂, energy and drug delivery open countless theranostic opportunities.

7.1. Delivery, or removal, of therapeutic gases other than oxygen

Gases other than O₂ can also be delivered by PFC NEs, MBs and other PFC constructs. A F-octane NE delivered, for example, nitric oxide as an antibacterial agent [384]. Likewise for F-propane/NO MBs [385]. Pulsed US-induced release of NO from F-propane-stabilized MBs induced vaso-relaxation in porcine carotid artery tissue [386]. Complementing graphene oxide sheets with an F-alkylated PEG improved the preservation of dissolved NO gas, thereby enhancing antibacterial activity against *E. coli* and *S. aureus* [387,388]. Mixed hydrogen/F-propane MBs delivered H₂ as an antioxidant against myocardial ischemia-reperfusion injury in a rat model [389]. Xe-containing F-propane MBs were prepared for US-triggered neuroprotective Xe release for treatment of stroke [390].

PFCs may also help retrieve undesired gases, such as N₂ for prevention and treatment of decompression sickness, but the results obtained under diverse conditions were uncertain. Administration of a F-pentane P-SNEs (*Echogen*) increased central venous O₂ tension and effectively enhanced tissue denitrogenation under O₂ breathing in pigs [391]. Another study with a F-pentane P-SNE found no effectiveness in rodents [392]. An undesirable increase of pulmonary artery pressure and no

effect on survival were observed with *Oxycyte*, also in swine [393]. Further options are being discussed [394]. Removal of arterial gas embolism-causing air bubbles formed during use of cardiovascular bypass machines has been demonstrated with PFC NEs [395]. PFCs can also help eliminate the excess O_2 that inhibits cell growth in certain bioprocesses. The aid of a *F*-octylbromide/*F*-surfactant NE led, for example, to a 5-fold increase in biomass production in microalgae cultivation [396]. The rapid scavenging of O_2 dissolved in an air-saturated fluid during US-activated droplet vaporization of a *F*-pentane/albumin P-SNE was investigated in vitro and modeled [397,398].

7.2. ^{19}F MRI, ultrasound and multimodal imaging for diagnosis of disease, guidance for procedure, assessment of treatment outcome

As noted, PFC NEs provide ideal ^{19}F NMR and MRI probes, including for labeling and tracking of circulating cells, while soft-shell MBs are ideal contrast agents for diagnostic US imaging. Targeting provides molecular imaging, and functionalization (Section 3.6) opens numerous other imaging modalities, including fluorescence, photoacoustic, sonophotoacoustic imaging, CT, PET and SPECT, ^1H MR imaging or guidance, many of which can be acquired simultaneously.

^{19}F magnetic resonance benefits from high sensitivity, second only to ^1H , from a broader range of chemical shifts, and from high specificity owing to the lack of endogenous molecular fluorine in the body. ^{19}F MRI originally required use of PFCs having numerous magnetically equivalent fluorine atoms, such as *F*-poly(ethylethers) **12** (e.g. $\text{CF}_3(\text{OCF}_2\text{CF}_2)_n\text{OCF}_3$), *F*-15-crown-5-ether **13**, PERFECTA, and other *F*-alkylated polymers and dendrimers [19,399–403]. Technical progress in equipment (RF coils) and MR sequences now permits use of more rapidly excreted and less expensive PFCs, such as *F*-octylbromide, although they have fewer equivalent ^{19}F nuclei [297,298,403,404]. Incorporation of paramagnetic elements can further enhance the sensitivity of ^{19}F probes [256,257,405]. Being paramagnetic, the O_2 dissolved in PFCs also impacts MR relaxation parameters, enabling quantitative mapping of tissue oxygenation [406]. ^{19}F MRI is thus emerging as a powerful imaging modality for detection of inflammation, myocardial infarct, thrombi, angiogenesis, ischemia, allograft rejection, quantification of tissue oxygenation (oxymetry), temperature, pH and other parameters [19,297,402,404,405,407]. For example, ^{19}F MR imaging and spectroscopy with *F*-octylbromide or *F*-15-crown-5-ether NEs allowed quantification of vascular damage in kidney following ischemia-reperfusion injury, as well as assessment of treatment with an anti-thrombin agent-loaded PFC NE [408].

PFC probes and ^{19}F MRI could also help advance the development and clinical translation of immune and stem cell-based therapies, a promising up-and-coming strategy for cancer treatment, by providing critical quantitative in vivo information on circulating immune cell status [409,410]. Labeling of circulating cells by internalization of PFC NE droplets, as through phagocytosis, allows direct ^{19}F MRI tracking of immune cells, such as inflammation macrophages for visualizing inflammatory processes, or adoptive transfer of T lymphocytes, NK cells, dendritic cells or peripheral blood mononuclear cells for cell-based therapies and vaccines [24,399,411,412]. In oncology, the main objective is to realize separation, identification and destruction of circulating tumor cells shed by primary tumors [413–417]. As noted, a series of commercial *F*-15-crown-5-ether and *F*-poly(ethylether) NE tracer agents is available from Celsense Inc. (Pittsburg) for cell labeling, including the FDA-approved *F*-polyether NE, CS-1000, simplifying cell-tracking investigations by ^{19}F MRI [412,414,418]. Uptake of *F*-15-crown-5-ether nanodroplet by cardiac progenitor cells was significantly enhanced when the cells were labeled with *FuGENE*, a reagent frequently used for DNA transfection [419]. Use of a *F*-*t*-butylcyclohexane emulsion as a tracer has also been suggested [420].

Concerning standard ^1H MRI probe capacity, it is easily conferred to PFC NEs by incorporation of paramagnetic metal complexes, such as gadolinium(III) chelate- or superparamagnetic iron oxide-based agents

[421,422]. Dendronized iron oxide NPs inserted in the phospholipid shell of *F*-hexane-stabilized MBs are being proposed for combined MRI/US imaging [34].

Overlaying ^{19}F and ^1H images provides detection and quantification of the ^{19}F -probe as “hot spots” in a specific site, while ^1H MRI depicts the anatomic context [423], allowing, for example, detection and localization of inflammation [424], or of venous thrombosis and diagnosis of pulmonary thromboembolism [425].

Elastic soft-shelled gas bubbles are ideal sound reflectors (Scheme 3). When subjected to US waves, soft-shelled MBs alternatively expand and contract, and resonate at a frequency that is inversely proportional to their radius and also depends on shell elasticity. Nonlinear MB expansion and contraction generate harmonic and sub-harmonic frequencies that can be filtered out and constitute the very signature of the MBs. Their detection and localization enable contrast-enhanced US imaging. Higher energy US pulses engender inertial MB cavitation and, eventually, can lead to disruption and release of energy (either thermal or mechanical) and cargo [67,107,108,115,277,426–428]. The capacity for manipulating MBs by US is unique and has reached utmost accuracy. Conversely, the advent of MBs has stimulated the development of numerous novel MB-specific imaging modes and procedures, such as cavitation imaging, harmonic power Doppler, pulse inversion, or amplitude modulation imaging that improve diagnostic sensitivity, specificity and reliability [428–431]. In view of their size, usually 1–4 μm , standard intravascularly administered contrast MBs are restricted to the vasculature and are therefore basically blood pool agents [87,88,107,137,278,431–434]. Several MB contrast agents have been in clinical practice for 20 years (Section 3.3). The initially approved indication concerned essentially left ventricular opacification and endocardial border delineation. Over the years, the range of indications has expanded, while most of the FDA's initial restrictions have been lifted. In Europe (and off-label in the US), contrast agents are also utilized for liver and breast masses characterization and other uses, including assessment of pancreatic masses, of the neovascularization of carotid plaques and detection of vulnerable ones, and evaluation of the gastrointestinal and urinary tracts [435–437].

Several clinical studies explore the use of these agents for further indications, especially tumor characterization. MB-delivered contrast can also help monitor and assess treatment efficacy [434,438–441]. A pilot study indicated that SF_6 MBs (*SonoVue*)-enhanced US could be as effective as contrast CT for evaluating radiotherapy efficacy in patients with liver metastasis [442]. The safety of PFC-based MB products has been surveyed in a meta-analysis involving over 200,000 patients, which found that contrast echocardiography was actually associated with a lower mortality than for control patients! [426,443] Use of contrast-enhanced US in emergency and critical care situations has been reviewed [444], and guidelines for clinical use of US contrast agents are regularly published [445,446].

As mentioned earlier, MBs can also, when fitted with appropriate implements, provide fluorescence [447] or photoacoustic [448,449] imaging, PET [228,450,451] or SPECT [383,452,453], or proton MRI imaging or guidance. For example, SF_6 MBs with gold NPs in their shell, when destroyed by US, enhanced the photoacoustic signal, allowing background-free photoacoustic imaging of tumor-bearing mice [448]. *F*-butane MBs (*Vevo MicroMarker*), along with focused US, enabled delivery of silica-coated gold nanorods across the BBB for photoacoustic diagnostic imaging and therapeutic monitoring of neurological conditions in mice [449]. Magnetically guidable iron oxide-loaded *F*-pentane NBs provided ^1H MRI/US contrast imaging [454]. *Definity* was used for US/ Gd^{3+} -enhanced MRI-guided BBB opening [455].

Molecular imaging detects, characterizes and quantifies molecular markers of diseases. It reveals specific biochemical changes produced at the cellular level. Therefore, the PFC NE droplet or MB is fitted with ligands that can target one or more biomarkers specific of the disease (Scheme 6b) [100,101,278,279,426,456–461]. Favored targets include atherosclerosis, microvascular inflammation, thrombosis, ischemic

injury and tumor neovascularization [277,278,462–468]. Molecular imaging should be particularly valuable for early cancer diagnosis and therapy. It could also help detect acute rejection after cardiac transplantation [469] and should provide precious means of monitoring treatment efficacy. US molecular imaging with BR55 could provide a predictive tool of anti-angiogenic mammary tumor treatment efficacy through early assessment of individual patient response [470]. Dual-targeted MBs enhance diagnosis specificity and reliability [471,472].

A clinical trial explores the use of macrophage-aiming phosphatidylserine-containing MBs (*Sonazoid*) for imaging of sentinel lymph nodes in breast cancer patients [473]. NEs could have an advantage over MBs in molecular imaging because of their much longer intravascular persistence, which increases the probability for binding to the targeted tissue.

Detection of metastases grown from circulating tumor cells (CTCs) shed by a primary tumor is key to diagnosis and prognosis of cancer. Properly targeted PFC NEs and MBs can provide effective ways for early detection, isolation, characterization and targeted treatment of such cells [283,474–477]. This technique offers a non-invasive liquid biopsy approach for metastatic cancer staging and treatment. PFC-labeled MBs can thus help biopsy sentinel lymph nodes in breast cancer, potentially replacing axillary surgery [478,479], and has entered routine practice in certain medical centers [480]. Targeted *USpheres* MBs were used for in vivo characterization of programmed death ligand-1 (PD-L1)-expressing cells in a mouse model [481].

7.3. Controlled delivery and channeling of therapeutic energy

Using PFC NEs, MBs or P-SNEs, controlled focalized US- or light-actuated delivery of thermal and/or mechanical therapeutic energy can be achieved in various forms, including heat, acoustic streaming and radiation force, stable and inertial cavitation and, for higher US intensities, MB explosion sound waves and jet of particles (Scheme 3). Energy can be focused, targeted and delivered on-demand at specific sites, thereby enabling a panoply of minimally invasive procedures such as thermal or mechanical tissue ablation, sonothrombolysis, embolotherapy, revascularization, sonoporation, sonobactericide and other procedures. MBs provide cavitation nuclei, help control the conversion of acoustic pressure into thermal and/or mechanical energy, reduce the level of energy required to initiate cavitation and reduce the duration of the intervention, thereby decreasing damage on nearby healthy tissues. *F*-colloids can help direct the procedure either towards production of heat as for coagulative thermal ablation, or towards mechanical tissue destruction (histotripsy).

One objective of high-intensity focused US is to raise the temperature of a specific targeted tissue volume above 55 °C to generate coagulative necrosis and tumor death. PFC-based SNEs increased the efficacy of focal energy deposition in a tissue-mimicking phantom study [482]. Another phantom study with a *F*-hexane P-SNE coated with human serum albumin, or with a fluorinated surfactant, provided controlled thermal ablation [483]. In a further phantom study, micron-sized *F*-octylbromide NEs stabilized with *F*-surfactants were proposed for focused US thermotherapy [191]. A *F*-pentane P-SNE allowed in vivo MR-guided focused US ablation of tumors in rabbit [484]. The efficacy of *F*-butane MBs and of *F*-pentane/*F*-butane P-NEs in rat liver ablation was compared [485]. A *F*-15-crown-5-ether NE enabled precise ¹⁹F MRI-guided thermal ablation of tumors in mice [486]. Lipid and poloxamer-shelled *F*-propane nanobubbles provided image-guided delivery of Pluronic L10 as a thermal sensitizer for facilitating radiofrequency tumor ablation [487]. Tumor ablation can also be achieved photothermally. Thus, *F*-pentane P-SNEs with a PLGA shell loaded with magnetic NPs allowed MRI/US imaging and photothermal tumor ablation in mice [488,489]. A *F*-pentane P-SNE loaded with gold nanorods produced cavitation effects that enhanced sonoporation and improved delivery of photothermal tumor treatment in mice [490]. A clinical study of MB (*SonoVue*)-assisted ablation of uterine fibroid

found that significantly lesser acoustic power and shorter exposure time were needed than in the absence of MBs [491].

Histotripsy is a non-invasive image-guided US technology that mechanically destroys targeted tissues. Unlike thermal ablative forms of therapeutic US, histotripsy relies on the mechanical action of bubble clouds for tissue destruction [492]. Applying short high pressure histotripsy pulses to *F*-pentane or *F*-hexane NEs effectively ablated prostate cancer spheroids embedded in tissue-mimicking gel phantoms [493]. Transcranial MRI-guided focused US with *Optison* MBs allowed ablation of tissue volumes in rat brain without long-term undesirable effects [494]. Phospholipid-shelled *F*-butane MBs under US were used for myocardial reduction therapy in a rat model of hypertrophic cardiomyopathy [495].

Sonothrombolysis, the destruction of vessel-obstructing blood clots, is being intensively investigated [496–498]. *Definity*, combined with US, enhanced the lytic activity of rtPA in an in vitro model of ischemic stroke [250]. US-induced inertial cavitation, but not stable cavitation, of *F*-butane MBs (*BR38*, Bracco), in combination with rtPA, achieved clot lysis and fibrin degradation in vitro [499]. Sonothrombolysis using *BR38* or *SonoVue* improved microvascular patency in a rat model of stroke [500]. A *F*-pentane P-SNE achieved clot lysis at reduced acoustic powers in rat [501]. A comparison of in vitro sonothrombolysis of aged clots with *F*-butane P-SNEs and MBs is available [502].

Clinical advances in sonothrombolysis have been reviewed [497,503]. A phase III clinical MB (*SonoVue*)-assisted sonothrombolysis study in acute stroke patients is ongoing in Norway [504]. A clinical trial using *Definity* improved recanalization rates and reduced infarct size, resulting in sustained improvements in systolic function in patients with acute ST-segment elevation myocardial infarction undergoing a percutaneous coronary intervention [505].

Embolotherapy aims mainly at the deliberate occlusion of tumor-feeding blood vessels. *F*-butane MBs were, for example, used to produce an antivasular effect that led to tumor inhibition in mice [506]. A potent reduction of tumor perfusion, accompanied by intratumoral immune activation, was obtained with *Definity*-assisted US under MRI monitoring in murine melanoma [507]. US vaporization of an integrin $\alpha_v\beta_3$ -targeted, phospholipid-shelled *F*-pentane P-SNE halted growth and induced necrosis in a rodent model of hepatocellular carcinoma [508]. Synergistic antitumor and antivasular effects were observed when US-stimulated MBs (*Definity*) were combined with radiation for breast tumor treatment in mice [509]. The effect of low-frequency low-intensity, MBs (*SonoVue*)-assisted US irradiation parameters on xenograft prostate cancer hypoxia were investigated in mice [510].

Safe blood-brain barrier crossing is another intensely pursued goal. MB (*Definity*)-assisted transcranial focused US alone may, without drug adjunction, help reduce amyloid plaque formed in neurodegenerative diseases and increase endogenous immunoglobulin levels and glial activation [511–513]. Improvement in spatial memory was noted [512,513], as well as a short term antidepressant effect in rodents [514]. Small pilot safety studies with *Definity*- or *SonoVue*-enabled, MR-guided focused US indicated that transient BBB opening in Alzheimer's patients [515] or amyotrophic lateral sclerosis patients [516] could be safe. Blood-spinal cord barrier opening using MB/US combinations has been investigated in rabbits with *Optison* [517] and *SonoVue* [518].

Tissue oxygenation can also be enhanced by dilating blood vessels, which can be produced by mechanical stimulation of NO synthesis. This was achieved by combining *F*-butane/lipid MBs and US exposure, and facilitated reperfusion of ischemic tissue after microvascular obstruction [519]. Likewise, US-mediated cavitation of such MBs markedly increased blood flow and reversed hind limb ischemia in a mouse model [520]. In both cases, the US-induced MB oscillations produced shear stress that caused endothelial mechanotransduction and NO release.

The compressibility of PFCs was put at work in “shock absorbers” consisting of large, centimeter-size polymer-enclosed PFC capsules (*Vesair* bladder control system, Solace Therapeutics, Framingham MA)

that are introduced in the bladder and are in advanced clinical trial for alleviating urinary stress incontinence [521,522].

7.4. Delivery of drugs, genes, tracers, nanoparticles, cells

PFC-based colloids can be loaded with drugs, genes, markers, nanoparticles, cells and other therapeutic devices for controlled delivery. This delivery approach has potential for image-guided, externally triggered, repeatable release of therapeutic cargo at an elected site in diseased tissues, thereby increasing drug efficacy whilst minimizing undesired effects for healthy tissues. Targeting and sonoporation are essential facets of this strategy, as well as concomitant contrast imaging for guidance and monitoring of treatment. Only a limited number of references, mainly reviews, can be given here [95,98,108,137,149,157,523–527]. PFC-promoted recognition, recruitment and transport of drugs, biomarkers and NPs, with enhanced efficacy for the fluorinated ones, could further contribute to safe and efficacious delivery [23,528,529].

Sonoporation, that is, forcing transient openings through the vascular endothelium or blood-brain barrier for passage of drugs, genes, markers, NPs, etc., can be achieved using US of sufficient intensity. Adjunction of MBs can greatly increase sonoporation efficiency, lower the acoustic energy required and mitigate untoward effects [530–534]. The mechanisms of sonoporation have been discussed [535–537]. Sonoporation with clusters associating negatively charged *Sonazoid* MBs and positively charged P-SNE droplets improved therapeutic efficacy of paclitaxel in a pancreatic tumor model in mice [538]. *MicroMarker* MBs, as well as polymer capsules, enhanced US-assisted sonoporation of drug-loaded liposomes [539]. A small clinical study indicated that MB (*SonoVue*)-mediated US applied during chemotherapy in patients with tumors in the digestive system was safe and could restrict tumor progression [540].

Safely crossing the tight blood-brain barrier to deliver therapeutics to specific targets within the brain is a major challenge. PFC colloids could assist focused US-induced BBB opening in a minimally invasive way by reducing and controlling the energy required for the process, while also providing the imaging, guiding and monitoring tools needed for efficacy and safety assessment [159,541–545]. The vaporization efficiency of *F*-propane and *F*-butane nanodroplets was investigated in relation with delivery of a protein-sized dextran [546]. A SF₆ MB/liposome complex delivered O₂ and brain-derived neurotrophic factor, showed neuroprotective effects and improved behavioral deficits in a mouse model of Parkinson's disease [547]. The use of MB-assisted focused US for BBB opening in experimental models of Parkinson's disease has been reviewed [548].

Cardiovascular drug and gene delivery with US and MBs has been reviewed [267,525]. Intraplaque neovascularization-targeting MBs (*Targestar*-derived), loaded with *Endostar* (a recombinant human endostatin, a broad-spectrum anti-angiogenesis agent), inhibited atherosclerotic plaque growth in a mouse model [549]. Antibody-targeted, resveratrol-loaded *F*-propane NBs slowed down intervertebral disk degeneration in rabbits [550].

Cancer treatment involving *F*-colloids is the focus of many reviews [117,551–554]. Permeabilization of cell membrane by MB/low intensity US treatment can facilitate drug penetration in tumors [555], and synergistic (mechanical) antiangiogenic and (chemical) antitumor effects [556]. Examples of cancer treatment studies implying PFC colloids include [346,557–561]. A US-responsive doxorubicin-loaded *F*-hexane NE released Na₂CO₃ for alleviation of lactic acidosis-mediated doxorubicin resistance [562]. A doxorubicin-loaded *F*-octylbromide NE, encapsulated in brain-targeting peptide-modified PLGA, increased intratumoral accumulation and allowed on-demand high intensity focused US-triggered drug release at the glioblastoma site in mice [563]. A *F*-pentane P-SNE with a shell of PLGA decorated with gold NPs for photothermal therapy, and fitted with a peptide ligand directed at a membrane receptor of melanoma cells for activating the immune system in the tumor microenvironment, enhanced anti-programmed

death-1 (anti-PD-1) antibody treatment efficacy [564]. Sequential release of a chemotherapeutic drug at different acoustic pressure levels from a mixture of soft and hard-shell *F*-pentane P-SNEs is also feasible [167]. Doxorubicin was loaded in a multifunctional *F*-hexane P-SNE that also incorporated Fe₃O₄ NPs and was folic acid-targeted, allowing MRI and US imaging-guided combined chemotherapy and high intensity focused US tumor ablation (Fig. 20) [565]. O₂-sparged, doxorubicin and paclitaxel-loaded *F*-butane MBs, which also incorporated the Rose Bengal sonosensitizer, demonstrated US-mediated, combined chemosensitization treatment efficacy on a spheroid model of human breast cancer and on a murine model of the disease [355]. Such *F*-butane MBs, functionalized with the antimetabolite gemcitabine, proved efficacy in the treatment of pancreatic cancer in mice [356].

Several clinical studies involve PFC-assisted focused US-induced BBB opening for treatment of central nervous system diseases. The MBs used are usually the commercially available ones. A small Phase I study in pancreatic cancer patients found that combining PFC MBs (*SonoVue*) with low intensity focused US to enhance gemcitabine delivery through sonoporation was safe [566]. Clinical Phase I/IIa trials revealed that an implantable US device (*Sonocloud*), in combination with *SonoVue*, allowed carboplatin treatment of patients with glioblastoma [567,568]. MR-guided low intensity focused US BBB opening with *Definity* combined with chemotherapy was deemed accurate, safe, reversible and repeatable in patients with glioma [569].

Gene delivery can be achieved either by gene-loaded, usually cationic *F*-colloids, or by co-administration of genes along with sonoporation-promoting MBs. Reviews on enhanced gene delivery using MBs and US are available [90,570,571]. SF₆ MB/liposome complexes were proposed for site- and time-specific plasmid DNA and doxorubicin delivery to tumor cells [559]. The effect of *F*-butane MB-enhanced sonoporation on the delivery of DNA was studied in mice [572]. RNA was delivered to the heart for cardiac gene expression knockdown in mice [573]. MR-guided focused US with *Definity* allowed targeted delivery of a gene silencing viral vector for Parkinson's disease treatment in mice [574]. Aptamer tumor-targeted *F*-propane/lipid siRNA-loaded NBs were effective for prostate cancer treatment in mice [575]. siRNA biotin-streptavidin bound to the surface of *F*-propane/lipid NBs, when subjected to US, enhanced siRNA transfection and reduced glioma tumor growth in mice [576]. Combined siRNA and doxorubicin loaded, targeted SF₆ MB-liposome complexes were investigated for US-guided prostate cancer treatment in mice [560]. Site-specific MB (*SonoVue*)-mediated delivery of a bone remodeling gene was investigated for bone fracture healing in mice [577]. Gene delivery for acute spinal cord injury treatment was achieved by US destruction of cationic *F*-propane NBs in rats [578].

Inhibiting immune checkpoints that block T-cell function is a promising cancer therapy approach. Delivery across the BBB of targeted natural killer (NK) cells to specific regions in the brain, using *Definity* MBs and MRI-guided focused US, improved survival in early treatment of brain tumors in rodents [579]. Likewise, the use of *USpheres* helped improve adoptively transferred NK cell infiltration in a mouse model of ovarian cancer [580]. An anti-vascular US-stimulated *F*-propane MB treatment, combined with anti-PD-1 therapy, increased tumor growth inhibition and survival in mice [581]. Numerous articles report use of *F*-15-crown-5-ether NEs for dendritic cell tracking.

Fighting bacterial infection by combining MBs or P-SNEs with US and antimicrobial drugs is an emerging strategy (sonobactericide) that could be particularly valuable in the context of increasing drug resistance of bacteria [582–584].

7.5. Pulmonary, ophthalmic and other applications

Neat liquid or gaseous PFCs are routinely being used in ophthalmic indications [585] and investigated for pulmonary ones [20]. A review on US-mediated ocular delivery of therapeutics cites a few studies on PFC MB-assisted gene transfer [586]. Aerosolized PFCs, primarily

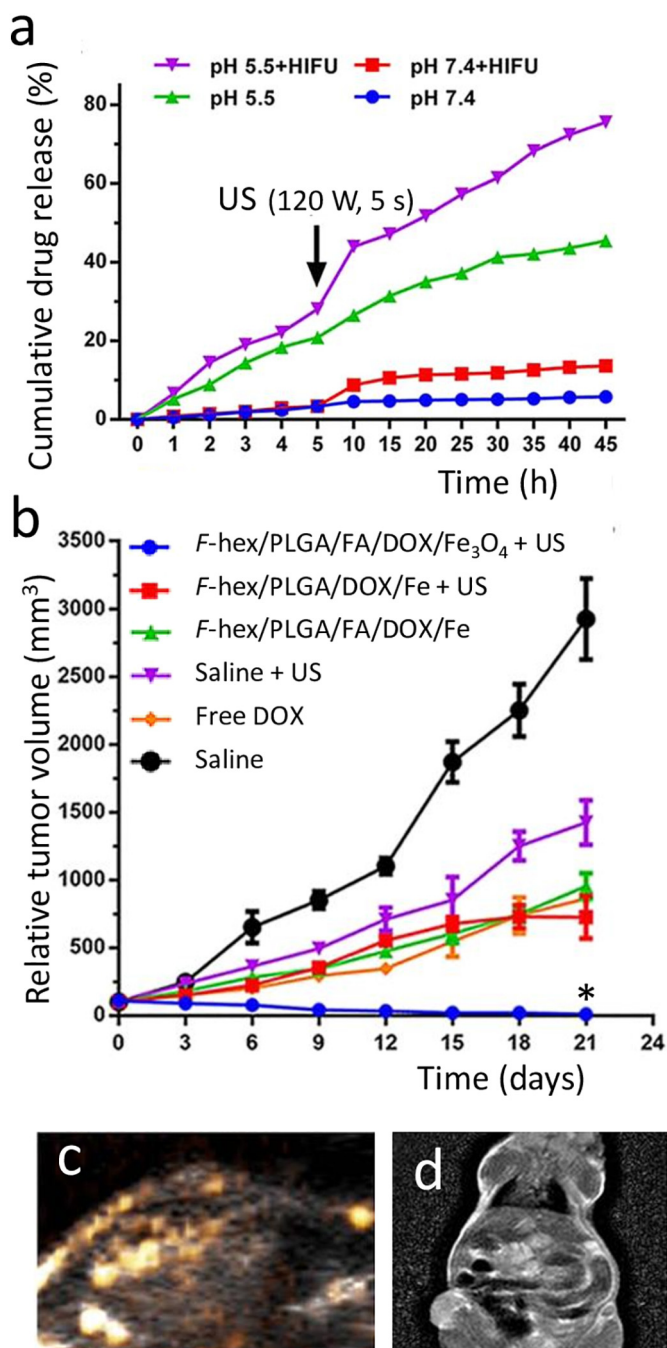


Fig. 20. Combined (theranostic) tumor ablation and chemotherapy using a US-triggerable phase-shift PLGA-shelled *F*-hexane composite loaded with doxorubicin (DOX) and superparamagnetic Fe₃O₄ NPs (Fe), and fitted with folic acid (FA) for targeting. a) Stimuli-responsive DOX-release at different pH and high-intensity focused US exposure conditions; b) variation of relative tumor volume with time after diverse treatments, (**p* < 0.01 vs. other treatment groups); c) and d) contrast US imaging and MRI, respectively, for image guidance. Adapted from [565].

F-hexane, were investigated as anti-inflammatory agents in acute lung injury models [20,587,588]. Pulmonary delivery of neat PFCs is being investigated for liquid ventilation and hypothermic organ protection after cardiac arrest [589], and as lung surfactant substitutes [30]. Intratracheal *F*-hexane is also being tested for attenuation of inflammation in smoke inhalation injury patients [590].

Pulmonary delivery of a *F*-decalin NE containing a fluorinated antagonist of a chemokine receptor and a siRNA was more effective than intravenous injection in the treatment of metastatic lung cancer in a

mouse model [591]. A *F*-octylbromide/CO₂ combination is undergoing clinical trials as a bronchodilator for treatment of acute asthma [592]. Water-in-PFC reverse emulsions are effective systems for delivery of hydrophilic drugs through the pulmonary route [20,199,203]. Pulmonary delivery of vasodilators was achieved via aerosolization of drug-loaded water-in-*F*-octylbromide NEs in rats, providing a potential treatment of high-altitude pulmonary edema and pulmonary artery hypertension [205].

In addition to delivering O₂ for alleviating isolated Langerhans islet necrosis, an *F*-octylbromide emulsion was found to prevent adhesion of Langerhans cells to Petri dishes, favoring pseudo-islet formation and preserving insulin production (Fig. 14) [352].

A notable use of a PFC as a processing aid in pharmaceutical engineering is that of a phospholipid/CaCl₂-shelled *F*-octylbromide emulsion for manufacturing spray-dried hydrophobic porous particles, *Pulmospheres*TM (initially developed by Alliance Pharm. Corp.) that enable uniform suspension and consistent aerosol delivery of a large variety of drugs as dry powders [593,594]. Commercially developed *Pulmosphere*-based formulations include inhalable insulin (*Exubera*, Pfizer, New York, USA) and tobramycin (*TOBY Podhaler*, Novartis, Basel, Switzerland). A ciprofloxacin antibiotic dry powder inhaler product (Bayer Healthcare, Berlin, Germany) for chronic lung infection treatment is in late-stage clinical development [595]. Re-branded *Aerospheres*, *Pulmospheres* are used to deliver fixed dose combinations of long-acting beta-agonist and muscarinic antagonist, or of corticosteroid budesonide/anticholinergic glycopyrrolate/long-acting beta-agonist formoterol fumarate from pressurized metered-dose inhalers for chronic obstructive pulmonary disease treatment (*Bevespi* and *Breztri Aerospheres*, AstraZeneca, London, UK).

7.6. A vocation for theranostics

As seen above, PFC colloids can fulfill multiple duties, often simultaneously (Table 8). Therapeutic delivery of O₂, energy or drugs is, owing to the specific properties of PFCs, accompanied by an aptitude for ¹⁹F or US imaging. Theranostic approaches have become trendy. They often involve several functionalization and targeting devices, multiple components, sophisticated preparation procedures and increasingly complex colloidal structures. These theranostic approaches will not be reviewed here. A profusion of recent reviews, a few of which are mentioned here, attests the attention raised by PFC-based theranostics. These reviews bear largely on cancer treatment and cover design of new PFC materials [269,554,596,597]; O₂ delivery for photo-triggered theranostics [99,314,316]; photoacoustic imaging and photothermal therapy [598]; focused US/targeted MB destruction for tissue permeation and image-guided local drug delivery [117,159,599]; MB dynamics and multiparameter interactions with US, cells and tissues, with implications for clinical translation [115,137]; MB-mediated BBB-opening and PET-monitored drug delivery [451]; use of targeted, stimuli-responsive ¹⁹F MRI probes [403,600]; and more. Examples of theranostic *F*-colloids can be found in papers cited earlier, such as [221,228,329,336,378,383,565], as well as in Figs. 16–20.

8. Prospects

The past decade has witnessed an amazing surge of the number of new PFC-based O₂-carrying colloids and medical indications investigated. Our knowledge of PFC colloid preparation, stabilization, physics and in vivo behavior has considerably progressed. Substantial O₂ delivery efficacy data further support the capacity of PFC nanoemulsions to help relieve tissue hypoxia, potentiate O₂-dependent cancer treatment, regenerate damaged tissues, preserve isolated organs, etc. In view of these data, we believe that the PFC NE-based O₂ delivery approach retains its assets. However, many of the NEs presently used for experimental and clinical investigations were designed in the late 1990s or earlier, when our understanding in the matter was still elementary.

Table 8
PFC-based nanomedicines: a vocation for theranostics.

Diagnostic ± Triggering ± Targeting ± Focusing	<i>Anatomic, dynamic</i> ± <i>Molecular imaging</i>	Multiple ultrasound imaging modes: B-mode, harmonics, Doppler, cavitation.... ^{19}F MRI ± ^1H MRI, hot spot detection ± Photoacoustic imaging ± Computed X-ray tomography ± PET, SPECT ± Fluorescence imaging	<i>Angiogenesis</i> <i>Inflammation</i> <i>Thrombi, plaques</i> <i>Infarcted myocardium</i> <i>Tumors</i> <i>Circulating tumor cells</i> <i>Liquid biopsy</i>
	± <i>O₂ delivery</i>	Bridging/emergency O ₂ supply Relieving hypoxia, protecting organs Enhancing O ₂ -dependent cancer therapies: radiotherapy, photo- & sonodynamic therapies chemo- & immunotherapies	<i>Resuscitation</i> <i>Emergency; trauma</i> <i>Stroke</i> <i>Myocardial infarction</i> <i>Cancer therapy</i> <i>Tissue regeneration</i> <i>Graft preservation</i>
	± <i>Energy delivery</i>	High intensity focused ultrasound radiation force, microstreaming, microjets, shock waves Photothermal therapy	<i>Tissue ablation</i> <i>Histotripsy</i> <i>Thrombolysis</i> <i>Embolotherapy</i> <i>Revascularization</i> <i>Sonoporation</i>
	± <i>Drug/gene delivery</i>	On-demand localized release Membrane permeation Barrier crossing: blood/brain/tumor/retina Tumor extravasation and retention effect Fluorine-fluorine recognition effect	<i>Focused drug delivery</i> <i>Transfection</i>
→ F-colloids offer countless functional combinations and theranostic opportunities			

Disease detection & characterization
 ± Image-guided procedures
 ± Enabling O₂, energy and drug-based therapies
 ± Monitoring/assessing therapy efficacy
 ↑ Personalized and precision medicine

Clearly, further progress in the field commands that any new product development effort takes into account and capitalizes on the vast amount of knowledge and experience, positive and negative, that has since been gained. Unfortunately, this has not always been the case, including for research projects and clinical trials bearing on otherwise innovative applications. Inappropriate selection of PFCs, formulations, emulsification techniques, characterization methods, has often led to suboptimal NEs, calling for suboptimal results. O₂-carrying NEs should obviously use the shortest dwelling time, highest O₂-carrying capacity PFCs, and should definitely avoid need for frozen storage. Much could also be learned from studies directed at other indications for PFC colloids than O₂ delivery, but that could help solve O₂ delivery issues. Investigation of advanced, re-formulated NEs is warranted.

In addition to NEs, the option of delivering O₂ through PFC-stabilized MBs and P-SNEs appears very promising. The commercially available MBs that were approved for US contrast imaging have demonstrated their safety and their ability to deliver O₂ to tissues. Use of approved MB products certainly encouraged and facilitated exploration of other uses and could ease clinical translation for O₂ delivery-based indications. But here again, most of these agents have been developed (and licensed) some 20 years ago. Numerous new MB systems investigated for various indications could also ensure focused, on-demand relief of tissue hypoxia. Integration of more recently accrued knowledge, and development of MBs more specifically adapted to the O₂ delivery function, are warranted. Modeling of MB behavior has progressed considerably. Theoretical models are now available to predict the behavior of MBs, facilitating the understanding and characterization of their physical properties, as well as their interactions with the ultrasound field and their environment. Nanobubbles provide further possibilities, although theoretical basis needs to be developed.

The P-SNE-based O₂ delivery approach is particularly interesting, in particular for tumor tissue penetration and blood-brain barrier crossing. PFCs constitute ideal phase-change agents. This option implicates, however, that the complex in vivo vaporization (phase-shift) process be

wholly mastered so that the size of the MBs generated upon activation be properly determined. Thorough consistent control of the phase-change event remains one of the major challenges of this desirable approach, and a key to product safety. Surmounting this challenge appears achievable through suitable PFC selection and shell formulation.

The findings that O₂ delivery from F-colloids can be manipulated non-invasively using US waves, laser light, or other stimuli, and that O₂ release can be image-guided and focused on selected tissues and triggered when ever required represent a precious advance. Most PFC-based colloids, in addition to carrying O₂, have other assets, such as ^{19}F probe quality for NEs, or US contrast imaging attributes for MBs, illustrating their outstanding aptitude for theranostic functions. Conversely, even when designed for other purposes, PFC-based colloids will constantly carry and help diffuse some amount of O₂. PFC-based colloids will thus always offer a O₂ delivery component. F-colloid functionalization and/or targeting (and multi-functionalization and multi-targeting) provide further tools for controlled in vivo colloid efficacy, augmented versatility, expanded theranostic capacities, and enhanced safety. The PFC can perform several tasks concurrently, including dissolving gases, generating bubbles, ensuring their stability, providing cavitation nuclei for phase shift, a sensitive probe for MRI, a protective environment for fragile species, thermal and mechanical energy transfer capacity, pressure buffering effects, and more. Some F-colloids can provide, simultaneously or successively, detection and characterization of illness, image guidance for surgical procedures, delivery of O₂, drug and gene, including across the blood-brain barrier, delivery of therapeutic energy, monitoring and assessment of treatment efficacy, and eventually, the prospect for personalized treatment. The abundance of recent reviews attests the proficiency of F-colloids for breeding multi-functionalized PFC-based theranostic nanomedicines.

The present surge of medically oriented PFC colloid research is characterized by a marked trend towards increased complexity, implicating numerous components and sophisticated design and engineering processes for the eventual generation of multiple simultaneous diagnostic

and therapeutic (theranostic) functions. One may wonder whether such complexity, and the manifold manufacturing, regulatory and marketing constraints that such constructs implicate may not accumulate obstacles on the road to timely pharmaceutical development, clinical translation and regulatory acceptance. Each component, and their association, each industrial process involved, each claimed function or indication will likely require demonstration of safety and efficacy. Although the cleverness of certain approaches is appealing, the downsides of increased complexity cannot be ignored.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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