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Role of Counterions in Controlling the Properties of Ultrasonically Generated Chitosan-Stabilized Oil-in-Water Emulsions

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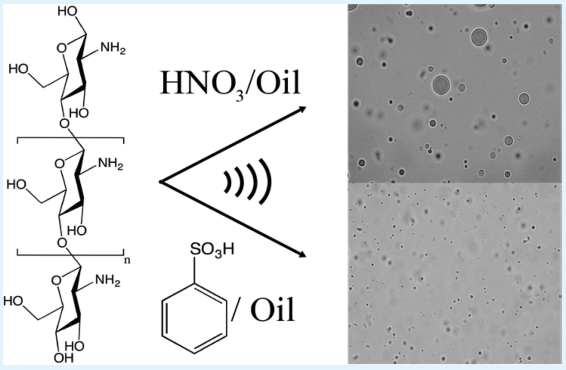
Supporting Information

ABSTRACT: An oil-in-water emulsion was ultrasonically prepared in aqueous chitosan solutions containing different counterions. Tetradecane was used as the oil phase in order to mimic nonpolar substances used in food processes. Various acids were used to dissolve chitosan, and we found that conjugate bases of the acids used, which act as counterions to neutralize the positive charges of ammonium ions present in the chitosan backbone, played a significant role in controlling the size, size distribution, and stability of chitosan-encapsulated tedradecane emulsion droplets (microspheres). The counterion effect is also found to be strongly dependent upon tetradecane (TD)/chitosan (CS) ratio and ultrasonic power. Key observations are: (i) for a given TD/CS ratio, the size and size distribution decrease when the acid is varied from nitric acid to benzenesulfonic acid at high TD/CS ratio, and the effect becomes less significant at low TD/CS ratio; (ii) for a given acid, the size and size distribution increase with an increase in TD/CS ratio; and (iii) at low TD/CS ratio the size and size distribution are significantly influenced by the viscosity of the system. A possible mechanism for the observed counterion effect is proposed. The role of counterions, solution viscosity, and ultrasonic power in controlling the physical and functional properties of ultrasonically generated chitosan-stabilized tetradecane microspheres is discussed in detail. The key new finding of this study is that it is possible to form stable emulsions without the addition of external emulsifiers and stabilizers, but only using chitosan with different acids to dissolve chitosan. This strategy could be used in the generation of stable food emulsions.



KEYWORDS: *counterions, chitosan microspheres, ultrasound, emulsion*

1. INTRODUCTION

Chitosan is a natural amino-polysaccharide (a copolymer of 2-glucosamine and *N*-acetyl-2-glucosamine) used in a wide range of applications in industries,[1] medicine,[2] and catalysis.[3] Chitosan has also attracted attention for its potential application in controlled release or delivery of drugs[4,5] because of its solubility in aqueous medium, high specific surface area, and structural features. In addition, chitosan has many positive attributes such as excellent biocompatibility and biodegradability and low toxicity.[6] It also has biological activities such as antimicrobial activity and low immunogenicity.[7]

Nutrient encapsulation has become a major focus in the past decade for the delivery of nutritional products in common food matrices. Many substances, such as dairy proteins, have been used for the encapsulation and delivery of nutrients as stable emulsions.[8] Chitosan has also been widely used in the production of microspheres (emulsion droplets) encapsulating oil-based materials.[9,10] The amino groups in chitosan offer several functionalities such as varying solubility in aqueous solutions as a function of solution pH. For this reason, chitosan has amphiphilic characteristics in acidic solutions. Such a surfactant-like property could be used to stabilize oil-in-water emulsions, viz., oil microspheres stabilized by a chitosan shell.

Chitosan microspheres have been used in a wide range of applications. Liu et al. have reported on the use of chitosan microspheres in metal ion adsorption.[11] Chitosan microspheres combined with calcium phosphate can be used as cement for bone regeneration, as reported by Meng et al.[12] Owing to such applications, developing synthetic methods to regulate the size, size distribution, and stability of chitosan microspheres is of great importance. For example, Biró et al.[13] have studied the size control of chitosan microspheres in the presence of cross-linkers, glutaraldehyde, and polysorbate 20.

Chitosan microspheres are generally prepared using a few emulsification-based experimental procedures. The methods used to prepare these microspheres can be summarized as water-in-oil emulsion,[13] oil(1)-in-water-in-oil(2) (O1/W/O2) double emulsion,[14] and air-in-water emulsion.[15] All these procedures use a cross-linker to strengthen the structure. The

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effect of two different cross-linkers, glutaraldehyde and sodium tripolyphosphate, on microspheres' shape has been studied by Zou et al.[16]

Generally, the procedure to obtain chitosan microspheres as an oil-in-water emulsion involves dissolving the polymer in acetic acid solution. Then, a nonpolar substance (oil) is dispersed in this solution under stirring for a few hours. This process allows the generation of oil droplets stabilized by chitosan molecules. This is followed by the addition of NaOH that results in the neutralization of the charges on amino groups leading to the formation of stable microspheres. A cross-linker is often used to strengthen the structure.

Other studies have improved the amphiphilic properties of chitosan by reacting amino and carboxyl groups of chitosan with molecules containing long hydrocarbon chains. In the study conducted by Elsabee et al., chitosan surfactant-like properties were improved by covalent linking of hydrocarbon chains to the polymer backbone.[17−19] The stability of emulsion droplets strongly depends upon the nature of stabilizers. When a surfactant is used as a stabilizer, the close packing of the molecules at the oil droplet−water interface is important. When ionic surfactants are used, close packing becomes an issue due to the repulsion between charged head groups. The repulsion could be minimized by the addition of a salt. In order to strengthen the interaction between chitosan molecules on the surface of oil droplets, a counterion can be used. Pignolet et al. have highlighted the influence of surfactant counterions during electrophoretic particle deposition.[20] In their study, polystyrene microspheres were surrounded by a surfactant, and they studied the effect of different counterions in the electrophoretic deposition. They discovered that the counterion influences the microspheres transport by electrophoretic forces.

The aim of this study is to explore the effect of counterions on the emulsifying properties of chitosan using ultrasound to generate the emulsions. As reported in many studies, the ultrasonic technique not only causes fast emulsification but also generates a homogeneous dispersion.[21] A detailed study has been carried out in order to explore the effects of processing parameters on the physical and mechanical properties of chitosan microspheres in the absence of cross-linkers. The experimental variables chosen were: ultrasonic power, tetradecane/chitosan ratio, and type of acid. We have observed that the type of acid used to dissolve chitosan plays a major role in controlling the physical and functional properties of chitosan microspheres.

2. MATERIALS AND METHODS

2.1. Materials. Formic acid (99%) was purchased from Chem-Supply PTY LTD (Australia). Acetic acid (100%) and sodium hydroxide (>99%) were purchased from Merck KGaA (Germany). Hydrochloric acid (37% water solution) and nitric acid (70% water solution) were purchased from Scharlab S.L. (Spain). Benzenesulfonic acid (97%), butanoic acid (>99%), and chitosan with low molecular weight and 75−85% degree of deacetylation, propionic acid (>99.5%), and tetradecane (>99%) were purchased from Sigma-Aldrich Co. LLC (Australia). Benzoic acid (>99%) was purchased from The British Drug Houses Ltd. (England). All the reagents were used without further purification. For chitosan labeling, fluorescein isothiocyanate was used, and for marking tetradecane (TD) Nile red was used. Both reagents were purchased from Sigma-Aldrich Co. LLC (Australia).

2.2. Preparation of Chitosan Solutions. Chitosan (CS) is not soluble in any organic solvent. However, CS is soluble in acidic solutions. Rinaudo et al.[22] have mentioned that a stoichiometric ratio between acetic acid and chitosan amine groups must be at least $[AcOH]/[CS\text{-}NH_2]$ = 0.6 in order to dissolve chitosan in acidic solutions. Another study reports that the concentration of protons needed is at least equal to the concentration of RNH_2 units involved.[23] In our experiments, the amount of acid used was equal to chitosan monomeric units. The chitosan sample used had a composition of 80% 2-glucosamine and 20% *N*-acetyl-2-glucosamine with an average monomeric molecular weight of 169.4 g/mol. Using this information, the molarity of $CS\text{-}NH_2$ and hence the amount of acid required were calculated to maintain the molar ratio between the amount of acid and chitosan monomeric units.

Eight different acids were used: nitric acid, hydrochloric acid, formic acid, acetic acid, propionic acid, butanoic acid, benzoic acid, and benzenesulfonic acid. An amount of 0.8 g of CS was added to 100 mL of 0.05 M acid solution and magnetically stirred for 2 days. From each acid stock solution three dilutions were made with concentration of 0.08 mg of CS/mL (corresponding to acid concentration: 0.5 mM), 0.04 mg of CS/mL (0.25 mM), and 0.005 mg of CS/mL (0.03 mM). The relative viscosities of these solutions were measured at 23 °C.

2.3. Preparation of Chitosan Microspheres. Solutions containing six different concentrations of CS (0.08, 0.04, 0.03, 0.02, 0.01, and 0.005 mg of CS/mL) were made in triplicate. For the preparation of TD-loaded chitosan microspheres, three different amounts of TD were used. Typically, 15, 25, and 35 μL of TD was added to 1 mL of chitosan at each concentration. Thus, 18 samples were prepared in total for each acid used.

In a typical experiment, 1 mL of chitosan containing an appropriate amount of TD was taken in a centrifuge tube. A 3 mm 20 kHz horn (BRANSON Digital Sonifier Generator Model 450-D) was placed at the interface between aqueous solution/TD phases, sonicated in most cases at 10.7 W acoustic power for 30 s. Immediately after sonication, 12 mL of 0.1 M NaOH solution was added. In order to study the effect of sonication power, the following samples were prepared: 0.08, 0.04, 0.01, and 0.005 mg of CS/mL with 15 μL of TD for the first one and 35 μL of TD for the rest. These samples were used to produce microspheres at acoustic power levels of 0.4, 2.9, 5.4, 10.7, and 16.7 W (calculated by calorimetric measurements).

2.4. Characterization of Chitosan Microspheres. In order to verify the formation of TD-loaded CS microspheres, additional experiments were carried out using CS labeled with fluorescein isothiocyanate (CSFITC) and TD containing dissolved nile red (TDNR). The CS labeling was carried out using the reaction between the FITC isothiocyanate group and the chitosan amine group. The reaction between these two molecules produces a thiourea-type compound. Instead, nile red is a fluorescent dye that is not soluble in water. With these probes, it was possible to label both chitosan and oil phase without interference between them during the analyses.

To prepare CSFITC, 20 mL of chitosan solution, dissolved in acetic acid, was taken in a beaker, and 80 mL of Milli-Q water was added. The solution pH was adjusted to 4.5 using 0.1 M NaOH solution, and 0.004 g of fluorescein isothiocyanate was added. The mixture was placed under magnetic stirring for 3 h. Then, CSFITC was precipitated by adding 0.1 M NaOH until the solution pH reached 8.5. The product was collected by centrifugation and washed 5 times with Milli-Q water and 1 time with acetone. The solid was dried under vacuum overnight. All samples were analyzed the day after their preparation using an IX71 Olympus Optical Microscope with an objective Olympus UPIanFLN 60x. The size distribution was determined using optical microscopic images by measuring at least 120 microspheres per sample using the software "analysis LS Research v3.1".

2.5. Stability Analyses. The samples analyzed were TD15−0.08 mg/mL; TD35−0.04 mg/mL; TD35−0.01 mg/mL; and TD35−0.005 mg/mL of each acid solution. The medium was kept at pH 7 in order to avoid any possible dissolution of the polymer, which occurs under pH 6. The analyses were performed measuring the size and size distribution at one-week intervals for a month in order to evaluate the oil leakage from the microspheres.

2.6. Analysis of the Microsphere Core. At the end of the experiments, an additional analysis was carried out to investigate the microsphere core. All samples with benzenesulfonic acid produced at

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acoustic power of 10.7 W were analyzed. Microspheres floating at the top of the solutions were collected and washed several times with Milli-Q water. It must be noted that the initial pH was 12, and the washing of the microspheres was performed using Milli-Q water until the solution pH was about 7. The washing process also eliminated all unreacted counterions and NaOH. Acetone was then added to the collected microspheres in order to break them. Then, the acetone and the oil were removed by evaporation. This procedure was repeated three times. At the end, a mixture of 5 mL of methanol:acetone (8:2) was added to the dried broken microspheres. The mixture was then concentrated by evaporation to reduce the volume to 1 mL and analyzed by Shimadzu SCL-10AVP high-performance liquid chromatography (HPLC) equipped with a Phenomenex column model "Jupiter 5u C18 300A" and with an ultraviolet detector (UV) set at 205 nm. All chromatograms were generated by LabSolution software (Shimadzu). The injection volume was 20 μL and, the flow rate was 1 mL/min with pure methanol as eluent.

3. RESULTS AND DISCUSSION

Three different TD-filled chitosan microsphere (CS) samples, FITC labeled CS, CSFITC, CS filled with NR labeled TD, CS/TDNR, and CSFITC TDNR were prepared. Nile red and FITC were used in TD and CS, respectively, as fluorescent probes in order to confirm the formation of TD-filled microspheres.

Figure 1 shows the fluorescence microscopic images of the microspheres. It can be seen in these images that CS forms a shell around TD droplets. Figure 1A shows the image of the CS microsphere labeled with FITC. The intense fluorescence confirms the formation of the CS shell. Figure 1B shows the image of a CS microsphere where TD was labeled with NR. Unlabeled CS was used in this case. An intense fluorescence from the TD droplet is observed, confirming the encapsulation of TD within the CS microsphere. In Figure 1C, both chitosan and oil labeled were used, and the microsphere appears as a hybrid of the previous two. This is evident by the strong fluorescence intensity throughout the microsphere. These images clearly demonstrate the formation of TD emulsion droplets stabilized by the CS shell.

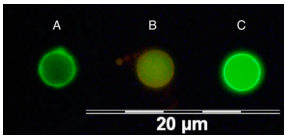

Figure 1. Comparison of the microspheres obtained with CSFITC/TD (A), CS/TDNR (B), and CSFITC/TDNR(C).

When CS is dissolved in an acid, the amino groups are protonated, resulting in the formation of positively charged ammonium ions. The conjugate base of the acid used acts as counterions. For example, when acetic acid is used to dissolve CS, acetate ions act as counterions. When the CS shell is formed around an emulsion droplet, the counterions remain in contact with charged CS to stabilize the shell structure. In order to see the role of the counterions in stabilizing the CS shell structure as well as in controlling the size and size distribution and stability of TD emulsion droplets, the nature of the counterions was systematically varied by choosing various acids containing different counterions. In addition to varying the nature of the acid, the ratio between the amount of TD and CS was also varied. This ratio was calculated as microliters of TD on milligrams of CS dissolved in the solution ($\mu L_{TD}/mg_{CS}$).

Figure 2 summarizes the results obtained in these experiments. The images show the images of the microspheres as a function of varying TD/CS ratio and acids, used to dissolve chitosan, in the order of their amphiphilicity. Starting from nitric acid, it is evident that increasing TD/CS ratio resulted in an increase in the size of microspheres. A similar trend is observed for samples obtained with different acids, with the exception of the last two acids, namely, benzoic and benzenesulfonic acids. In effect, a small decrease in size is observed with an increase in TD/CS ratio for these acids. Analyzing the images vertically at a TD/CS value of 7000, it can be seen that there is a decrease in the mean size of microspheres when the acid is changed from nitric acid to benzenesulfonate acid. This trend can be seen also at TD/CS ratios of 3500 and 875, with an exception at 187.

For clarity, the results are summarized in Figure 3.

The following inferences could be made from the optical microscopic images (Figure 2) and the data shown in Figure 3:

(i) For a given TD/CS ratio, the size and size distribution decrease when the acid is varied from nitric acid to benzenesulfonic acid at high TD/CS ratio, and the trend becomes less significant at low TD/CS ratio.

(ii) For a given acid, the size and size distribution increase with an increase in TD/CS ratio.

Considering point (i) mentioned above, it is evident that the nature of counterions strongly influences the size and size distribution of the microspheres. It is important to consider the nature of counterions used in this study. Nitrate and chloride ions are simple counterions derived from strong acids with no amphiphilic property. Formate, acetate, propionate, and butyrate ions are derived from their corresponding conjugate weak acids. The amphiphilicity of the counterions varies in the order, butyrate > propionate > acetate > formate. The last two counterions are aromatic in nature with higher amphiphilicity than the counterions mentioned above. In order to understand the amphiphilicity, HLB value (hydrophilic lipophilic balance) was calculated using Griffin's method for each counterion. Griffin's method has some limitations and does not consider the aromaticity or the spatial distribution of the substituents. The data are shown in Table S-1 (Supporting Information).

Figures 2 and 3 show that there is a strong correlation between the TD/CS ratio and mean size and size distribution. TD/CS ratio was calculated as μL/mL of tetradecane on mg/mL of chitosan. Its value gives information about the quantities of oil and polymer used in the experiments. A low ratio represents that the system has a high concentration of CS and a low quantity of oil and vice versa. It can be observed from the data that the viscosity plays a significant role in controlling the size and size distribution of microspheres. Relative viscosities of chitosan solution, at 0.08 mg_{cs}/mL, are 1.19, 1.83, and 1.33 when the acids used for dissolution are nitric, acetic, and benzenesulfonic acids, respectively. Instead, when the chitosan concentration is 0.005 mg/mL, the relative viscosity values are 1.01, 1.04, and 1.02, for these three acids, respectively. At low TD/CS ratio, the concentration of chitosan solution is high, and consequently the solution viscosity is high. Conversely, a high TD/CS ratio corresponds to a lower viscosity solution. Figure 3 shows that when chitosan solution viscosity decreases both size and size distribution increase when the acid is varied from nitric acid to butyric acid. This trend is not observed for benzoic and benzensulfonic acids.

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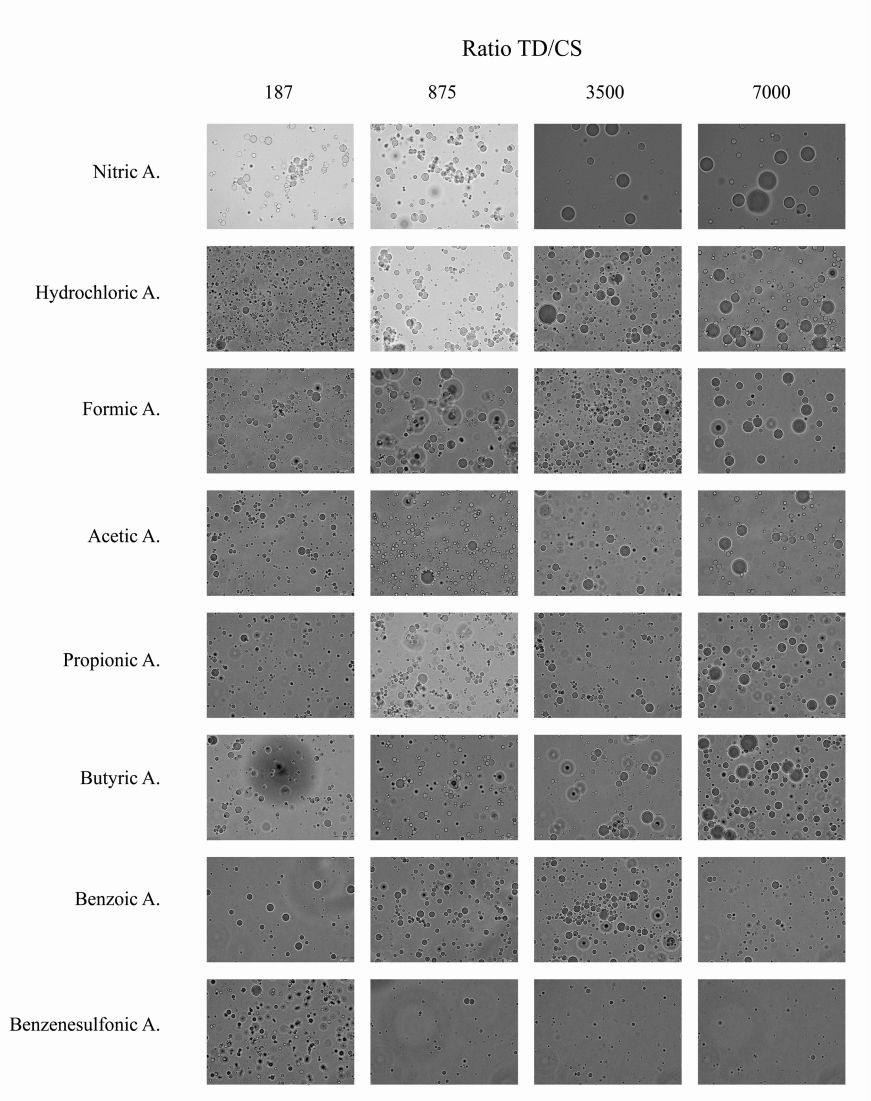

Figure 2. Comparison of different microspheres obtained with different chitosan solutions at different TD/CS ratio. Scale: all images reported have a size of 110 μm × 147 μm.

To summarize, there are three interdependent parameters that regulate the formation of microspheres: The counterion, amount of oil, and viscosity of the system.

During sonication, the oil is dispersed as microdroplets, and the difficulty to disperse them in a medium increases with an increase in the viscosity. Also, during sonication, the oil droplets can undergo continuous breaking and coalescence due to strong physical forces generated. At the end of the sonication, the presence of an amphiphilic molecule plays a fundamental role in stabilizing the emulsion. However, if the stabilizer is weak, coalescence between droplets could dominate. As a result, a strong stabilizer can be expected to generate emulsion droplets with small mean size and a narrow size distribution. Instead, a weak stabilizer would generate larger sized microspheres with a broader size distribution.

In a high viscous system, the breaking and dispersion of the oil phase to microdroplets are relatively difficult to achieve. Once broken into microdroplets, coalescence will be slower due to the high viscosity of the medium. As a result, the oil droplets would be generally smaller and the size distribution narrower. Instead, a strong stabilizer would generate droplets with a smaller size irrespective of the solution viscosity.

As mentioned earlier, when CS is dissolved in nitric or hydrochloric acid solutions, its emulsion-stabilizing property is not improved. Formate, acetate, propionate, and butanate ions slightly improve the stabilizing property due to their weak amphiphilic nature. Benzoate and benzenesulfonate ions are strong stabilizers and hence can improve the stabilizing properties of CS. Indeed, these two counterions show an opposite trend that highlights the line of demarcation between strong and weak amphiphilic molecules. Figure 4 shows the size distribution of the solutions obtained from chitosan dissolved in nitric and benzenesulfonic acids. The broken lines show the size distribution of weak (CS-nitric acid solution: HNO_3 − TD/CS 187) and strong stabilizers (CS-benzenesulfonic acid solution: B. A. − TD/CS 187) when the system has high viscosity (chitosan concentration 0.08 mg/mL). The two curves show the predicted trend: when the stabilizer is weak the mean size is larger and the size distribution broad; when the stabilizer is strong the mean size is smaller and the size distribution narrow.

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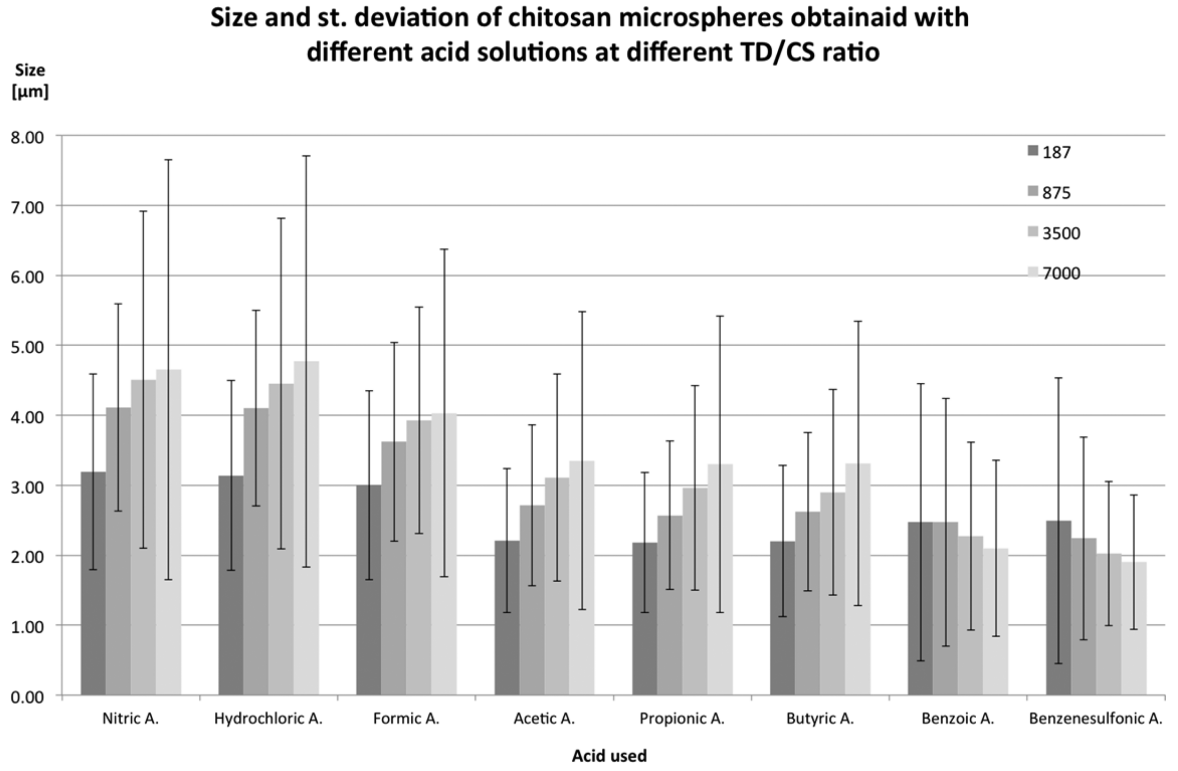

Figure 3. Mean size and size distribution of the microspheres obtained with different solutions at different ratios of TD/CS: 187, 875, 3500, and 7000. The mean size data for additional ratios is shown in Figure S-2 (Supporting Information).

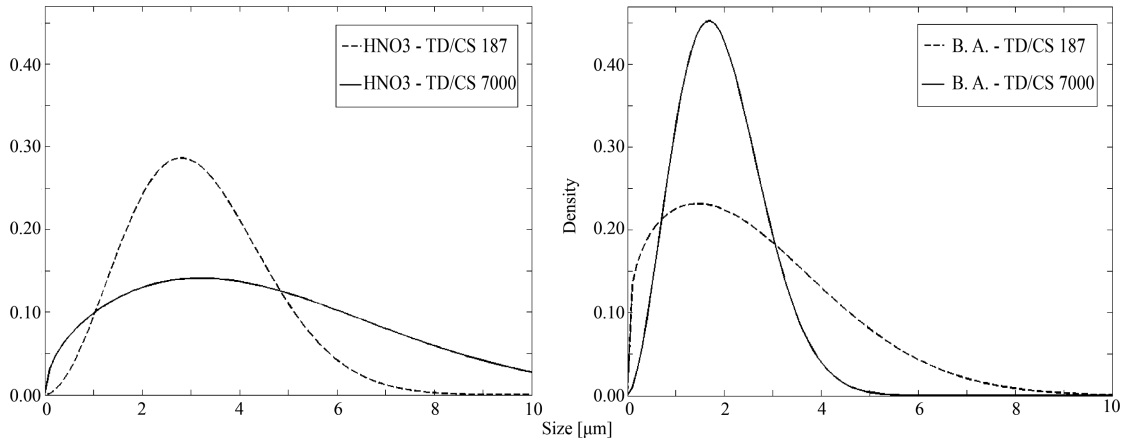

Figure 4. Microsphere distribution at the ratio TD/CS of 187 and 7000 obtained from chitosan dissolved with nitric acid (on the left) and benzenesulfonic acid (on the right).

Size distributions of microspheres are shown in Figure 5 in terms of standard deviation as a function of the TD/CS ratio.

The data shown in Figure 5 could be discussed based on the explanation given earlier. Except for the solutions made using benzoic and benzenesulfonic acids, all other acid solutions follow a similar trend, but to a different extent. The curves of nitric and hydrochloric acids are similar, which could be due to their hydrophilic nature. As a result, these two solutions are only driven by the chitosan's amphiphilicity, which is very low. The formic acid curve shows a transition from hydrophilic to weak amphiphilic counterions. Its behavior is similar to the hydrophilic ones when the viscosity is high, but it becomes similar to the weak amphiphilic counterions when the viscosity is decreased at higher amount of oil. The transition between weak and strong amphiphilicity is clearly demonstrated by benzoic and benzenesulfonic acid curves. Also, the difference between benzenesulfonic and benzoic acid curves suggests that the benzenesulfonate ion has a higher amphiphilicity than the benzoate ion. In summary, the results confirm that the nature of counterions and the TD/CS ratio have an important role during the microsphere formation and that the mean size and size distribution can be greatly influenced by both of these variables.

The microsphere samples were kept in a basic solution for additional experiments. After the stability analyses as described in the Materials and Methods section, the microspheres were collected and analyzed for the presence of the counterions in the shell of the microspheres. Figure S-3 in the Supporting Information confirmed the presence of benzenesulfonate in the

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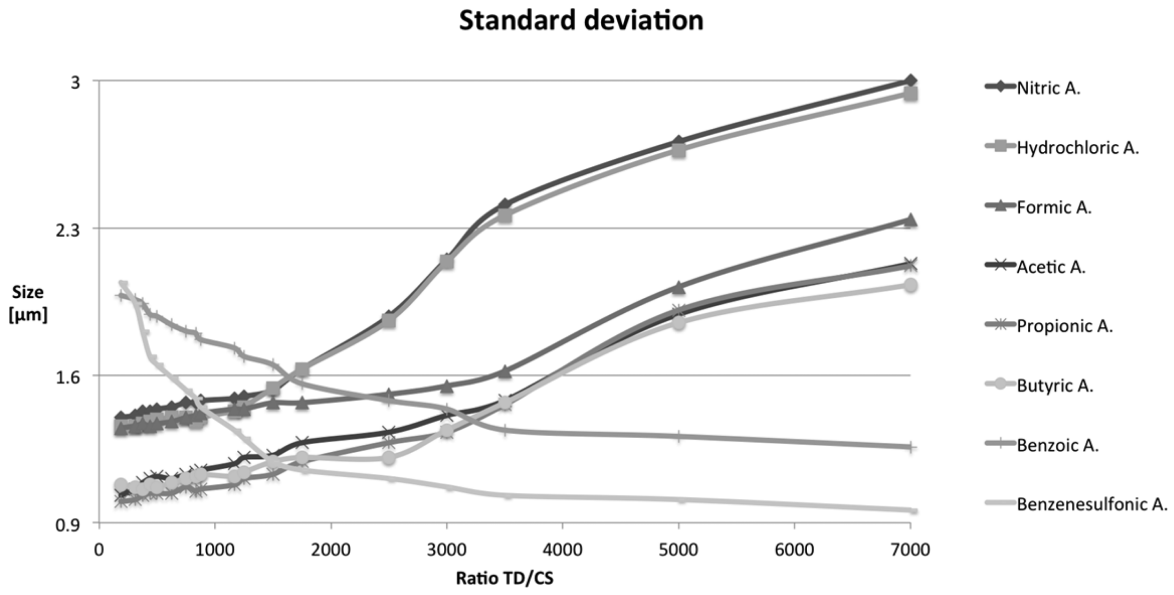

Figure 5. Standard deviation in the size of microspheres obtained with different solutions at different ratios of TD/CS.

shell. The benzenesulfonic acid series was chosen to understand the mechanism of microsphere formation (Figure 6).

Chitosan, in its dried state, can be compared as a coil kept intact by its intramolecular hydrogen bonds (A). However, when chitosan is added into an acidic medium, protonation of amine moieties occurs, and the counterions move close to CS in order to compensate the positive charges on the ammonium ions. During this process, the polymer is forced to open itself to counterbalance the charges just formed. At this stage, the hydrogen bonds between the chitosan monomeric units are replaced by hydrogen bonds formed between the monomeric units and water molecules. This allows the polymer to stretch out as an elongated chain to minimize the electrostatic repulsion. This process is identical whether the acid is amphiphilic (B.A.) or not (HNO_3) (Sections B1 and B2 in Figure 6). When the oil is added and the emulsification is carried out by sonication (Section C in Figure 6), different scenarios could be considered. Chitosan surrounds the oil droplets generated spontaneously due to its amphiphilic properties. However, when a more amphiphilic counterion is present (B. A.), the coverage process will be faster. Moreover, the role of the counterion is not limited to the initial stabilization of the oil droplets. Indeed, the counterions are linked to chitosan by electrostatic interactions. As a result, the counterion is not only faster in the stabilization process but also drives the chitosan adsorption around the oil droplets (Section D). This process greatly influences the mean size and size distribution because it can prevent coalescence. When the stabilizer is strong, the microspheres would maintain their size and size distribution as generated at the end of sonication. However, when the stabilizer is weak, coalescence between droplets can change mean size and size distribution of the microspheres.

When NaOH is added and the CS charges are neutralized (Section E), chitosan becomes insoluble in water. HPLC analysis (Figure S-3, Supporting Information) showed that benzenesulfonate remained in the microspheres, possibly at the oil−chitosan interface (E). In this case, the addition of sodium hydroxide neutralized the majority of the charges on the chitosan but not the ones at the interface. The neutralization of chitosan charges increases its hydrophobicity, resulting in a better stabilization of emulsion droplets.

3.1. Stability. In order to understand the stability of the microspheres, a series of experiments were carried out. The samples analyzed were TD15−0.08 mg/mL; TD35−0.04 mg/mL; TD35−0.01 mg/mL; and TD35−0.005 mg/mL. Generally, microspheres prepared without a cross-linker have a soft shell. As a consequence, the oil will be slowly released, and the time necessary for this process depends on the capacity of the polysaccharide to stabilize the oil inside the sphere. In effect, a layer of oil was observed on the surface of samples prepared from nitric, hydrochloric, and formic acid solutions after 1−2 weeks; other samples showed a similar behavior after 3 weeks with exception for the samples obtained using benzoic and benzenesulfonic acids, where oil separation was not observed at least for one month. The samples were periodically analyzed, once a week for a month by measuring the mean size and size distribution. The changes observed were homogeneous independent of TD/CS ratios. For this reason, an average of the changes in percentage was calculated for each series every week, and the values are reported as a function of storage time in Figure 7.

The microspheres prepared using nitric and hydrochloric acids were the most unstable. Their mean size changed by about 35%. Microspheres obtained using formic acid solution changed their size by about 29%. Instead, when the amphiphilicity is increased, size changed by 12% (microspheres obtained with acetic, propionic, and butyric acids). For benzoate and benzenesulfonate counterions, the mean size changed only around 4% after a month. Also in this case, it is evident that the stability follows the counterion amphiphilicity. However, these data must be considered in terms of encapsulation capacity. The volume changes calculated are ≈73% for nitric and hydrochloric acids; 64% for formic acid; 33% for acetic, propionic, and butyric acids; and 11% for benzoic and benzenesulfonic acids. In other words, the stability of the most amphiphilic group is around seven times the stability of the first group, which has lost almost three-quarters of the oil during storage.

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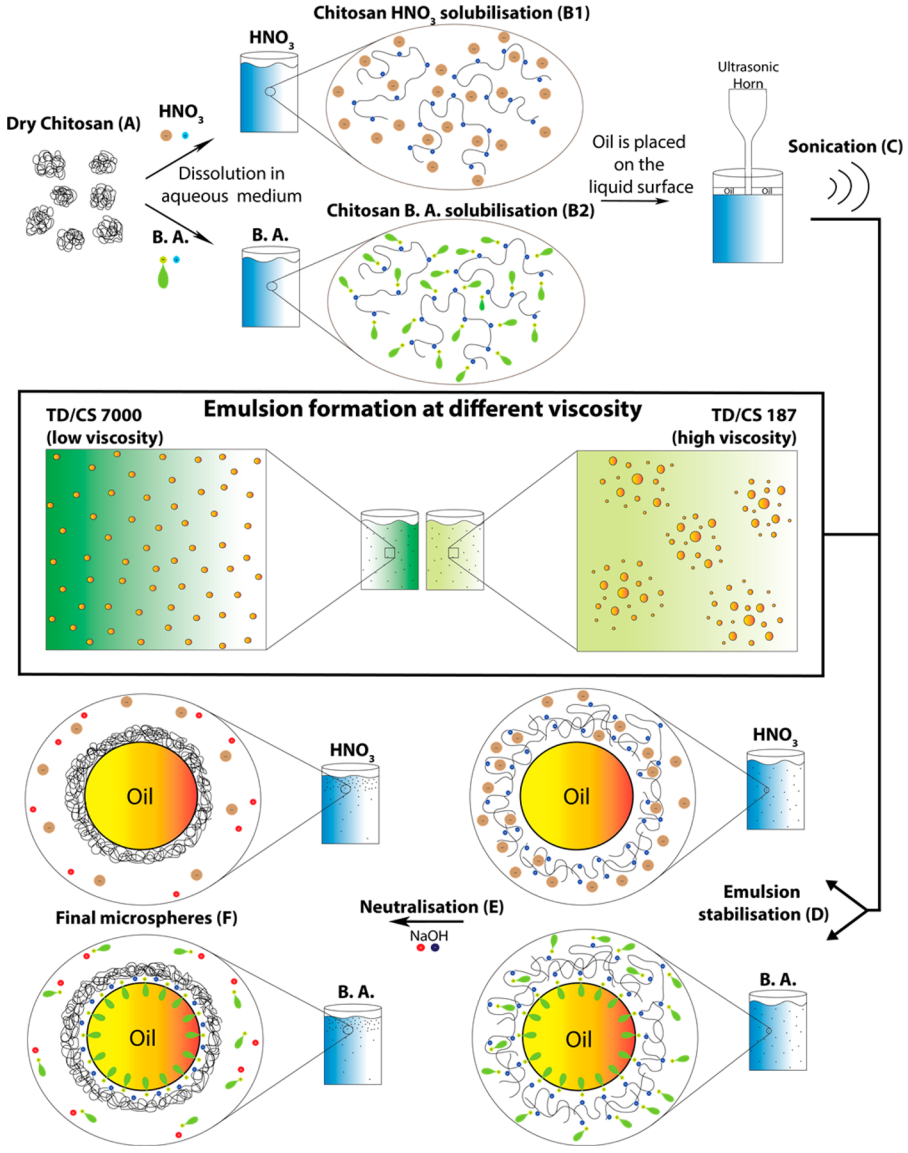

Figure 6. Mechanism of microsphere formation from two chitosan solutions obtained with benzenesulfonic acid (B. A.) and nitric acid (HNO_3). A: Dry chitosan; B1 and B2: Chitosan dissolved with nitric acid and benzenesulfonic acid, respectively; C: Sonication process; TD/CS 187 and TD/CS 7000 show the result of the emulsion at high and low viscosity, respectively; D: Stabilization of an oil droplet by chitosan; E: neutralization of chitosan charges; F: Final product.

Figure S-4 in the Supporting Information shows the changes in standard deviation as a function of storage time. All the curves show a decreasing trend, which indicate that larger microspheres were reduced in number. This could have been caused by two factors: first, the shell of the larger microspheres could be thinner compared with the small ones, which leads to the easier release of oil; the second reason could be the release of oil due to the porous nature of the chitosan shell of larger microspheres.

3.2. Effect of Ultrasonic Power. As mentioned earlier, the counterions play a fundamental role in the microsphere formation. In order to support this proposition, a series of experiments were carried out at different sonication powers using 4 TD/CS ratios (187, 875, 3500, and 7000) for each group. Then, for each sonication power, an average of mean size and size distribution were made and presented in Figure 8.

Figure 8 and Figure S-5 (Supporting Information) show the general expected trend. At low sonication power (0.4 W), very large emulsion droplets were produced, due to weaker shear forces generated during cavitation, leading to a wider size distribution and larger mean size. At higher power levels, the shear forces increase, resulting in a narrower size distribution and smaller mean size. However, at very high power levels, the shear forces not only break oil droplets but also increase collision between droplets. As a result, the mean size and size distribution increase. However, the influence of the counterion is clear. For instance, at a sonication power of 0.4 W the mean size is around 8 μm when the counterion is nitrate and decreases to about 2.5 μm when the counterion is benzenesulfonate. Then, the microsphere size decreases with an increase in acoustic power up to 5.4 W, beyond which a larger size range is observed. This is not the case when the counterion is benzoate or benzenesulfonate, for which the minimum is reached at 2.9 W.

Figure 8 also shows the standard deviation of data. The trends observed are consistent with the previous explanations.

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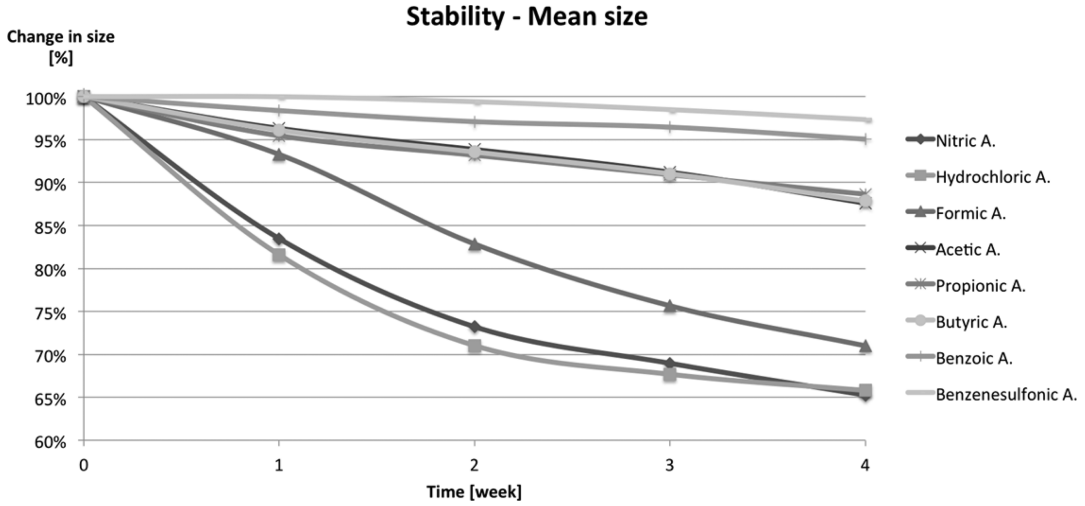

Figure 7. Microsphere size variation as a function of storage time.

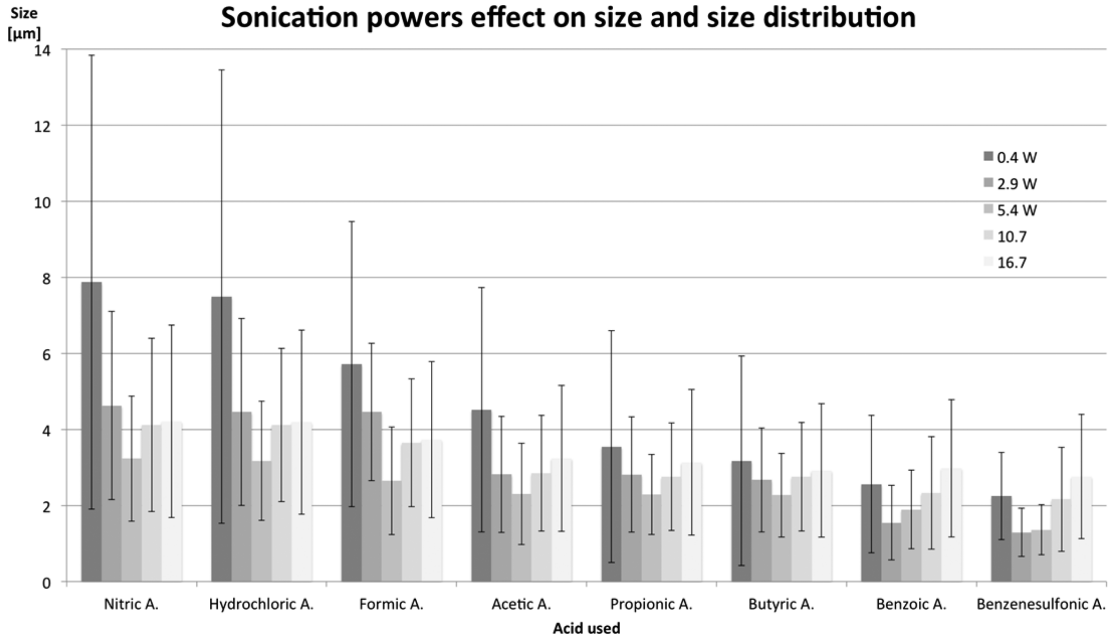

Figure 8. Mean size of microspheres as a function of sonication power and acid used to dissolve chitosan.

In particular, it confirms that the inversion point with regard to the size occurs at an acoustic power 5.4 W for all the counterions, except for benzoate and benzenesulfonate ions, where the inversion occurs at 2.9 W acoustic power. It is also worthwhile to note that the amphiphilicity trend could be better observed at a sonication power of 0.4 W. At this point, the standard deviation decreases from 6 μm for nitrate to 1 μm for the benzenesulfonate ion.

4. CONCLUSIONS

Chitosan microspheres were produced in aqueous solutions of different TD/CS ratios and different sonication powers. The experiments have shown a new approach to control the size, size distribution, and stability using different acids, with different characteristics. The study has also proven that the counterion has an important role not only in controlling mean size and size distribution and their stability but also in the energy required for the microspheres preparation. The acid used in this study may not be suitable for drug or food delivery. However, the concept developed could be applied to choose appropriate acids for such applications in order to tune the physical and functional properties of microspheres.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table S-1 (HLB calculation); Figure S-2 (Microspheres mean size as a function of counterions and TD/CS ratio); Figure S-3 (HPLC analysis); Figure S-4 (Standard deviation as a function of time), Figure S-5 (Standard deviation as a function of sonication power of the microspheres produced with different counterions). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b02773.

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Notes

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■ ABBREVIATIONS

BA, benzenesulfonic acid; CS, chitosan; CSFITC, chitosan labeled with FITC; FITC, fluorescein isothiocyanate; HLB ,hydrophilic lipophilic balance; HPLC, high performance liquid chromatography; NR, nile red; TD, tetradecane; TDNR ,tetradecane with NR as probe.

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