Opinion

Breaking free from vascular confinement: status and prospects for submicron ultrasound contrast agents

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The development of encapsulated microbubbles (~1–6 μm) has expanded the utility of ultrasound from soft tissue anatomical imaging to not only functional intravascular imaging, but therapeutic interventions, with compelling studies of elicited biological effects. The large diameter of these bubbles has confined their utility to the vasculature, but converging interdisciplinary research pathways are giving rise to new submicron ultrasound contrast agents capable of extending their effects beyond the vascular compartment. This article reviews the status and prospects of exogenous agents including nanobubbles, echogenic liposomes, gas vesicles, cavitation seeds, and nanodroplets, and assesses outstanding criticisms preventing their advance.

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INTRODUCTION

Ultrasound is a widely and routinely used modality, forming images from acoustic signals scattered by tissue. An important application of ultrasound is to assess blood flow, which can be readily achieved in larger vessels but is difficult in microvasculature due to tissue motion artifacts and low signal strength from blood. In 1968, Gramiak and Shah discovered that tiny air bubbles coincidentally produced during rapid saline injections enhanced delineation of aortic blood flow,[1] leading to the development of microbubbles for contrast enhancement. Amongst the first ultrasound contrast agents commercially available were air bubbles stabilized by various means, but the enhancement they provided was too transient for practical use.[2] Second generation agents utilized various self-assembling surfactant shell materials such as proteins, polymers, or lipid monolayers to reduce interfacial tension and slow diffusion, and improved microbubble persistence in circulation to the order of minutes with cores of high molecular weight and low-solubility gases instead of air.[3]

The high compressibility of the gas core enables microbubbles to oscillate volumetrically in response to ultrasound with scattering cross-sections 10^3 times larger than their geometric cross-sections, and 10^{10} times that of a rigid sphere of the same radius.[4] In addition to linear backscatter, microbubbles can be induced to oscillate nonlinearly, displaying a rich resonant structure with integer and sub-integer multiples of the transmit frequency that can be used to further improve contrast enhancement over its mainly linear surroundings.[5] Bubbles stimulated near their resonant frequency are highly effective acoustic emitters. Fortuitously, bubbles that are resonant in the diagnostic ultrasound frequency range of 1–10 MHz are on the order of the size of red blood cells (1–6 μm) and can pass freely through the capillary bed, rendering them effective intravascular contrast agents. At present, microbubbles are approved

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worldwide for echocardiography, and are additionally variably sanctioned in specific countries for characterization of breast, kidney, liver, spleen and pancreatic masses, and gastrointestinal and urogenital tracts.[6,7]

Due to their rich dynamic behavior when exposed to ultrasound, microbubbles have become a subject of interest for their ability to not only enhance imaging, but also produce a range of therapeutically relevant biological effects, illustrated in Figure 1. Under low amplitude stimulation, bubbles undergo gentle oscillations of amplitudes dependent on the relationship between ultrasound frequency and bubble resonant frequency, which is affected by shell properties.[8] Moderate oscillation amplitudes result in over-expansion of vessels and circulating fluid flow called 'microstreaming' with consequently enhanced drug transport and permeabilization.[9,10] These oscillations become increasingly large and nonlinear with increasing pressure until the inertia of inrushing fluid during expansion results in bubble collapse with accompanying shock waves, jet formations, temperature elevations and production of reactive oxygen species.[8,10]

A plethora of compelling preclinical studies have harnessed these effects to demonstrate enhanced vascular permeability,[9,11,12] inflammation,[13] thrombogenesis,[14,15] thrombolysis,[16,17] angiogenesis,[18,19] and microvascular shutdown.[20,21] Ongoing clinical trials are investigating sonothrombolysis,[16] the use of acoustically stimulated microbubbles to break through blood clots—arguably the application closest to clinical adoption with significant efforts toward its translation since 2006—and transient disruption of the blood–brain barrier for spatially targeted drug delivery to the brain,[22,23] which entered clinical trials in 2015. Efforts have also been made to enhance drug delivery to pancreatic tumors, with the first clinical trial for this application in 2016.[24] It is anticipated that these clinical trials are the first of many; that ultrasound-stimulated microbubbles will serve as the 'magic bullet', with the potential to enhance treatment for a spectrum of diseases.

While first generation agents pioneered the way to clinic, second generation advances brought microbubbles to the forefront of ultrasound as not only contrast agents, but as 'theranostic' (simultaneous *thera*peutic and diag*nostic*) mediators. Recently, there has been growing interest in further expanding ultrasound utility to increasing frequency ranges (>15 MHz) for intravascular ultrasound, molecular imaging, ophthalmic applications, and preclinical small-animal imaging, shifting focus to smaller bubbles in recognition of their higher resonant frequencies. Concurrent with this came the realization that these nanoscale agents could extend ultrasound to extravascular targets, breaking free of their vascular confinement for oncological applications, depicted in Figure 2. While commercial bubble formulations have demonstrated nonlinear scattering at high frequencies associated with a subpopulation of smaller bubbles,[25–27] bubbles of this dimension comprise only a minor portion of the volume fraction, prompting efforts to reduce formulation size. Here we introduce and evaluate the status and potential of the next generation of ultrasound contrast agents on the submicron scale.

THE NEXT GENERATION: SUBMICRON ULTRASOUND CONTRAST AGENTS

Nanoparticles exhibit novel properties distinct from bulk materials, with structural versatility, improved pharmacokinetic profiles, and versatile multifunctionality for

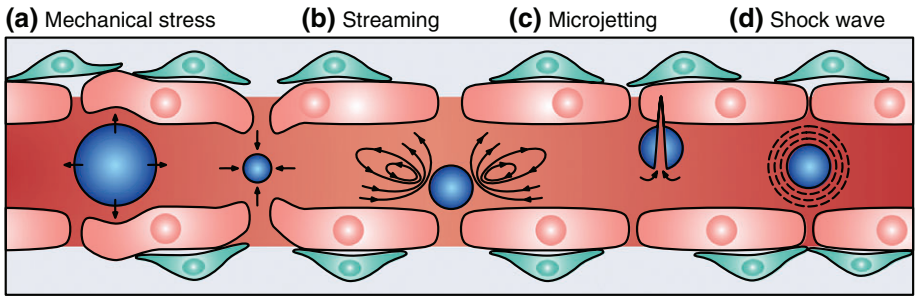


FIGURE 1 | Schematic of ultrasound-stimulated microbubble behaviors and biophysical effects. (a) Mechanical stresses of bubble oscillation cause overexpansion and contraction of the vessel walls, and (b) circulating fluid flow patterns or acoustic streaming around the bubble give rise to shear stress at the lumen surface of endothelial cells. Larger oscillation amplitudes can lead to inertial cavitation and bubble collapse, with (c) microjetting capable of perforating the vessel wall, and (d) violent shock waves with accompanying temperature and pressure elevations and the release of reactive oxygen species.

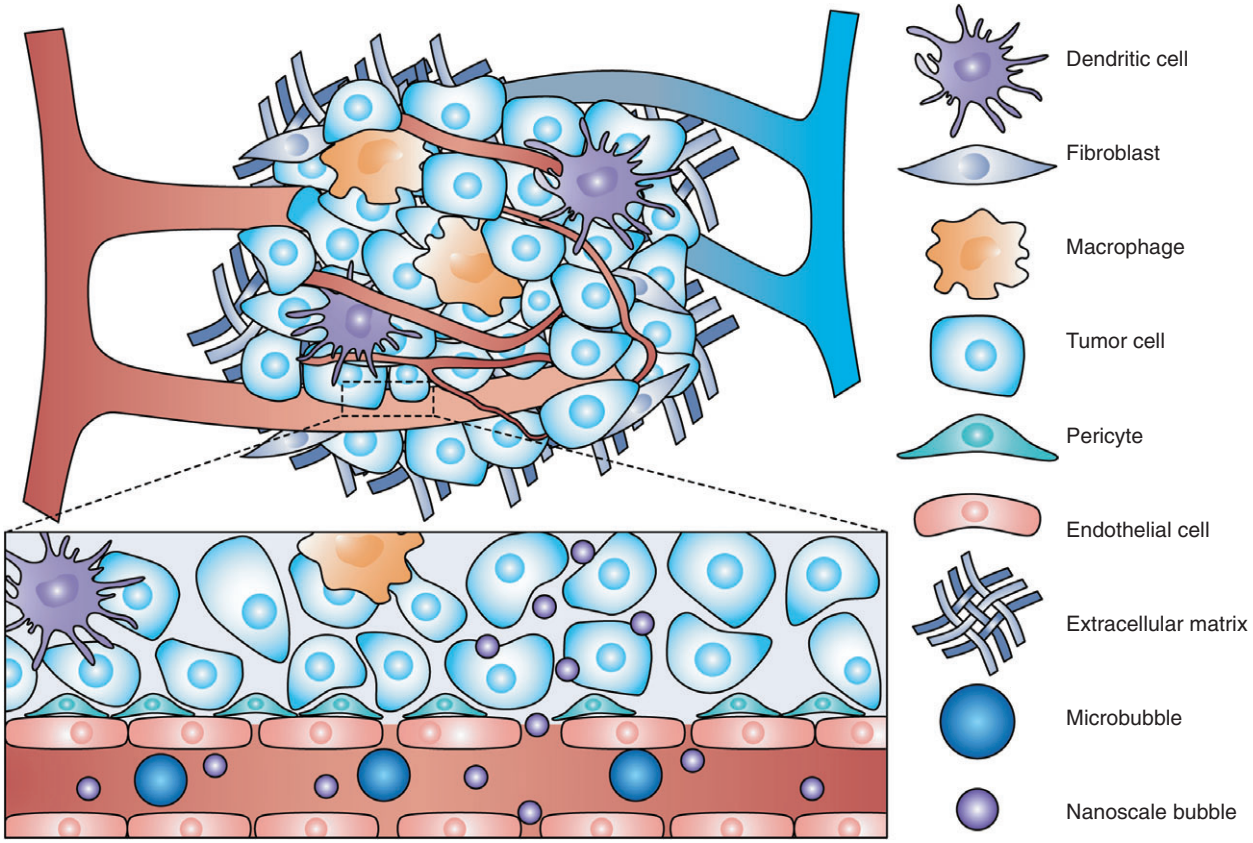


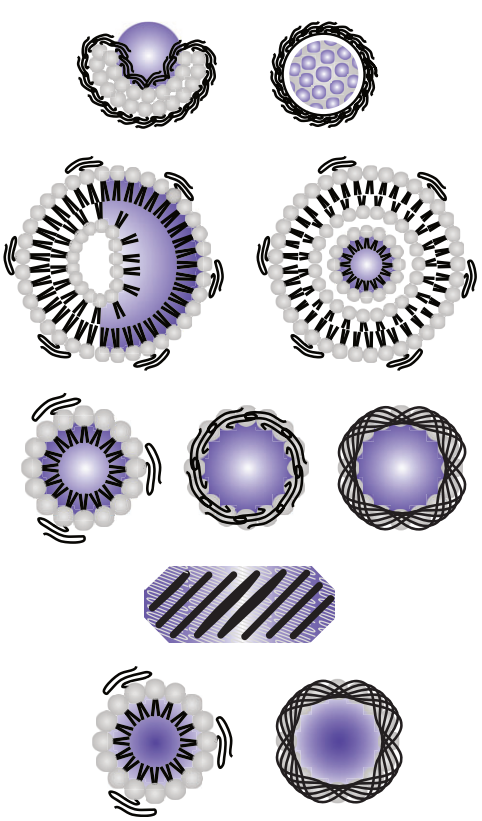
FIGURE 2 | In contrast to healthy tissue, tumors demand a high supply of nutrients and oxygen, and will invade existing vessels and develop new ones to meet these demands. However, uneven angiogenic signaling from the tumor results in vessels abnormal in form and architecture, characterized by haphazard dilated, tortuous channels lacking the organization and hierarchy of healthy blood vessels. This vascular heterogeneity is coupled with structural abnormalities; a defective endothelium barrier, with larger gaps and poorly adherent pericytes. While microbubbles are confined to remain in the vasculature, physiological consequences of tumor microvasculature afford opportunities for nanoscale agents to escape the intravascular space and expand bubble-mediated potential.

advanced delivery strategies. Consolidating the abilities of traditional nanoparticles with ultrasound contrast agents by shrinking to the nanoscale can have profound effects on bubble behavior, bringing new possibilities as well as challenges.

Nanobubbles

Epstein and Plesset theoretically predicted that unencapsulated bubbles smaller than 1 μm should dissipate in less than 0.02 seconds[28]—before they can be detected or characterized, and long before passing from a peripheral vein to the end organ 12 seconds later.[29] The presence of an encapsulating shell reduces this surface tension and inhibits diffusion thereby prolonging circulation times, and there is evidence that with the appropriate shell material selection, persistence in circulation can be considerable enough to permit practical applications.[30] Decreasing bubble size from micro to nano also significantly affects behavior as the diameter no longer falls within the size range resonant at clinically relevant low frequencies.[25,31] Shrinking in size results in an increase in stiffness and thus a rise in resonant frequency, and an increase in viscosity and therefore damping; eventually a loss of resonance is predicted with system overdamping.[25,31] This is more pronounced for encapsulated bubbles, where linear bubble theory predicts profoundly reduced scattering and resonant frequencies that are well in excess of those employed in biomedical ultrasound.[32] In the initial stages of research, this perspective led to echogenic nanobubbles being treated with skepticism.

However, many reports of nanobubbles have now been made for intravascular and extravascular imaging applications,[30,33–41] demonstrating both stability and signal from size isolated subpopulations or interesting alternative shell components, such as the copolymer Pluronic proposed by Krupka et al.,[30]



Agent Type	Shell	Core	Size (Diameter)
Cavitation seed	Lipid, polymer	Solid core with gas pockets	20 - 500 nm
Echogenic liposome	Lipid bilayer	Aqueous core with gas pockets (perfluorocarbon)	100 nm - 10 um
Nanobubble	Lipid, polymer, protein	Gas core (perfluorocarbon)	100 - 800 nm
Gas vesicle	Protein	Gas core	45 - 250 nm (diameter) 100 - 600 nm (length)
Nanodroplet	Lipid, polyer	Liquid core (perfluorocarbon)	200 - 400 nm

FIGURE 3 | Classes of submicron ultrasound contrast agents, including nanobubbles, echogenic liposomes, gas vesicles, cavitation seeds, and nanodroplets. Various encapsulating shells have been reported, including lipids, polymers, and proteins, and perfluorocarbon cores. These exogenous agents vary in size from 20 nm to several microns.

which controlled bubble size, enhanced stability and echogenicity, and acted as a chemo- and thermal-sensitizer by altering target cell membrane fluidity. Generally, there are two explanations for the seemingly anomalous observations of nanobubbles: First, the thick polymer or thin surfactant-based encapsulating layer[42] introduced in the second generation of bubbles can facilitate the generation of nonlinear signals above a pressure threshold.[32] This is well established for bubbles down to a single micron and below, and appears to be a key factor in enabling nonlinear bubble imaging at higher frequencies. A second important perspective is that although microbubbles have a larger scattering cross-section than submicron bubbles, many more smaller bubbles may be present in a given volume fraction, proportional to $1/r^3$, which can offset individual bubble scattering differences.

These agents provide particular potential to combine both passive and active targeting strategies for deeper penetrating imaging and therapy by virtue of their small size and ability to incorporate a variety of targeting moieties. However, while microbubble behavior has been extensively studied, dynamics of nanobubbles cannot simply be extrapolated from their micron-scale counterparts: Dynamics of nanobubbles as a function of size, shell properties, and exposure parameters are complex and not well understood.[43]

Echogenic Liposomes

The ability to trigger bubble-mediated theranostics comes at the expense of drug loading capacity, as echogenicity is derived from the presence of a gas core. To reconcile echogenicity of bubbles (which have a gas core) with the drug carrying capacity of liposomes (containing an aqueous core), researchers have reported 'echogenic liposome' constructs ranging in size from 100 nm to a few microns, with gas pockets trapped within the lipid bilayer or stabilized within monolayers in the liposome core for imaging, therapy, and delivery of therapeutic gases and drugs.[44–53] Given the small size of entrapped air and presence of multiple encapsulating layers capable of absorbing cavitation energy, acoustic contrast from echogenic liposomes is predicted to be negligible, yet their high backscattering coefficient and low

attenuation reported by Coussios et al.[48] points to effective contrast in terms of a high scatter-to-attenuation ratio; with good scattering to improve image visualization, and low attenuation to image underlying biological structures.[54]

These agents have the potential to facilitate delivery of a spectrum of therapeutics due to their hybrid liposome-bubble structure. However, exact mechanisms for cavitation of echogenic liposomes are not completely understood, as the location and volume fraction of gas pockets within the liposomes remains elusive.

Gas Vesicles

A unique class of biologically derived protein nanostructures, gas vesicles were discovered and characterized biophysically over a century ago,[55] but had yet to be exploited as an ultrasound contrast agent until 2014 by Shapiro et al.[56] Naturally formed by prokaryotic cells in lakes and oceans to regulate buoyancy for harvesting light,[55] gas vesicles are cylindrical or biconical in shape, with maximal diameters of 45–250 nm and lengths of 100–600 nm depending on their genetic host.[56] Since the size and shape are genetically encoded, assembly of the protein shell is tightly regulated, and engineering at the level of the constituent proteins can form nanoscale imaging agents with widely varying but replicable mechanical—and by extension, acoustic—properties.[57]

Gas vesicles have demonstrated echogenicity at clinically relevant and high frequency ranges[56–60] with nonlinear behavior that makes them amenable to established microbubble methods of amplitude modulation for detection.[58] While they have demonstrated acoustic contrast, gas vesicles interact with gases in a fundamentally different way from microbubbles: Microbubbles trap a pre-loaded gas, while gas vesicles exclude water from their hydrophobic core but permit gas from the surroundings to freely diffuse in and out of the shell.[55,56,61] As a result, the interior and exterior are in equilibrium, and the lack of a pressure gradient confers inherent stability despite the nanometer size. While their nanoscale dimensionality provides admittedly low echogenicity,[58] gas vesicles expand the range of ultrasound to extravascular and intracellular targets, and enhance contrast at concentrations representing the gas content of typical microbubble injections—which contain 1000 times more gas per particle—due to their prolonged stability and clustering.[56]

These agents may provide new opportunities as reporter genes[56] for tracking stem cells and monitoring gene therapy, and a deeper-penetrating alternative to optogenetics. However, the degree to which eukaryotic cells are able to express and assemble these prokaryotic DNA sequences is unknown. Further, the shape, shell composition, elastic properties, and shell permeability differ from those of extensively explored microbubbles, and the physical mechanism for nonlinear acoustic responses from such anisotropic rigid structures remains relatively unexplored.

Nanoparticle Cavitation Seeds

Bubbles can be nucleated in water at much lower stresses than theoretically predicted,[62] with a wide range of thresholds reported by different investigators. It is generally believed that the reason for these discrepancies is the presence of small inhomogeneities in the liquid that are generally stable and trap gas in crevices. The generation of cavitation from such nuclei was theorized by Harvey et al.,[63] Apfel,[64] Atchley and Prosperetti,[65] and Crum,[66] and experimentally demonstrated with various nanoparticle seeds, including hydrophobic mesoporous silica nanoparticles,[67–69] solid polytetrafluoroethylene (PTFE) nanoparticles,[70] and polystyrene nanocups.[71,72] These works established the requirements of hydrophobicity and surface roughness or porosity to trap and stabilize gas against dissolution in the bloodstream, and indicated nucleation threshold dependence on crevice shape and surface chemistry.

While acoustic signals for cavitation nuclei at clinically relevant frequencies have been reported to be significantly below that of microbubbles, it should be noted that microbubbles are quickly destroyed by ultrasound exposure on the order of seconds, limiting their theranostic capacity particularly for monitoring and enhancing the delivery of drugs that circulate on the order of tens of minutes. Although multiple microbubble injections are possible in small animal testing, such a protocol would rapidly exceed the maximum dose for human administration (0.06 mL/kg, with typical maxima of 2 mL per injection and two injections per visit[71]). Cavitation nuclei have demonstrated contrast 20 min post-injection,[68] and cavitation activity four times longer than existing microbubble constructs.[71] Superhydrophobic nuclei, which cannot be deactivated except via direct wetting from a jet course into the pit during collapse, have also been reported,[62] enabling truly sustained cavitation. There is very little understanding of the effects of nanoparticle properties on nanobubble stabilization and acoustic cavitation to date.

Nanodroplets

Droplets represent a novel class of phase-change agents combining the advantages of nanoparticle size to extravasate into the interstitial space with the acoustic properties of microbubbles,[73,74] and are arguably the primary nonbubble based agent under investigation. These agents are comprised of liquid phase perfluorocarbon droplets coated with surfactant[75] or lipid[76] shells, and are superheated above their boiling points at physiologic temperatures to a metastable state.[77] Their liquid core enables prolonged circulatory persistence over commercial microbubble agents,[77] but results in poor imaging contrast potential unless targeted to accumulate in layers, demonstrated by Lanza and coworkers.[78,79] Alternatively, when droplets absorb stimulating energy—in the form of heat by ultrasound or in some cases light with multimodal droplets as demonstrated by Paproski et al.,[80] or when stimulated by appropriate pressures—droplets can change phase, expanding to form echogenic bubbles on the order of 5–6 times larger than their precursor droplet size.[81] The conversion pressures required are frequency dependent in a way that can depend on droplet formulation type and size,[77,82,83] and are critical to identify to spatially control biological effects and optimize treatment *in vivo*. Earlier reports converted droplets using longer pulses, which stimulated conversion and subsequent potentially violent oscillations of the resultant bubbles.[83,84] More recent work has focused on conversion with short duration pulses and subsequent detection with imaging length pulses.[85] The mechanisms of conversion are not fully understood, but the consensus is that cavitation can be nucleated within droplets, facilitated (at least for larger droplets) by harmonics of the transmit frequency arising from nonlinear propagation.[86]

Larger droplet formulations are under investigation for selective blood flow occlusion,[87] enhancement of thermal ablation,[88] and as point targets for phase aberration correction.[82,89] Submicron formulations are being pursued as vascular[90,91] and extravascular agents,[92–96] for the latter enabling metrics of vascular permeability, targeting of extravascular epitopes, and facilitating drug delivery. However, production of monodisperse submicron droplets is both challenging and crucial for controlled activation: The size of the converted bubbles and thus their behavior is strongly dependent on the size of its precursor.[97]

PERSPECTIVES

While classical linear bubble theory predicts that submicron agents are acoustically occult and therefore difficult to image, numerous reports now suggest otherwise. The next generation of echogenic agents present novel properties and opportunities to expand the utility of ultrasound to intravascular, extravascular, and intracellular imaging and therapeutic applications with improved pharmacokinetic profiles over their microbubble counterparts, yet none of the submicron echogenic agents have been widely adopted.

Characterization Considerations

Studies are often rightly criticized for poor characterization of agent size and volume distribution. The dimensions of the agent dictate how it interacts with ultrasound, and can thus drastically affect interpretation of acoustic response if, for example, there is a subpopulation of larger bubbles. Larger bubbles will dominate by their high scattering cross-section, which is proportional to r^6, and strongly dependent on the ratio between the insonating and resonant frequencies. Further, many reports are not accompanied by injection amounts or ultrasound system parameters: The high concentration of smaller agents present in a given volume fraction in the vasculature may result in detection as a consequence of clustering, coalescence, copious target epitopes, or simply by means of agent growth and violent oscillations due to inappropriate imaging settings. Of course, characterization of submicron agents is complicated, with the primary distribution existing near or below brightfield resolution limits of microscopy; and standard nanoscale sizing approaches estimate the particle distribution indirectly.[98] A new generation of sizing instruments may circumvent this issue with direct measurements, but submicron sizing should be coupled with microscale sizing to eliminate outliers beyond the sensitivity of the submicron sizing instrument.[77]

Submicron Agent Behavior

The decrease in size from micron to submicron is predicted to profoundly influence bubble behavior. Smaller bubbles experience increased damping effects, with the most prominent component being associated with shell damping, proportional to $1/r^3$.[25,99] This should act to increase the pressure levels required to attain substantial bubble oscillations necessary for imaging and therapeutic applications, however, this may be fortuitously offset by shell viscosity which appears to reduce with decreasing size and increasing frequency.[25,100] Another determining factor of encapsulated bubble behavior is nonlinear surface rheology. There is extensive

evidence, primarily anchored in high-speed optical imaging of individual bubbles above 2 μm in size, that asymmetric 'compression' dominated oscillations associated with shell buckling are a major factor in nonlinear behavior.[29,101] Such experiments cannot readily be done for submicron agents due to optical resolution limits, however there is evidence derived from single bubble acoustic experiments that 'expansion' dominated oscillations occur with smaller bubbles down to approximately 1 μm in size, at least at higher frequencies.[43] It is thus hypothesized that the behavior of submicron ultrasound contrast agents is driven by nonlinear surface rheology that is not only dependent on size, but also the exposure parameters.[43,102] Separate from these experiments which employ shorter imaging type pulses, it is further possible that these agents are experiencing growth during longer therapeutically relevant pulses, resulting in an increase in their echogenicity and presumably their capacity to elicit biological effects. Indeed, initial reports using submicron agents for therapeutic applications have demonstrated biological effects accompanied by substantial levels of detected cavitation.[103,104] Continued modeling efforts with meticulously characterized agents for experimental data acquisition may improve future understanding of the complex relationships between agent size, shell properties, exposure parameters, and resultant dynamics.

Evidence of Extravasation

The low echogenicity of nanoscale agents and limited knowledge of submicron bubble behavior additionally makes it difficult to conclusively prove their extravasation. Arguably, this may be further due to limitations in resolution that preclude visualizing nanoscale agent spatial relationships with the microvascular lumen, and so the field must rely on other indirect proof. One approach is to determine whether contrast in images remains static over time, as agent in the vasculature would wash out with the associated signals decaying accordingly. The challenge with this evidence relates to previous difficulties of poor agent characterization: Populations may contain subpopulations of larger agents that can lodge within microvessels and prevent wash-out. A second basis for evaluation is to assess for the presence of agents in an organ where they are expected to remain within the vasculature, and then in tumors where it is hypothesized that the agent can leak out. If the signal decays in the vascular organ but is still detected in the tumor, this would be consistent with leakage out of the vasculature. A third approach is to employ histology to detect encapsulating material in the extravascular space. While most studies follow this approach and indeed provide concrete evidence of shell material extravasation, verifying that these materials remain structurally intact with contained gas is more challenging, and remains inconclusive to date.

Behavior beyond Vascular Confinement

The performance of extravascular agents is further altered by the viscoelasticity of the surrounding environment: For example, microbubbles within vessels experience higher damping, shifted resonant frequencies, complex flow patterns, and asymmetrical collapse when near the vessel wall.[8,105,106] While there have been many studies on microbubble response near boundaries and in confining vessel-like geometries,[105] the extravasation of nanoscale echogenic agents into tissue places them in an entirely different physical environment that remains to be incorporated with vigor (i.e., considering anisotropic properties) in simulation and experimental approaches. It is presently believed that newly discovered acoustic signatures and elucidated relationships of bubble properties with their surroundings and sonication parameters will enhance sensitivity of detection methods.

Problems with Passive Delivery

It is important to note that many of the studies outlined are reliant on physiological consequences of tumor vascular heterogeneity and structural abnormalities—a defective endothelium barrier, with larger gaps and poorly adherent pericytes—to passively infiltrate tumors while sparing surrounding normal tissue.[107] Despite being the cornerstone of nanomedicine and thereby widely applied and established in pre-clinical mouse models,[108] the relevance of the enhanced permeability and retention effect as a ubiquitous targeting strategy is clinically disputed.[109,110] Interesting alternatives include using ultrasound to trigger the conversion of microbubbles to nanobubbles, demonstrated by Huynh et al.,[111–113] where the conversion process actively enhanced the accumulation and retention of *in situ* generated nanostructures in tumor tissue. But with reduced echogenicity of nanoscale agents, this platform remains to be expanded by either reverting to the microscale, as demonstrated by Blum et al.[114] via exposure of the nanoparticulate products from microbubble destruction to high intensity focused ultrasound, or by determining alternative imaging and therapeutic strategies for the utilization of nanoscale echogenic agents at lower intensities and frequencies

such that conventional clinical ultrasound systems can be used.

Moving Forward

It is clear from the complexity of bubble dynamics and their elicited biological effects that interdisciplinary approaches are required to advance nanoscale agents. To date, a myriad of submicron ultrasound contrast agents have been formulated, but the field lacks standardized size distribution characterization methods, has little understanding of nanoscale echogenic agent behavior, and subsequent cellular response is largely unexplored. However, unraveling these behaviors, and particularly the links between them is challenging: Bubbles vibrate on a timescale of nanoseconds to microseconds, much faster than subsequent physiological (milliseconds) and biological (seconds to minutes) effects, let alone that of clinical relevance (hours to days).[12] While these effects have been studied independently, their combination necessitates the challenging integration of different imaging systems capable of high resolution evaluation of the same region of interest. One possible path is to combine cavitation mapping, high-speed imaging, and intravital microscopy: Passive cavitation detection and optical methods have facilitated advances in characterization of bubble behavior,[115] while window chamber models and specialized transducer development have enabled intravital microscopy studies capable of subcellular resolution imaging in real-time.[116,117] Such a combination would afford direct observation of bubble dynamics as a function of their size, composition, and acoustic stimulation parameters while under the physical influence of biological factors—and link such behavior to subsequent physiological and biological effects.

CONCLUSION

Microbubbles have expanded the utility of ultrasound from anatomical to functional imaging, and are currently on the cusp of enormous clinical impact with compelling evidence of elicited biological effects. Here we reviewed the status and prospects of the next generation of echogenic agents on the submicron scale, with motivations of expanding ultrasound utility to high frequency ranges, and breaking free of vascular confinement. The development of these agents is fairly recent, but early studies have highlighted an enticing breadth of potential. Further progress toward their clinical realization will require the convergence of interdisciplinary efforts in chemistry, physics, engineering, biology, and clinical medicine.

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