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One-Step Preparation of Multifunctional Chitosan Microspheres by a Simple Sonochemical Method

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Abstract: Chitosan is a biodegradable natural polymer with great potential for pharmaceutical applications due to its biocompatibility, high charge density, nontoxicity, and mucoadhesion properties. Processing techniques for the preparation of chitosan microspheres have been extensively developed since the 1980s. The present paper describes for the first time a fast and one-step process for the preparation of stable chitosan microspheres by a simple sonochemical method. The microspheres were characterized by their particle size, surface morphology, stability, and drug-entrapment efficiency. The average size of the microspheres was found to be around 1 μm with a narrow size distribution, which enabled them to be used for in vivo applications. The encapsulation of different dyes into these microspheres was readily achieved with more than 75% efficacy by dissolving them into the organic phase before sonication. The chitosan microspheres demonstrated excellent stability toward acidic and basic conditions ranging from pH 4 to 9, thereby indicating their implementation as possible therapeutic and diagnostic agents. The stability of these microspheres appears to be contributed from intermolecular imine cross-linking in addition to other noncovalent interactions. The ability of the surface-exposed amino groups of chitosan microspheres to undergo chemical conjugation with potential drugs and/or targeting vectors was determined by their reaction with fluorescein isothiocyanate (FITC) and fluorescamine followed by confocal microscopy.

Keywords: chitosan · drug delivery · fluorescence · microspheres · sonochemistry

Introduction

Over the past few years, many methods have been developed for the preparation of a large variety of microspheres. The search for new synthetic routes has been motivated by the wide range of microsphere applications including microencapsulation of dyes, flavors and fragrances, drug-delivery systems, and contrasting agents.[1–7]

Among the different methods that have been developed to synthesize microspheres, preparation of proteinaceous microspheres (PM) has gained considerable attention. A modified polymerization method for the preparation of such microspheres was first developed by Rhodes et al. in the late 1960s.[8–10] Suslick et al. found a remarkably easy sonochemical technique for the preparation of both air-filled microbubbles and nonaqueous liquid-filled PM.[11] Using this approach, bovine serum albumin,[12] hemoglobin,[13] and human serum albumin[12] microspheres were generated in only a 3 min sonication process. The PM were filled with *n*-dodecane, *n*-decane, *n*-hexane, cyclohexane, or toluene.

The mechanism proposed by Suslick for the formation of the microspheres relied on the presence of cysteine thiol groups in the native protein,[11,12] which are easily oxidized by the superoxide radical generated under sonication conditions[12,14,15] into S–S bonds, thereby holding together the 2–5 μm-sized sphere. However, Gedanken et al. demonstrated that microspheres can be produced sonochemically even from streptavidin or polyglutamic acid, which do not contain cysteine residues in their structure.[16] Intermolecular inter-

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actions between the proteins (hydrogen bonding, van der Waals, hydrophobic, and electrostatic interactions) after the initial ultrasonic emulsification were suggested to assist microsphere formation.

The present work reports on the sonochemical preparation of microspheres from the non-sulfur-containing polyglucosamine, chitosan. Chitosan (poly-[1→4]-β-D-glucosamine) is a unique basic polysaccharide obtained by deacetylation of the natural polymer chitin. With a primary amino group at C2 and a hydroxyl group at the C3 and C6 positions, chitosan undergoes a series of chemical reactions under mild conditions. In recent years, chitosan has gained considerable attention due to its high potential in biomedical and pharmaceutical applications,[17,18] including hybrid antitumor drugs,[19,20] immunostimulators,[21–23] and biocompatible and biodegradable tissue-engineering materials.[24–26] Moreover, it exhibits potent antimicrobial activity[27,28] and has been widely used in biomedical and agricultural areas[29–31] for its nontoxicity.

Chitosan microspheres have been prepared by various methods,[32] such as interaction with anions,[33,34] complex coacervation,[35] modified emulsification and ionotropic gelation,[36,37] cross-linking with a naturally occurring agent (genipin),[38] glutaraldehyde cross-linking,[39] and thermal cross-linking.[40] However, limited control of the size and size distribution of the microspheres, poor mechanical strength in cases of physical cross-linkers, denaturation of protein and peptide drugs, and difficulties in the release and degradation of the chitosan carrier at physiological conditions due to excessive chemical cross-linking are still challenging.

The present paper describes a novel, fast, one-step process for the preparation of stable chitosan core–shell microspheres (CTSM) with narrow size distribution using ultrasound radiation. The microspheres can be prepared in solutions with a wide range of pH values (4–9), and the capability of the microspheres to encapsulate different compounds or drugs was demonstrated by entrapping a fluorescent dye inside the microspheres. Moreover, the free amino groups of the chitosan on the shell of the microsphere were conjugated, under mild conditions, with different fluorescent dyes, thereby suggesting possible modification of the surface of the microsphere with different targeting vectors such as peptides, proteins, or small molecules for different therapeutic or diagnostic purposes.

Results and Discussion

Chitosan microspheres were prepared sonochemically from an aqueous solution of different sources of chitosan with different degrees of N-deacetylation. Vegetable oil, such as soybean, or dodecane was used as the cosolvent, and the sonochemical process was continued for only 3 min. Chitosan microspheres were effectively generated in solutions that had a pH ranging from 4 to 9, even though the solubility of chitosan at pH 9 is very low. The low solubility of chitosan in natural and basic pH actually limits its applications in many biomedical and biopharmaceutical fields, and therefore considerable efforts have been directed toward the synthesis of chitosan derivatives with enhanced solubility in solutions with neutral pH.[41–43] The successful generation of microspheres in solutions with neutral-basic pH is probably related to the spherization process, which, according to the Le Chatelier rule, forces new molecules to dissolve. In all cases, the microspheres had a spherical shape as observed by microscope images and electron microscopy analysis, similar to the proteinaceous microspheres obtained by Suslick and coworkers.[11] The microspheres demonstrated a narrow size distribution, as revealed by optical micrograph and SEM analysis (Figure 1). The Gaussian size distribution of the microsphere generated either with dodecane or soybean oil is plotted in Figure 2. The average size of the microspheres prepared using soybean oil as the cosolvent was found to be somewhat smaller than those prepared in dodecane (0.78 vs. 1.24 μm), which basically allows their application for in vivo applications.

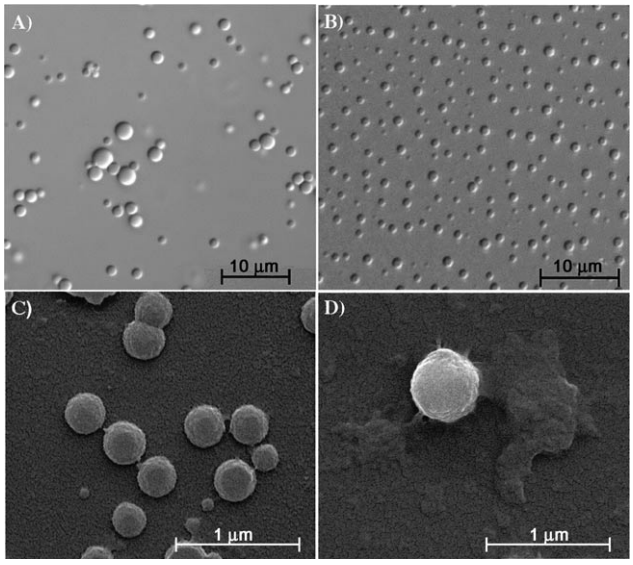

Figure 1. Microscope images (×100) of chitosan microspheres prepared in A) dodecane or B) soybean oil, and corresponding SEM pictures prepared in dodecane (C, D).

The cross-sectional TEM analyses revealed that the observed microspheres are core–shell structures (Figure 3). To further verify that these microspheres are indeed core–shell structures, the chitosan solution was sonicated with dodecane containing Nile red as a fluorescent dye that dissolves only in the organic phase. The generated microspheres were then reacted with fluorescein isothiocyanate (FITC) under basic conditions (pH 8.5) to label the chitosan shell. Figure 3 shows the cross-sectional TEM and fluorescent images of fluorescently labeled chitosan microspheres. The images illustrated that the core of the microspheres is filled with the organic solution, whereas the shell is composed of the polymeric chitosan. These data also demonstrated that the free

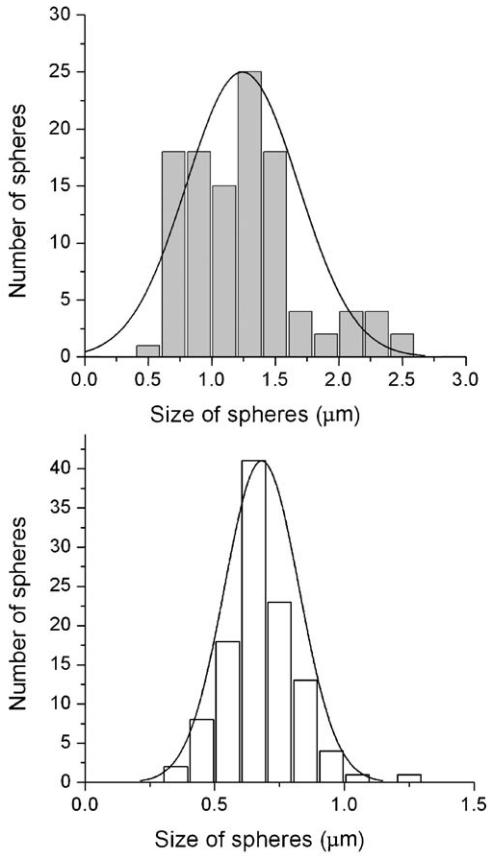

Figure 2. Particle size distribution of an aqueous suspension of chitosan microspheres measured with an optical microscope, and processed by Scion image software.[50] Chitosan microspheres were prepared with either dodecane (top) or with soybean (bottom) as the cosolvent.

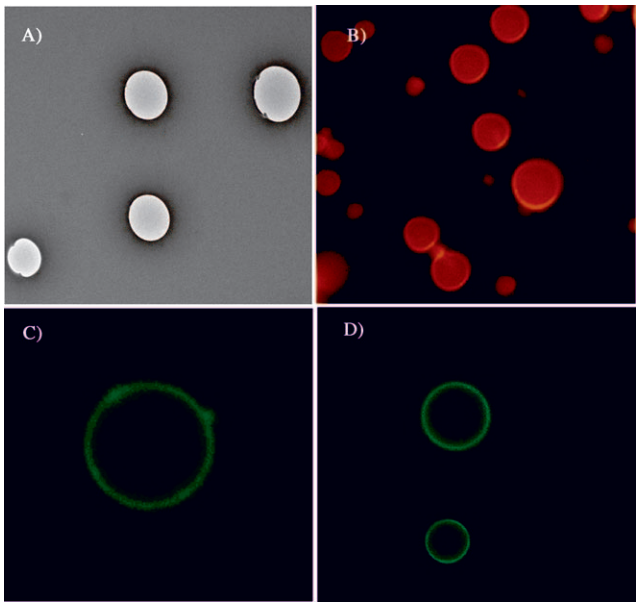

Figure 3. The chitosan microspheres generated in the presence of soybean or dodecane are spherical core–shell structures as show by A) the cross-sectional TEM analysis of embedded microspheres generated in soybean and B) a fluorescent image of microspheres encapsulated with Nile red and visualized by using a fluorescent microscope. Chitosan microspheres were reacted with C) FITC or D) fluorescamine and visualized by fluorescent microscopy (×100). The free amine groups of microspheres are reactive toward different electrophiles.

amine groups of the chitosan could undergo chemical interactions, such as nucleophilic interaction with appropriate electrophiles. The nucleophilic reactivity of the amine groups of the chitosan microspheres was further confirmed by their reaction with the nonfluorescent probe fluorescamine, which fluoresces upon its interaction with free amines. Figure 3D shows the fluorescent micrographs of chitosan microspheres following their interaction with fluorescamine. The fluorescent signal appeared mainly on the shell of the microspheres, thereby suggesting that the amine functionality of the chitosan can be conjugated to different targeting vectors, such as small molecules, or even peptide or protein vectors for targeting specific organs or cancer cells. The attachment of the proteins or peptides to chitosan microspheres can be potentially carried out by thiolation of the free amino groups of the chitosan by using, for example, 2-iminothiolane followed by the addition of peptides or proteins analogues containing a maleimido group.[44] Recently, Suslick et al. have reported the preparation of proteinaceous microspheres coated with the Arg–Gly–Asp (RGD) peptide for targeting cancer cells that overexpress integrin receptors. Although the peptide was not covalently attached to the microspheres, the fluorescence microscopy studies revealed that these modified microspheres are selectively bound and taken up by HT29 human colon cancer cells in vitro.[45]

Chitosan microspheres were also characterized by FTIR spectroscopy (Figure 4), which clearly shows the peaks of dodecane (Figure 4B) encapsulated in the chitosan core. A

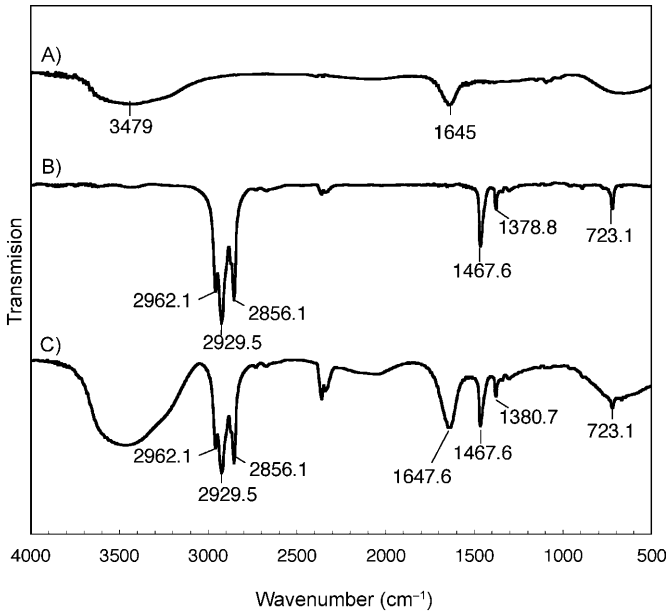

Figure 4. FTIR spectra of A) free chitosan, B) dodecane, and C) chitosan core–shell microspheres.

shift of approximately 2.5 cm^{-1} (from 1645 to 1467.6 cm^{-1}) was observed for the chitosan peak of the microspheres as compared to free chitosan, which possibly arises from the formation of an imine bond (−C=N) between free amine groups and the reduced end (aldehydic form) of the sugar moiety.[46] Therefore, we assume that intermolecular imine bond formation in addition to other interactions, such as hydrogen bonding, van der Waals, hydrophobic, and electrostatic interactions, are responsible for the generation of chitosan microspheres.

The stability of the chitosan microspheres under different physiological conditions is a prerequisite for their successful application. Thus, the pH-dependent stability of the microspheres was determined in different buffer solutions at 37 °C. The samples were shaken continuously and the volume of the microsphere was observed for 7 days. Although the microspheres demonstrated high stability toward basic conditions (pH 9) for 7 days, they partially dissolved in natural and acidic media (pH 7.5 and 4) after a few days, probably due to the low stability of the imine bond at low pH.

To investigate the efficacy of the sonochemical process toward microsphere generation, increasing amounts of chitosan (1–10 mg) were sonicated in the presence of soybean or dodecane, and the amount of free chitosan in the aqueous solution was then determined by a fluorescentic method by using fluorescamine. A standard curve was generated from the reaction of known concentrations of chitosan with fluorescamine; it was found to be linear in the ranges we used. The data suggest that more than 85 % of the chitosan was consumed in the generation of the microspheres, irrespective of the concentration used. The encapsulation efficacy of the chitosan microspheres was evaluated by using a water-insoluble dye such as Sudan III as the encapsulating agent. Following the completion of the reaction and separation of the two phases, the total volume of the dodecane containing the dye was reduced to 25 % of its original volume and the concentration of the dye in dodecane was not changed. Therefore, we concluded that 75 % of the dye was encapsulated in chitosan microspheres. Moreover, the amount of the dye encapsulated inside the microspheres was quantified after they were destroyed by centrifugation at high speed. We have found that the amount of the encapsulated dye could reach as high as 0.1 mg per milliliter of microspheres.

Conclusion

High-intensity ultrasound was used to prepare core–shell chitosan microspheres. The size of the microspheres was found to be around 1 μm. The microspherization process involves the formation of imine bonds, in addition to hydrogen bonds, which stabilize the structure of the microspheres. The formation of the chitosan microspheres reveals that the role of the disulfide bonds that accounted for the formation of proteinaceous microspheres is limited only to these microspheres. The current results open a wide range of possibilities for creating microspheres from polysaccharides and other biochemical sources that do not contain cysteine or sulfhydryls in their structure.

The major advantage of the sonochemical method, compared with other techniques used for the preparation of chitosan microspheres, is the ease and shorter reaction time. For example, the preparation of chitosan microspheres by means of emulsification and ionotropic gelation[47] requires about 3 h, whereas in the sonochemical process the microspherization of chitosan takes only three minutes. Another advantage of the sonochemical process is that cross-linking chemicals such as tripolyphosphate[34] or glutaralgehyde[48] are not required for the spherization process. The average microsphere size of approximately 1 μm with a narrow size distribution is much smaller than the 20–70 μm for the chitosan microspheres prepared by emulsification and ionotropic gelation.[49] The other advantage of the sonochemical process over the other microspherization techniques is that by using sonochemistry chitosan microspheres can be obtained even at a pH of 9, whereas all the other techniques are limited to pH 7.5.[41–43] Finally, the surface of the microspheres could be easily modified by different electrophiles to generate a covalent bond between microspheres and other molecules, such as tissue- and tumor-targetable peptides and proteins.

Experimental Section

Unless otherwise stated, all the solvents and reagents used in this study were purchased from Sigma–Aldrich and used as received. Chitosan (originating from *Agaricus bisporus*) with a molecular weight of 50.17 kDa and an 87 % degree of N-deacetylation was purchased from KitoZyme (Herstal, Belgium). Medium-molecular-weight and low-molecular-weight chitosan (originating from shrimp shell) with 75–85 % degree of deacetylation was obtained from Sigma–Aldrich.

Preparation of chitosan microspheres: Chitosan (2.5–11 mg) obtained from different sources was dissolved in double-distilled water (30 mL, pH 5.5) in a cylindrical vessel and a layer of dodecane or soybean oil (20 mL) was placed over the top. The tip of a high-intensity ultrasonic probe was placed at the aqueous–organic interface, and the mixture was sonicated (150 $W cm^{-2}$, 20 kHz) for 3 min, while it was cooled by an ice/water bath. After the synthesis, the microspheres were separated from the unreacted chitosan by leaving the reaction mixture at 4 °C for 24–36 h, and washed with sufficient volumes of distilled water by centrifugation at 800 rpm for 15 min.

Characterization of chitosan microspheres: The shape and surface of the microspheres were determined using scanning electron microscopy, SEM (FEI Quanta 200 FEG, Hillsboro, Oregon, USA) and an optical microscope (Apo-Tome AxioImager.Z1 microscope, Zeiss). SEM samples were prepared by applying a drop of the suspension of the microsphere onto the glass wafer, followed by drying and gold sputtering. For cross-sectional TEM images, the microspheres were embedded in epoxy resin before sectioning with a microtome (RMC PT-X & PT-XL PowerTome Ultramicrotomes, Arizona, USA). The morphology of the microsphere slices was characterized by TEM (FEI Tecani G2, Hillsboro, Oregon, USA). The size of the microspheres was determined by optical microscopy. A Scion image software program[50] was applied to measure the distribution of sizes of the particles captured with an optical microscope. The statistics were derived from the measurement of more than 100 particles. The FTIR spectra of the microspheres were recorded using a Nicolet FTIR spectrometer (Impact 410), using KBr pellets.

Preparation of Sudan III-encapsulated microspheres: Chitosan microspheres were loaded with different concentrations of Sudan III as a model for different water nonsoluble compounds. Various amounts of Sudan III (0.5–3 mg) were directly dissolved in dodecane and irradiated in the presence of aqueous solution of chitosan. The microspheres were washed with water as described above.

Quantitative measurement of the yield of microsphere synthesis: The amount of free chitosan before and after microsphere generation was determined by reacting it with a fluorescamine probe. Briefly, a sample of chitosan (25 μL) was diluted with an equal volume of tris(hydroxymethyl)aminomethane (Tris) buffer (10 mM, pH 8.5) and reacted with fluorescamine solution (10 μL, 1 mg mL^{-1} DMF) in a black 96-well plate. The mixture was incubated at 37 °C for 1 h and the fluorescent signal was then measured by a fluorescent plate reader (Tecan, Switzerland) using an excitation of 390 nm and emission of 480 nm. The amount of free chitosan in samples taken before and after microsphere preparation was extrapolated from a standard curve generated from known concentrations of chitosan solutions reacted under the same conditions with fluorescamine. The yield of the microsphere preparation was then determined by comparing the amount of chitosan before and after the sonication.

Preparation of FITC- and fluorescamine-labeled chitosan microspheres: Chitosan microspheres (200 μL) were reacted either with fluorescein isothiocyanate (FITC, 200 μL, 0.2 mg/1 mL) in phosphate-buffered saline (PBS) solution (75 mM, pH 7.4) or fluorescamine (20 μL, 1 mg mL^{-1} DMF) in Tris/HCl buffer (10 mM, pH 8.5), and the mixture was shaken at room temperature for 2 h. The microspheres were then separated from unreacted FITC and fluorescamine by centrifugation and washed with water (3 times) to remove the residue of unreacted probes. The microspheres were then visualized using a fluorescent microscope (Apo-Tome AxioImager.Z1, Carl Zeiss), by employing appropriate filter sets.

Determination of encapsulation efficiency and loading: The encapsulation efficiency was determined by sonicating different amounts of Sudan III (3–10 mg) in dodecane (20 mL) and chitosan (5 mg) in aqueous solution (30 mL) as described, followed by separation of the aqueous solution from dodecane and measuring the amount of the dodecane left. Sudan III was dissolved exclusively in dodecane and was encapsulated together with dodecane in the core of the chitosan microspheres. The concentration of Sudan III in dodecane was determined spectroscopically before and after sonication using the known excitation coefficient of the Sudan III (28766 M^{-1} cm^{-1}) at 523 nm and was found to remain unchanged after the sonication process. Therefore, the encapsulation efficacy (*EE*) was calculated by comparing the volume of the dodecane before and after sonication as follows [Eq. (1)]:

$$EE = \frac{V_0 - V}{V_0} \times 100\% \quad (1)$$

in which V_0 is the total volume of dodecane and V is the amount of the dodecane that was left after the sonication process.

To calculate the amount of Sudan III encapsulated in the microspheres, a known volume of loaded microspheres (1 mL) was destroyed by centrifugation at 13400 rpm for 10 min, and the amount of the free dye in the solution was determined by UV spectroscopy. All samples were analyzed in duplicate.

Measurement of the stability of the microspheres in different pH solutions: The stability of chitosan microspheres prepared with either dodecane or soybean oil was determined by shaking the microspheres (0.5 mL) under different acidic and basic conditions, including formic acid buffer (100 mM, pH 4.0), PBS buffer (100 mM, pH 7.4), and Tris/HCl buffer (100 mM, pH 9), and the changes in the volume of the microspheres were monitored for 7 days.

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