

SONOCHEMICALLY PRODUCED HEMOGLOBIN MICROBUBBLES

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ABSTRACT

Using high-intensity ultrasound, we have developed a method for the synthesis of air-filled hemoglobin (Hb) microbubbles ($\approx 2.5 \mu\text{m}$ in diameter). Transmission electron, scanning electron, and optical microscopy show spherical particles with a shell thickness of approximately 35 nm, or roughly six protein molecules thick. The mechanism of microbubbles formation has been determined to involve both the dispersion of gas into micron-sized bubbles and the chemical cross-linking of cysteine residues between protein molecules. The primary oxidizing agent is superoxide (HO_2), which is sonochemically produced from oxygen and water during acoustic cavitation. The Hb microbubbles possess many of the desired characteristics of a blood substitute. The microbubbles are smaller than red blood cells and will not block capillaries. The microbubbles are air-filled and provide a large O_2 carrying capacity. The hemoglobins of the microbubbles retain their ability to bind oxygen reversibly. In addition, the oxygen affinities are similar to those of native Hb. Even more surprisingly, microbubbles show extensive cooperativity, as indicated by Hill coefficients as high as 18, which means that in the microbubble shell, there is communication between several of the *crosslinked Hb tetramers* upon binding oxygen. The Hb microbubbles show minimal degradation ($< 25\%$) after storage for six months at 4 °C.

INTRODUCTION

Previous work by Suslick and coworkers have shown that micron-sized, gas or liquid filled proteinaceous microspheres can be produced from serum albumin with high-intensity ultrasound [1-2]. These microspheres are formed by chemically crosslinking cysteine residues of the protein with HO_2 around a micron-sized gas bubble or non-aqueous droplet. We have now extended this work to form functional, gas filled hemoglobin (Hb) microspheres. These Hb microbubbles have many of the ideal characteristics needed for use as a blood substitute. Previously studied blood substitutes include perfluorocarbons [3], chemically cross-linked oligomers of Hb [4], stroma-free Hb [5], and genetic engineered Hb [6]. The major drawbacks of previous blood replacements include low oxygen carrying capacity, low temperature storage conditions, and high renal and hepatic toxicity [7-9].

There are multiple critical criteria for an ideal blood replacement [10-11]. First, a useable blood substitute must fully bind oxygen in the lungs and efficiently unload oxygen in the tissues, which implies positive cooperativity in oxygen binding. Second, it should respond to the body's own allosteric effectors (such as phosphates) to modify its binding properties *in vivo* in response to metabolic needs. Third, the blood substitute should possess adequate oxygen-carrying capacity. Fourth, it must not trigger an immunogenic response or damage the kidneys or other organs. Fifth, the blood substitute must be stable under storage conditions.

EXPERIMENTAL

Native human or bovine Hb (Sigma) were purified as described previously [12]. In a typical synthesis of Hb microbubbles, the ultrasonic horn (Heat Systems XL 2020) was positioned at the air-solution (2.5% w/v Hb) interface with an initial cell temperature of 55 °C and pH 6.8. The solution was irradiated for 3 minutes at a power output of $\approx 150 \text{ W/cm}^2$. Size and concentration of the Hb microbubbles are very sensitive to the experimental configuration and should be optimized for any specific apparatus with respect to protein concentration, cell temperature, and sonication time.

To separate the microbubbles from denatured or unreacted Hb, a Centricom centrifuge filter with a molecular-weight cut-off of 100 kilodaltons (kD) was used. Centrifugation of the microbubbles solution at 1000 G for 5 minutes allowed most of the native and denatured Hb (64.5 kD) to pass through the membrane.

Size distributions of the microbubbles were determined in electrolyte solutions with a zeta-potential sizer (Particle Data, Elzone 180XY). Optical and electron microscopy confirmed the spherical morphology of the microbubbles. The Hb microbubbles have excellent stability and show minimal degradation (< 25%) after storage for six months at 4 °C.

The purified Hb microbubbles are initially in the met-Fe(III) form, which of course cannot bind oxygen. The reduction system of Hyashi *et. al.* [13] was used to reduce Fe(III) to Fe(II). The reduction system consists of a complex solution of glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, ferredoxin, ferredoxin reductase, and catalase. Before each oxygen binding experiment, the reduction system was added to the microbubbles and left at 4 °C for 24-36 hours and then removed by centrifugal filtration. All binding experiments were done at 25 °C in Tris-buffer (pH 7.4).

Oxygen binding curves for the native Hb and Hb microbubbles were determined using an apparatus similar to that described previously [14]. A microcomputer was interfaced to the Clark electrode and photomultiplier output of a spectrophotometer to yield oxygen pressure and percent saturation simultaneously. Hill coefficients (n) were calculated using the formula:

$$n = \Delta \log(Y/1-Y) / \Delta \log P_{O_2} \quad (1)$$

where Y = fraction saturation and P_{O_2} = oxygen partial pressure.

RESULTS AND DISCUSSION

Characterization and Morphology

Particle size distribution analysis of the solution revealed typical total concentrations of roughly 3×10^{19} microbubbles/ml with an average diameter of $\approx 3.0 \mu\text{m}$. The morphology of the microbubbles has been determined using transmission electron microscopy (TEM). Figure 1 shows a TEM micrograph of a cross-sectional slice of a Hb microbubble. The microbubble has been fixed with glutaraldehyde, stained with osmium tetroxide and potassium ferrocyanate (to provide contrast in regions of high protein concentration), embedded in a low viscosity resin, and ultra-microtomed (slice thickness $\approx 75 \text{ nm}$). Since some shrinkage in the overall diameter and expected during this process, the true diameter some shape distortion of the microbubble are of the microbubble is best represented by the solution particle size distribution, rather than direct

measurements from the TEM micrograph. A closer look at the TEM micrograph in Figure 1 shows three distinctive regions: a clear central region; a dark, thin layer; and a loosely attached, diffuse, speckled gray region. The dark, thin layer is the microsphere shell. It contains a high density of protein, and during staining procedure develops the most contrast. The loosely attached, gray region appears to be native protein that adheres to the microsphere shell during the fixation step in the sample preparation. Initial measurements from this and many other micrographs indicate the shell thickness of the Hb microbubbles to be 25-35 nm. The Hb tetramer is a roughly spherical protein with a diameter of 5.5 nm [15]. Thus, the protein shell of the Hb microbubbles corresponds to a thickness of roughly six crosslinked tetramers.

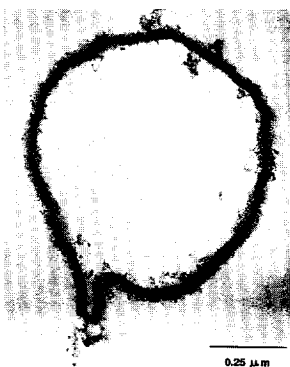


Figure 1. TEM of Hb Microbubbles

Mechanism of Microbubbles Formation

The mechanism for the Hb microbubble formation is similar to that previously determined by Suslick and coworkers for the sonochemically produced serum albumin microspheres. Ultrasonic irradiation of liquids is well known to produce emulsification of immiscible liquids [16], dispersion of gases [16], and acoustic cavitation [17]. In microbubble formation, the air is dispersed into the aqueous protein solution to form micron-sized bubbles. Such dispersion, however, is insufficient by itself to form long-lived microbubbles. Dispersions produced by vortex mixing, instead of ultrasonic irradiation, do not produce stable microbubbles.

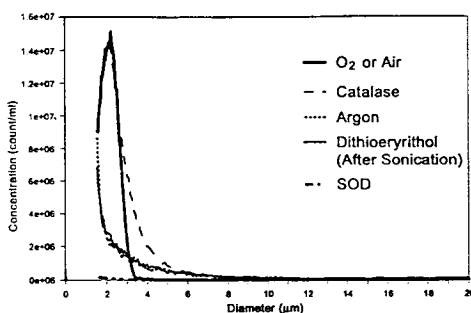


Figure 2. Sonochemically generated Hb microbubbles under various chemical conditions.

High concentrations of microbubbles are only observed when the mixture is well oxygenated (Figure 2). If the reaction is run under an inert atmosphere (He, Ar, or N₂) microbubbles are *not* formed. If thermal or solvent denaturation were responsible, the atmosphere would have no effect on microsphere formation.

Another *chemical* process must be involved. Ultrasonic irradiation of liquids generates acoustic cavitation: the formation, growth, and collapse of bubbles in a liquid creates intense local heating which can drive high energy chemical reactions [17]. Specifically, the sonolysis of water produces OH• and H• [18-20]. These radicals form H₂, H₂O₂, and (in the presence of O₂)

superoxide, HO₂ [20]. Hydroxyl radicals, superoxide, and peroxide are all potential protein cross-linking agents.

To identify the specific oxidant involved, the formation of microbubbles was examined in the presence of chemical traps (Figure 2). The effects of radical traps, of catalase (which decomposes hydrogen peroxide to water and oxygen [21]), and of superoxide dismutase (which decomposes superoxide to hydrogen peroxide and oxygen [22]) were tested. Microbubble formation was strongly inhibited by superoxide dismutase, but *unaffected* by catalase. This result indicates that HO₂ (and not H₂O₂) is the important oxidant involved in microbubbles formation.

Cysteine is easily oxidized by superoxide [23]. Ultrasonic irradiation of proteins has been shown to oxidize cysteine residues [24]. If the microbubbles are held together by inter-protein disulfide bonds from cysteine oxidation, Hb and myoglobin (Mb) provide an interesting test. The two proteins have similar sequences and monomeric structures; however, a Hb tetramer contains six cysteine residues whereas Mb has none. Ultrasonic irradiation of Mb solutions does not form microbubbles: *Hb does*. In other tests, the addition of a disulfide bond cleavage reagent, dithioerythritol [25], destroys the previously formed Hb microbubbles (Figure 2). Similarly, alkylation of the Hb cysteines prior to sonication suppresses microsphere formation [1]. These results confirm the significance of disulfide bond formation in microsphere formation.

Physical Properties

After formation and reduction, the hemoglobin molecules that make up our microbubbles retain their ability to bind oxygen reversibly. Figure 3 shows the visible spectra of the microbubbles in met-Fe(III), oxy-Fe(II), and deoxy-Fe(II) forms. The spectra are closely similar to native Hb. The microbubbles can be cycled between the deoxy and oxy states for more than ten cycles without significant degradation. This is important because it indicates that the environment surrounding the active heme site has not been altered significantly in the process of making the microbubbles.

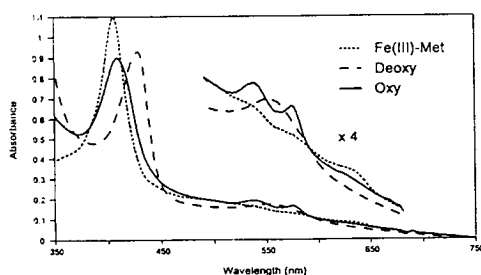


Figure 3. Visible spectra of Hb microbubbles in Fe(III)-met, Fe(II)-deoxy, and Fe(II)-oxy forms.

The $P_{1/2}$ values (the partial pressure of O₂ at which half of the available binding sites on Hb are bound by oxygen) are very similar for both native and sonicated Hb (Table I). This result indicates that the microbubbles can bind and release oxygen at the same oxygen pressures as native Hb. Strikingly, the maximum Hill coefficient n (indicating the level of cooperativity between oxygen binding sites) of the microbubbles is significantly higher (Table I) than the native Hb solution (2.7 versus 10.0 in the absence of phosphates).

Allosteric effectors of native Hb such as inositol hexaphosphate (IHP) have been shown to increase both $P_{1/2}$ (i.e., lower oxygen affinity) and to enhance cooperativity [26]. The same effects are seen in the microbubbles. Even though the $P_{1/2}$ values are increased by the same amount, a more dramatic effect is seen in the cooperativity of the microbubbles. The Hill coefficient has increased roughly six-fold over that of native Hb in the presence of 1.7 mM IHP (Table I).

Table I. Summary of $P_{1/2}$ and Hill coefficients for native Hb and Hb microbubbles with and without inositol hexaphosphate (IHP).

IHP Conc. (mM)	$P_{1/2}$ (torr)		Maximum Hill Coefficient (n)	
	Native	Microbubbles	Native	Microbubbles
0	22.4	21.2	2.7	10.0
1.7	36.7	39.7	2.8	18.0

The increase in cooperativity apparently is due to the disulfide linkages between Hb tetramers within the microbubbles' shell. The Hill coefficient cannot be greater than the number of interacting binding sites. The value of approximately 2.8 in native Hb reflects the cooperativity in one tetramer. However, in the microbubble shell, there is communication between several of the cross-linked tetramers (from the formation of disulfide bonds) upon binding oxygen. The interactions with nearest-neighbor tetramers are likely to be strongest; additional weaker interactions between tetramers further away may also exist. Essentially, the large Hill coefficient is an indication that multiple tetramers cooperate in switching from the low-affinity, deoxy-T state to the high-affinity oxy-R state within the microbubble shell upon binding oxygen. As discussed earlier, TEM micrographs of the Hb microbubbles reveal a shell thickness of about six Hb tetramers, which means that a 3.0 μm diameter bubble would contain about 10^6 Hb molecules.

We have also recently calculated the oxygen carrying capacity of the microbubbles as compared to whole blood and the fluorocarbon emulsion Fluosol-DA 20% (an FDA approved blood substitute). The volume of O_2 gas carried by whole blood is roughly the same as in air: 0.2 ml O_2 /ml blood [27-28]. The O_2 carrying capacity is considerably lower in the fluorocarbon emulsion: 0.05 ml/ml [27]. In the Hb microbubbles, if the inside of the microbubbles was filled with pure O_2 (as it would be if pure O_2 were being inhaled), then the gas carrying capacity of the microspheres would be 0.32 ml/ml. This is 50% better than whole blood.

CONCLUSION

In summary, we have used high-intensity ultrasound to produce Hb microbubbles a few microns diameter at high concentrations with narrow size distributions. The process involves crosslinking of cysteine residues oxidized by HO_2 produced during sonolysis. The Hb microbubbles retain its ability to reversibly bind oxygen at pressures similar to native Hb. In addition, the microbubbles exhibit significantly higher cooperativity, an indication of communication between crosslinked tetramers. The Hb microbubbles have greater oxygen carrying capacity than fluorocarbon emulsions and show significant stability.

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