Characterization of sonochemically prepared proteinaceous microspheres

K.S. Suslick*, M.W. Grinstaff, K.J. Kolbeck and M. Wong

School of Chemical Sciences, University of Illinois at Urbana-Champaign, 505 S. Mathews Ave., Urbana, IL 61801, USA

Aqueous suspensions of albumin and haemoglobin microspheres can be synthesized using high-intensity ultrasound. The particle size distributions are Gaussian with mean diameters of a few micrometers. These microspheres can contain either nonaqueous liquids (i.e. microcapsules) or air (i.e. microbubbles). The chemical processes involved in the synthesis and some physical properties of the microspheres have been elucidated. Yields and size distributions of albumin microbubbles as a function of experimental parameters indicate that the microspheres are held together primarily by disulphide cross-linking of cysteine residues between protein molecules. The primary oxidizing agent is superoxide (HO_2), which is generated from water and oxygen during acoustic cavitation. Physically, the protein within the microsphere shell is not significantly denatured; the heme held within haemoglobin, for example, is fully retained. Optical, scanning electron, and transmission electron microscopy reveals spherical particles with diameters between 1 and 3 μm, and an approximate shell thickness of roughly 30 nm.

Keywords: microspheres; albumin microbubbles; protein shell

Microscopic vesicles have wide ranging applications, including microencapsulation of dyes, flavours, and fragrances, drug delivery systems, and magnetic resonance contrast (and imaging) agents[1-8]. The vesicles currently in use can be divided into two categories: liposomes and microspheres. Liposomes encapsulate materials via a lipid bilayer, and are generally held together by weak hydrophobic forces. Microspheres use polymer shells for encapsulation, in which covalent bonds stabilize the particles. The use of covalently cross-linked proteins as the microsphere shell has particular benefits for biomedical applications[9-11]. The advantages of polymer microspheres over liposomes include improved robustness and facile chemical modification of the shell. These post-synthetic modifications can increase circulation times *in vivo*, improve shelf-life *in vitro*, and alter other physical and chemical properties (including solubility, reactivity, etc.)

Synthesis and morphology of protein microspheres

Proteinaceous microspheres have been synthesized with a high-intensity ultrasonic probe (Heat Systems, W375 or XL2020, 20 kHz, 0.5 in. Ti horn) from various proteins including bovine serum albumin (BSA), human serum albumin (HSA), and bovine haemoglobin (Hb) as described in detail elsewhere[9-12]. In a typical synthesis of nonaqueous liquid-filled proteinaceous microcapsules, the organic liquid was layered over a 5% w/v protein solution, a high-intensity ultrasonic horn positioned at the interface, and the mixture irradiated for 3 min at an acoustic power of ≈ 150 W cm^{-2} with an initial reaction cell temperature of 23 °C at pH 7.0. For air-filled microbubbles, a similar procedure was used with the horn positioned at the air–water interface. The yield of microspheres strongly depends on the temperature profile of the solution during irradiation; optimization of the initial temperature must be made for each specific experimental configuration.

After the synthesis, the microspheres remain as a suspension in the native protein solution. In order to separate the microspheres from the untreated protein, we have successfully used both filtration and centrifugation. The mixture was filtered through an Anotop syringe filter with 0.2 μm diameter pore size (Whatman Inc.) The filter was washed with several volumes of water, until the filtrate contained very little or no protein (as determined by UV spectroscopy near 200 nm). The microspheres were 'backwashed' out of the filter and resuspended in an equivalent volume of water. Air-filled and most nonaqueous liquid-filled microspheres have a density less than water, and centrifugation of the mixture at approximately 1000 g for 5 min is usually sufficient to separate the microsphere-containing phase from the rest of the mixture. The aqueous phase (containing native protein only) is removed, and the microspheres are resuspended in water. Purification by this procedure requires multiple centrifugations and resuspensions. A third purification

1350-4177/94/010065-04

procedure combines these two techniques with the assistance of an Amicon centrifuge filter with a molecular weight cut-off of 100 000 D. The combined centrifugation filtration technique is generally our preferred method of purification.

The morphology of these materials was determined using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and light microscopy. TEM micrographs show a cross-sectional slice of the particles. Staining techniques darkened the regions of high protein concentration. A TEM micrograph of a single air-filled Hb microsphere is given in *Figure 1*, and a highly concentrated sample of perfluorononane-filled BSA microspheres in *Figure 2*.

Sample preparation for TEM micrographs consisted of three major steps: fixation and staining (for contrast), embedding, and sectioning. The protein in the microspheres was extensively cross-linked with glutaraldehyde and stained with both osmium tetroxide and potassium ferrohexacyanate. The samples were resuspended in alcohol solutions (25%, 50%, 75%, twice with 100%) for dehydration. Polypropylene oxide was added to assist in dissolving the embedding media. A nonpolar epoxy was added and cured by heating at 90 °C for 4 h. The samples were then microtomed to suitable thickness and imaged on a JEOL-100 TEM instrument. Some shrinkage of the microspheres is expected during this process. The true diameter of our microspheres is therefore best represented by the solution particle sizing (*Figure 3*), rather than directly from the electron micrographs. Confirming data from scanning electron microscopy and visible-light microscopy demonstrate the spherical shape of the microspheres.

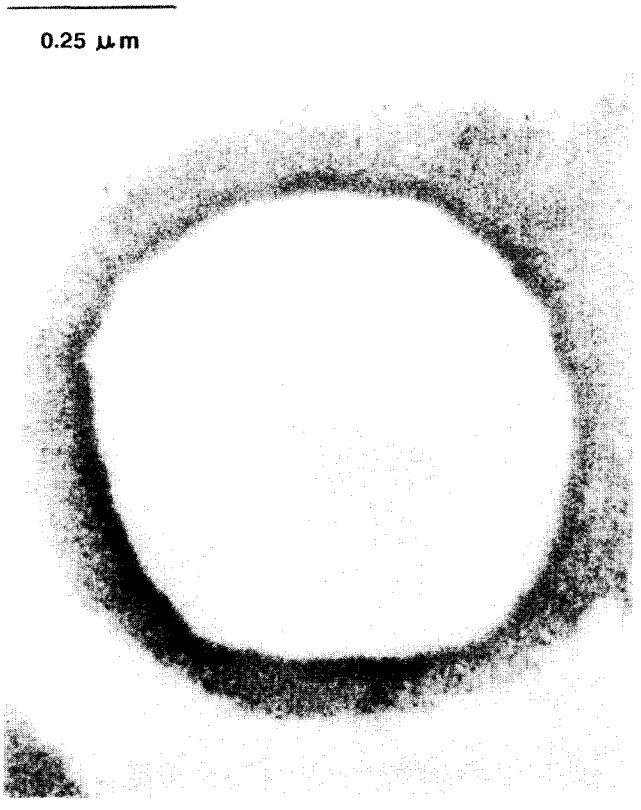

Figure 1 Transmission electron micrograph of air-filled proteinaceous microbubbles made from haemoglobin

Figure 2 TEM micrograph of highly concentrated perfluorononane-filled microcapsules made from bovine serum albumin

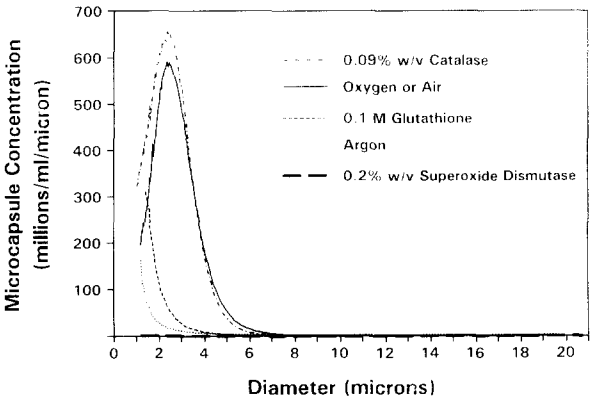

Figure 3 Particle distribution of an aqueous suspension of proteinaceous microspheres, determined with an Elzone particle counter (Model 180XY)

Mechanism of the sonochemical synthesis of microspheres

We have developed a detailed understanding of the chemical mechanisms that form the microspheres and that hold them together. Ultrasonic irradiation of liquids is well known to produce both emulsification[13] and cavitation[14–16]. In microsphere formation, the nonaqueous liquid (or air) is emulsified as a micrometre-sized dispersion into the aqueous protein solution. Emulsification alone, however, is insufficient to form long-lived microspheres. Emulsions produced by vortex mixing, instead of ultrasonic irradiation, do not produce stable microspheres. The vortex emulsions phase separate quickly, while protein microspheres are stable for days at room temperature and for several months at 4 °C.

Hydrophobic or thermal denaturation of the protein after the initial ultrasonic emulification is another possible explanation for microsphere formation. High concentrations of microspheres, however, are only observed when the mixture is well oxygenated. If the reaction is run under an inert atmosphere (He, Ar or N_2) microcapsules are *not* formed (*Figure 3*). If thermal or solvent denaturation were responsible, the atmosphere would have no effect on microsphere formation.

Another *chemical* process must be involved. As mentioned above, ultrasonic irradiation of liquids generates acoustic cavitation: the formation, growth and collapse of bubbles in a liquid[14-16]. The collapse of these bubbles produces high energy chemistry. Specifically, aqueous sonochemistry produces OH· and H·[17-19]. These radicals form H_2, H_2O_2 and (in the presence of O_2) superoxide, HO_2[19-21]. Hydroxyl radicals, superoxide and peroxide are all potential protein cross-linking agents.

To identify the specific oxidant involved, the formation of microspheres was examined in the presence of radical traps (*Figure 3*). The effects of nonspecific traps (e.g. 2,6-di-*t*-butyl-4-methylphenol or glutathione), and specific traps, catalase (which decomposes hydrogen peroxide[22]) and superoxide dismutase (which decomposes superoxide[23]), were tested. Both microcapsule and microbubble formation were inhibited by the nonspecific traps and by superoxide dismutase, but *not* by catalase. Catalase activity was confirmed after ultrasonic irradiation (i.e. irradiation did not destroy the functioning of this enzyme). These results indicate that the important oxidant involved in microsphere formation is superoxide.

To determine how superoxide affects the formation of microspheres, several experiments were performed. Cysteine is easily oxidized by superoxide[24] and is present in BSA, HSA and Hb. Ultrasonic irradiation of proteins has been shown to oxidize cysteine residues[25]. If the microspheres are held together by inter-protein disulphide bonds from cysteine oxidation, Hb and myoglobin (Mb) provide an interesting test. Although the two proteins have very similar sequences and monomeric three-dimensional structures, only Hb contains cysteine residues. Ultrasonic irradiation of Hb solutions forms microspheres; Mb does not. In other tests, the addition of a disulphide bond cleavage reagent, dithioerythritol[26], rapidly destroys Hb–toluene or BSA–toluene microcapsules. Finally, the oxidation of cysteine residues can be inhibited by alkylation with *N*-ethylmaleimide[27]. Microsphere formation from alkylated Hb solutions is greatly reduced. These results confirm the significance of disulphide bond formation in microsphere formation.

Physical and chemical properties

We are actively exploring the physical and chemical properties of these sonochemically synthesized microspheres. Of particular interest are the enzymatic functioning of the proteins once they are part of a microsphere, the molecular structure of the proteins making up the shell, and the physical nature of the proteinaceous shell.

In order to explore the ability of the proteins to continue to function after incorporation into the microspheres, we carefully isolate the microspheres from the free protein containing supernatant, by microfiltration or centrifugation (as described earlier). The Hb microspheres are an especially important system for such examination. We are able to prepare reduced, deoxy-Hb microspheres by using the enzyme-reducing system of Hyashi *et al.*[28] to reduce the heme to Fe(II). Upon exposure to O_2, these microspheres bind O_2 and form oxy-Hb microspheres whose spectrum is identical to normal oxy-Hb solutions. Upon flushing with Ar, the O_2 is removed and reversible formation of deoxy-Hb microspheres is observed. The optical spectra of the states of Hb microspheres is shown in *Figure 4*.

In order to monitor the effects of microsphere formation on the structure of the proteins, we have employed circular dichroism as a probe. Circular dichroism is commonly used to assist in determining the secondary structure of proteins (e.g. fraction of α-helix present). BSA contains a high percentage of α-helix in its native form, as indicated by the large negative ellipticity around 220 nm. *Figure 5* shows the normalized circular dichroic spectra of BSA in solution and of air-filled BSA microspheres. The overall shape of the microsphere spectrum shows only minor changes in the α-helical content and does not indicate extensive denaturation of the protein. The intensity differences in the ellipticity of the solution and microsphere spectra are due to increased light scattering from the microspheres.

The microsphere shell thickness can be obtained from the TEM micrographs. Close inspection of *Figure 1*

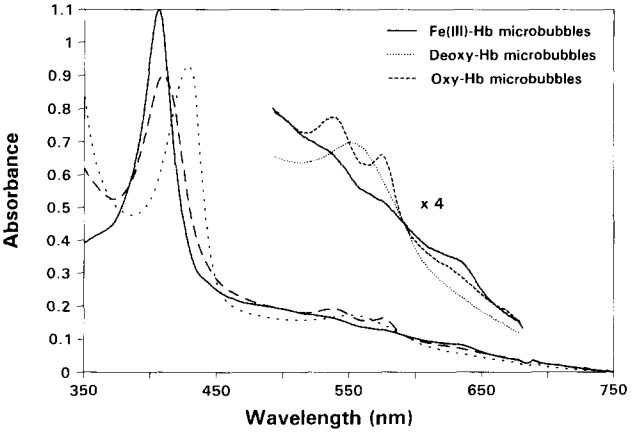

Figure 4 Absorbance spectra of Hb microspheres

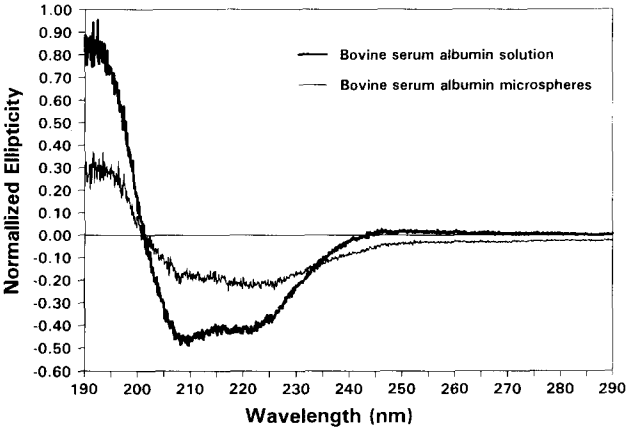

Figure 5 Normalized circular dichroism (CD) spectra of bovine serum albumin (BSA) microbubbles. The secondary structure of BSA is primarily α-helix and is nearly unchanged after incorporation into the microbubble shell. The difference in intensity of the ellipticity is due to increased light scattering by the microbubbles

reveals three separate regions: an empty, clear central region; a dark, thin layer of the shell; and a loosely attached, diffuse grey layer surrounding the shell. The dark, thin layer is the microsphere shell. It contains a high density of protein, and during the staining procedure, develops the most contrast. The loosely attached, grey layer appears to be native protein that adheres to the microsphere shell during the fixation step in the sample preparation. With extensive purification, the loosely held material can be eliminated. Measurements of the microsphere shell range from 25 to 35 nm in all samples observed. Haemoglobin is a roughly spherical protein with a diameter[29] of 5.5 nm. Thus, our protein shell corresponds to a thickness of roughly 4 to 7 protein molecules!

Summary

Ultrasound can produce proteinaceous microcapsules and microbubbles a few micrometres in diameter at high concentrations with narrow size distributions. The process involves two separate acoustic phenomena: emulsification and cavitation. Stable protein microspheres are produced by the combination of the dispersion of gas or nonaqueous liquids into the protein solution followed by the chemical cross-linking of cysteine residues from superoxide produced during cavitation. Initial characterization of the protein in the microsphere shell indicates that the protein is not significantly denatured and still retains important functional properties. The protein shell is quite thin, roughly 30 nm, and is therefore roughly six protein molecules thick.

Acknowledgements

This work was supported by a special creativity extension award from the NSF (CHE-8915020) and the NIH (HL-25934) - M.W.G. gratefully acknowledges receipt of the Procter and Gamble Graduate Fellowship of the Colloid and Surface Chemistry Division of the American Chemical Society. We also thank Lou Ann Miller of UI Veterinary Medicine for her assistance with the TEM micrographs.

References

1 **Langer, R.** *Science* (1990) **249** 1527
2 **Lee, T.K., Sokoloski, T.D. and Royer, G.P.** *Science* (1981) **213** 233
3 **Heller, J. and Bake, R.W.** *Controlled Release of Biomaterials* Academic Press, New York, USA (1980)
4 **Burgess, D.J., Davis, S.S. and Tomlinson, E.** *Int J Pharm* (1987) **39** 129
5 **Morimoto, Y., Sugibayashi, K. and Kato, Y.** *Chem Pharm Bull* (1981) **29** 1433
6 **Jalsenjak, V., Stolink, S. and Jalsenjak, I.** *Acta Pharm Jugosl* (1988) **38** 297
7 **Risch, S.J. and Reineceius, G.A.** *Flavor Encapsulation* American Chemical Society, Washington, DC, USA (1988)
8 **Thaku, M.L., Vinitski, S., Mitchell, D.G., Consigny, P.M., Lin, S., Defulvio, J.D. *et al.*** *Magnetic Resonance Imaging* (1990) **8** 625
9 **Keller, M.W., Feinstein, S.B. and Briller, R.A.** *J Ultrasound Med* (1986) **5** 493
10 **Suslick, K.S. and Grinstaff, M.W.** *J Am Chem Soc* (1990) **112** 7807
11 **Grinstaff, M.W. and Suslick, K.S.** *Proc Natl Acad Sci USA* (1990) **88** 7708
12 **Suslick, K.S and Grinstaff, M.W.** In *Macromolecular Assemblies* (Ed. Stroeve, P. and Balazs, A.C.) American Chemical Society, Washington, DC, USA (1992) 218–226
13 **Rooney, J.A.** In *Ultrasound: Its Chemical, Physical, and Biological Effects* (Ed. Suslick, K.S.) VCH, New York, USA (1988) 74–96
14 **Suslick, K.S.** *Science* (1990) **247** 1439
15 **Suslick, K.S.** *Sci Am* (1989) **260** 80
16 **Suslick, K.S.** (Ed.) *Ultrasound: Its Chemical, Physical, and Biological Effects* VCH Publishers, New York, USA (1988)
17 **Makino, K., Mossobo, M.M. and Reiz, P.** *J Phys Chem* (1983) **87** 1369
18 **Riesz, P., Berdahl, D. and Christman, C.L.** *Environ Health Perspect* (1985) **64** 233
19 **Weissler, A.** *J Am Chem Soc* (1959) **81** 1077
20 **Del Duca, M., Jeager, E., Davis, M.O. and Hovarka, F.** *J Acoust Soc Am* (1958) **30** 301
21 **Lippitt, B., McCord, J.M. and Fridovich, I.** *J Biol Chem* (1972) **247** 4688
22 **Felton, R.H.** In *The Porphyrins* (Ed. Dolphin, D.) Academic Press, New York, USA (1978) Vol. 5 111–115
23 **Fee, J.A.** In *Metal Ion Activation of Dioxygen* (Ed. Spiro, T.G.) Wiley, New York, USA (1980) 209–239
24 **Asada, K. and Kanematsu, S.** *Agr Biol Chem* (1976) **40** 1891
25 **Bronskaya, L.M. and El'piner, I.Ye.** *Biophys* (1963) **8** 344
26 **Jocelyn, P.C.** *Biochemistry of the SH Group* Academic Press, New York, USA (1976)
27 **Haugaard, N., Cutler, J. and Ruggieri, M.R.** *Anal Biochem* (1981) **116** 341
28 **Hyashi, A., Suzuki, T. and Shin, M.** *Biochim Biophys Acta* (1973) **310** 309
29 **Stryer, L.** *Biochemistry*, 3rd edn W.H. Freeman, New York, USA (1988) 151 152