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Sonochemical synthesis of liquid-encapsulated lysozyme microspheres

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ABSTRACT

Liquid-encapsulated lysozyme microspheres were successfully synthesized using a sonochemical method. The encapsulation of four different liquids, namely, sunflower oil, tetradecane, dodecane and perfluorohexane on the formation, stability and morphology of the lysozyme microspheres was studied. Among the four different liquids used for encapsulation, perfluorohexane-filled microspheres were found to be most stable in the dried state with a narrow size distribution. In order to explore the possibility of encapsulating biofunctional molecules (e.g., drugs) within these microspheres, liquids containing a fluorescent dye (Nile red) were encapsulated and the ultrasound-induced release of these dye-loaded liquids was studied. The fluorescence data for the liquid-filled lysozyme microspheres demonstrated the potential use of the sonochemical technique for synthesizing these "vehicles" for the encapsulation and the controlled delivery of dyes, flavours, fragrances or drugs.

1. Introduction

Protein microspheres have attracted much research interest in the biomedical field because of their biodegradability and biocompatibility. Applications such as ultrasound contrast agents, magnetic resonance-imaging, optical coherence tomography, oxygen and drug delivery have been considered [1–4]. Several types of protein encapsulation have been reported, including a protein matrix [5], hollow protein microbubbles [6,7] and core–shell protein microspheres [8]. The loading capability of these microspheres can be improved by modifying the outer surface of the protein microspheres, the interior core or the matrix structure. Various techniques can be employed to form the protein microspheres, including spray drying [9], an oil in water emulsion method [10] and a sonochemical approach [11]. Suslick and co-workers [6,12] have carried out pioneering work on the ultrasonic synthesis of protein microspheres. Suslick proposed that for sulphur containing proteins (e.g., bovine serum albumin (BSA) [11], human serum albumin (HAS) [6] and haemoglobin (Hb) [7]), the mechanism of the sonochemical formation of stable oil- and gas-filled microspheres involves the cross-linking of cysteine residues through disulphide bond formation. For sulfhydryl-free polypeptides (e.g., polyglutamate), hydrogen bonding or ion pairing between carboxylates [13] provided the mechanism for stabilization of oil-filled microparticles. Avivi and Gedanken [14] suggested that hydrophobic interactions in acidic medium are responsible for the fabrication of poly(glutamic acid) and streptavidin microspheres; –SH functional groups are absent in both of these systems.

It has been claimed that the one-step sonochemical encapsulation process is extremely effective in producing a core–shell structure with high encapsulation efficiency [1]. Bovine serum albumin (BSA) is the most commonly used shell material for entrapping a variety of oils. In 1990, Suslick [11] reported the ultrasonic synthesis of proteinaceous microcapsules of BSA filled with *n*-dodecane, *n*-hexane and cyclohexane.

Hen egg white lysozyme, an enzyme present in many animal and vegetable tissues, contains four intramolecular disulphide bonds that can be partly broken using reducing agents, such as dithiothreitol. This enhances the probability of intermolecular disulphide bond formation leading to cross-linking between lysozyme molecules. Based on this strategy, lysozyme air-filled microspheres have been successfully synthesized using chemically denatured lysozyme under high-intensity ultrasound [15]. Upon sonication, new disulphide linkages are formed enabling inter-protein cross-linkage to occur leading to the formation of lysozyme microspheres with a thick (0.1–0.8 μm) protein shell that are stable for several months. The lysozyme microbubbles represent a multifunctional ultrasound-responsive systems endowed with antimicrobial and biodegradability features. When tailored with targeting moieties, the lysozyme-shelled microspheres are promising candidates for controlled drug delivery applications. However, loading a sufficient amount of therapeutic agent onto the micro-

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bubbles is a current limitation for these ultrasound contrast agent-based drug delivery systems. A better alternative is to encapsulate an oil-phase containing the drug which increases their lipophilic drug loading capacity.

In this study, we have explored the possibility of using cross-linked lysozyme as the shell material to ultrasonically encapsulate a vegetable oil and some non-polar organic liquids, which make them suitable reservoirs for lipophilic compounds. The morphologies of lysozyme microspheres filled with oil and organic liquids have also been studied. The effective encapsulation of the liquid materials within the microspheres has been confirmed by ultrasonically breaking the microspheres loaded with liquids containing an organic dye and studying the fluorescence properties of the dye.

2. Experimental details

2.1. Materials

Lysozyme from hen egg white, DL-dithiothreitol (DTT) and perfluorohexane were purchased from Sigma–Aldrich and tris(hydroxymethyl)amino methane (Tris buffer) from Mallinckrodt. Nile red ($\geqslant$98.0%), tetradecane (olefine free; >99%) and dodecane (>99.8%) were from Fluka. Sunflower oil was commercial-grade cooking oil purchased from a local supermarket. High-purity water Milli-Q filtered water was extracted from a Millipore system (18.2 MΩ/cm at 25 °C). A Branson 20 kHz ultrasound generator with a standard 1 cm diameter horn and adjustable power intensity was employed for the emulsification and cross-linking of protein molecules in an aqueous medium [15].

2.2. Synthesis of lysozyme microspheres

Protein solutions of 5% w/v lysozyme were prepared in 5 ml Tris buffer (0.1 M) at pH 8. The lysozyme was denatured by adding DTT (0.15 g) and stirring for 2 min. The organic solvents (4% (v/v)) saturated with a dye (Nile red) were layered on the surface of the protein solution prior to sonication. The tip of the high-intensity ultrasonic horn was positioned at the solvent-protein solution interface. After ultrasonic irradiation at an applied acoustic power of 300 W/cm^2 for 30 s, a 250 ml separation funnel was used to wash out DTT and the residual protein. The washing procedure involved mixing the sonicated solution with excess Milli-Q water in the separation funnel and leaving the mixture to settle for 4–6 h. For low density solvents, the lysozyme microspheres floated to the surface. The microspheres were collected after repeating this washing procedure five times. Perfluorohexane-filled lysozyme microspheres were collected from the bottom of the separation funnel as the density of perfluorohexane is higher than that of water (Table 1).

2.3. Characterization

For imaging, the microspheres were first air-dried and then sputter-coated with a thin gold film. The morphology of the lysozyme microspheres was observed using a scanning electron microscopy (SEM) (FEI Quanta) and an inverted Olympus IX71 wide field fluorescence microscope with a 60× objective lens and green filter cube. A CCD camera (Cool SNAP fx, Photometrics, Tucson, AZ) was mounted on the left-hand port of the microscope. Fluorescence spectral measurements were carried out using a RF-5301PC spectrophotometer (Shimadzu). Optical microscopy was used to record the images of microspheres dispersed in water. The average size and the size distribution of the liquid-filled lysozyme microspheres were evaluated by measuring over 200 microspheres per system using optical microscopic and SEM images.

3. Results and discussion

3.1. Morphology of encapsulated microspheres

In this study, the sonochemical synthesis of liquid-filled lysozyme microspheres was successfully achieved. The mechanism for the lysozyme microsphere formation has been discussed in our previous report [15]. In brief, there are three processes involved in the formation of liquid-filled lysozyme microspheres: the emulsification of the liquid to form a suspension of microdroplets in an aqueous solution, aggregation of lysozyme at the liquid–water interface and the chemical cross-linking of cysteine residues of the partially denatured lysozyme. The inter-protein cross-linking is caused by the superoxide generated during the acoustic cavitation process leading to the encapsulation of the liquid [11].

The oil-filled lysozyme microspheres could be stored for several weeks as aqueous dispersions. The morphology of these microspheres varied with the type of the encapsulation material (Fig. 1 and Table 1). It can be seen in Table 1 that the mean size and size distribution of lysozyme microspheres are significantly different for the different encapsulated materials. Among the four solvents used, tetradecane- and perfluorohexane-filled microspheres showed smaller mean sizes and narrow size distributions. The average size of the lysozyme microspheres filled with perfluorohexane and tetradecane was about 2.5 and 3 μm, respectively. The size of dodecane-filled lysozyme microspheres is around 3–5.5 μm, while the size has a broad range of 4–13 μm for sunflower oil. This might be due to other additives present in the cooking oil which could affect the stability of the microemulsion prior to cross-linking lysozyme.

The perfluorohexane-filled microspheres were found to be the most stable system when dried (Fig. 1). Other solvent-filled lysozyme microspheres were broken during air drying or under vacuum in the SEM chamber and appeared as broken shells in the SEM images (Fig. 1). Since perfluorohexane is a low boiling liquid (Table 1), the evaporation of the liquid during the drying process may not account for the observed instability of other liquid-filled microspheres. One possible reason might be the ability of protein adsorption at the liquid/water interface prior to cross-linking. It has been suggested [15] that the adsorption property of the proteins plays a critical role in cross-linking the proteins. For example, in order to produce air-filled microspheres, the adsorption of the protein molecules at the bubble solution interface is critical. Simi-

Table 1
Characteristics of sonochemically synthesized liquid-filled lysozyme microspheres.

Liquid	Molecular weight (g/mol)	Boiling point (°C)	Surface tension at 25 °C (mN/m)	Density (g/ml)	Average size (μm)	Size distribution
Sunflower oil	–	–	–	0.917	4–13	Nonuniform
Tetradecane	198.40	253.5	26.5	0.762	3	Uniform
Dodecane	170.34	216.3	25.3	0.749	3–5.5	Nonuniform
Perfluorohexane	338.04	56.6	11.9	1.71	2.5	Uniform

Note: oil concentration in sonication solution equals 4 w/v%.

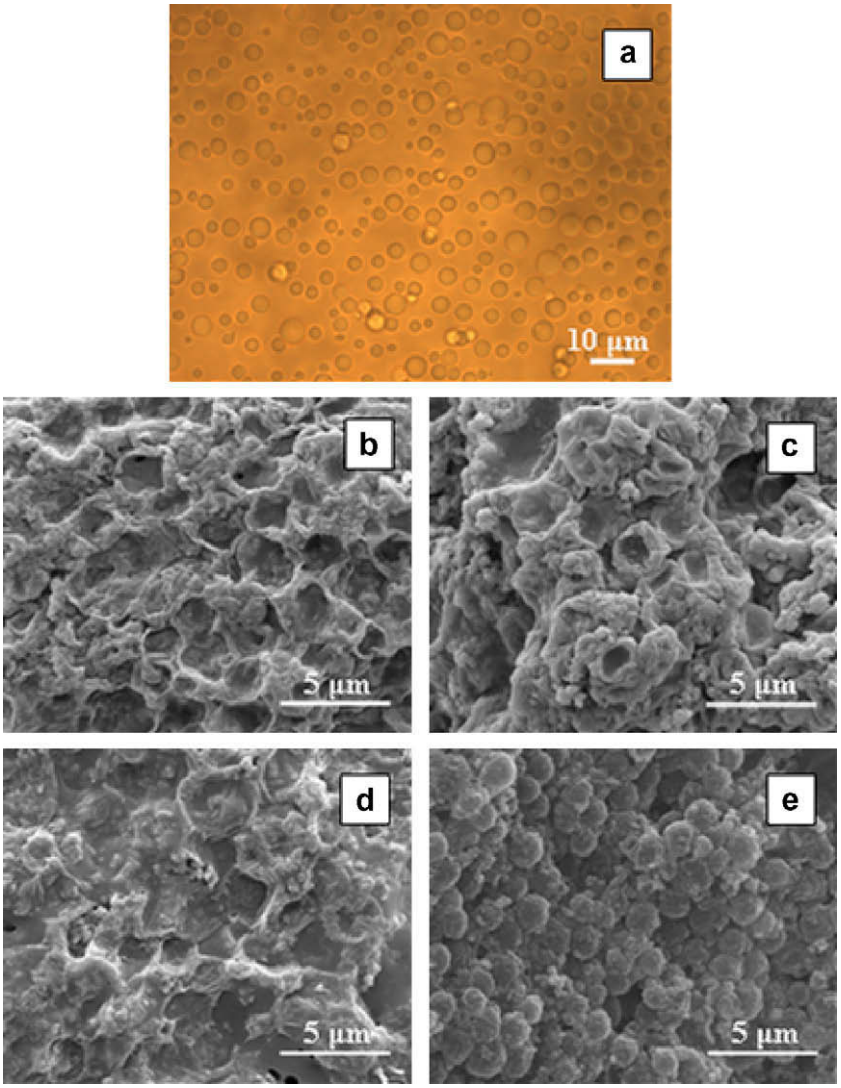

Fig. 1. (a) Optical microscopic image and SEM images of (b) tetradecane, (c) dodecane, (d) sunflower oil and (e) perfluorohexane-filled lysozyme microspheres.

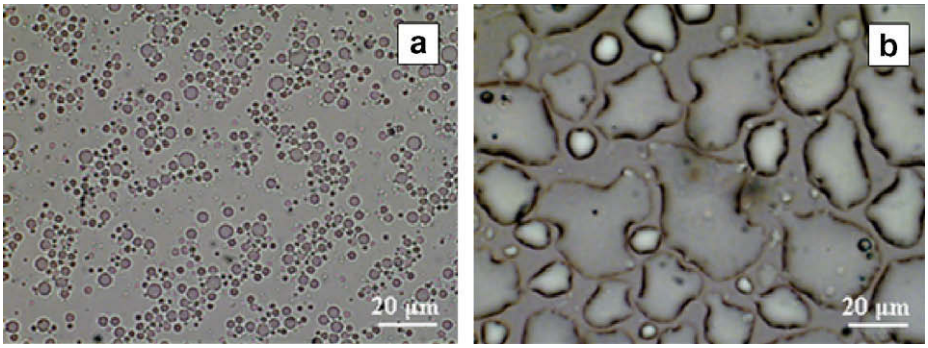

Fig. 2. Optical microscopic images of perfluorohexane-filled lysozyme microspheres (a) before and (b) after heating at 57 °C.

larly, the adsorption of lysozyme at the organic liquid/water interface might be critical for effective cross-linking. It can be speculated that lysozyme adsorption at the liquid/water interface (when an emulsion is produced by sonication) is effective in perfluorohexane system due to the lower surface tension of this liquid (Table 1), which leads to the formation of a relatively stronger/thicker shell wall when the microspheres are produced. The adsorption of lysozyme at the liquid droplet/water interface might be weaker with other organic liquids. This leads to a relatively weaker cross-linked (or porous) shell that collapses upon solvent evacuation.

Separate experiments by heating the microspheres up to 57 °C were carried out in order to find out if perfluorohexane is present in the gas phase within the microspheres at higher temperatures. It can be expected that perfluorocarbon microemulsion may partly undergo the phase transition from liquid to gaseous states upon sonication and local heating. The optical microscopic analysis (Fig. 2) of heated microspheres showed the release of the perfluo-

rohexane in the liquid form, which coalesce to large liquid droplets.

3.2. Probing the liquids encapsulated inside the lysozyme microspheres

Further experiments were carried out in order to confirm the encapsulation of the liquid inside the microspheres, where a fluorescent dye, Nile red, was used. Nile red is an uncharged heterocyclic molecule and thus is soluble in organic solvents, but its solubility in water is less than 1 μg ml^{-1}. This property makes Nile red suitable for probing the encapsulation process. The fluorescence microscopic picture in Fig. 3a shows the uniform size distribution of Nile red/tetradecane filled microspheres. The confocal laser scanning microscopy (CLSM) image of Nile red/tetradecane-filled lysozyme microsphere (Fig. 3) shows that the fluorescence colour observed is distributed homogeneously across the microsphere's cross-section, indicating that the microsphere is filled with tetradecane containing Nile red.

3.3. Ultrasound-induced release of liquid

In order to evaluate the possibility of using the ultrasonic encapsulation technique for "delivery" applications (e.g., flavour, drug, etc.), the Nile red/solvent-encapsulated microbubbles were ultrasonically broken and the release of the liquid into the aqueous medium was monitored using the solvent-dependent fluorescence properties of the dye [16–19]. The emission spectrum of Nile red exhibits one band in methanol and ethanol [17,18], whereas two bands in hexane [17] and dodecane [19]. The ratio of the emission intensity of the first to the second band (I_1/I_2) is found to be dependent on the solvent environment. Fig. 4a shows the fluorescence spectrum of Nile red in tetradecane. The emission spectrum in the range 500–700 nm shows two distinct band maxima at 532 and 573 nm. The ratio of intensity of the first band (I_1:532 nm) to the second band (I_2: 573 nm) is ~1.5 in pure tetradecane.

In Fig. 4b, the I_1/I_2 ratios observed in different solution environments are shown. First, consider the case where tetradecane containing Nile red was mixed with water at different proportions. The I_1/I_2 ratio for tetradecane:water 50:50 is about 1.5, which is similar to that observed in pure tetradecane. However, this ratio increases to about 2.8 when tetradecane:water ratio is changed to 25:75. This change could be due to the direct contact of solvent containing Nile red molecules with water or due to the additional contribution of emission form Nile red dissolved in water. As shown in Fig. 4b, the I_1/I_2 ratio observed in pure water is about 2.1. In summary, if Nile red/tetradecane is isolated from water, then the I_1/I_2 ratio can be expected to be closer to 1.5 and if Nile red/tetradecane is in contact with water, then the I_1/I_2 ratio can be more than 2.

In fact, when Nile red/tetradecane is encapsulated within the protein shell, the solvent is not in direct contact with water and

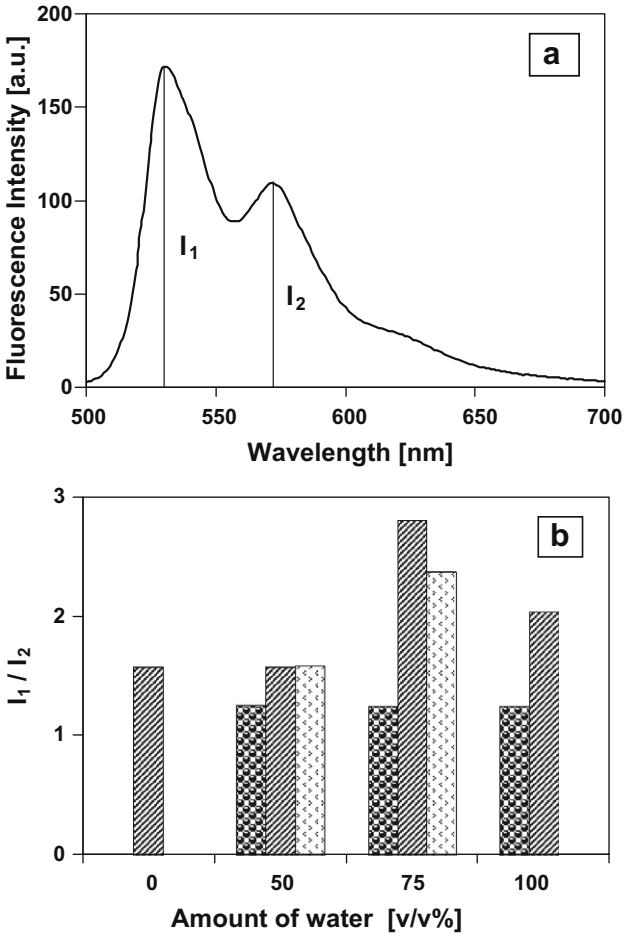

Fig. 4. (a) Fluorescence spectrum of Nile red in tetradecane and (b) The intensity ratios of peak at 532 nm (I_1) and peak at 573 nm (I_2): ▨ – Nile red in tetradecane mixed with 0, 50, 75, and 100 v/v% water. ▩ – unbroken Nile/tetradecane-filled lysozyme microspheres in tetradecane and water mixtures v/v % 50:50, 25:75 and 0:100. ▨ – broken Nile/tetradecane-filled lysozyme microspheres in tetradecane and water mixtures v/v% 50:50 and 25:75.

hence a I_1/I_2 ratio of about 1.5 can be expected if the encapsulated microspheres are dispersed in 50:50 tetradecane:water mixture. Indeed, the I_1/I_2 ratio shown in Fig. 4b for the unbroken Nile red/tetradecane microspheres is similar to that observed for Nile red in tetradecane without the microsphere. This indicates that the organic solvent is encapsulated within the microsphere and the protein shell acts as a barrier between the solvent and water.

This argument can be further supported by breaking the microsphere shell and releasing the encapsulated content into the water

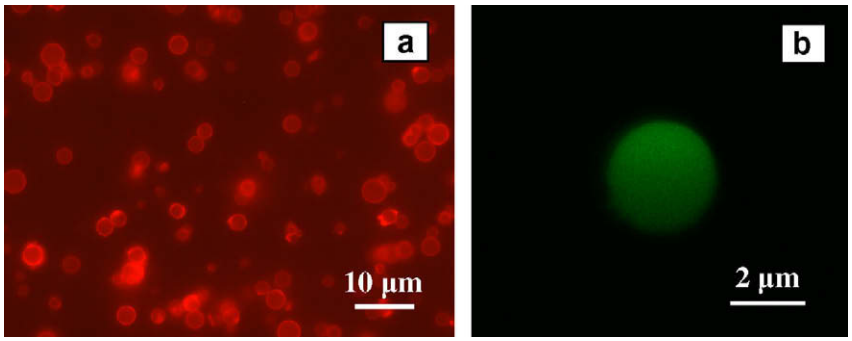

Fig. 3. (a) Fluorescence and (b) confocal laser scanning microscopic images of Nile red/tetradecane-filled lysozyme microspheres.

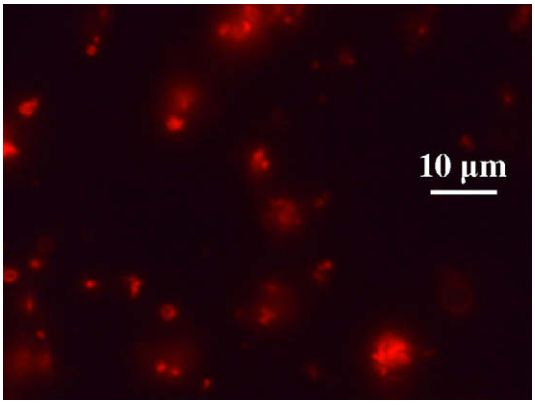

Fig. 5. Fluorescence microscopic image of Nile red/tetradecane-filled lysozyme microspheres after 10 min sonication at 355 kHz.

phase. When the microspheres were broken by sonicating at 355 kHz at 30 W for 10 min and the liquid was released into water, the ratio changes to about 2.5, which is similar to that observed when Nile red was dissolved in tetradecane and mixed with 75% water in the absence of the microspheres. Also, the fluorescence microscopic picture shown in Fig. 5 supports the argument that the microspheres were broken during sonication. Compared to the microscopic picture shown in Fig. 3a, the microspheres shown in Fig. 5 do not show the spherical shape. Thus, the observation that I_1/I_2 ratios are similar for Nile red in tetradecane/water mixture after the microspheres were broken illustrates that the encapsulated materials can be released into specific sites by ultrasonically breaking them.

4. Conclusions

Liquid-encapsulated lysozyme microspheres were obtained by the sonochemical method. The size and the stability of these microbubbles were found to be dependent on the nature of the encapsulated materials. Using an oil-soluble fluorescent solute, the potential use of this technique for encapsulating specific materials within the microspheres and the controlled release of these materials from the microspheres have been demonstrated. This method can be conveniently adopted into many potential applications in medicine and food industry, since the liquid could be replaced by a great of variety of water-insoluble drugs or food ingredients.

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