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Chemistry

Air-filled proteinaceous microbubbles: Synthesis of an echo-contrast agent

(ultrasound/sonochemistry/microencapsulation/sonography/echocardiography)

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ABSTRACT **Air-filled microbubbles are in clinical use as echo-contrast agents for sonographic applications. The synthesis of aqueous suspensions of air-filled proteinaceous microbubbles involves the ultrasonic irradiation of aqueous protein solutions in the presence of O_2. Yields and size distributions of human and bovine serum albumin microbubbles have been determined as a function of various experimental parameters. The chemical nature of these microbubbles and the origin of their remarkably long lifetimes have been explored. The microbubbles are held together primarily by interprotein cross-linking of cysteine residues. The principal cross-linking agent is superoxide created by the extremely high temperatures produced during acoustic cavitation.**

Two-dimensional contrast echocardiography has become a valuable tool in diagnosing cardiac disease (1–3) and monitoring myocardial perfusion (4–6). This technique uses the reflection of ultrasound to image heart tissue *in vivo*. To enhance image quality, a solution containing microbubbles may be injected intravenously to perfuse the cardiovascular system; these microbubbles change the acoustic impedance of the blood flow, resulting in dramatically improved echo contrast with the surrounding tissues (7, 8). To permit unimpeded motion through the circulatory system, the optimum diameter for such microbubbles is <10 μm. Gas-filled bubbles this small, however, are usually very short-lived because of rapid gas dissolution. Air-filled microbubbles have been formed in various aqueous solutions [including those of H_2O_2, meglumine sodium diatrizoate (Renografin-76), 50% dextrose, 70% sorbitol, and SHU-454]. These systems suffer from short storage life, low microbubble stability, or high toxicity (9–13).

Recently, the synthesis of long-lived air-filled microbubbles was reported (14–18) from the ultrasonic irradiation of human serum albumin (HSA). HSA microbubbles are nontoxic, have optimal size, and have been used successfully in transpulmonary contrast echocardiography (19–21). These proteinaceous microbubbles are under commercial development and have U.S. Food and Drug Administration approval for clinical trials (22). The chemical nature of these microbubbles, however, has not been previously explored. We describe here the chemical mechanism by which high-intensity ultrasound creates air-filled proteinaceous microbubbles from aqueous protein solutions.

MATERIALS AND METHODS

Bubble size distributions in aqueous solution were analyzed with a particle counter (Elzone 180XY). Light microscopy confirmed the particle size distribution. Bovine catalase, bovine superoxide dismutase, sperm whale myoglobin, human hemoglobin (Hb), and fraction V powders of HSA and bovine serum albumin (BSA) were purchased from Sigma and used without further purification. Proteinaceous microbubbles were synthesized from BSA and HSA using procedures described elsewhere (14–18), resulting in concentrations as high as $\approx 3 \times 10^9$ microbubbles per ml. Microbubbles formed from BSA and HSA have similar size distributions. Solutions were irradiated with a high-intensity ultrasound horn (Heat Systems W375, 20 kHz, 1.27-cm Ti horn) for 3 min at an acoustic power of $\approx$200 W/cm^2, at pH 7.0 with an initial temperature of 50°C.

RESULTS AND DISCUSSION

Ultrasonic irradiation of solutions can produce emulsification (23). Ultrasonic emulsification creates the microscopic dispersion of gas into the protein solution to form the proteinaceous microbubbles. Alone, however, emulsification is insufficient: emulsions produced by vortex mixing instead of ultrasonic irradiation produce no long-lived microbubbles, as shown in Fig. 1.

Ultrasonic irradiation of liquids also can produce cavitation: the formation, growth, and implosive collapse of bubbles (24). The compression of such bubbles creates transient hot spots with enormous peak temperatures (24, 25). One might propose that thermal denaturation of the protein from this localized heating is responsible for microbubble formation. If so, then the effects of dissolved gases on cavitation should increase microbubble yields for Ar versus O_2 or N_2 (because of decreased heat capacity ratios, C_p/C_v), whereas the latter two gases would be equivalent. Experimentally, however, high concentrations of microbubbles are synthesized only when the reaction is run under O_2 or air. Sparging the solution with Ar or N_2 before ultrasonic irradiation substantially diminished the formation of microbubbles (Fig. 1). Thus, proteinaceous microbubbles are not formed from heat denaturation of the protein but instead result from specific chemical reactions involving O_2.

Acoustic cavitation in water generates H· and OH·, which have been detected by ESR spin-trapping experiments (26). These radicals can react further to produce H_2O_2 or (in the presence of O_2) HO_2 (27–29). Thus, one might hypothesize that OH·, H_2O_2, or HO_2 could be oxidizing the protein during formation of the proteinaceous microbubbles. To determine the nature of such sonochemical reactions in the synthesis of microbubbles, we examined the effects of various chemical traps. The addition of nonspecific radical traps, such as glutathione or 2,6-di-*tert*-butyl-4-methylphenol, completely inhibits the synthesis of microbubbles (Fig. 2). To determine specifically which oxidant is important, separate reactions were run in the presence of catalase [which rapidly decomposes H_2O_2 to water and oxygen (30)] or in the presence of

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Abbreviations: HSA, human serum albumin; BSA, bovine serum albumin.

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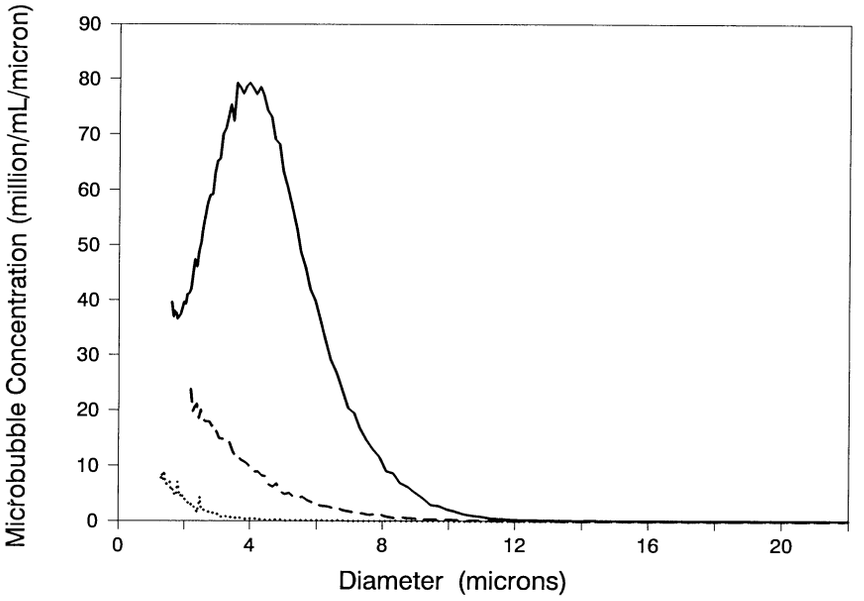

FIG. 1. Particle size distribution analysis of aqueous suspensions of proteinaceous microbubbles. ——, Aqueous solution of 5% (wt/vol) BSA irradiated with ultrasound under air; - - -, aqueous solution of 5% (wt/vol) BSA irradiated with ultrasound under Ar; ·····, aqueous solution of 5% (wt/vol) BSA vortex agitated under air.

superoxide dismutase [which rapidly decomposes superoxide to H_2O_2 and water (31)]. Microbubble formation is inhibited by superoxide dismutase but *not* by catalase. In fact, the presence of catalase increases the yield of microbubbles, presumably by decreasing protein destruction from sonochemically produced peroxide. Fig. 2 shows the inhibition of microbubble formation by superoxide dismutase. Thus, we propose that superoxide is the principal oxidant responsible for microbubble formation.

Superoxide is known to oxidize free cysteine (32). We find that air-filled proteinaceous microbubbles can be formed only from proteins that have cysteine residues. For example, BSA, HSA, and Hb (all of which have cysteine residues) form microbubbles, whereas myoglobin (which has no cysteine residues) does not (33–35). If the cysteine residues are alkylated with *N*-ethylmaleimide (36, 37) to prevent oxidation and the formation of disulfide bonds, we observe a dramatic decrease in microbubble formation (Fig. 2). Furthermore, the oxidation of aqueous solutions of cysteine by ultrasound has been previously observed (38). Thus, we believe that protein cysteine residues are oxidized during microbubble formation, creating interprotein disulfide bonds that cross-link the proteins and hold the bubbles together.

The mechanism responsible for forming the proteinaceous microbubbles is a combination of two acoustic phenomena: emulsification and cavitation. Dispersion of gas into the protein solution, coupled with *chemical* cross-linking of the protein at the bubble interface results in the formation of

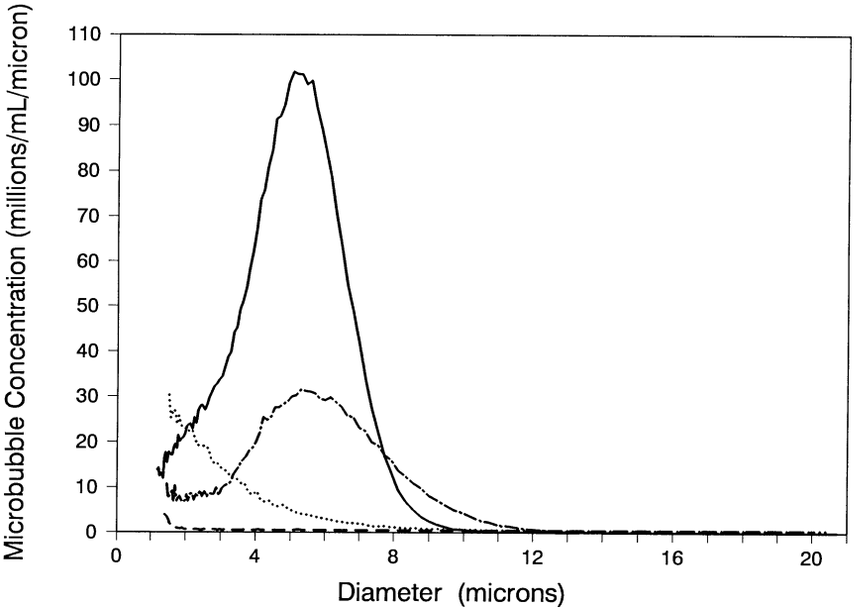

FIG. 2. Radical trapping and cysteine modification prevent the formation of proteinaceous microbubbles during ultrasonic irradiation under air. ——, Aqueous solution of 5% (wt/vol) BSA; -·-·-, aqueous solution of 5% (wt/vol) BSA and 0.2% superoxide dismutase; ·····, aqueous solution of 5% (wt/vol) BSA and 0.04 M *N*-ethylmaleimide; - - - -, aqueous solution of 5% (wt/vol) BSA and 0.1 M glutathione. Inhibition of microcapsule formation also occurs with 2,6-di-*tert*-butyl-4-methylphenol, another common radical trap.

long-lived microbubbles useful as sonographic echo-contrast agents. We have recently discovered that a similar process can also be used to prepare water suspensions of protein-coated microcapsules filled with various nonaqueous liquids (39).

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1. Kerber, R. E., ed. (1988) *Echocardiography in Coronary Artery Diseases* (Future, New York).
2. Pieroni, D. R., Varghese, J., Freedom, R. M. & Row, R. D. (1979) *Catheter. Cardiovasc. Diagn.* **5,** 19–29.
3. Kerber, R. E., Kioschos, J. M. & Laver, R. M. (1974) *Am. J. Cardiol.* **34,** 722–727.
4. Kaul, S., Oliner, J., Kelly, P. & Watson, D. D. (1987) *J. Am. Coll. Cardiol.* **9** (Suppl. A), 2A.
5. Tei, C., Kondo, S., Meerbaum, S., Ong, K., Maurer, G., Wood, F., Sakamaki, T., Shimoura, K., Corday, E. & Shah, P. (1984) *J. Am. Coll. Cardiol.* **3,** 39–46.
6. Kaul, S., Pandian, N. G., Okada, R. D., Pohost, G. M., Weyman, A. E. & Lutrario, D. (1984) *J. Am. Coll. Cardiol.* **4,** 1272–1282.
7. Gramiak, R. & Shah, P. M. (1968) *Invest. Radiol.* **3,** 356–366.
8. Powsner, S. M., Keller, M. W., SanIIe, J. & Feinstein, S. B. (1986) *Am. J. Physiol. Imag.* **1,** 124–128.
9. Feinstein, S. B., Ten Cat, F. J., Zwehl, W., Ong, K., Maurer, G., Tei, C., Shah, P. M., Meerbaum, S. & Corday, E. (1984) *J. Am. Coll. Cardiol.* **3,** 14–20.
10. Smith, M. D., Kwan, O. L. & Reiser, H. J. (1984) *J. Am. Coll. Cardiol.* **3,** 992–998.
11. Gillam, L. D., Kaul, S., Fallon, J. T., Levine, R. A., Hedley-Whyte, E. T., Guerrero, J. L. & Weyman, A. E. (1985) *J. Am. Coll. Cardiol.* **6,** 687–694.
12. Kemper, A. J., O'Boyle, J. E., Cohen, C. A., Taylor, A. & Parisi, A. F. (1984) *Circulation* **70,** 309–317.
13. Armstrong, W. F., Mueller, T. M., Kinney, E. L., Tickner, E. G., Dillon, J. C. & Feigenbaum, H. (1982) *Circulation* **66,** 166–173.
14. Keller, M. W., Feinstein, S. B. & Briller, R. A. (1986) *J. Ultrasound Med.* **5,** 493–498.
15. Dick, C. D. & Feinstein, S. B. (1988) *Pract. Cardiol.* **14,** 69–95.
16. Feinstein, S. B., Keller, M. W. & Dick, C. D. (1987) *J. Am. Coll. Cardiol.* **9,** 111A.
17. Keller, M. W. & Feinstein, S. B. (1988) in *Echocardiography in Coronary Artery Disease*, ed. Kerber, R. E. (Future, New York), pp. 443–465.
18. Feinstein, S. B. (1985) U.S. Patent 805975.
19. Keller, M. W., Feinstein, S. B. & Watson, D. D. (1987) *Am. Heart J.* **114,** 570–575.
20. Feinstein, S. B., Keller, M. W. & Dick, C. D. (1987) *J. Am. Coll. Cardiol.* **9,** 111a.
21. Keller, M. W., Feinstein, S. B. & Watson, D. D. (1987) *Clin. Res.* **35,** 290a.
22. Molecular Biosystems (1990) *Albumex Preclinical Investigators Brochure* (Molecular Biosystems, Inc., San Diego).
23. Rooney, J. A. (1988) in *Ultrasound: Its Chemical, Physical, and Biological Effects*, ed. Suslick, K. S. (VCH, New York), pp. 65–96.
24. Suslick, K. S. (1990) *Science* **247,** 1439–1445.
25. Suslick, K. S., Hammerton, D. A. & Cline, R. E., Jr. (1986) *J. Am. Chem. Soc.* **108,** 5461–5462.
26. Makino, K., Mossobo, M. M. & Reiz, P. (1983) *J. Phys. Chem.* **87,** 1369–1377.
27. Weissler, A. (1959) *J. Am. Chem. Soc.* **81,** 1077–1081.
28. Del Duca, M., Jeager, E., Davis, M. O. & Hovarka, F. (1958) *J. Acoust. Soc. Am.* **30,** 301–307.
29. Lippitt, B., McCord, J. M. & Fridovich, I. (1972) *J. Biol. Chem.* **247,** 4688–4690.
30. Felton, R. H. (1978) in *The Porphyrins*, ed. Dolphin D. (Academic, New York), Vol. 5, pp. 111–115.
31. Fee, J. A. (1980) in *Metal Ion Activation of Dioxygen*, ed. Spiro T. G. (Wiley, New York), pp. 209–239.
32. Asada, K. & Kanematsu, S. (1976) *Agr. Biol. Chem.* **40,** 1891–1892.
33. Carter, D. C., He, X., Munson, S. H., Twigg, P. D., Gernert, K. M., Broom, M. B. & Miller, T. Y. (1989) *Science* **244,** 1195–1198.
34. Dugiaczyk, A., Law, S. W. & Dennison, O. E. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 71–75.
35. Dickerson, R. E. & Geis, I., eds. (1983) *Hemoglobin: Structure, Function, Evolution, and Pathology* (Benjamin/Cummings, Menlo Park, CA).
36. Jocelyn, P. C., ed. (1976) *Biochemistry of the SH Group* (Academic, New York).
37. Haugaard, N., Cutler, J. & Ruggieri, M. R. (1981) *Anal. Biochem.* **116,** 341–343.
38. Bronskaya, L. M. & El'piner, I. Ye. (1963) *Biophys. J.* **8,** 344–348.
39. Suslick, K. S. & Grinstaff, M. W. (1990) *J. Am. Chem. Soc.* **112,** 7807–7809.