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An Easy Sonochemical Route for the Encapsulation of Tetracycline In Bovine Serum Albumin Microspheres

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Protein microspheres have a wide range of biomedical applications, including their use as echo contrast agents for sonography,[1] magnetic resonance-imaging contrast enhancement,[2−4] and oxygen and drug delivery.[5−6] In the late 1960s, a modified polymerization method for the preparation of proteinaceous microspheres was developed by Rhodes, Scheffel, Wagner, Zolle, and co-workers.[7−9] The microsphere formation was accomplished by a modified emulsion polymerization method, using either heat denaturation at various temperatures, or by a cross linking agent such as gluteraldehyde. However, this method yielded microspheres with a short storage life, low stability, and high toxicity.

A sonochemical method has been developed by Suslick and coworkers for the synthesis of nonaqueous liquid-filled microcapsules and air-filled microbubbles. Suslick has shown that micrometer-sized gas- or liquid-filled proteinaceous microspheres can be produced from various kinds of proteins such as bovine serum albumin (BSA),[10−12] human serum albumin (HAS),[13] and hemoglobin (Hb).[14]

The mechanism for the preparation of proteinaceous microspheres using the sonochemical method has been discussed previously.[15] According to this mechanism, the microspheres are formed by chemically cross-linking cysteine residues of the protein with an HO_2 radical formed around a micrometer-sized gas bubble or a nonaqueous droplet. The chemical cross-linking is responsible for the formation of the microspheres and is a direct result of the chemical effects of ultrasound radiation on an aqueous medium. Recently, we have demonstrated that this mechanism is not general and cannot be applied to streptavidin, since the latter does not contain cysteine residues in its structure.[16] Proteinaceous microspheres are excellent drug delivery vehicles, especially albumin microspheres. Albumin is an attractive macromolecular carrier and widely used to prepare microspheres due to its availability in pure form and its biodegradability, nontoxicity, and nonimmmunogenicity.[17] A number of studies have shown that albumin accumulates in solid tumors[18,19] making it a potential macromolecular carrier for the site-directed delivery of antitumor drugs. In the current work BSA microspheres were used as an antibiotics carrier. The antibiotic used for the encapsulation was tetracycline (TTCL). TTCL antibiotics have a broad spectrum of activity, are relatively safe, can be used by many routes of administration, and are widely used. TTCLs bind reversibly to the small subunits of bacterial (and eukaryotic) ribosomes where they interfere with binding of charged-tRNA to the "Acceptor" site. Various methods of delivery systems for the controlled release of TTCL have been investigated before. Poly (L-lactide) fibers,[20] poly (L-lactide) barrier membrane,[21] PLGA films,[22] and Bioerodible Hydrogel[23] were used for this purpose. Chitosan microspheres[24] and poly (DL-lactide-*co*-glycolide) microspheres[25] have also been investigated.

In the current work, we have loaded TTCL molecules inside BSA microspheres using the sonochemical method.[14−15] This was achieved in a one-step process starting with the native BSA and TTCL. The product was analyzed and characterized by SEM and DLS measurements. The amount of TTCL loaded in the microspheres was also determined.

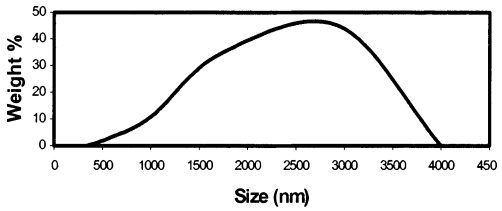

Figure 1. Particle distribution of an aqueous suspension of TTCL-loaded bovine serum albumin microspheres, determined with Coulter particle analyzer (N4 plus).

TTCL loaded in BSA microspheres was prepared using the sonochemical method.[26] Mesitylene (20 mL, 97% Aldrich) was layered over 30 mL of a 5% w/v aqueous BSA (albumin, bovine fraction v, Sigma) solution. (The details are presented in the Supporting Information section.) A separation flask was used to separate the product from the mother solution. The separation was accomplished within a few minutes, due to the lower density of the microspheres as compared to water. We waited 24 h to ensure a complete phase separation. We have repeated the preparation of TTCL-loaded microspheres by using a different concentration of the drug in the precursor solution. The amount of TTCL loaded in the microspheres was determined by subtracting the amount of the drug in the microspheres' residue phase (the lower phase in the separation flask) from its total amount in the precursor solution. The sonochemistry did not destroy the TTCL as evidenced by the very small changes in the TTCL concentration (less than 5%) which occur during sonication in the absence of BSA. No residues of TTCL were found in the excess mesitylene (the upper phase). The amount of TTCL loaded was assayed using a Cary 100 spectrophotometer at 350 nm, and the actual values were calculated using a calibration graph. The concentration of TTCL was computed in water as grams in 30 cm^3 of liquid solution.

Size determination was conducted by dynamic light scattering (DLS) measurements, employing a Coulter particle analyzer instrument (N4 plus). The size of the microspheres was measured at a 90° scattering angle and at 20 °C. The computations were based on taking viscosity and refractive index values of 1.002 centipoises and 1.33299, respectively.

The sensitivities of the tested bacterial strains to TTCL were determined by the agar diffusion technique on nutrient agar plates.[27] Two reference strains, *Staphylococcus aureus* and *Escherichia coli*, were employed in these tests.

The distribution of the sizes is determined by the DLS measurements and is presented in Figure 1. This figure shows that there is a wide distribution of particle sizes ranging between 400 and 2800 nm, falling more sharply between 3000 and 4000 nm. The average diameter is 2.5 μm. This size distribution is almost identical to that for microspheres which were prepared without the addition of TTCL.

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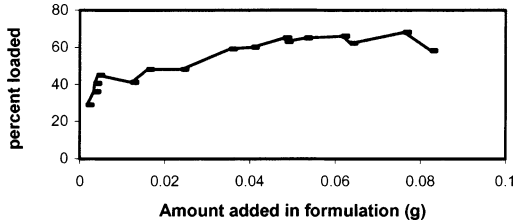

Figure 2. The amount of TTCL (in percent) that was encapsulated in BSA microspheres

The TTCL loading studies showed that the maximum TTCL loading capacity was found to be 65%. The percentage of the loaded drug in the BSA microspheres increased with the increase in the concentration of the TTCL in the original solution (Figure 2). However, this behavior changed when the concentration of TTCL reached 3.6×10^{-3} M. At this concentration saturation is obtained, and the percentage of the drug in the microsphere does not grow with an increase of its concentration in the precursor solution, The increase in the amount of TTCL that was loaded in the microspheres can be explained by understanding the sonochemical method. The microspheres are formed by chemically cross-linking cysteine residues of the protein with an HO_2 radical formed around a micrometer-sized gas bubble or a nonaqueous droplet. The TTCL was not very soluble in the mesitylene solution; however, it was found that TTCL partially dissolves in mesitylene after the sonochemical reaction. These results were found when applying the sonochemical reaction to a liquid solution which does not contain BSA. In this experiment, TTCL was found in the excess mesitylene. (The minimum ratio of TTCL concentration in the residue aqueous phase to the concentration in the excess mesitylene phase was 22:1.) The droplet of the solution trapped upon the collapse of the bubble encapsulates the mesitylene and the TTCL molecules. The higher the TTCL concentration in the solution, the higher is the amount of TTCL loaded in the microspheres, until it reaches a maximum, However, since the solubility of TTCL in mesitylene is limited, saturation is obtained when this limit is reached. The saturation reached at a certain percentage of entrapped TTCL is due to molecules of TTCL leaving the microspheres via the walls, and the equilibrium that is attained between leaving and entering the molecules.

The following steps were carried out to find whether the measured amount of tetracycline is due to molecules adsorbed on the outer surface of the sphere or due to the TTCL molecules encapsulated inside the microspheres. The first experiment ivolved heating (50 °C) the as-prepared solution in a separation flask for 5 h. TTCL that is adsorbed on the surface of the microspheres is know to dissolve in hot water. After a complete separation the amount of TTCL in the residue phase (water) was measured. The average of a few experiments yielded 4% of TTCL adsorbed on the surface of the microspheres. The second experiment was carried out by measuring the absorbance of a sample of TTCL microspheres before and after washing with ethyl acetate/diethyl ether (6:4).[28] The results yielded a value of 1−3% of TTCL adsorbed on the microsphere surface. The differences between the results of these two experiments can be explained by assuming that even during a gentle heating (the first experiment) some microspheres can be destroyed. Nevertheless, these results indicate that most of the TTCL molecules are found inside the microspheres and only a very small amount on the surface. The high capacity of the microspheres, which were produced by the sonochemical method, can be used in the future for antibiotic treatment.

The antimicrobial activity of the TTCL loaded in BSA microspheres was tested on two bacterial strains that are sensitive to TTCL (see Figure 3). One strain, *S. aureus*, represents the Gram-positive bacteria and the other, *E. coli*, represents the Gram-negative bacteria. Each of the strains was spread on nutrient agar plates,

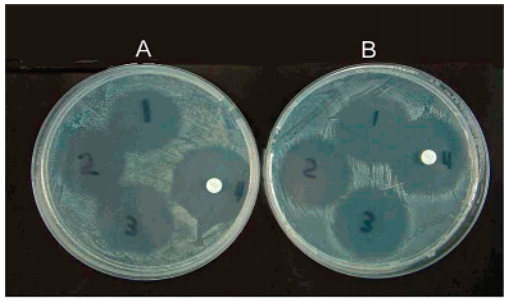

Figure 3. Zones of inhibition of *S. aureus* (A) and *E. coli* (B) on agar plates as a result of: (1) Tc freed from micospheres, (2) Tc trapped in microspheres, (3) fresh Tc, (4) Tc.

and 20 μL of each of the tested samples was put on the seeded plates. Microspheres loaded with TTCL have shown an inhibition zone around both bacteria of 30 mm. This inhibition zone was almost equal to the inhibition zones obtained by the TTCL that was freed from the microspheres by increasing the temperature or the zone of freshly prepared TTCL at the same concentration (30 μg). A TTCL disk that contain also 30 μg of the antibiotic and was used in clinical diagnostic, showed the same inhibition zone. It seems that the TTCL trapped within the microsphere and released to the medium is equally as active as the TTCL freed from the microspheres by heating. Both sonochemically treated TTCLs are active as antimicrobial agents to the same degree as TTCL, which was not sonochemically, treated.

Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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