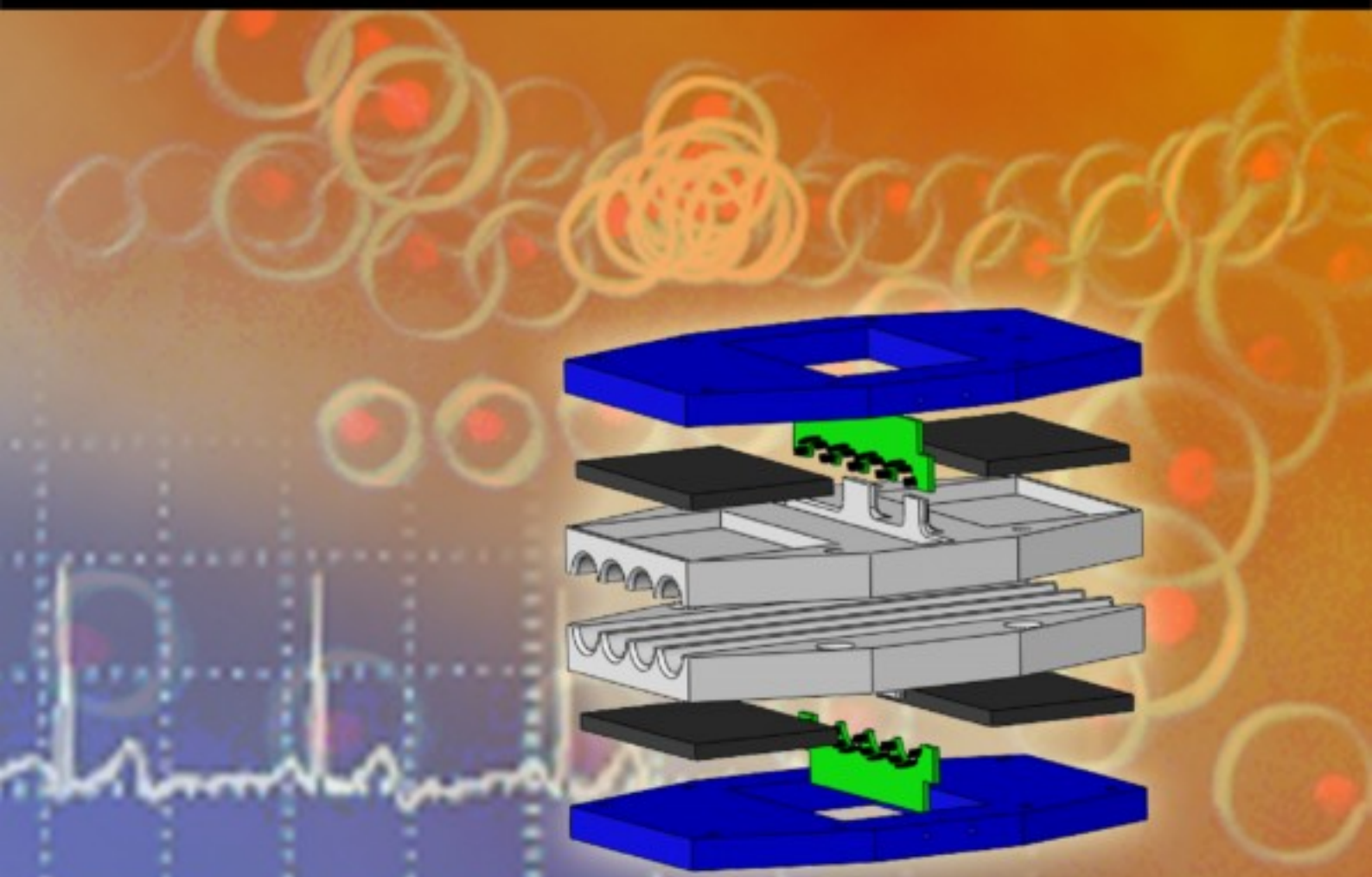


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12

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Contents

Volume 9
Special Issue
December 2010

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ISSN 1726-5479

Research Articles

Modern Sensing Technologies - III

Subhas Chandra Mukhopadhyay, Aime Lay-Ekuakille, Anton Fuchs 1

Composition and Detection Rate of a Symmetry Axis Localization Algorithm for Digital Images

Norbert Eidenberger, Daniel C. H. Schleicher and Bernhard G. Zagar 1

Formulation and Characterization of Cu Doped ZnO Thick Films as LPG Gas Sensor

A. V. Patil, C. G. Dighavkar, S. K. Sonawane, S. J. Patil and R. Y. Borse 11

Characterization of Microbubble Contrast Agents for Echographic Imaging through Time-Scheduled Size Distribution Measurements

Francesco Conversano, Roberto Franchini and Sergio Casciaro 21

Production and Characterisation of Multifunctional Textile for Masonry Retrofitting and Health Monitoring

Angela Coricciati, Paolo Corvaglia, Alessandro Largo, Michele Arturo Caponero, Giovanni Fardin 28

Al-doped TiO₂ Thick Film Resistors as H₂S Gas Sensor

Chandrakant Dighavkar, Arun Patil, Sunil Patil and Ratan Borse 39

Ultrasound Signal Analysis Applied to Determine the Optimal Contrast Dose for Echographic Examinations

Roberto Franchini, Francesco Conversano, Antonio Greco, Raffaella Verrienti, Sergio Casciaro 48

Extended Phase Accordance Method: A Real-time and Accurate Technique for Estimating Position and Velocity of Moving Objects using Ultrasonic Communication

Tomohiko Sato, Shigeki Nakamura, Masanori Sugimoto and Hiromichi Hashizume 56

Magneto-inductive Sensors for Metallic Ropes in Lift Application

Aldo Canova, Francesco Ficili and Daniel Rossi 71

Studies on Gas Sensing Performance of Pure and Surface Chrominated Indium Oxide Thick Film Resistors

D. N. Chavan, V. B. Gaikwad, S. D. Shinde, D. D. Kajale, G. E. Patil, G. H. Jain 82

Effect of Annealing Temperature on Gas Sensing Performance of SnO₂ Thin Films Prepared by Spray Pyrolysis

G. E. Patil, D. D. Kajale, S. D. Shinde, R. H. Bari, D. N. Chavan, V. B. Gaikwad, G. H. Jain 96

Measurement Using Conductive Polymeric Fibers in a Wearable Sensor Platform

Ram Manoj Sarda, Thomas Donnelly, Mansour Taherinahzahdi and Michael Haji-Sheikh 109

Three Dimensional Measurement of Aquatic Organisms Using a Single Video Camera

Kikuhito Kawasue, Satoshi Nagatomo and Yuichiro Oya 118

127

Pain Sensing System for Animals <i>Ibrahim Al-Bahady, Subhas Mukhopadhyay and Khalil Alkhumaisi</i>	
Experimental Assessment of a Pneumatic Level-sensing Method for Closed Tanks Applied to Water and Wooden Pellets <i>Gert Holler, Rudolf Brunnader, Bernhard Schweighofer, Hannes Wegleiter</i>	151
Synthesis and Characterization of Nanostructured ZnO Thick Film Gas Sensors Prepared by Screen Printing Method <i>R. Y. Borse and V. T. Salunke</i>	161
Sensitivity Limits of a Magnetometer with an Air-core Pickup Coil <i>Kunihisa Tashiro, Shin-ichiro Inoue and Hiroyuki Wakiwaka</i>	171
A Survey on Unobtrusive Measurements of the Cardiovascular Function and their Practical Implementation in Wheelchairs <i>Eduardo Pinheiro, Octavian Postolache, Pedro Girão</i>	182
Multi-Sensor SLAM Approach for Robot Navigation <i>Sid Ahmed Berrabah, Yvan Baudoin, Hichem Sahli</i>	200
ZigBee Test Harness: An Innovative Tool for ZigBee Node Testing <i>Andrea Ranalli, Claudio Borean</i>	214
Effect of Firing Temperature on the Composition and Structural Parameters of Screen Printed ZrO₂ Thick Film Sensors <i>S. J. Patil, A. V. Patil, C. G. Dighavkar, R. Y. Borse</i>	223
Wide-band Induction Magnetometers Stability <i>Vira Pronenko and Yevhen Vasiliev</i>	233
Experimental Performance Measurements of Home Photovoltaic Plants: A Case Study <i>C. Calò, C. Chiffi, G. D'Aniello, A. Lay-Ekuakille, P. Vergallo, A. Trotta</i>	240


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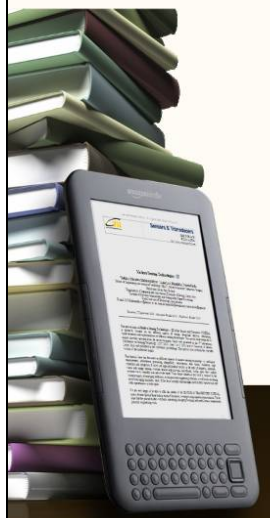
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- Complex Systems



Characterization of Microbubble Contrast Agents for Echographic Imaging through Time-Scheduled Size Distribution Measurements

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Abstract: Aim of this work was to study the dissolution behaviour of the phospholipid-shelled perfluorobutane microbubbles of an experimental contrast agent for echographic imaging through an innovative methodology based on time-scheduled size distribution measurements. Two different contrast handling procedures were employed and temporal evolution of corresponding microbubble populations was monitored for several hours. Dissolution behaviour of shelled microbubbles resulted to be qualitatively analogous to that theoretically predicted for unshelled perfluorobutane bubbles, with a much longer lifetime due to the shell effect. In particular, mean microbubble diameter, initially around 2 μm , first increased to more than 2.6 μm and then gradually reduced to less than 1.7 μm , with corresponding variations of the effectively employable ultrasound frequencies for imaging purposes. We also demonstrated that excess lipid material is diffused as submicron particle shedding. Finally, we discussed the implications of these results for diagnostic and therapeutic applications involving the studied microbubbles. *Copyright © 2010 IFSA.*

Keywords: Ultrasound contrast agents, Microbubble sizing, Microbubble counting, Microbubble dissolution, Echographic imaging.

1. Introduction

Currently available ultrasound (US) contrast agents consist of encapsulated gas-filled microbubbles, generally between 1 and 10 μm in diameter. When located in an US field, microbubbles oscillate

because of the compressibility of their gas core. This oscillating behavior is the primary source of the high scattering strength of these agents, which makes them well suited to provide contrast enhancement in diagnostic US imaging [1].

Typical echographic scanning protocols make use of US frequencies in the range 2-10 MHz, both in presence and in absence of contrast agent employment. In fact, for most of applications, the best suitable US frequency falls in the mentioned interval as a consequence of the compromise between image resolution (which is improved using higher frequencies) and penetration depth requirements (attenuation of the US beam increases with the square of the adopted frequency, so limiting the maximum frequency employable to image deep organs).

Numerous recent studies [2-7] have also emphasized an increasing interest in the use of US contrast agents at higher frequencies, for applications in superficial tissue and transesophageal imaging, interventional procedure guidance, ophthalmology and in combination with intravascular ultrasound (IVUS) systems. Therefore, in the last years, several techniques (e.g. decantation, mechanical filtration) have been employed to modify in a predictable way the mean diameter of microbubble populations, in order to shift bubble resonance in the desired frequency range, so maximizing the corresponding contrast effectiveness [2, 3, 8, 9]. To date very little work has been focused to a quantitative evaluation of the spontaneous dissolution behavior of contrast microbubbles, which is due to gas diffusion phenomena in turn influenced by shell dynamics.

In this paper we present a phenomenological study of such a behaviour for an experimental US contrast agent (BR14, Bracco Research SA, Geneva, Switzerland), consisting of phospholipid-shelled perfluorobutane microbubbles. Two different ways of preparing contrast solutions were compared and the temporal evolution of microbubble size distribution was monitored for several hours by direct measurements, performed through a MultisizerTM III Coulter Counter[®] (Beckman Coulter, Fullerton, CA, USA).

2. Materials and Methods

2.1. Contrast Agent Handling

BR14 vials supplied by Bracco Research SA were resuspended, as indicated by the manufacturer, by injecting 5 mL of sterile saline and vigorously shaking the vial for 30 seconds. The solutions that underwent the subsequent measurements were then prepared in two different ways, in order to compare the effects of two different contrast agent handling procedures, as described in the next paragraphs.

Withdrawal of a constant volume from a continuously stirred beaker. In the first case, we aimed to monitor the temporal evolution of a microbubble population at a given volume dose. Native BR14 was diluted 1:20000 in a continuously stirred 500-mL beaker. Starting 30 s after the injection of BR14 into the beaker, a 20-mL sample of contrast solution was withdrawn every 20-25 minutes and underwent size distribution measurements (Fig. 1). Employed volumes and adopted time scheduling had been chosen in such a way that microbubble floatation effects could be neglected, so the obtained diameter trends were attributable to gas diffusion phenomena.

Withdrawal of a constant bubble number from the vial. In this second case, we did not operate at constant volume dose, but at constant microbubble number. Every 20-25 minutes from BR14 vial reconstitution a new sample solution was prepared through a direct withdrawal from the vial itself (Fig. 2) followed by a two-step dilution, in which the second step was each time adjusted to assure, by means of a quick combined MultisizerTM analysis, the presence of $(1\pm 0.2)\cdot 10^4$ microbubbles/mL in

each solution undergoing the subsequent size distribution measurements. This means that we assumed a number concentration of 10^4 microbubbles/mL to be both statistically significant to monitor the evolution of microbubble population inside the vial and sufficiently diluted for effective Multisizer™ analyses.



Fig. 1. Sample solution withdrawal from a continuously stirred beaker.



Fig. 2. Sample solution withdrawal from contrast agent vial.

2.2. Size Distribution Measurements

Both the described handling techniques were separately employed on different BR14 vials and microbubble population evolution was monitored for several hours. Each considered sample solution, just after preparation, underwent triplicate MultisizerTM analyses whose average resulting distribution was corrected by subtracting the corresponding background particles, resulting after microbubble destruction through a 10-s immersion in a bath sonicator. The whole analysis of each sample took about 20-25 minutes.

Granulometric analyses performed by MultisizerTM exploit the “Coulter Principle”, also known as ESZ (Electrical Sensing Zone) Method [10], which is briefly summarized in the next paragraph.

The Coulter principle. Particles suspended in a weak electrolyte solution are drawn through a small aperture, separating two electrodes between which an electric current flows. The voltage applied across the aperture creates a “sensing zone”. As particles pass through the aperture (or “sensing zone”), they displace their own volume of electrolyte, momentarily increasing the impedance of the aperture and so producing a pulse. The Coulter Principle states that the pulse is directly proportional to the tri-dimensional volume of the particle that produced it. Analyzing these pulses enables a size distribution to be acquired and displayed. In addition, a metering device is used to draw a known volume of the particle suspension through the aperture and a count of the number of pulses then yields the concentration of particles in the sample.

3. Results and Discussion

3.1. Microbubble Dissolution Behaviour

Fig. 3 shows the obtained diameter-time curves for both the employed contrast handling procedures. We can note that the two observed diameter trends are qualitatively similar, with an initial transient diameter growth due to the ingression of air into the bubbles and a subsequent slower diffusion out of perfluorobutane. In this context, both the presence of the shell and the absence of insonification act as slowing factors for migrations of gas molecules, with respect to theoretical predictions for unshelled perfluorobutane bubbles [11] and to our previous results for insonified BR14 microbubbles [12]. On the other hand, the employment of magnetic stirring seems able to speed up the diffusion kinetics of the involved gases. These overall trends indicate anyway that, also in the case of phospholipid-encapsulated perfluorobutane microbubbles, temporal variations in microbubble diameter take place due to spontaneous gas diffusion phenomena.

Obtained results on temporal evolution of BR14 microbubble population are emphasized also in Fig. 4, showing the measured size distributions at some significant time instants when employing the second handling procedure.

Our data suggest that a reduction in mean diameter, with the corresponding increase in resonance frequency, can be achieved by simply waiting for a certain time interval and, in case, adding a mechanical stirring of the contrast solution in order to speed up the involved processes.

Moreover, we demonstrated that BR14 microbubble population spontaneously reaches the maximum value of mean diameter about 50-60 minutes after agent reconstitution and then diameter slowly decreases back, reaching again its initial value within the next 2-3 hours and becoming even smaller for longer time intervals. Therefore, since diameter variation rate is definitely higher in the increasing phase than in the decreasing, contrast agent employment could be improved by using the agent during

the decreasing diameter phase: in this way the clinician will be simply asked to choose the proper waiting time to be sure of injecting a contrast agent with the desired mean diameter, in turn determined by the ultrasound frequency to be adopted.

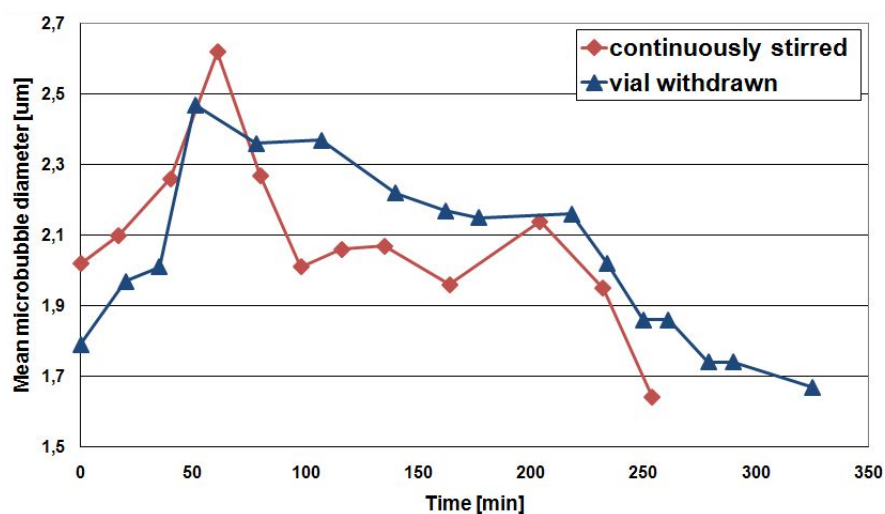


Fig. 3. Diameter-time curves obtained for the two employed contrast handling procedures.

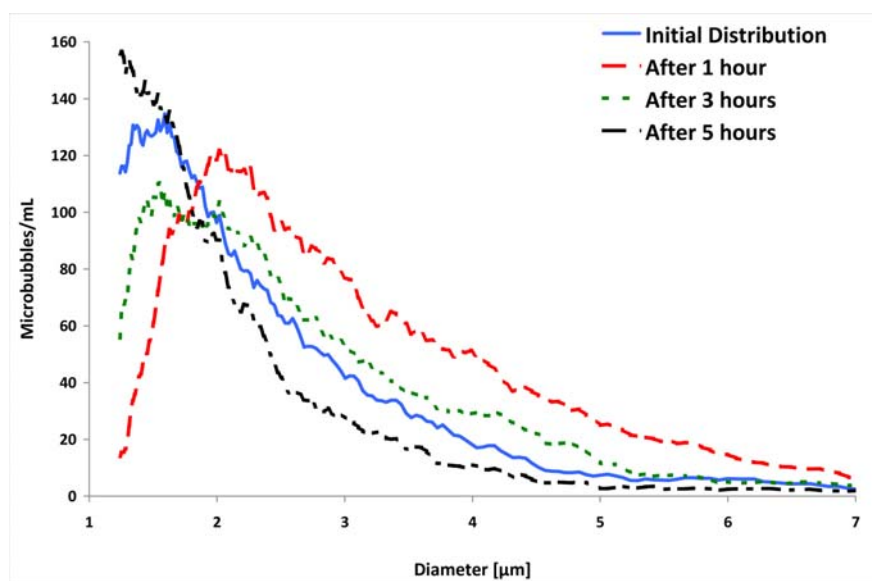


Fig. 4. Temporal evolution of microbubble size distribution for suspensions with constant microbubble number.

3.2. Shell Deterioration Mechanisms

Since the irreversible diameter decreasing phase must be accompanied by an ejection of excess lipid material from the monolayer shell, we compared obtained results on BR14 spontaneous dissolution with the main literature-available models for microbubble lipid shedding [13], which are schematically illustrated in Fig. 5: a) microbubbles shed excess material as diffuse submicron particles; b) microbubbles form micron-sized buds that rapidly shed; c) microbubbles exhibit buds that grow and detach less readily, significantly increasing the bubble surface area; d) microbubbles exhibit buds and strings that build up on the original shell.

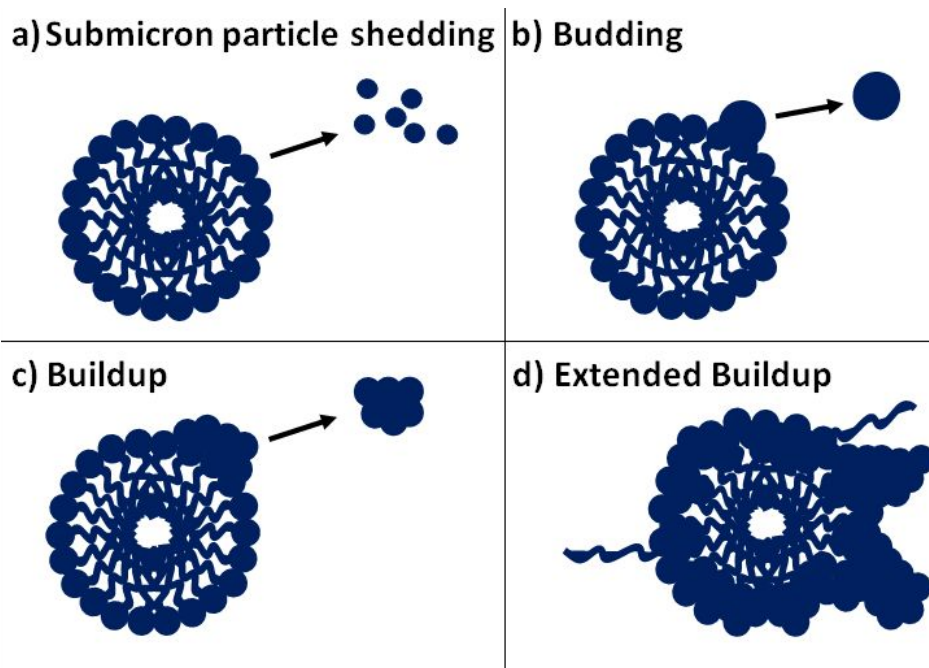


Fig. 5. Schematic representation of possible mechanisms of excess lipid shedding by microbubbles during phospholipidic shell deterioration (see the text for details). (Adapted from [13]).

The significant diameter variations we observed suggest that an extended buildup on the microbubble shell (Fig. 5d) is not the case of BR14; moreover, in presence of either budding (Fig. 5b) or limited buildup (Fig. 5c), when considering the diameter decreasing phase, we should have found somewhere a variation in the shape of background particle distribution and/or an increase in microbubble number, but this was not the case in turn. Hence, we hypothesized that excess lipid material is shed by BR14 microbubbles as diffuse submicron particles (Fig. 5a).

This shedding mechanism, in particular, is characteristic of shells constituted by phospholipids with relatively short alkyl chains, which could be efficiently used also for specific drug delivery purposes where a rapid release throughout the target volume is required.

4. Conclusions

We have investigated the dissolution behaviour of BR14 microbubbles through time-scheduled size distribution measurements performed on saline-diluted contrast solutions prepared with two different approaches, showing that in both cases temporal diameter variations take place due to spontaneous gas diffusion phenomena combined with a specific shell deterioration mode.

Our findings suggest the introduction of two novel contrast handling techniques: first, the choice of an appropriate waiting time between vial reconstitution and contrast injection to obtain microbubble populations with a mean diameter higher than the initial one, and so more effective at lower frequencies; second, the employment of mechanically stirred contrast solutions, in order to speed up the gas diffusion phenomena and obtain a microbubble population with a reduced mean diameter, more useful for innovative applications at higher frequencies.

Finally, by comparing our experimental results with available models for microbubble lipid shedding, we were able to hypothesize that BR14 could be efficiently used also for drug delivery purposes, especially for applications requiring a rapid release throughout the target volume.

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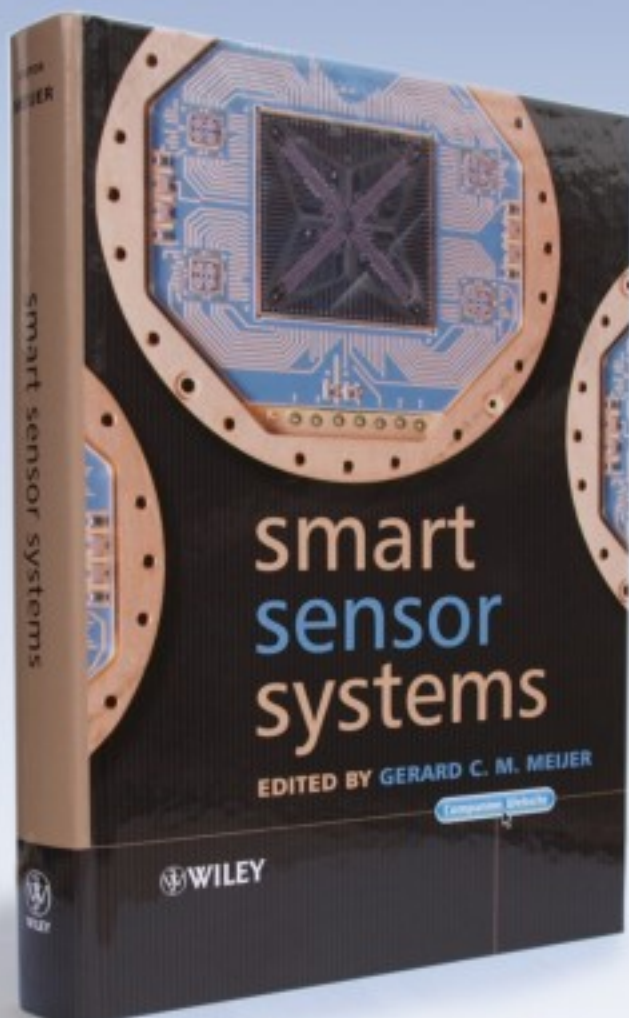
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