NOTE

Physical Characteristics of Ultrastable Lipid-Coated Microbubbles

Surfactant-stabilized microbubbles can be rapidly and easily produced in high concentrations within the narrow size range of 1–5 μm. Such synthetic microbubbles, which find use in medical and other applications, persist in various aqueous media for periods up to one year. Microbubble size distribution analyses at different salt concentrations and after the addition of thiourea clearly indicate that electrostatic interactions are much less important than hydrogen bonding in stabilizing the lipid monolayer surrounding each microbubble. Direct optical confirmation of the typical (1–3 μm) microbubble size and lack of bubble agglomeration was provided by phase-measurement interferometric microscopy data. In addition, freeze-fracture electron microscopy data further indicated a high degree of size uniformity for the lipid-coated microbubbles.

INTRODUCTION

Surfactant-coated microbubbles, technically referred to as stable gas-in-liquid emulsions (1), have various applications within both medicine and industry (2). For example, industrial applications currently under development include production of ultrauniform insulating polymer foams and structural (i.e., load-bearing) foams, water reconditioning via microflotation, and control of hydrodynamic and/or acoustic cavitation. Since this gas-in-liquid emulsion technology (i.e., microbubble technology) can be utilized in oil-based as well as aqueous media (2), a more immediate industrial application has been replacement of the glass microballoons, by much less costly surfactant-stabilized microbubbles, in emulsion-type slurry explosives commonly used in mining and construction activities (2, 3).

Most studied are several medical applications of this gas emulsion technology (1, 4). To date, the vast majority of the clinical research with gas microbubbles by other investigators has centered upon the cardiovascular system, particularly the heart (see Refs. (1) and (2) for summaries). However, another important class of medical applications has been the successful use of intravenously injected, lipid-coated microbubbles for earlier ultrasonographic identification and localization of tumors in the brain and other organs (5–8).

In order to further expand such applications development work concerning the gas emulsion technology, data collection on the general physicochemical properties of the ultrastable lipid-coated microbubbles as well as direct microscopy of individual coated microbubbles were undertaken in the present study.

MATERIALS AND METHODS

Microbubble Preparation

Lipid-coated microbubbles were prepared (2) by admixing 20 mg of a powdered mixture of selected lipid surfactants with 100 ml of standard, isotonic NaCl solution (0.9% w/v in water) or a given test solution. The nonionic surfactant mixture used contains a total of 5 low molecular weight lipids, comprising glycerides, cholesterol, and cholesterol esters (4). The total surfactant concentration is well above the critical micelle concentration needed to form any mixed micelles, but is below the point of significant phase separation. The resultant saturated solution was shaken vigorously for 10 s in air (at room temperature) forming high concentrations of stable gas microbubbles. Subsequently, centrifugation or filtration was used to remove any solid, supracolloidal particulate matter, yielding a final total lipid concentration of approximately 0.25 mg per ml in the lipid-coated microbubble suspension (Filmix™, CAV-CON Inc., Farmington, CT).

Size Distribution Analyses

The average diameter and size distribution of the lipid-coated microbubble population were determined in all test solutions at 20°C by electroimpedance-sensed volumetric sizing using a Coulter Multisizer (Coulter Electronics, Inc., Hialeah, FL), in conjunction with the data handling software provided by the manufacturer.

Phase-Measurement Interferometric Microscopy

A ZYGO (Middlefield, CT) Maxim 3D laser interferometric microscope, or phase-measurement interferometric microscope (PMIM), was used to image the lipid-coated microbubbles (residing in a thin aqueous film). Topographical features of a given test surface are measured by the microscope using optical phase-measurement interferometry. PMIM measures the phase, ϕ, of monochromatic, coherent light with wavelength λ reflected from the test substrate relative to light reflected from an optical reference surface. Differences in phase, $\Delta\phi$, between the two locations on the surface are proportional to the optical height, h, between these two locations:

$$\Delta\phi = 4\pi h/\lambda. \quad [1]$$

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An optical image of the surface can thus be constructed by measuring the phase as a function of coordinates in a plane. For surfaces with uniform optical properties, the optical image obtained (in air) is equivalent to the substrate topography (9).

Freeze-Fracture Electron Microscopy

Freeze-fracture replica films were prepared with a Balzers BAF 400 device. After mixing a microbubble solution with glycerol (3:1 water:glycerol by volume) and standing 30 min, an aliquot of the solution was vitrified in liquid Freon cooled by liquid N_2. Under reduced pressure, the vitrified specimen was fractured at −130°C; the fractured surface was shadowed by the evaporation of platinum metal at an angle of 45° and then deposited with carbon at 90°. The replica film was washed using a sodium hypochloride solution and water.

Transmission electron microscopic observation was carried out at room temperature on a JEOL 1200EX apparatus operating at 100 kV with direct magnifications of ×12,000–×30,000.

RESULTS

The particle size analysis of the lipid-coated microbubbles in standard solution (isotonic saline) routinely yields the following results: Mean microbubble diameter was approximately 2 μm, over 95% of the population was under 4.0 μm, and 100% of the microbubbles were under 6 μm in diameter. The above microbubble size distribution limits were also observed in the glycerol solutions (i.e., glycerol: isotonic saline ratio of 1:2 or 1:3) used to prepare the coated microbubbles for freeze-fracture electron microscopy (see below). In addition, one-by-one nonoptical counting of particles by the Coulter Multisizer consistently results in a calculated total concentration of 540,000 ± 15% microbubbles/ml in standard, isotonic saline. Consistent with earlier observations (5), this concentration range for the standard microbubble suspension and the above microbubble size specifications remain constant at room temperature for more than 6 months.

In Fig. 1, each set (a–c) of histograms represents three repeat particle size distribution assays of 0.05-ml samples of a given test solution containing the maximum concentration of coated microbubbles. It is apparent that a considerable change in ionic strength had little effect on the size distribution and concentration of the stable (1-day-old) lipid-coated microbubbles. Specifically, comparison of Fig. 1(a) with Fig. 1(b) demonstrates that when the NaCl concentration of the medium was increased from 0.15 M (isotonic) to 1.0 M, the result was a minor increase in microbubble mean diameter (increased 12.9%, i.e., 1.905 to 2.150 μm) and somewhat decreased average concentration (decreased 14.4%, i.e., 521,420 to 446,280 microbubbles/ml).

However, the presence of 1 M thiourea was observed to have a profound effect on both the particle size distribution and concentration of the synthetic microbubble suspension. Figure 1(c) displays the effect of adding 7.6% w/w (1 M) thiourea to a microbubble suspension in isotonic NaCl (two hours old). Comparison of Fig. 1(a) with Fig. 1(c) shows that the (neutral) thiourea results in a large increase in microbubble mean diameter (increased 134%, i.e., 1.905 to 4.466 μm) and a similarly drastic decrease in average concentration (decreased 89.4%, i.e., 521,420 to 55,320 microbubbles/ml).

As concerns typical microbubble sizes in standard isotonic saline, direct confirmation was obtained by phase-measurement interferometric microscopy. Figure 2 displays computer-generated pictures from direct imaging of individual lipid-coated microbubbles, residing in a thin aqueous film. In the figure, individual microbubbles can be seen widely spaced from each other (lower right panel).

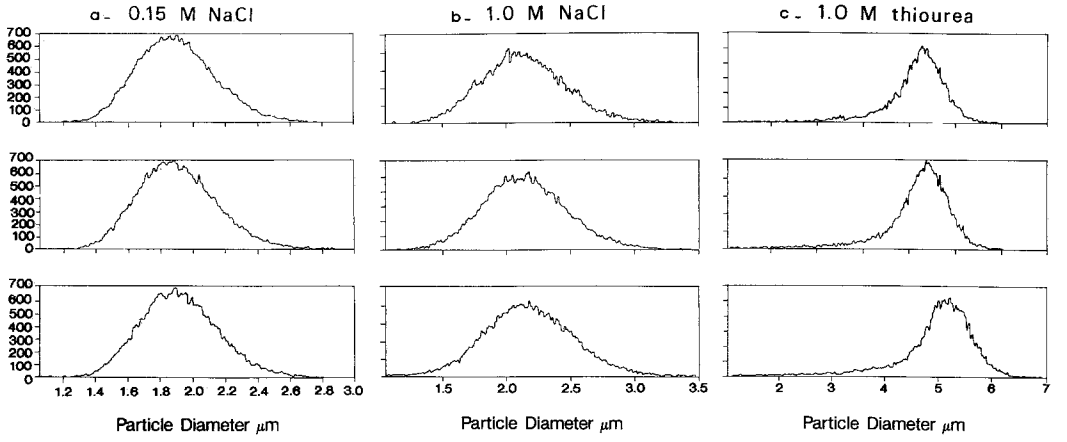

FIG. 1. Size distributions of lipid-coated microbubbles in: (a) 0.15 M (isotonic) NaCl; (b) 1.0 M NaCl; and (c) 7.6% w/w (1 M) thiourea in isotonic NaCl.

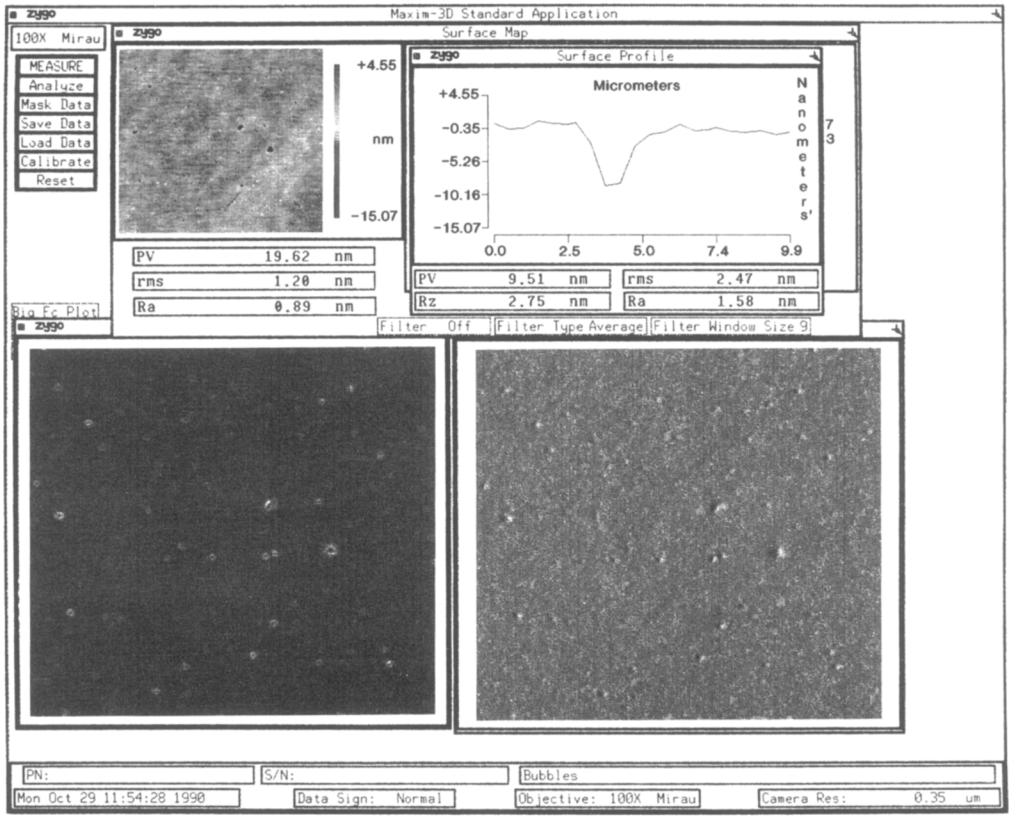

FIG. 2. Direct optical imaging of lipid-coated microbubbles by phase-measurement interferometric microscopy (see text).

An "edge-enhanced" version of the same micrograph further emphasizes the bubble outlines and their apparently random spacing (lower left panel), while the graph displays the surface profile of a typical microbubble yielding a diameter of approximately 2 μm.

Direct microscopical evidence of the uniformity of microbubble sizes was obtained in greater detail by also using freeze-fracture electron microscopy. The circular or spherical-looking microstructures were observed to have highly uniform diameters.

DISCUSSION

Judging from the fact an increase in salt concentration from 0.15 *M* (isotonic) to 1.0 *M* NaCl had little effect on the size distribution and concentration of the lipid-coated microbubbles (Figs. 1(a),(b)), it appears clear that electrostatic interactions play only a very minor role in the intermolecular bonding helping to stabilize the surfactant monolayer surrounding each microbubble. This finding parallels the physicochemical stabilization mechanism operating within naturally occurring surfactant-stabilized microbubbles found in various surface waters; specifically, the surfactants in the microbubble's monolayer coating are reversibly held together by hydrogen bonding and nonpolar interactions (10, 11).

The suspected major role of hydrogen bonding within also the artificial monolayer coating of the synthetic microbubbles, examined in this study, was clearly supported by the striking effects of 1 *M* thiourea on the synthetic microbubble suspension (Fig. 1(c)). At room temperature, molar or higher concentrations of (neutral) thiourea are known to very effectively disrupt hydrogen bonding within complex molecules and supramolecular structures (12). From the very marked changes encountered in the size distribution and maximum concentration of synthetic microbubbles upon addition of 7.6% w/w (1 *M*) thiourea to the microbubble suspension (Fig. 1(c)), it appeared evident that the reagent successfully broke (accessible) hydrogen bonds within the lipid monolayer surrounding and stabilizing the synthetic microbubbles.

It is also useful to note that both the increase in salt concentration and addition of thiourea tended, although to very different extents, to increase the diameter of the

lipid-coated microbubbles while reducing their maximum concentration. These observed trends in the physicochemical data clearly argue *against* the particle size distributions representing *flocs* of ultrasmall microbubbles held together either by electrostatic interactions (refuted by the 0.15 and 1.0 *M* NaCl data) or even hydrogen bonding (refuted by the 1.0 *M* thiourea data).

In addition, direct confirmation of the typical (1–3 μm) microbubble size and lack of bubble agglomeration was provided by the phase-measurement interferometric microscopy data (Fig. 2). The surface profile of a representative microbubble (Fig. 2, graph) revealed a smooth structure of approximately 2 μm in diameter, while individual microbubbles generally appeared widely and randomly spaced. These optical imaging findings contraindicate any appreciable microbubble agglomeration or fusion taking place. At the same time, these findings are also in agreement with the freeze-fracture electron microscopy data, which revealed a high degree of size uniformity for the lipid-coated microbubbles.

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