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● *Original Contribution*

PHYSICOCHEMICAL CHARACTERISTICS OF SONAZOID™, A NEW CONTRAST AGENT FOR ULTRASOUND IMAGING

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Abstract—The objective of the current work is to describe the physicochemical characteristics of Sonazoid™, a new ultrasound contrast agent for detection and characterisation of focal liver lesions. It has been demonstrated that Sonazoid™ powder for injection consists of microspheres of perfluorobutane (PFB) stabilised by a monomolecular membrane of hydrogenated egg phosphatidyl serine, embedded in an amorphous sucrose structure. Upon reconstitution with sterile water, stabilised microspheres of PFB are released in a predefined amount and size into a low viscosity, isotonic sucrose solution with a neutral pH. Sonazoid™ reconstituted product contains approximately 8 μl microspheres/ml with volume median diameter of approximately 2.6 μm. The product contains approximately 1.2 billion microspheres/ml of which less than 0.1% are larger than 7 μm. The acoustic properties of Sonazoid™ such as attenuation efficacy, fundamental and second harmonic backscatter efficacy are all well correlated to the microsphere volume concentration. The stability of Sonazoid™ after reconstitution is good, with no significant changes in physicochemical properties 2 h after reconstitution. Pressure stress is well tolerated by both concentrated and diluted Sonazoid™ with no permanent effects of pressures up to 300 mm Hg. The level and consistency of the investigated physicochemical properties demonstrate that Sonazoid™ should be well suited as a contrast agent for medical imaging with ultrasound. (E-mail: per.sontum@ge.com) **© 2008 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Contrast agents, Microspheres, Physicochemical properties, Acoustic properties, Perfluorobutane, Phosphatidyl serine.**

INTRODUCTION

Over the past two decades, the pharmaceutical industry has shown a great interest in developing a safe and efficacious ultrasound contrast agent (USCA) for medical imaging. This effort has so far resulted in bringing a few products to the market as well as a number of candidates late in the development pipeline. The principal physical contrast creating mechanism of all these USCAs is the scattering of ultrasound (US) from small envelopes of gas as they undergo volume oscillations in the sound beam. Due to the high compressibility of gases and their ability to resonate when insonated, a population of microspheres is very effective in scattering incident US compared with surrounding blood or tissue. During ultrasound scanning, body cavities and compartments containing microspheres will thus appear white compared with regions without contrast agent.

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The main drive for the development of a safe and efficacious compound has been in producing stable microspheres of a predefined, biologically acceptable, size that enables passage through capillary beds and thus allows for imaging in the entire cardiovascular system. The average diameter of the lung capillaries has been reported to approximately 7 μm with approximately 95% being larger than 4 μm (Hogg 1987). Hence, to optimise for free flowing properties and avoid potential capillary embolism, the size of microspheres in USCAs should preferably be smaller than approximately 4 μm. In addition, the content of larger microspheres should be minimised. To this point, it should be noted that the functional diameter of the capillaries for any given vesicle may be different from the geometric diameter; *e.g.*, surface properties and deformability may influence their retention in the microcirculation.

Various concepts have been explored in developing an USCA with suitable properties. Early products investigated the stabilisation of air bubbles, either by release *in vivo* from micro-porous crystalline sugar structures as with Levovist® (registered trademark of Schering AG)

or by stabilising individual envelops of air by a shell of denatured human serum albumin as with Albunex® (registered trademark of Mallinckrodt Inc.) (Christiansen et al. 1994; Sontum et al. 1997). Both these concepts were successful in bringing microspheres through the capillary bed of the lungs, producing contrast in the bulk volume of the left heart chamber after an i.v. injection. Their stability *in vivo* (*i.e.*, contrast persistency), however, was not sufficient to track contrast into other organs of the body. A primary cause for the insufficient stability of these air-based USCAs is that the small molecules of the air easily diffuse across the stabilising structure. Additionally, as air is highly soluble in the surrounding matrix (*i.e.*, blood), the microspheres simply dissolve too quickly after injection. In trying to improve the stability of the microspheres, a concept of encapsulating air with a more solid, polymer based, shell was investigated (Bjerknes et al. 1997). In this case, however, the thickness/stiffness of the shell lowered the physical, *in vitro* acoustic efficacy of the compound to such a degree that the substance did not deliver the necessary imaging quality (Hoff et al. 2000). In an effort to increase the stability of microspheres without including a rigid stabilising structure, a more slowly diffusing, low solubility gas such as sulphur-hexafluoride or low molecular weight perfluorocarbons may be utilised (Dugstad et al. 1996). These gases, in combination with various flexible stabilising structures, are now used in several products (*e.g.*, Definity®, registered trademark of Bristol-Myers Squibb, SonoVue™, registered trademark of Bracco and Optison™, registered trademark of GE Healthcare.)

As apparent from above, for USCAs the physical state of the active ingredient may be more important to product performance than its content or chemical composition. To facilitate a sufficient understanding of a product and to assure consistent performance both regarding efficacy and safety, comprehensive physicochemical characterisation and control is imperative. The objective of the current work is to give a thorough description of important characteristics of Sonazoid™ (registered trademark of GE Healthcare), a new USCA recently approved in Japan for detection and characterisation of focal liver lesions. During clinical use of Sonazoid™, imaging is performed in two stages; vascular-phase imaging, while the contrast is predominantly in the blood-pool and delayed-phase imaging, when microspheres have been taken up or trapped by Kupffer cells. Vascular phase imaging is performed shortly after administration and arterial-phase information can be obtained by monitoring the first passage of contrast into the liver. Because of the increased arterial vasculature surrounding malignant lesions, these become "bright rimmed" and easily detectable from normal tissue. The dynamics and nature of vascular contrast enhancement can be used to distinguish between different kinds of malignant lesions, such as hepatocellular carcinoma and metastasis, and different types of benign lesions, such as hemangioma and focal nodular hyperplasia, *etc.* Kupffer-phase imaging is performed after typically 10 min when the hepatic parenchyma will appear homogeneously bright (*i.e.*, filled with contrast) whereas malignant lesions will appear dark; the entire liver can be easily imaged in this phase and scanned for the presence of suspicious lesions. The mechanism behind the contrast enhancement observed during Kupffer-phase imaging has been shown to be due to the trapping of microspheres by the Kupffer cells present in the hepatic parenchyma (Miyahara et al. 2006; Watanabe et al. 2007). These cells are not present in malignant lesions which hence will appear as clear contrast defects in the image. The safety and clinical utility of Sonazoid™ have been demonstrated (Gordon 2006; Moriyasu 2006).

Sonazoid™ consists of perfluorobutane gas (PFB) microspheres stabilised by a membrane of hydrogenated egg phosphatidyl serine (HEPS). The product is formulated and presented as a lyophilised powder for injection that before use is reconstituted with sterile water to release stabilised microspheres of PFB in a predefined concentration and size distribution in an isotonic sucrose solution.

MATERIALS AND METHODS

Materials

Sonazoid™ is formulated as a powder for injection consisting of lyophilised sucrose entrapping HEPS stabilised PFB microspheres under a PFB headspace. Sonazoid™ is aseptically produced by continuous homogenisation of PFB in an aqueous dispersion of HEPS. After the initial microsphere generation, the concentration and size distribution of microspheres is adjusted through a series of controlled separation steps. The final dispersion, targeted to yield 8 μl microspheres per ml in the reconstituted product, is made isotonic by addition of sucrose. Two ml of the dispersion is filled into 10 ml glass vials and lyophilised. After lyophilisation, the vial head space is back-filled with PFB before stoppering. Before use, the product is reconstituted by addition of 2 ml of water through a supplied vented filter (5 μm) spike (Codan Chemoprotect® Spike, Codan GmbH & Co., Germany) followed by manual mixing for 1 min. After reconstitution, the product appears as a milky white, homogeneous dispersion. As the dispersion is nontransparent, visual inspection for extraneous particles is difficult. To ensure the absence of such particles, the product is withdrawn through the filter spike into the syringe before administration. After reconstitution, if left nonagitated the microspheres will start to segregate by flotation and form a

cream layer on top of the liquid phase. If not used immediately after reconstitution, the product should be re-homogenised prior use by manual mixing for 10 s.

Methods

To obtain information on physicochemical aspects of Sonazoid™ powder for injection and Sonazoid™ reconstituted product, a number of analytical techniques have been utilised. Some of these techniques have been part of the routine quality control regime and employed on all investigated batches; others have been used in limited studies to evaluate typical properties of a representative set of batches.

Dry powder morphology was investigated by scanning electron microscopy (SEM) utilising a Jeol JSM 840 (Jeol Ltd., Tokyo, Japan). Samples were coated with a thin gold layer (200 Å) and SEM micrographs of surface and transverse cut of the lyophilised cake were recorded.

In addition, lyophilised product was investigated by wide angle X-ray diffractometry with a Siemens D5000 Diffractometer (Bruker AXS Ltd., Coventry, UK) with monochromatic CuKα radiation.

The morphology of the lyophilised product was also investigated by optical microscopy utilising a Zeiss Axioskop Plus light microscope with a HAL 100 halogen lamp (Carl Zeiss Jena GmbH, Jena, Germany). The microscope illumination was optimised using the transmitted-light Brightfield setting for the 40X objective. A Soft Imaging System F-view II black and white digital camera and AnalySIS software with extended focal imaging analysis was used for visualisation. A thin slice of lyophilised sample was mounted on a glass slide and covered with a cover slip before generation of micrographs.

The morphology of the microspheres in the reconstituted product was investigated by transmission electron microscopy (TEM) utilising a Jeol 100S transmission electron microscope (Jeol Ltd., Tokyo, Japan). Samples of reconstituted product were centrifuged for 5 min at 2000 rpm and the microsphere rich (cream) layer on top of the infranatant was used for further sample preparation. Samples were fixed in 1% OsO_4 for 1 h, dehydrated in acetone and then embedded in liquid epoxy resin. After hardening, the resin blocks were sectioned in 50 nm sections and stained in uranyl acetate and lead citrate before uptake of micrographs.

Microsphere concentration and size distribution was determined on a routine basis with a Coulter Multisizer Mrk II or III (Beckman Coulter, Inc., Fullerton, CA, USA) fitted with a 50 μm aperture with a nominal measuring range of 1.0 to 30 μm. A suitable sample volume was diluted in 200 ml of Isoton II (Beckman Coulter, Inc., Fullerton, CA, USA) electrolyte at ambient room temperature and stirred for 5 min before analysis.

PFB content in the reconstituted product was determined by gas chromatography-mass spectroscopy (GC-MS) utilizing a Fisons Instrument MD800 (Fisons GmbH, Egelsbach, Germany) with a Crompack CPProaPlot-Q 25 m/25 mm column (Crompack International B.V., Middelburg, The Netherlands) and a DANI HSS 3950 headspace sampler (DANI, Milano, Italy). For some samples, analysis was also performed on the liquid matrix phase (infranatant) after complete segregation of microspheres by gravitational flotation 24 h.

Microsphere membrane lamellarity was assessed by measuring the distribution of phospholipid between the microspheres and the liquid matrix phase, in combination with determination of microsphere surface area. The phospholipid content quantified by HPTLC utilising a Camag III system (Camag, Muttenz, Switzerland) with 200 μm silica gel HPTLC plates. Microsphere surface area was calculated from the Coulter analysis (see above). Analysis was performed on whole samples of reconstituted product as well as on liquid matrix phase (infranatant) after complete segregation of microspheres by gravitational flotation for 24 h.

Microsphere membrane surface charge was determined as a function of pH by laser Doppler velocimetry utilising a Malvern Zetasizer IV (Malvern Instruments Ltd., Malvern, UK). Measurements were performed in 10 mM saline pH adjusted with NaOH or HCl in the range of 6 to 8.

Osmolality, pH and viscosity of reconstituted product were determined utilising a FISKE one-ten freezing point depression osmometer (Advances Instruments Inc., Norwood, MA, USA), an ATI Orion PherpHect 350 pH-meter with a Ross Sureflow electrode (ATI ORION Laboratory Products Group, Boston, MA, USA) and a Bohlin VOR Rheometer with a DG 24/27 measuring system (Malvern Instruments Ltd., Malvern, UK), respectively.

Pressure stability was assessed on a routine basis during measurement of the acoustic attenuation spectrum as detailed in Sontum et al. (1999a). The attenuation spectrum of a diluted sample was measured before, during and after application of 120 mm Hg pressure above ambient for 30 s. As a numeric pressure stability parameter, the recovery of the attenuation at 3.5 MHz after pressure application, in percent of attenuation before pressure application, was calculated. In addition to testing at 120 mm Hg, some batches were tested during pressurisation up to 300 mm Hg in the same set- up.

To mimic the pressure stress induced during injection of the reconstituted product, some batches were tested for pressure stability in the undiluted state at pressures up to 300 mm Hg for 60 s.

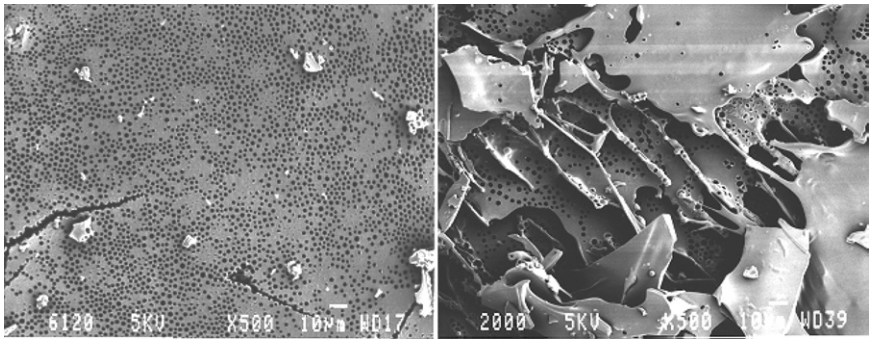

Fig. 1. Typical images from SEM on Sonazoid™ powder for injection. Surface view (left) and transverse cut (right) of lyophilised cake. Size bars are 10 μm.

RESULTS AND DISCUSSION

Dry Powder Morphology

Figure 1 shows typical results from SEM on Sonazoid™ powder for injection. The surface view shows evidence of microspheres to appear as a population of circular perforations. As the SEM analysis is performed under vacuum, it is possible that some of the microspheres close to the sucrose surface are disrupted before the image is taken. The relatively high density of microspheres in the surface view is caused by flotation before the dispersion is frozen before lyophilisation, which results in a high microsphere concentration in the upper layer. From the transverse cut, it may be observed that the lyophilised powder consists of an amorphous, macroporous solid structure with evidence of microspheres observed as perforations or shadows of vesicles embedded in the sucrose. The amorphous structure of the lyophilised product was also confirmed by X-ray diffraction analysis, which gave a broad, diffuse maximum typical for amorphous solids (results not shown).

Figure 2 shows typical results from optical microscopy on Sonazoid™ powder for injection. As can be seen from this figure the Sonazoid™ microspheres appear as spherical cavities, typically 1 to 5 μm in diameter, individually embedded in the solid sucrose structure. This demonstrates an important aspect of Sonazoid™; that the microspheres are formed during the primary production. Hence, their concentration and size are predefined and not formed during the reconstitution procedure or influenced by the end user. Upon reconstitution, when the sugar dissolves, gas filled cavities with phospholipid lining will be released as stabilised microspheres of a predefined amount and size into the sucrose solution.

Microsphere Concentration, Shape and Size Distribution

The active ingredient of USCAs is normally defined as the physical entity of the stabilised microsphere hence; the microsphere content is the natural assay (content of active) parameter for this class of products. The content of microspheres may be expressed as number or volume concentration. In addition to content, the size of the microspheres is critical for product efficacy (see Acoustic Properties section) and also important for biological behaviour (*e.g.*, capillary retention). For Sonazoid™ the microsphere volume concentration has been shown to be a relatively precise predictor of product efficacy and, as this parameter also describes the mass content of PFB, volume concentration has been chosen as the parameter for designation of strength.

Determination of concentration and size is an important part of the routine quality control of Sonazoid™. Both these parameters may conveniently be determined by Coulter counting, a technique that has earlier been shown to yield results of acceptable quality when applied to similar systems (Sontum and Christiansen 1994). The accuracy of the results obtained from Coulter analysis on Sonazoid™ has been investigated by comparative analysis using the alternative techniques; microscopy/image analysis and laser diffraction. The results from both these

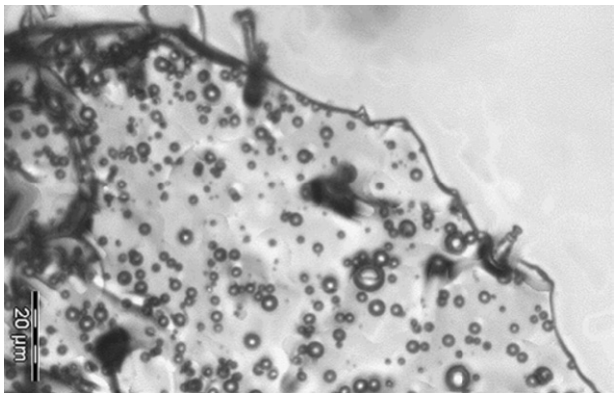

Fig. 2. Typical image from optical microscopy on Sonazoid™ powder for injection. View through thin fragment of the lyophilised sucrose matrix in which the microspheres are embedded. Size bar is 20 μm.

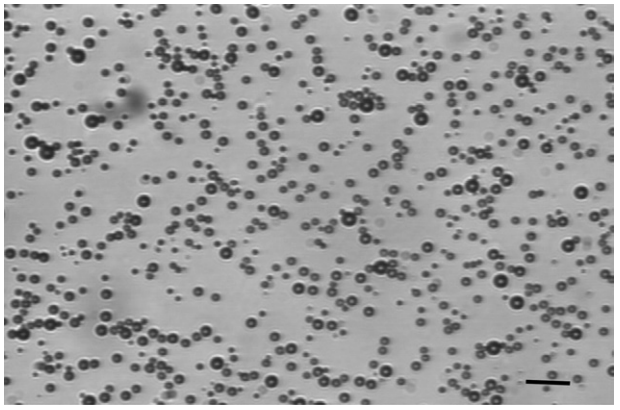

Fig. 3. Typical images from optical microscopy on Sonazoid™ reconstituted product. Size bar is 10 μm.

techniques (not shown) where insignificantly different from the results obtained by the Coulter analysis and, hence, corroborate the accuracy of the latter.

Figure 3 shows a typical image of microspheres in Sonazoid™ reconstituted product as visualised by optical microscopy. Figure 4 shows the number and volume size distributions for a typical sample of Sonazoid™ reconstituted product as determined by Coulter counting. Typical results for various numeric parameters routinely determined by the Coulter analysis are given in Table 1.

As can be seen from Fig. 3, Sonazoid™ reconstituted product consists of spherical microspheres with diameters typically in range of 1 to 5 μm. As apparent from Fig. 4, the microsphere size distribution is well defined in a narrow mono-modal peak. As determined by Coulter counting, the microsphere volume concentration

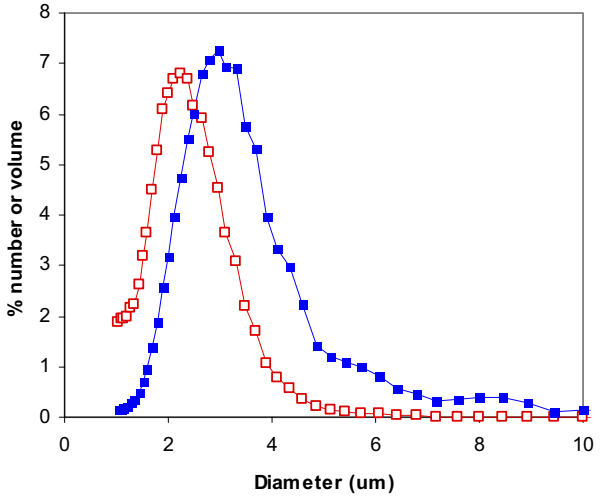

Fig. 4. Number (open squares) and volume (filled squares) size distributions of microspheres in a typical batch of Sonazoid™ reconstituted product as determined by Coulter counting.

Table 1. Typical values for various physicochemical parameters of Sonazoid™

Parameter	Average ± SD*
Microsphere volume concentration (il/ml)	8.0 ± 0.6
Microsphere number concentration (billions/ml)	1.2 ± 0.1
Microspheres > 7 μm (%)	0.05 ± 0.02
Microsphere volume median diameter (im)	2.6 ± 0.1
Microsphere number mean diameter (im)	2.1 ± 0.1

* Average ± SD for 10 vials from each of 10 batches.

in Sonazoid™ reconstituted product is 8.0 ± 0.6 μl microspheres/ml, equivalent to 1.2 ± 0.1 billion microspheres/ml. The volume median diameter is 2.6 ± 0.1 μm and the number mean diameter is 2.1 ± 0.1 μm. Less than 0.1% of the total number of microspheres has a diameter larger than 7 μm, hence, the product should be free flowing with minimal retention in capillary beds. The lack of significant variance in these parameters (batch to batch and vial to vial) ensures a consistent product efficacy and biological behaviour.

Encapsulated PFB Content

The Coulter analysis does not distinguish between solid, liquid or gaseous entities, nor does this technique distinguish between PFB- filled microspheres and microspheres containing other gases. To ensure that the particles detected during the Coulter analysis are in fact filled with PFB in a reproducible manner, the amount of encapsulated PFB in the liquid layer of reconstituted Sonazoid™ has been determined on a routine basis by GC-MS analysis. The results from analysis on 72 vials from seven different batches gave a content of PFB in the liquid layer (expressed as the ratio between PFB content in liquid layer and microsphere volume concentration) of 9.7 ± 0.5 μg PFB/μl microspheres. In this study, the content of PFB in the liquid matrix (infranatant of Sonazoid™ reconstituted product completely segregated by flotation) was determined to approximately 2 μg/ml. The amount of PFB dissolved in the liquid matrix is, hence, negligible (~ 2% to 3%) compared with the amount encapsulated in the microspheres. To evaluate the presence of non gas filled material larger than 1 μm (*e.g.*, liquid filled phospholipid vesicles), for some samples the Coulter analysis was also performed on the infranatant of fully segregated Sonazoid™. The volume concentration observed for these samples was typically 2% to 3% of the total volume concentration, demonstrating the limited presence of such structures.

The density of PFB can be calculated from a molecular weight of 238 g/mol and the molar volume of an ideal gas at 22°C of 24.3 l/mol, to 9.7 μg/μl. The correspondence between the measured ratio between en-

capsulated PFB and microsphere volume concentration and the density of the gas, reveals several aspects of the fundamental physicochemical properties of Sonazoid™. Most importantly, it demonstrates that the microspheres measured by Coulter counting are in fact filled with PFB at approximately 1 atmosphere and that other gases are present in a negligible amount. In addition, the presence of liquid filled phospholipid vesicles larger than 1 μm must be limited (as demonstrated by the Coulter analysis on infranatant samples) and that the volume of the stabilising membrane must be negligible compared with the total microsphere volume. Had such components contributed to the total volume measured, a significantly lower PFB content per volume would have been expected.

Membrane Lammelarity

In an aqueous liposome system, to minimise the thermodynamically unfavourable contacts between the hydrophilic and hydrophobic parts, phospholipids form bi-layer structures. In such structures, the hydrophilic head group is always oriented to the water phase (either external matrix or encapsulated volume) whereas the hydrophobic tails are contained in the middle of each bi-layer. In a foam such as Sonazoid™, however, the encapsulated phase consists of a hydrophobic gas. In such a system, the thermodynamic gain from forming bi-layers is lost and structures where the hydrophilic head group is oriented to the water phase and the hydrophobic tail groups are oriented to the gas phase would constitute the thermodynamically favoured situation. In its simplest form, such a structure would be a mono-layer of phospholipids, although any odd numbered layer (*e.g.*, tri-layer) would satisfy this condition.

As a qualitative investigation of membrane morphology/lamellarity, TEM studies of Sonazoid™ reconstituted product have been performed, utilising a staining sample preparation technique. A typical TEM image for a stained sample is visualised in Fig. 5 showing a resin-free interior surrounded by darker material. These results are interpreted as the original gas-filled microsphere surrounded by an intact membrane wall that has survived sample preparation. In this case, a single electron dense line with a fuzzy coat surrounds the interior of the microsphere. The thickness of this single layer is approximately 2 to 3 nm. This type of image, observed for a number of intact microspheres, indicate that the gas filled microspheres are surrounded by a monomolecular phospholipid layer.

To further investigate the lamellarity of the microsphere membrane, the distribution of components in the system was studied by chromatographic determination of phospholipid content in both homogeneous reconstituted product and in infranatant from fully segregated samples (*i.e.*, liquid matrix phase). To estimate phospholipid mass per area, the microsphere surface was determined by Coulter counting. These analyses were performed on 27 samples from five different batches and the phospholipid mass per microsphere surface area was determined to 3.6 ± 1.0 mg/m^2. The molecular areas of phosphatidyl serine in an air/water mono-layer have been reported to 38 Å^2/molecule (Paptil et al. 1979; Beitinger et al. 1989) corresponding to 3.4 mg/m^2. For Sonazoid™, the observed value is thus in good agreement with the assumption of a single mono-layer, 2 to 3 nm thick, surrounding each microsphere, confirming the indications from the TEM analysis.

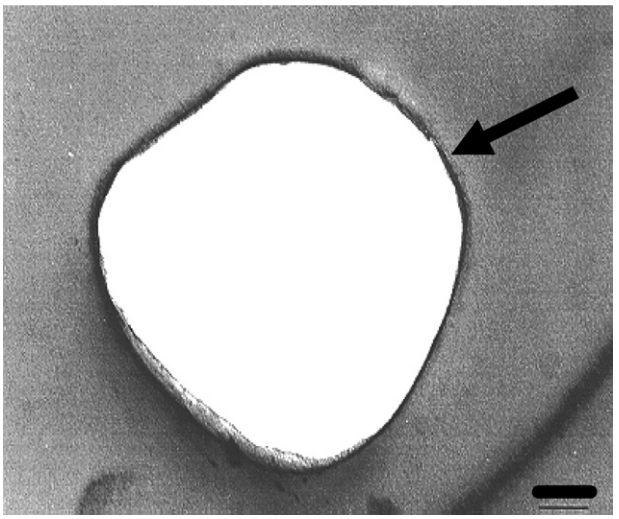

Fig. 5. Typical image from TEM on Sonazoid™ reconstituted product. Stained sample showing void from intact microsphere. Arrow shows electron dense lining surrounding the void. Size bar is 100 nm.

Based on the observations on size, shape, PFB content and lamellarity discussed in the previous sections the physicochemical structure of Sonazoid™ microspheres can be visualised as shown in Fig. 6.

Membrane Surface Charge

To further characterise the membrane surrounding the Sonazoid™ microspheres, their surface charge was measured as a function of pH. Over the pH range of 6 to 8 the zeta potential was relatively stable with results varying from -76 mV to -82 mV. The most important implication of this property is that the high negative charge in this pH range ensures consistent physical stability after reconstitution and at physiological pH due to the electrostatic repulsion between the microspheres.

Acoustic Properties

The acoustic properties of an USCA are dependent on microsphere size as well as on their viscoelastic properties. To provide a basis for choice of parameters for optimal

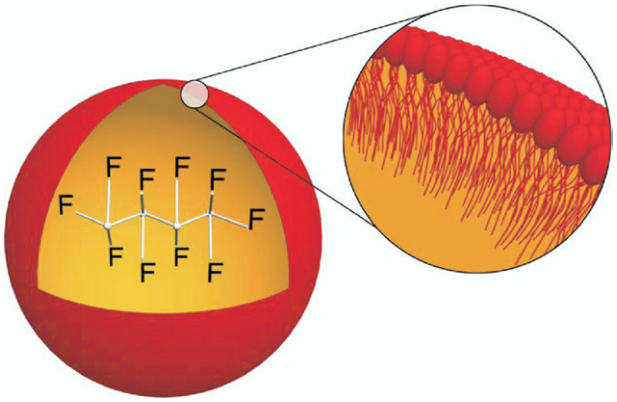

Fig. 6. The physicochemical structure of Sonazoid™ microspheres; PFB stabilised with a 2 to 3 nm thick, monomolecular membrane of HEPS.

control of product efficacy and to indicate ranges for these parameters that ensure an optimal contrast efficacy per unit of active ingredient, a precise knowledge of the relationship between the acoustic properties and microsphere size is essential. However, even with such knowledge and with a tight control of microsphere content and size, potential variation in the acoustic properties of the product may still occur if the mechanical properties of the stabilising membrane structure should vary. To ensure consistent acoustic properties, the attenuation spectrum and the backscatter (fundamental and harmonic) efficacy of Sonazoid™ has been measured on a routine basis throughout the development phase of the product. The covariance between the acoustic properties and the microsphere size distribution of Sonazoid™ has been extensively studied, utilising both theoretical and empirical approaches. In addition, the mechanical properties of the stabilising membrane have been estimated and the consistency of these (batch to batch) has been demonstrated. Results from these investigations have been comprehensively reported previously (Hoff et al. 1996; Hoff and Sontum 1998; Sontum et al. 1999a, 1999b; Hoff et al. 2000, Østensen et al. 2000; Sontum 2006) and will only be briefly reviewed in the following section.

The covariance between microsphere size and the acoustic scattering efficacy is a complex function with distinct maxima dependent on the US frequency. At 5.0 MHz the optimal microsphere size is approximately 3.2 μm. To illustrate the strong size dependency, at 5.0 μm the efficacy is about 40% of optimum and at 2.0 μm it is only approximately 10% of optimum. Hence, to produce a product with consistent acoustical properties a rigorous control with microsphere size is imperative. From a pure efficacy perspective the size of the microspheres in Sonazoid™ should preferably have been slightly larger, especially when low frequency US is used. However, to produce a monodisperse microsphere exactly at the optimal diameter has proven to be impossible. As a tailing of the population toward larger microspheres is inevitable, the size is a trade-off between a sufficient efficacy and a biological acceptable size with regards to potential capillary retention.

Due to the size dependency of the scattering efficacy, the microsphere volume concentration is a better predictor of product efficacy than number concentration. Results for the relative standard error in the slope from linear regressions between three different efficacy parameter reported in Sontum et al. (1999b) and microsphere volume and number concentration are stated in Table 2. As can be seen from these data, the volume concentration is well correlated to product efficacy whereas number concentration is not. The relative error in the linear regressions versus volume concentration is less than 10% which is comparable to the precision of the analytical methods used for determination of the correlated responses. Similar conclusions have been reported for other USCAs (Gorce et al. 2000). Comprising results from 29 (for fundamental backscatter) and 39 (for attenuation efficacy and harmonic backscatter) batches; the consistency of these data also demonstrates that the mechanical properties of the stabilising membrane do not display a significant batch to batch variance. Hence, with the low variance in size and volume concentration displayed by Sonazoid™, the consistency in biological behaviour and clinical efficacy is also assured.

Osmolality, pH and Viscosity

The osmolality of samples from seven batches of Sonazoid™ reconstituted product was measured to 269 ± 11 mOsm/kg whereas the pH was measured to 6.7 ± 0.4. The viscosity of five batches was measured to 1.32

Table 2. The relative standard error in the slope from linear regressions of microsphere number and volume concentration versus acoustic attenuation efficacy (2 MHz), fundamental and two-harmonic backscatter. Data taken from Sontum et al. (1999b)

	Attenuation (2 MHz) vs.		Fundamental backscatter vs.		2-Harmonic backscatter vs.	
	Number conc.	Volume conc.	Number conc.	Volume conc.	Number conc.	Volume conc.
Standard error in slope (%)	33.3	9.8	21.2	8.8	24.5	9.4

mPa s. The product is, hence, isotonic, has a neutral pH and a low viscosity which is comparable with that of an isotonic sucrose solution.

Stability

The product is formulated as a powder for injection and displays good shelf life stability at room temperature. No significant changes in any of the routinely monitored physicochemical parameters have been observed after 36 mo of storage at 25°C (results not shown). Of equal importance to the user is the stability after reconstitution and injection and the product ruggedness toward normal handling. To this point, it should be noted that the reconstituted product segregates by flotation if left nonagitated. If not used immediately after reconstitution it should be re-homogenised by 10 s manual shaking before use. The stability of microsphere content and acoustic attenuation efficacy after reconstitution has been studied. No significant changes in the investigated parameters were observed in any of these systems after 2 h storage at ambient room temperature (results not shown).

Due to their noncompact nature and the limited mechanical strength of the stabilising structure, all USCAs are potentially prone to damage from increased hydrostatic pressures, encountered for example during handling (*i.e.*, mechanical shock or injection pressure) or within the cardiovascular system. For the earlier products, utilizing air, this was a major problem which limited their user friendliness and their stability *in vivo* severely. By utilising the more slowly diffusing and low solubility PFB encapsulated in a flexible phospholipid membrane, problems with microsphere stability are limited. To assure a consistent performance of the product, the pressure stability of Sonazoid™ has been routinely monitored during determination of the attenuation spectrum, *i.e.*, after an approximately 1:5.000 dilution in saline. Figure 7 visualises typical results from such testing; during 120 mm Hg pressure stress a marked decrease and shift to higher frequencies in the attenuation spectrum is observed, as could be expected from an elastic compression of the microspheres. After decompression the recovery of the original attenuation spectrum has been almost complete: 97 ± 2% at 3.5 MHz for the 37 batches investigated. At 300 mm Hg, the effects are similar only with a more pronounced decrease during pressurisation but still with a complete recovery after decompression.

To investigate a situation more relevant to the stress induced during injection, the effect of pressure stress on concentrated product was also studied. Samples of reconstituted product was exposed to pressures up to 300 mm Hg for 60 s with no significant effects on the microsphere content or size distribution (Fig. 8, left) nor

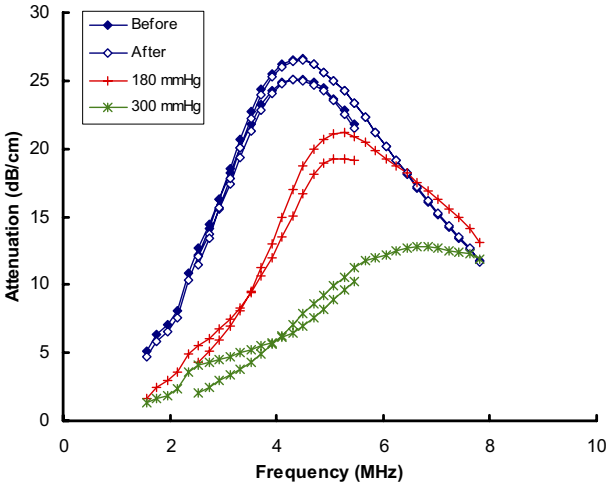

Fig. 7. Effect of increased hydrostatic pressure on the acoustic attenuation spectrum of a diluted (1:5000 in saline) sample of Sonazoid™ reconstituted product. Before, during and after pressurisation at 180/300 mm Hg for 30 s.

on the acoustic attenuation efficacy. During pressurisation, the microspheres are compressed but the full recovery of both acoustic properties and concentration and size distribution after decompression demonstrates the reversibility of this effect and the flexible nature of the microspheres in Sonazoid™. From these observations, it is concluded that pressure stress up to 300 mm Hg is consistently and well tolerated both by concentrated and diluted Sonazoid™. Hence, the product stability toward pressures induced during normal handling, such as injection, or experienced *in vivo* should be consistent and fit for purpose.

Potential effects on microsphere content and size of the filtration through the supplied Codan chemoprotect spike have also been studied. Figure 8 (right) shows the microsphere volume distribution pre and post filtration applying a relatively rapid withdrawal rate of >1.0 ml/s. As can be observed, the filtration procedure has no significant effects on microsphere content and size. The fact that microspheres well above 5 μm passes through the filter unchanged confirm their flexible but yet stable nature as they would have to deform significantly during this process.

SUMMARY

It has been demonstrated that Sonazoid™ powder for injection consists of PFB microspheres, stabilised by a monomolecular membrane of HEPS, embedded in an amorphous, macroporous sucrose structure. Upon reconstitution, stabilised microspheres of PFB are released in a predefined amount and size into a low viscous, isotonic sucrose solution with a neutral pH. Sonazoid™ reconsti-

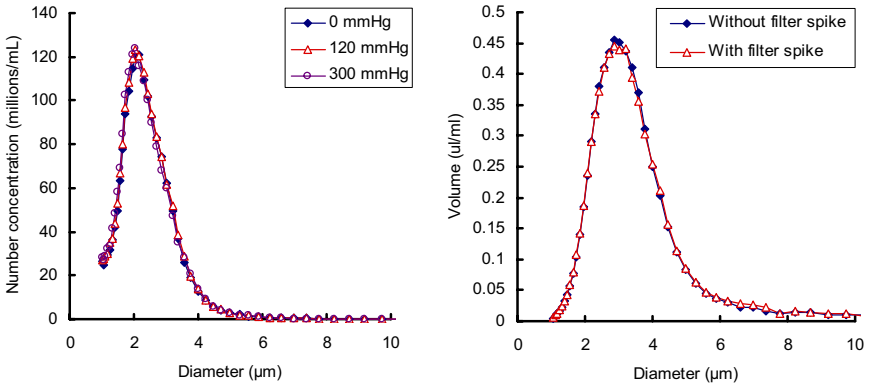

Fig. 8. (Left) effect of increased hydrostatic pressure on the microsphere content and size distribution of Sonazoid™ reconstituted product. Samples have been pressurised for 60 s at stated pressure before release and re-analysis. (Right) Effect of filtering of Sonazoid™ through the supplied Codan Chemoprotect® Spike on the microsphere content and size distribution.

tuted product contains approximately 8 μl microspheres/ml with volume median diameter of approximately 2.6 μm. The product contains approximately 1.2 billion microspheres/ml of which less than 0.1% are larger than 7 μm.

The good agreement between microsphere volume concentration and the amount of PFB in the liquid phase demonstrates that the microspheres are filled with PFB at approximately 1 atmosphere.

The acoustic properties of Sonazoid™ such as attenuation efficacy, fundamental backscatter efficacy and harmonic backscatter efficacy, are all well correlated to the microsphere volume concentration.

The stability of Sonazoid™ after reconstitution is good, with no significant changes in physicochemical parameters 2 h after reconstitution. Pressure stress is well tolerated by both concentrated and diluted Sonazoid™ with no permanent effects of pressures up to 300 mm Hg.

The level and consistency of the investigated physicochemical properties demonstrate that Sonazoid™ should be well suited as a contrast agent for medical imaging with ultrasound.

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