Published in final edited form as:

Adv Colloid Interface Sci. 2018 December ; 262: 39–49. doi:10.1016/j.cis.2018.10.004.

Reverse Engineering the Ultrasound Contrast Agent

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Abstract

In this review, a brief history and current state-of-the-art is given to stimulate the rational design of new microbubbles through the reverse engineering of current ultrasound contrast agents (UCAs). It is shown that an effective microbubble should be biocompatible, echogenic and stable. Physical mechanisms and engineering calculations have been provided to illustrate these properties and how they can be achieved. The rational design paradigm is applied to study current FDA-approved and commercially available UCAs. Given the sophistication of microbubble designs reported in the literature, rapid development and adoption of ultrasound device hardware and the growing number of revolutionary biomedical applications moving toward the clinic, the field of Microbubble Engineering is fertile for breakthroughs in next-generation UCA technology. It is up to current and future microbubble engineers and clinicians to push forward with regulatory approval and clinical adoption of advanced UCA technologies in the years to come.

Keywords

microbubble; coalescence; surface forces; dissolution; imaging; drug delivery

A brief history

The field of ultrasound contrast agents (UCAs) was born in the late 1960's with the observation by Gramiak and Shah of enhanced ultrasound signals from the aortic valve after the injection of dyes [1]. They determined that bubbles formed by agitation of the dye solution were responsible for enhancing the ultrasound echoes. This important discovery opened the possibility of improving the ultrasound scattering contrast between blood and tissue to image cardiac and vascular structures. Research on injectable microbubbles progressed over the ensuing years, and the first UCA for echocardiography was approved in the 1990's for clinical use. First-generation UCAs comprised an albumin shell and air core (Albunex). These microbubbles were sufficiently stable and small ($<10\ \mu\text{m}$ diameter) to pass through the lung capillaries to the left ventricle [2]. Second-generation microbubbles comprising perfluoropropane gas (Optison) significantly extended contrast signal duration [3]. Shortly thereafter, lipid-coated microbubbles were developed and approved for

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echocardiography and radiology [4,5]. Currently, clinical and research studies employ lipid-coated microbubbles more than any other formulation.

Early on, microbubbles were conjugated to targeting ligands for ultrasound molecular imaging [6]. At the same time, the remarkably strong response of microbubbles to ultrasound inspired studies examining *in vitro* sonoporation and *in vivo* drug delivery, in which microbubble cavitation induces pore formation in the cellular plasma membrane and vascular endothelium, respectively [7]. Parallel research investigated the use of ultrasound and microbubbles to dissolve blood clots *in situ* [8]. Around the turn of the twentieth century, ultrasound-mediated drug delivery research had expanded as far as enabling measurable gene transfection in tissue [9], as well as transient, noninvasive and targeted disruption of the blood-brain barrier [10]. Microbubble Engineering began to mature as a field during this time, and researchers began to explore new methods to load drugs and genes onto UCAs [11]. Thus, the microbubble emerged as a drug carrier that could transport and release therapeutic agents into tissue on-demand by the targeted application of ultrasound. Microbubble UCAs also play a key role in the utilization of novel plane-wave imaging strategies, including super-resolution ultrasound localization microscopy of deep vascular structures [12]. On the therapeutic side, microbubble-assisted chemotherapy was recently shown to significantly improve outcomes for human pancreatic cancer patients [13], and microbubble-assisted blood-brain-barrier disruption is now in human clinical trials for treating brain cancer in both Europe and Canada (clinicaltrials.gov).

The development of microbubble engineering principles for advanced imaging and drug delivery has spawned new applications outside the field of ultrasound. Researchers have employed the methods to synthesize perfluorocarbon microbubbles to encapsulate other gases, such as oxygen [14]. Microbubble encapsulation of oxygen has provided a biocompatible formulation for direct injection into the bloodstream or body cavities [15,16]. Oxygen and other bioactive gases can thus be delivered passively by diffusion or actively by stimulation with ultrasound to improve the effects of radiotherapy, for example [17]. Until recently in the field of molecular diagnostics, UCAs have long been handicapped by potentially immunogenic conjugation chemistries, which are essential to the attachment of targeting moieties and probes to the microbubble surface. The state-of-the-art in non-toxic conjugation schemes, such as azido click-chemistry, has recently been applied to pre-clinical UCAs with demonstrable biocompatibility in a large animal model [18]. Currently, we witness the advent of promising near-clinical applications for blood-brain-barrier disruption and drug-delivery in the treatment of neurological diseases such as brain cancer and Alzheimer's [19–21]. These applications often repurpose existing UCAs such as Sonovue and Definity to translate a purely thermal (and potentially unsafe) process involving the use of focused ultrasound (FUS) alone, into a safer mechanical process by using microbubbles as actuators of acoustic forces. In pursuit of such applications, Microbubble Engineering has emerged as an important discipline within the field of colloid science.

Two approaches to microbubble development have been employed. Clinicians and health-technology professionals began by developing simple UCA formulations to achieve regulatory approval, and now use them based on their regulatory status with the aim of increasing translational potential. At the same time, researchers have taken to engineering,

validating and optimizing novel microbubble formulations for a particular application, such as molecular imaging or drug delivery. As expected of efforts surrounding clinical translation, the field has begun to favor convergence of these two approaches, in which parametric considerations and their bioeffects *in vivo* produce validating, but not necessarily expository data regarding paths for microbubble engineering. This lack of exposition, and significant pockets of misunderstanding regarding the fundamental principles of microbubble engineering, has resulted in debates regarding dose metrics for therapeutic studies [22,23], acceptable doses [24,25] and even safety in established imaging applications [26,27]. We therefore offer the following review of UCA microbubbles to stimulate greater sophistication in microbubble design and insights into their ultimate clinical translation.

Reverse Engineering

Future UCAs face significant barriers to adoption, yet offer the expected advantages of next-generation theranostic agents. The cost and time involved in regulatory approval processes are sizeable, but at the same time it is not difficult to identify significant obstacles in the repurposing of existing UCA formulations for future applications. For example, the use of fluid volume as a dose metric and the absence of an effect-associated dose metric impedes comparisons of safety and efficacy in theranostic applications [24,25]. Indeed, significant differences in bioeffects due to microbubble size alone can be seen in both therapeutic and imaging applications [22,28,29]. Such effects, paired with batch-to-batch inconsistencies in microbubble concentration between competing formulations (or even the same formulation) are imposing factors when considering the cost of implementing new formulations and evaluating safety for clinical use [30]. It is up to investigators to engineer not only an agent, but to accommodate prospective questions and concerns regarding the adoption of the microbubble design, be it wholly novel or a modified formulation.

The purpose of this review is to facilitate the rational design of next-generation microbubbles through reverse engineering of UCAs that are currently approved for clinical use. The review follows a paradigm set forth in an illuminating book chapter by David Needham [31], featuring another colloidal system - low-temperature-sensitive liposomes - which is deconstructed to teach the development and validation steps and gain deeper insight. Needham argued that undertaking the process of reverse engineering helps to develop deeper learning, problem solving skills, critical thinking and inventiveness. According to Needham, reverse engineering involves finding answers to a few key questions in order to identify the essential design features of a device, product or process:

- What is it for?
- How should it work?
- What is it made of?
- How is it made?
- What are the characteristics of the material?
- Has anybody made something similar?

- Does it really work?

These simple questions become relevant as we witness the continued application of primitive UCAs for more sophisticated applications, such as the repurposing of diagnostic UCAs for therapeutic applications. Indeed, the feasibility and efficacy of innovations in ultrasound theranostics will require novel UCA formulations. This review article aims to guide the reader through the reverse engineering process, highlighting critical functions expected of UCA formulations, and interrogating the current state-of-the-art from an engineering perspective. Such an approach provides an objective method for evaluating both existing and new microbubble products, and outlines a streamlined path towards optimizing microbubbles for future applications. Below we provide answers to each of these reverse-engineering questions, in turn.

Application of UCAs in biomedicine

Ultrasound is unique among the imaging techniques in that it uses mechanical waves rather than electromagnetic waves to image the structures and processes of the body. A typical ultrasound image acquisition cycle uses the pulse-echo method, in which a high-frequency ultrasound pulse is transmitted from a transducer probe into the body, and the resulting reflected and backscattered waves are received and processed to form an image. The speed of sound in tissue is quite fast (1540 m/s), so frame rates can be as high as 1500 Hz. The high frame rate allows high temporal resolution of dynamic processes, such as turbulent flow in the carotid artery. Alternatively, the high frame rate provides high sensitivity to detect anatomical features through signal averaging and super-resolution methods. Ultrasound is also portable and inexpensive compared to other imaging modalities. The main drawback of ultrasound is the image quality, which is limited by the inherent scattering properties of tissue. Blood in particular is a poor ultrasound scatterer. The comparatively high scattering of interstitial tissue makes it difficult to distinguish echoes from vascular structures within the tissue, or to visualize cardiac structures. The purpose of the UCA is to increase acoustic scattering of the blood beyond that of tissue, and thus create sufficient contrast between vascular structures and interstitial tissue (Fig. 1).

In theranostic applications, the purpose of UCAs is to serve not only as an imaging probe, but also as a mechanical cell and tissue disruptor, a microfluidic mixer and a drug delivery vehicle. By applying ultrasound at an elevated intensity, a microbubble undergoes larger amplitudes of oscillation than those seen in contrast-enhanced ultrasound. Consequently, these oscillations effect mechanofluidic permeabilization of tissue within approximately a diameter's range of the microbubble [34,35]. This behavior is advantageous in the context of transient and tunable blood-brain barrier disruption, a primary focus of current therapeutic UCA applications [19,21,20]. As a delivery vehicle, the purpose of UCAs is to localize release of drug molecules, which may be dissociated from the microbubble via these same large-amplitude oscillations [36,37]. Compared to other technologies in drug delivery, UCAs offer the significant advantage of spatio-temporally targeting. The combined capability to sense and actuate the microbubble with ultrasound provides unprecedented capability for feedback-control of pharmaceutical delivery.

Properties of the UCA

Safety for intravenous injection

For obvious reasons, a contrast agent should be safe to inject into the bloodstream. It should be small enough ($<10\ \mu\text{m}$ diameter) to traverse the capillary vessels without occluding flow. This sets the upper limit for a microbubble to about the size of a red blood cell. It also should be nontoxic and biologically inert - that is, it should not induce any unintended physiological processes, such as activation of the immune system or the thrombotic cascade. Therefore, a contrast agent should be non-immunogenic, and it should only be made of biocompatible components that can easily be metabolized and excreted from the body. The current FDA-approved and commercially available UCAs have been tested extensively for safety, in both retrospective and prospective trials [38,39]. Names and characteristics for three UCAs currently on the market are shown in Table 1.

As the table shows, microbubble stabilization has been accomplished by encapsulation of low-solubility, biologically inert gases (SF_6 and C_3F_8) within a biologically degradable shell of lipid or protein. The necessity of a gas core is discussed next, in the context of acoustic scattering. Dissolution of the gas core is unavoidable owing to ventilation/perfusion mismatch of oxygen at the alveolus, but the process can be delayed long enough for an ultrasound examination by using low-solubility gases. Of course, the gas itself must be nontoxic and biologically inert. As will be shown below, a shell is needed to stabilize the microbubble against coalescence and rapid dissolution. From the standpoint of safety, the shell is necessary to protect the thrombogenic gas surface from clotting factors in the blood.

Echogenicity

The UCA should have a large acoustic scattering cross section in order to scatter sound waves more strongly than blood and interstitial tissue structures. Scattering occurs when an object is much smaller than the acoustic wave. This limit is expressed mathematically for a sphere by the relation $kR \ll 1$, where R is radius and k is acoustic wavenumber ($k = 2\pi f/c$; where f is frequency and c is sound speed). Red blood cells are the main endogenous acoustic scatterers in blood. The echogenicity of a particle is quantified by the scattering cross section (m^2), defined as the ratio of the scattered power (W) and the incoming sound intensity ($W\ \text{m}^{-2}$). Rayleigh derived an expression for the acoustic scatter (σ_s) of a particle in terms of its size, mass density and compressibility [41]:

$$\sigma_s = 4\pi R^2 (kR)^4 \left[\left(\frac{K - K_0}{3K} \right)^2 + \frac{1}{2} \left(\frac{\rho - \rho_0}{2\rho + \rho_0} \right)^2 \right] \quad (1)$$

where ρ and ρ_0 are the mass densities of the particle and medium, respectively, and K and K_0 are the compression bulk moduli of the particle and medium. The term πR^2 is the particle's geometric cross section, and the term $(kR)^4$ gives the frequency dependence. Overall, the scattering cross section scales with particle size as R^6 . Thus, a single 1- μm particle scatters as much as one million 100-nm particles. It is therefore advantageous to use

larger microbubbles, provided they are small enough to traverse the capillaries (<10 μm diameter).

Additionally, the scattering cross section scales as f^4 , but frequency is often limited by the range of clinical ultrasound (1–10 MHz). The properties ρ_0 and K_0 of blood are fixed to the values of water. This leaves particle properties ρ and K as the only adjustable parameters to maximize the value of σ_s . The effect of particle density on scatter is quite small, typically less than 10% [42]. On the other hand, particle compressibility has a very large effect, especially if the particle is highly compressible (low K). This is perhaps the *raison d'être* of the gas core in UCA design. No other state of matter lends such high compressibility: a microbubble is the most echogenic particle that can be achieved. For a given particle size and ultrasound frequency, the Rayleigh scattering cross section for gas is $\sim 10^9$ fold greater than for liquid (glycerin) and $\sim 10^8$ fold greater than for solid (steel) [42].

The microbubble echo is further amplified by resonance phenomena. For small-scale oscillations ($R/R_0 \ll 1$), the linearized resonance frequency for a bubble is given by the following expression [42]:

$$f_0 = \frac{1}{2\pi} \sqrt{\frac{1}{\rho_0 R^2} \left[3\kappa P_0 + \frac{2\gamma(3\kappa - 1)}{R} \right]} \quad (2)$$

where γ is the gas/water interfacial tension, κ is the polytropic gas constant and P_0 is the hydrostatic pressure. For a free (unshelled) microbubble, the surface tension is $\gamma = 0.072$ N/m, which gives a resonant radius $R_0 \approx 4 \mu\text{m}$ at 1.0 MHz and $R_0 \approx 0.6 \mu\text{m}$ for 10 MHz. Fortunately, microbubbles resonate at or near frequencies used by clinical ultrasound scanners. The scattering cross section for small-amplitude (linear) oscillations is given by [42]:

$$\sigma_s = 4\pi R^2 \frac{\Omega^4}{(1 - \Omega^2)^2 + (\delta\Omega)^2} \quad (3)$$

where $\Omega = f/f_0$ is the ratio of acoustic driving frequency to the bubble resonance frequency and S is the damping constant. Damping includes contributions from acoustic re-radiation (δ_{rad}), viscous dissipation of the medium (δ_{vis}) and thermal hysteresis (δ_{th}). At resonance, approximate equations for these damping terms are [43]:

$$\delta = \delta_{rad} + \delta_{vis} + \delta_{th} \quad (4a)$$

$$\delta_{rad} = \frac{2\pi f_0 R}{c} \quad (4b)$$

$$\delta_{th} \approx \delta_{vis} = \frac{4\mu}{R^2 \rho_0 2\pi f_0} \quad (4c)$$

where μ is the medium viscosity. Thermal damping is of similar magnitude to the viscous dissipation damping for UCAs, so one can (if expedient) omit δ_{th} , double δ_{vis} , and achieve near-parity [43]. These equations allow one to estimate the scattering cross section for an unshelled bubble driven at its resonance frequency. Figure 2 shows a plot of the scattering cross section versus radius for liquid and solid particles, as predicted by Rayleigh theory, compared to resonant microbubbles. The graph shows that at 1 MHz, the lower limit for clinical diagnostic ultrasound, the resonant microbubble scatters $\sim 10^{11}$ fold more than an equivalently sized liquid drop. At the upper limit of 10 MHz, the resonant microbubble scatters $\sim 10^9$ fold more than an equivalently sized liquid drop. The enormous amplification in acoustic backscatter is precisely why microbubbles are the dominant UCA.

Stability against coalescence

A contrast agent should be stable long enough in suspension for storage, handling and injection, as well as *in vivo* for the imaging exam (5–30 min). In the preceding section, it was illustrated that a microbubble is an ideal UCA owing to its excellent echogenicity. Unfortunately, unshelled microbubbles are neither biocompatible nor stable. The free gas/water interface is highly thrombogenic due to its propensity for rapid plasma protein adsorption. In addition, unshelled microbubbles are prone to the destabilizing mechanisms of coalescence and dissolution. The problem of coalescence can be addressed by considering the range and magnitude of the surface forces involved as two microbubbles approach one another. As two bubbles collide, the probability of coalescence is given by [44]:

$$P = \exp(-\tau_d/\tau_c) \quad (5)$$

where τ_d is the drainage time and τ_c is the contact time. The drainage time τ_d is the characteristic time it takes to drain the thin liquid film of medium between the two bubbles brought together. This analysis is useful to examine coalescence under conditions of fluid flow, e.g. in a flow focusing device, where collisions are brought about by convection. On the other hand, microbubble stability during storage in the vial can be considered from an alternative viewpoint of the energetics. It is generally considered safe to assume that the microbubbles are stable against coalescence if an energy barrier exists between them that exceeds $25 kT$, where k is Boltzmann's constant and T is absolute temperature [45].

First, consider the interaction between two unshelled microbubbles. All particles interact by the ubiquitous and usually attractive van der Waals force, which arises from a discontinuity in the dielectric properties of the intervening medium. For two spheres of equal radius R separated by a distance x , the van der Waals interaction energy is given by [45]:

$$W_{vdw}(x) = -\frac{AR}{12x} \quad (6)$$

where A is the Hamaker constant (3.7×10^{-20} J for two air bubbles in water). The negative sign denotes an attractive force. A second interaction between two unshelled microbubbles in water is the attractive hydrophobic force, where the interaction energy is given by [46]:

$$W_{hb}(x) = -2\pi R x_0 \gamma_i e^{-x/x_0} \quad (7)$$

where γ_i is the interfacial energy and x_0 is a characteristic decay length, approximately equal to the size of one water molecule (0.3 nm). Uncoated bubbles experience a large attraction because γ_i is equal to the surface tension of water, 72 mN/m at 25 °C. Figure 3a shows the theoretical interaction potential for two approaching 3- μ m diameter bubbles that are uncoated. The van der Waals and hydrophobic attraction forces combine to give a strong, long-range attraction, and the bubbles readily coalesce.

Coalescence can be avoided by adding a shell onto the microbubble that diminishes the attractive hydrophobic force, while also imposing new repulsive surface forces that overpower the van der Waals surface force. Current UCAs comprise a surfactant shell (protein or lipid) that has hydrophilic chemical groups facing the aqueous medium. Recall that Table 1 above shows a list of currently FDA-approved and commercially available UCAs, as well as their composition, surface potential (charge), size and concentration.

All of the commercial UCAs listed in Table 1 have an anionic surface charge [40]. The charged surface results in an enriched “double layer” of counter ions in the intervening medium that yields an osmotic repulsion, whose interaction energy for two spheres is given by [45]:

$$W_{es}(x) = \frac{1}{2} R Z e^{-\kappa_d x} \quad (8)$$

where κ_d^{-1} is the Debye length (~ 0.7 nm in physiological phosphate buffered saline) and Z is an interaction constant [$Z = 64\pi\epsilon\epsilon_0(kT/e)^2 \tanh^2(ze\psi_0/4kT)$; ϵ is the dielectric constant, ϵ_0 is the dielectric constant for water, e is the electronic charge, z is the counter ion charge valence and ψ_0 is the surface potential, related to the surface charge σ by the relation $\psi_0 = \sigma/\epsilon\epsilon_0\kappa_d$]. However, an analysis of the relative contributions of the electrostatic and van der Waals forces shows that a surface potential exceeding -50 mV would be necessary to stabilize the microbubbles under physiological conditions. Indeed, the surface charge is mainly used to mimic the erythrocytes, which have a negatively charged glycocalyx, in order to minimize interactions between the microbubbles and other cells in the cardiopulmonary system.

It turns out that the commercial UCAs listed in Table 1 are stabilized by alternative mechanisms other than charge. For example, Optison microbubbles are stabilized by a protein shell, and it is well documented that proteins exhibit short-range “hydration” repulsion [47]. The hydration force is analogous to the hydrophobic force in that it exhibits an exponential decay, but instead it is a repulsive force between hydrophilic surfaces. The force arises from the work required to remove the layer of tightly bound water molecules (the hydration shell) from the protein’s hydrophilic surface, in order to advance the surfaces into contact. The hydration interaction potential between two spheres is given by [45]:

$$W_h(x) = \pi R \lambda_h W_h e^{-x/\lambda_h} \quad (9)$$

where λ_h is a characteristic decay length (~ 0.6 nm) and W_h is an interaction energy (~ 30 mJ m⁻²). The hydration force is sufficient to stabilize protein-shelled microbubbles, such as Optison, against coalescence. Figure 3b shows the theoretical interaction potential between two 3- μ m diameter Optison bubbles based on the equations above. The surface of Optison is negatively charged ($\psi_0 = -9.5$ mV) [40], but it is the hydration force that provides stability.

The other commercial UCAs listed in Table 1, and many of the synthesized microbubbles described in the literature [48], are coated with lipids. Lipid membranes are known to exhibit short-range repulsive forces owing to out-of-plane protrusions and membrane undulations (the latter can be neglected for the gel-state lipids used to coat microbubbles). A repulsive force arises from the entropy loss as the protrusions are confined while the two surfaces approach. The protrusion interaction potential between two spheres is given by [45]:

$$W_p(x) = 2.7\pi R \Gamma kT \lambda_p e^{-x/\lambda_p} \quad (10)$$

where Γ is the lipid surface density and λ_p is a characteristic decay length (~ 0.1 nm). The protrusion force is sufficient to stabilize lipid-coated microbubbles, such as Sonovue. Figure 3c shows the theoretical interaction potential between two 3- μ m diameter Sonovue bubbles. The surface of Sonovue is negatively charged ($\psi_0 = -28.3$ mV) [40], but it is the protrusion force that provides stability.

Definity microbubbles also comprise lipids, but 8 mol% of the lipids are conjugated to methyl-terminated poly(ethylene glycol) (mPEG) of ~ 5000 Da molecular weight (average value due to polydispersity of the PEG). The methyl termination is critical to limiting immunogenicity, as the unstable thioester group on complement protein C3b is known to bind to free hydroxyl groups [49]. PEG is a hydrophilic and dynamic polymer, which extends away from the lipid surface into the aqueous medium. The inter-PEG distance for Definity is ~ 2.4 nm, which is smaller than the Flory radius of ~ 6.0 nm, so the PEG molecules form a hydrated brush on the microbubble surface. The length L of the brush is ~ 11 nm. Collision of two microbubbles leads to compression of the brush layers, and the interaction energy is given by [45]:

$$W_b(x) = 32R \Gamma^{3/2} L^2 kT e^{-\pi x/L} \quad (11)$$

where Γ is the PEG surface coverage (1.76×10^5 molecules/pm²) and L is the brush length (11.1 nm). Figure 3d shows the theoretical interaction potential between two 3- μ m diameter Definity bubbles. The PEG brush yields a long-range steric repulsion that extends tenfold longer than the hydration or protrusion forces.

These calculations show how the various shells of commercial UCAs can add repulsive forces (electrostatic, hydration, protrusion and polymer-steric) to overwhelm the van der Waals attraction. In each example, the repulsive forces produced an energy barrier far in excess of $25kT$, thereby stabilizing the microbubbles against diffusion-induced coalescence. This should lead to long-term stability, provided the microbubbles are stable against dissolution, which is considered below. Finally, it should be noted that microbubbles can be made to coalesce by other mechanisms, such as ultrasound-induced oscillations [50].

Stability against dissolution

The second destabilization mechanism for microbubbles is dissolution of the gas into the surrounding aqueous medium. For populations of densely concentrated microbubbles, this same mechanism can lead to Ostwald ripening [51], where gas diffuses from smaller to larger bubbles until the gas phase separates completely. A model for microbubble dissolution into an isotropic, infinite medium was first derived by Epstein and Plesset [52]:

$$\frac{dR}{dt} = -DH \left[\frac{1 - F + \Lambda/R\rho_\infty}{1 + 2\Lambda/3R\rho_\infty} \right] \left[\frac{1}{R} + \frac{1}{\sqrt{\pi Dt}} \right] \quad (12)$$

where again R is bubble radius, t is time, D is gas diffusivity in the aqueous medium, H is Henry's constant ($H = c_s/p$; c_s is the mass concentration of the gas solute in the aqueous medium and p is the mass concentration of gas in the contacting gas phase), F is the ratio of dissolved gas concentration to that at saturation ($F = c_s/c_{sat}$), Λ is a function of surface tension ($\Lambda = 2M\gamma/RT$; M is the gas molecular weight and R is the universal gas constant) and ρ_∞ is the mass concentration of gas in the gas phase for a flat interface at system pressure P ($\rho_\infty = MP/RT$). The time-dependent term in the second brackets of equation (12) can be neglected due to rapid establishment of the gas concentration profile in the aqueous phase surrounding the bubble [53].

An uncoated gas microbubble has surface tension ($\gamma \approx 0.07$ N/m), which as seen in equation (12) leads to a finite value for Λ and therefore rapid dissolution even in a saturated solution ($F = 1$). The dissolution rate ($-dR/dt$) can be reduced by the choice of low-solubility gases, such as SF₆ or C₃F₈. Figure 4a shows the lifetimes of 3- μ m diameter bubbles containing different gases, such as those used in the commercial UCAs (Table 1). Note that SF₆ only slightly increases stability over air in comparison to the perfluorocarbons. Microbubble dissolution within seconds remains unavoidable due to surface tension. However, in a multi-

gas environment, such as when an SF₆ bubble is injected into blood containing N₂ and O₂, the lifetime is extended significantly by the process of gas exchange [54,55].

Microbubble coatings used for commercial contrast agents inhibit dissolution by diminishing (or possibly eliminating) surface tension [56,53]. According to equation (12), $dR/dt \rightarrow 0$, and UCAs are thus stable in saturated media. Upon injection into the body, however, microbubbles experience hemodynamic pressure variations [57] and under-saturation of total dissolved gases due to ventilation-perfusion mismatch [58]. While these forces ultimately ensure complete bubble dissolution, the rate is hindered by the limited permeability [59] and elasticity [60] of the shell, thereby allowing sufficient stability for an imaging exam. Coated microbubbles dissolve according to a modified Epstein-Plesset relation [61]:

$$\frac{dR}{dt} = -H \left[\frac{1 - F + \Lambda/R\rho_{\infty}}{1 + 2\Lambda/3R\rho_{\infty}} \right] \left[\frac{1}{\Omega_w + \Omega_{shell}} \right] \quad (13a)$$

$$\Lambda = \frac{2M}{BT}(\gamma_0 + E\beta) \quad (13b)$$

where Ω_w is the mass transfer resistance of water ($\Omega_w = R/D$), Ω_{shell} is the mass transfer resistance of the shell, γ_0 is the shell surface tension at the resting radius R_0 and E is the shell elasticity ($E = E_0 \cdot \exp(-\alpha\beta)$; E_0 and α are material constants $\beta = (R/R_0)^2 - 1$) following work by Kausik Sarkar's group [62]. In theory, the permeation resistance and elasticity of a lipid shell can stabilize a microbubble even in a degassed environment, as shown in Figure 4b. In reality, however, the lipid monolayer shell is unstable to sustained compression and periodically collapses [59,63], leading to complete dissolution in an under-saturated medium. Here, the chemical potential gradient for the gas provides the work necessary to compress, buckle and fold the lipid shell. The process occurs in multiple discrete crumpling-to-smoothing transitions. In saturated media, the chemical potential gradient vanishes, and the microbubble is stable indefinitely.

UCA composition

The components of commercial UCAs are listed in Table 1 and shown schematically in Figure 5. Optison microbubbles consist of a perfluoropropane core coated with human serum albumin protein. Serum albumin is the most abundant protein in blood, which in principle should make the agent biocompatible for intravenous injection. During the manufacturing process, the heat-denatured protein acts as a surfactant and adsorbs to the gas/water interface. Proteins spontaneously attach to gas/water interfaces owing to their amphiphilicity and internal ordering, which is relieved when the protein adsorbs and partially unravels (increasing entropy). The aggregated protein shell is estimated to be ~15 nm thick [65]. A vial of Optison contains up to $\sim 8 \times 10^8$ /mL with a volume fraction of

~3.5% [66]. Note that the size distribution is highly polydisperse, and both size and concentration vary from batch-to-batch [30].

Defnity and Sonovue both have lipid shells primarily comprising zwitterionic phosphatidylcholine and an anionic phospholipid. The acyl chains are fully saturated to give a straight (all *trans*) configuration to allow close packing and enhanced cohesion. Sonovue has palmitic acid, which is a single-chain, anionic lipid. Defnity has a phospholipid-anchored hydrophilic PEG polymer, which as discussed above provides steric stabilization against coalescence. The lipids self-assemble into a monolayer at the gas/water interface, with their headgroups facing the aqueous medium and their tails facing the gas phase. Surface tension-driven dissolution forces the lipids to pack tightly on the interface; the estimated area per molecule is $\sim 0.45 \text{ nm}^2$ [67,68]. A vial of Sonovue contains up to $\sim 5.6 \times 10^8$ /mL with a volume fraction of 0.65%, and a vial of Defnity contains up to $\sim 1.2 \times 10^{10}$ /mL with a volume fraction of $\sim 4.4\%$ [66]. Again, note that the size distributions of these agents are highly polydisperse, and both size and concentration vary significantly from batch-to-batch.

UCA synthesis

Each commercial UCA is manufactured differently, and these manufacturing methodologies become increasingly important in high-volume applications such as oxygen delivery [15], or in formulations which require efficient drug-loading or conjugation of costly biologics. Optison is made by heating a 0.5–3% (w/v) solution of human serum albumin to 65–80 °C and applying mechanical shearing and hydrodynamic cavitation in the presence of C_3F_8 by a milling process to produce the microbubbles (Figure 6a), which are then cooled and stored under refrigeration [69]. The Optison microbubbles come to the user already prepared and stored in a sealed vial. One gently agitates the vial to disperse the microbubbles. While extracting the microbubbles with a syringe, an extra syringe needle is placed in the rubber septum to serve as a vent, in order to avoid inducing a vacuum that could destabilize the microbubbles. The recommended dose for human clinical imaging is 0.5 mL, corresponding to a microbubble volume dose of $\sim 22 \text{ }\mu\text{L}$.

Defnity microbubbles are made on demand at the patient bedside, via “activation” with a vial shaker [70] (Figure 6b). Prior to activation, each vial contains 6.52 mg/mL C_3F_8 in the headspace, and each mL of the clear liquid contains 0.75 mg lipid blend (consisting of 0.401 mg DPPC, 0.304 mg DPPE-mPEG₅₀₀₀ and 0.045 mg DPPA), 103.5 mg propylene glycol and 126.2 mg glycerin. Microbubbles are formed by mechanical agitation as the vial is shaken back and forth at a speed of $\sim 4500 \text{ Hz}$ for 45 s. The newly formed microbubbles are ready to be extracted via a syringe and vent for intravenous injection. The recommended bolus dose is 10 pL/kg corresponding to a microbubble volume dose of $\sim 44 \text{ }\mu\text{L}$ (for an average adult).

Sonovue microbubbles are also made on demand from freeze-dried lipid [71]. Each vial contains a white powder consisting of 24.56 mg of free PEG₄₀₀₀, 0.19 mg DSPC, 0.19 mg DPPG and 0.04 mg PA, as well as 60.7 mg SF_6 gas in the headspace. Microbubbles form spontaneously when 5 mL of diluent is added to the vial (Figure 6c), and are ready to be

extracted via a syringe and vent for intravenous injection. The recommended dose for echocardiography is 2.0 mL, corresponding to a microbubble volume dose of ~16 μ L.

UCA Material Properties

All of the commercial UCAs are echogenic and provide adequate contrast for their clinical imaging indications. Optison was the first of the current commercial UCAs to be approved. The C_3F_8 gas clears relatively quickly from the body through the lungs. In a clinical trial described in the package insert, a single intravenous dose of 20 mL of Optison was given to healthy volunteers, and most of the C_3F_8 was eliminated through the lungs within 10 min. The recovery was $96\% \pm 23\%$ (mean $\pm$ SD), and the pulmonary elimination half-life was 1.3 ± 0.69 min. The C_3F_8 concentration in expired air peaked approximately 30–40 s after administration. In another reported clinical trial, the median duration of Optison contrast enhancement for each of four doses (0.2, 0.5, 3.0 and 5.0 mL) was approximately one, two, four and five min, respectively. Albumin-coated microbubbles are known to bind complement protein C3b [72], which may reduce circulation persistence and can lead to hypersensitivity. Complement fixation may be why Optison microbubbles are avidly taken up by liver Kupffer cells [73]. When driven by ultrasound, albumin-coated microbubbles are known to exhibit “sonic cracking”: during expansion the shell ruptures and an uncoated gas bubble emerges that then rapidly dissolves owing to surface tension [74,75]. The protein shell is expected to be metabolized by the normal human routes for serum albumin.

Definity was the first lipid-coated microbubble to be approved for human use. Clinical trials show similar behavior with Optison, which has the same gas core species. Following an intravenous injection, the C_3F_8 was not detectable after 10 min in most subjects either in the blood or in expired air. C_3F_8 concentrations in blood were shown to decline in a mono-exponential fashion with a mean half-life of 1.3 min in healthy subjects. The lipid shell is thought to be metabolized to free fatty acids. The duration of useful contrast detection at the recommended dose for a bolus injection is 3.4 min. The PEGylated shell is believed to reduce complement C3 binding [76], but the PEG brush may also induce an antibody response after multiple administrations [77]. The acoustic behavior of lipid-coated microbubbles is markedly different from that of protein-coated agents: the shell is highly compliant to both compression and expansion, and the bubble remains encapsulated and stable after the pulse. As the mechanical index is increased, lipid-coated microbubbles may experience acoustically driven dissolution, presumably via lipid shedding [78], and fragmentation [74].

Sonovue (Lumason) is the most recent contrast agent to be approved in the US for echocardiography. Interestingly, despite the prediction of rapid dissolution in Figure 4, the SF_6 gas has a similar half-life to the perfluorocarbon used in the other agents. This may be due to a gas-exchange process between the SF_6 core and dissolved gases in the blood [54,55]. According to the package insert for Sonovue, after intravenous bolus injections of 0.03 mL/kg and 0.3 mL/kg, corresponding to approximately 1 and 10 times the recommended doses, concentrations of SF_6 in blood peaked within 1–2 min for both doses. The terminal half-life of SF_6 in blood was approximately 10 min for the 0.3 mL/kg dose. The area-under-the-curve of SF_6 was dose-proportional over the dose range studied. Twenty

minutes following injection, the mean cumulative recovery of SF₆ in expired air was 82 ± 20% at the 0.03 mL/kg dose and 88 ± 26% at the 0.3 mL/kg dose. SF₆ undergoes first-pass elimination within the pulmonary circulation; approximately 40% to 50% of the SF₆ content was eliminated in the expired air during the first minute following injection. Following the first appearance of contrast within the left ventricle, the mean duration of useful contrast effect ranged from 1.7 to 3.1 minutes. Sonovue showed markedly less uptake by liver Kupffer cells than Optison [73]. According to a study performed by researchers at Bracco [66], the acoustic behavior and stability of Sonovue is similar to Definity, as both are coated with phospholipids, and superior to Optison. Sonovue was the first UCA approved by the FDA for liver imaging and pediatric imaging.

Off-Market and Preclinical UCAs

Other UCAs have been approved in the US, but are not currently on the market. This includes lipid-coated, C₅F₁₂/N₂-filled Imagent (Alliance Pharmaceutical), as well as the air-filled agents Levovist (lipid/galactose shell) and Albunex (albumin shell), the latter of which was replaced by the longer circulating Optison. Other UCAs have only been approved outside the US, such as lipid-coated, C₄F₁₀-filled Sonazoid (GE Healthcare). Yet more UCAs have been under development, but not yet approved, such as the polymer-coated Sonovist (Scherring) and Cardiosphere (Point Biomedical).

One exciting development in the ultrasound molecular imaging community is the experimental agent BR55 (Bracco): a lipid-coated, C₄F₁₀-filled microbubble decorated with peptides targeted to VEGFR2, a biomarker for tumor angiogenesis [79]. The peptides on the microbubble surface bind to VEGFR2 molecules overexpressed on the endothelium, causing the microbubble to adhere to the neovessels. The molecular imaging agent has been shown to improve diagnosis of prostate cancer [80] and breast cancer [81] in human patients. This new agent may open the gateway for more targeted UCAs in the future. Fujifilm Visualsonics also markets non-targeted and target-ready Micromarker (Bracco) for preclinical use with high-frequency small-animal scanners.

Another exciting development is the commercialization of monodisperse microbubbles. Advanced Microbubble Labs (Boulder, CO) now markets size-isolated microbubbles (SIMB) for research use. The lipid-coated, C₄F₁₀-filled SIMBs are packaged ready-for-use in various sizes, from 1–2 µm to 6–8 µm diameter (Figure 7a). Additionally, Tide Microfluidics (Enschede, The Netherlands) now markets a microfluidic device to generate highly monodisperse microbubbles. The device makes use of recent advances in microfluidic flow-focusing to generate microbubbles with precise size and shell properties (Figure 7b).

Performance of UCAs in imaging and therapy

UCAs have been used in millions of patients throughout the world, and they have proven safe and efficacious for diagnostic monitoring through a rigorous regulatory process [38,39]. Unfortunately, all of the current commercial UCAs were developed almost three decades ago, mainly for echocardiography. Owing to intellectual property and regulatory constraints, these agents have changed very little from their original formulations. At the same time,

microbubble technology has made enormous advancements. There are now vast numbers of UCA synthesis techniques and designs described in the research literature, including targeted microbubbles, drug/gene-loaded microbubbles, multi-modal imaging agents, phase-change agents and many others. At the same time, our understanding of microbubble physical, chemical and biological behavior has improved tremendously. It is now clear that the currently approved UCAs are somewhat crude in terms of material characteristics, physical properties and biomedical performance compared to next-generation microbubble designs, and there is a need to translate these advanced formulations through regulatory approval and to clinical adoption.

At the same time, ultrasound devices have gotten smaller, faster and smarter than ever. For example, the iQ Butterfly device was approved by the US FDA in 2018 for 13 applications [82]. This revolutionary ultrasound imaging probe plugs into your smart phone, employs machine learning to optimize images and costs only ~\$2,000. As another example, MRI-guided focused ultrasound devices have been approved by the US FDA for treatment of uterine fibroids, prostate cancer, bone metastasis and other indications [83]. While such “hardware” technologies have been readily approved and adopted, the “software” technology (microbubble UCAs) has been largely neglected when it comes to clinical translation. This is problematic, as it could be argued that the microbubble, which is injected into the patient, is just as important as the external ultrasound device. Clinical applications employing microbubbles are therefore using sub-optimal technology because clinical adoption of microbubble technology has lagged that of ultrasound devices. The translation of more sophisticated microbubble designs to clinical practice will require a paradigm shift in the attitude of engineers and clinicians to value the importance of UCA “software” as much as ultrasound device hardware and their novel applications.

Conclusions

In this review, a brief history and current state-of-the-art is given to stimulate the rational design of new microbubbles through the reverse engineering of current UCAs. It is shown that an effective microbubble should be biocompatible, echogenic and stable. Physical mechanisms and engineering calculations have been provided to illustrate these properties and how they can be achieved. The rational design paradigm championed by David Needham [31] is applied to study current FDA-approved and commercially available UCAs. Given the sophistication of microbubble designs reported in the literature, rapid development and adoption of ultrasound device hardware and the growing number of revolutionary biomedical applications moving toward the clinic, the field of Microbubble Engineering is fertile for breakthroughs in next- generation UCA technology. It is up to current and future microbubble engineers and clinicians to push forward with regulatory approval and clinical adoption of advanced UCA technologies in the years to come.

Acknowledgements

Funding for this work was provided by NIH grant R01 CA195051.

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Highlights:

- Ultrasound contrast agents are echogenic microbubbles used in medical diagnostics.
- Their components can be deconstructed using reverse engineering principles.
- The components act to stabilize against coalescence and dissolution.
- Overall, their characteristics are dated and rather crude from an engineering perspective.
- Better designs must be promoted to keep pace with device hardware and applications.

Figure 1.

Ultrasound image enhancement with microbubble UCAs. Shown are a b-mode image of the rat kidney and Cadence Pulse Sequencing (CPS) mode images before and after UCA injection taken with a Siemens Sequoia ultrasound scanner. Note that the green boundary defining the kidney as the region of interest is drawn from the anatomical B-mode image, and that contrast is observed outside the kidney owing to nearby vasculature. Images from [33].

Figure 2.

Particle acoustic scattering cross section versus radius. Rayleigh theory (equation 1) was applied to estimate the cross sections for liquid (glycerin) and solid (steel) microspheres. The simple oscillator model (equations 2–4) was used to estimate the cross section for resonant gas bubbles - that is, the bubble is driven at its resonance frequency for each size. Solid lines show the cross sections at 1 MHz for liquid and solid particles. Dashed lines show the cross sections at 10 MHz for liquid and solid particles. Arrows show the cross sections for resonant gas bubbles at these frequency limits.

Figure 3.

Theoretical energy-distance profiles between two 3- μm diameter bubbles. a) Uncoated: van der Waals and hydrophobic interactions yield a purely attractive (negative) potential. b) Optison: the protein shell removes the hydrophobic attraction and imposes electrostatic and hydration repulsions, generating an energy barrier to coalescence. c) Sonovue: the lipid shell imposes electrostatic and protrusion repulsions, generating a purely repulsive (positive) potential. d) Definity: the PEG brush on the lipid shell yields a long-range repulsion.

Figure 4.

Theoretical dissolution curves for a 3- μm diameter bubble. a) Uncoated microbubbles containing various gases in a saturated medium dissolving due to surface tension; gas parameters taken from [60]. B) Lipid-coated vs. uncoated air bubbles dissolving in an undersaturated medium ($F=0.8$); lipid shell permeability is taken from [64] and elasticity is taken from [61].

Figure 5. Cartoon schematic of shell and gas components for Optison, Sonovue and Definity. Molecular structures are not shown to scale.

A) Optison

mechanical
cavitation
with a mill

B) Defnity

mechanical agitation
with a shaker

C) Sonovue

Figure 6.

Methods of UCA manufacture: A) Optison is made by mechanical cavitation (mechanical shearing and hydrodynamic cavitation) in a rotor-stator homogenizer mill. B) Defnity is made by shaking back-and-forth at 4–5 kHz in a vial shaker. C) Sonovue is made by rehydrating a freeze-dried lipid powder.

A) Size-Isolated Microbubbles (SIMBs)

B) Microfluidic Microbubbles

Figure 7. Commercialized next-generation microbubbles: A) Size-Isolated Microbubbles (SIMBs); graphic taken from AMB Labs (www.advancedmicrobubbles.com). B) Microfluidic microbubbles; graphic taken from Tide Microfluidics (www.tidemicrofluidics.com).

Table 1.

Currently FDA-approved and commercially available UCAs.

UCA	Gas	Shell	Size (Diameter)	Concentration
Optison™ (GE Healthcare)	C ₃ F ₈	<u>Protein</u> Human serum albumin $\psi_o = -9.5 \text{ mV}$	3.0 – 4.5 μm (max 32 μm) 95% less than 10 μm	$5.0\text{--}8.0 \times 10^8 \text{ /mL}$
Definity® (Lantheus)	C ₃ F ₈	<u>Phospholipid</u> DPPC: 0.401 mg/mL DPPA: 0.045 mg/mL DPPE-mPEG ₅₀₀₀ : 0.304 mg/mL $\psi_o = -4.2 \text{ mV}$	1.1 – 3.3 μm (max 20 μm) 98% less than 10 μm	$1.2 \times 10^{10} \text{ /mL}$
Sonovue® aka Lumas on® (Bracco)	SF ₆	<u>Phospholipid</u> DSPC: 0.038 mg/mL DPPG: 0.038 mg/mL PA: 0.008 mg/mL $\psi_o = -28.3 \text{ mV}$	1.5 – 2.5 μm (max 20 μm) 99% less than 10 μm	$1.5\text{--}5.6 \times 10^8 \text{ /mL}$

DPPC = 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPA = 1,2-dipalmitoyl-*sn*-glycero-3-phosphate; DSPC = 1,2-distearoyl-sn-glycero-3-phosphocholine; DPPE-mPEG5000 = 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000]; DPPG = 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol); PA = palmitic acid; ψ_o is the zeta potential measured [40].