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Ultrasonically synthesized organic liquid-filled chitosan microcapsules: Part 1: Tuning physical & functional properties

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KEYWORDS: microcapsule· microsphere · chitosan · ultrasound · AFM · stiffness · modulus

Abstract:

This study reports on the synthesis of tetradecane-filled chitosan microcapsules in acetic acid aqueous solutions using high intensity ultrasound at 20 kHz. The size, size distribution, and stability of microcapsules were tuned by varying the concentration of acetic acid from 0.2% to 25% v/v. After long-time storage at room temperature (more than 3 months), the microcapsules maintained their shell-core structure where the volume of microcapsules at 0.2% only decreased by 8.3%. Microcapsules were consistently spherical and had a smooth shell surface, however, their shell thickness varied with acetic acid concentrations. The relaxation behavior of individual microcapsules to an applied constant stress was measured by atomic force microscopy (AFM) to probe the shell strength and extent of crosslinking. The effect of acetic acid on relative viscosity of chitosan aqueous solutions played a major role on microcapsule size control at low acid concentrations. With constant addition of acetic acid, amino groups in chitosan chains were acetylated partially under ultrasonic irradiation. This reduced the amphiphilicity of shell material and therefore influenced the size, size distribution, stability and mechanical strength of microcapsules. Apart from the acetylation effect, the counter-ion effect and the formation of covalent bond crosslinks also made contributions to the formation of stable chitosan microcapsules.

1. Introduction

Core-shell microcapsules have attracted sustained interest since their physical properties were first reported in 1961 by Hughes and Brown[1]. The double functionalities of cores and shells that can be independently tuned resulted in a large number of applications[2]. A wide range of substances such as lysozyme[3], bovine serum albumin (BSA)[4], human serum albumin (HSA)[5], poly(L-lactide-co-glycolide) (PLGA)[6], poly(vinyl alcohol) (PVA)[7], polymers[8], alginates[9, 10] and chitosan [11] have been used as shell materials. The core materials may consist of air[12], aerosols[13], liquids[3], and solids[14].

From above mentioned shell materials, chitosan is a possible option to be used in applications such as and pharmaceutical products. As a natural amino-polysaccharide, chitosan is an excellent shell material already used in various fields such as heavy metal ion removal[15], cartilage tissue engineering[16] and drug delivery [11]. The amino groups in chitosan endow the material with controllable water solubility dependent on solution pH. In acidic aqueous solutions, chitosan is protonated and hence soluble as a polymer with a long carbon chain, leading to an amphiphilic property, which facilitates the encapsulation and stabilization of oil droplets within its shell. Additionally, chitosan has numerous merits of biocompatibility, biodegradability, low toxicity[17], antimicrobial activity, low immunogenicity[18], high specific surface area and surface features[19, 20]. Based on the advantages mentioned above, many production methods for solid, hollow and liquid-filled chitosan microcapsules have been developed. Lu et al. [21] have studied the size distribution, pH and ionic strength dual-response for multilayer chitosan hollow microcapsules prepared via Layer-by-Layer(L-b-L) assembly technique. Biró et al. [22] have reported the size control of chitosan solid microcapsules by water-in-oil (w/o) emulsion crosslinking method. Zhou et al. [23] have synthesized chitosan-TiO_2 microcapsules filled with tetradecane using ultrasound. Colombo et al. [24] have investigated the influence of various counter ions on the size distribution, and stability of ultrasonically synthesized chitosan microcapsules filled with tetradecane. Chitosan has a great potential to be used as a shell material for synthesizing microcapsules for edible applications. For synthesizing stable biopolymer shelled microcapsules exhibiting an extended shelf life and better release properties in drug or flavour delivery applications, the raw materials used must be non-hazardous and edible. However, the use of all food grade raw materials to synthesize chitosan shelled microcapsules has been elusive so far. Introducing crosslinkers or chemical modification is a conventionally used method to enhance the stability and strength of biopolymer shelled microcapsules. Crosslinkers for chitosan include genipin, tripolyphosphate, ethylene glycol, diglycidyl ether, diisocyanate and glutaraldehyde, which are toxic if swallowed or inhaled

according to their MSDS. (Material Safety Data Sheet). Chemical modification of chitosan was carried out by Tan et al.[25]. A synthetic chemical (DL-N-Acetylhomocysteine thiolactone) was used to prepare thiolated chitosan to achieve chitosan crosslinking. However, DL-N-Acetylhomocysteine thiolactone still cannot be used for food applications as it is toxic based on its MSDS. Acetic acid is a widely-used substance to dissolve chitosan into aqueous solutions. Acetic acid is used in food industry as it is not very expensive and food-grade. Acetic acid treatment of chitosan in our work is completely different from modifying chitosan via thiolation by a synthetic chemical. Acetic acid treatment not only produced stable and crosslinked microspheres which can be used for food (cheese, yoghurt and disserts), drug delivery applications but also enabled to vary the functional properties of microspheres by simply adjusting the dosage of acetic acid. The simplicity, safety and tunability is the novelty in the current work.

The control and tunability of the size, size distribution and shell thickness/strength of chitosan microcapsules have not been explored in previous reports. For many applications, the control over the physical properties is required. For example, microcapsules used in food applications should be about 1-5 μm.

The aim of our study is to use acetic acid to tune the capsule size distribution and the shell strength of chitosan-tetradecane microcapsules in the absence of additional crosslinking agents, by a one-step ultrasonic methodology that will instead form crosslinks between inter-chain functional groups on the chitosan. The physical forces generated during acoustic cavitation have been used to emulsify tetradecane in an aqueous chitosan solution. Tetradecane was used as a model core material. Many actives including small molecule pharmaceuticals, nutrients and other bio-actives have a hydrophobic nature and are only oil soluble causing difficulties in the delivery step. Tetradecane is a nonpolar substance, which can be an appropriate medium for these compounds and there may be more appropriate to specific actives, tetradecane serves as a well characterized model oil.

2. Experimental section

1.1 Materials

Chitosan and acetic acid (AA) were purchased from Sigma-Aldrich. Tetradecane (olefine free; >99%) was from Fluka. Milli-Q water was obtained from a Millipore system (18.2 MΩ/cm at 25°C).

1.2 Synthesis

Chitosan was dissolved in aqueous acetic acid solution at 2.5% w/v. The concentration of acetic acid was varied in the range 0.2%, 2%, 16%, 20% and 25% v/v. Tetradecane containing Nile red was chosen as a model organic liquid to demonstrate the versatility of the encapsulation procedure. Tetradecane-filled chitosan shelled microcapsules were obtained by layering tetradecane on the surface of chitosan solution and sonicating at 160 W for 30 s by placing a horn (microtip 3 mm in diameter; 20 kHz Branson sonifier) at the oil/solution interface. Following microcapsule formation, excess acetic acid was neutralized using strong NaOH solution. The microcapsules were then collected and washed 5 times with Milli-Q water and stored in an aqueous solution at pH 7.

1.3 Characterization

Optical microscopy (Olympus) and scanning electron microscopy (SEM, FEI Quanta) were used to examine the morphology and size of the microcapsules. The average capsule size and size distribution were evaluated by measuring over 400 microcapsules per system using optical microscopic and SEM images. Fluorescence optical microscopy (Olympus) was used for detecting tetradecane encapsulated inside chitosan microcapsules. Nile red was used as a fluorescence probe. The relative viscosity was measured by Ubbelohde Viscometry. Atomic force microscopy (AFM) (Asylum Research MFP-3D AFM in an acoustic isolation hood) was used to measure the stiffness, Young's modulus and stress-relaxation behavior of microcapsules. The AFM measurements employed a colloidal probe with diameters ranging from 18 to 35 and AFM V-shaped MLCT cantilevers (Bruker) with cantilever spring constants that ranged from 0.03 to 0.15 N/m, measured using the thermal method of Hutter-Bechhoefer [26]. FTIR spectra were recorded in a Bruker spectrometer using the KBr pellet technique.

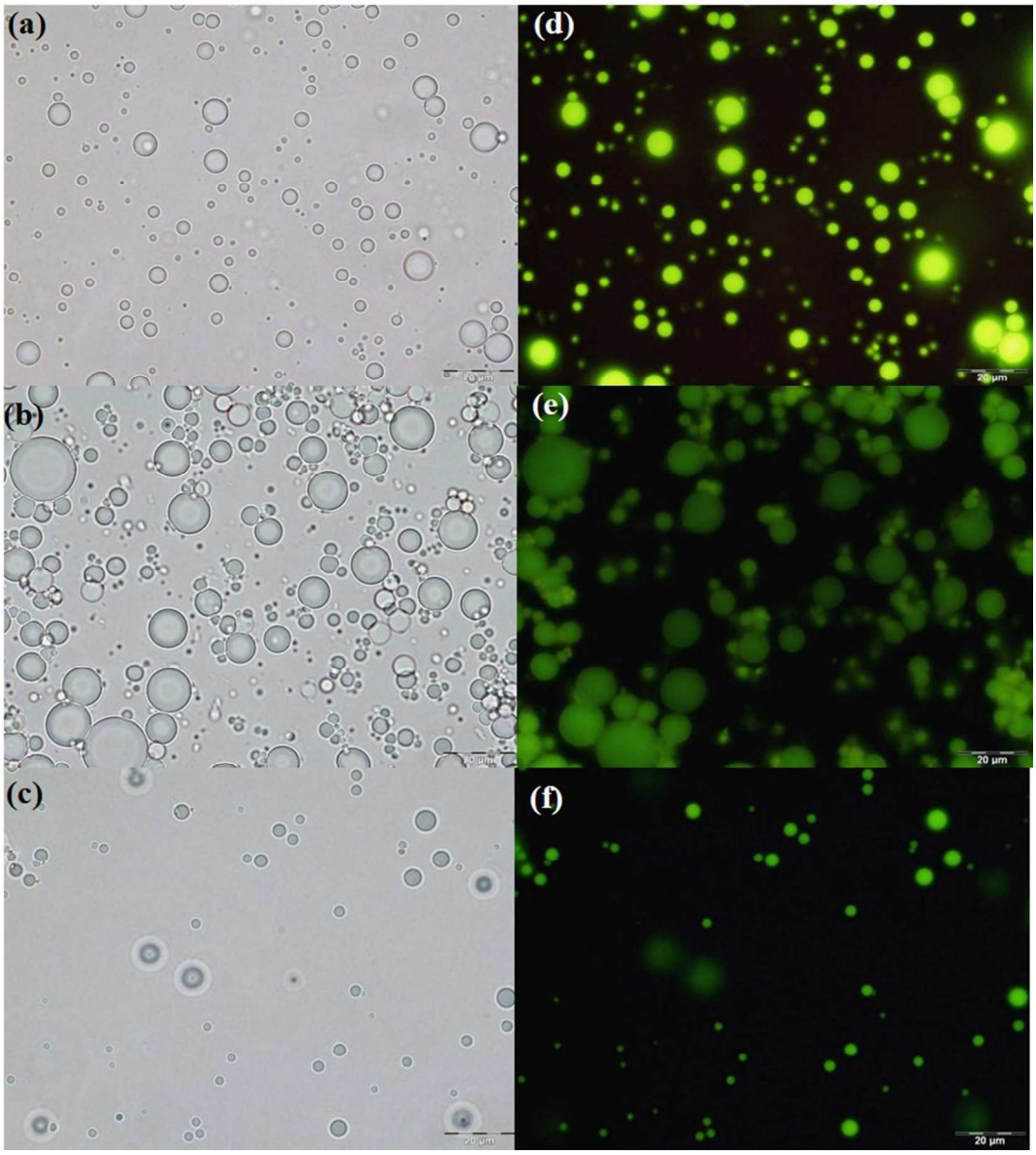

Figure 1. Images of tetradecane (dyed with Nile red) filled chitosan microcapsules: Optical microscopy (OM) (a. 0.2 %, b. 2 %, c. 25 %) and fluorescence OM (d. 0.2 %, e. 2 %, f. 25 %). Scale: all images reported have a size of 110 µm × 147 µm.

3. Results and Discussion

2.1 Size and size distribution of chitosan microcapsules

When chitosan is dissolved in an acidic solution, the amino groups are converted into positively charged ammonium ions due to protonation, where the extent of protonation directly influences the dissolution of the polymer [27, 28]. In addition, chitosan possesses a propensity to aggregate to form complexes in aqueous solvents[29]. In this investigation, acetic acid was systematically varied in order to mediate both chitosan solubility and aggregation to identify the

role of acetic acid in tuning the size distribution and stability of tetradecane-filled chitosan microcapsules as well as in affecting the mechanical strength of chitosan shell.

Six different types of tetradecane-filled chitosan microcapsules were prepared where the chitosan was protonated to different levels by varying the acetic acid concentration at 0.2%, 2%, 10 %, 16%, 20%, 25% v/v acetic acid. The optical and fluorescence microscopic images of the microcapsules as a function of varying concentrations of acetic acid are shown in figure 1.

The mean microcapsule size increased from 3.7±1.5 μm to 4.9±2.0 μm with an increase in acetic acid concentration from 0.2 to 2%. Further increasing the acetic acid concentration led to a monotonic decrease in microcapsule size, with a minimum microcapsule size of 3.5±1.6 μm at 25 % v/v concentration of acetic acid. The microcapsule size distributions are summarized in figure 2. There is a primary peak in the size distribution ranging from 2 to 4 μm and a tail in the distribution up to 10 to 11 μm. The presence of the tail in these distributions is most likely the result of a small amount of droplet coalescence during the ultrasound encapsulation process.

In aqueous chitosan solutions, the amount of acetic acid regulates the degree of protonation of the chitosan chains. Consequently, the intra-chain electrostatic repulsive force between positive ammonium ions groups controls the folding and unfolding transition of the polymer chain leading to two different configurations of the chitosan that play a vital role in the solution viscosity. A relative viscosity measurement in figure 3 shows a rapid decrease from 1.5 to 1.0 when the concentration of acetic acid increased from 0.2 to 2 % v/v, respectively. It indicates that during sonication the decrease in relative viscosity facilitates coalescence of dispersed oil droplets, instead of continuous breaking, owing to the strong physical forces generated. This results in an increase of microcapsule size. With constant addition of acid, however, the reducing trend of relative viscosity becomes slow, from 0.91 at 12 % v/v to 0.89 at 25 % v/v. At higher acetic acid concentrations this suggests that the folding and unfolding transition due to the protonation degree of chitosan amino groups exerts a minor influence on the relative viscosity of the system, compared to the dilution effect of the acetic acid.

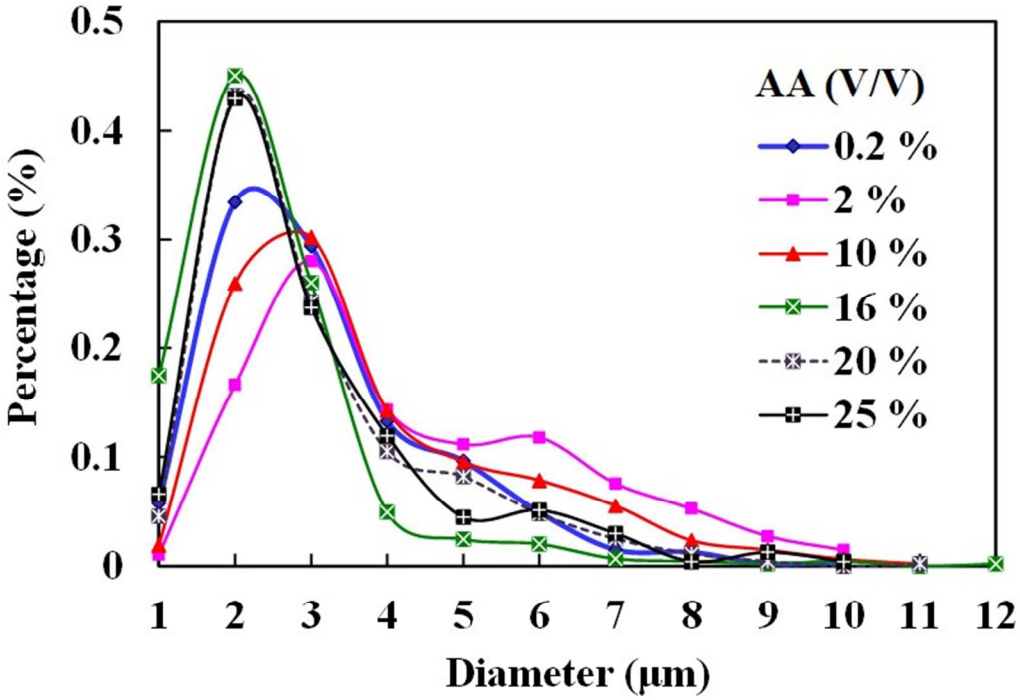

Figure 2. Size distributions of tetradecane with Nile red-filled cross linked chitosan microcapsules.

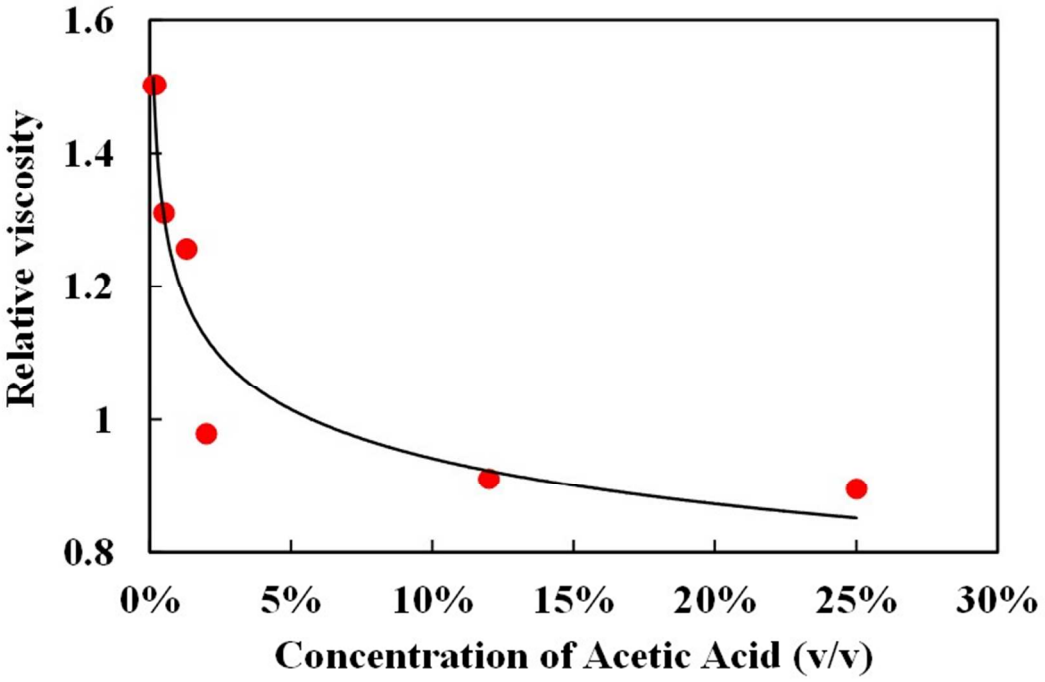

Figure 3. Relative viscosity of chitosan aqueous solutions with varying acetic acid concentrations, measured at 22.3±0.2 °C.

2.2 Stability of chitosan microcapsules.

The stability of the chitosan microcapsule size distribution was monitored for three months for the lowest and highest acetic acid concentrations. figure 4 shows that microcapsules

size distributions, prepared at two different acid concentrations, 0.2 and 25 % v/v, shifted to a larger mean size and broader distribution, most likely due to coalescence and leaking during the more than 3 month storage time. The mean volume of the microcapsules at 0.2 % v/v increased by approximately 8.3 % after 3 months. This may have resulted from the porous and flexible structure of chitosan shell. For the microcapsules prepared at 25 % v/v, the mean volume reduced by nearly 59 % and a layer of oil was observed on the surface of the sample after 3 weeks. This may be due to an increase of the degree acetylation of the chitosan shell after sonication at high acid concentrations, which is discussed in mechanism section below. Consequently, the stabilization capacity of the polysaccharide decreased, leading to a significant release of oil.

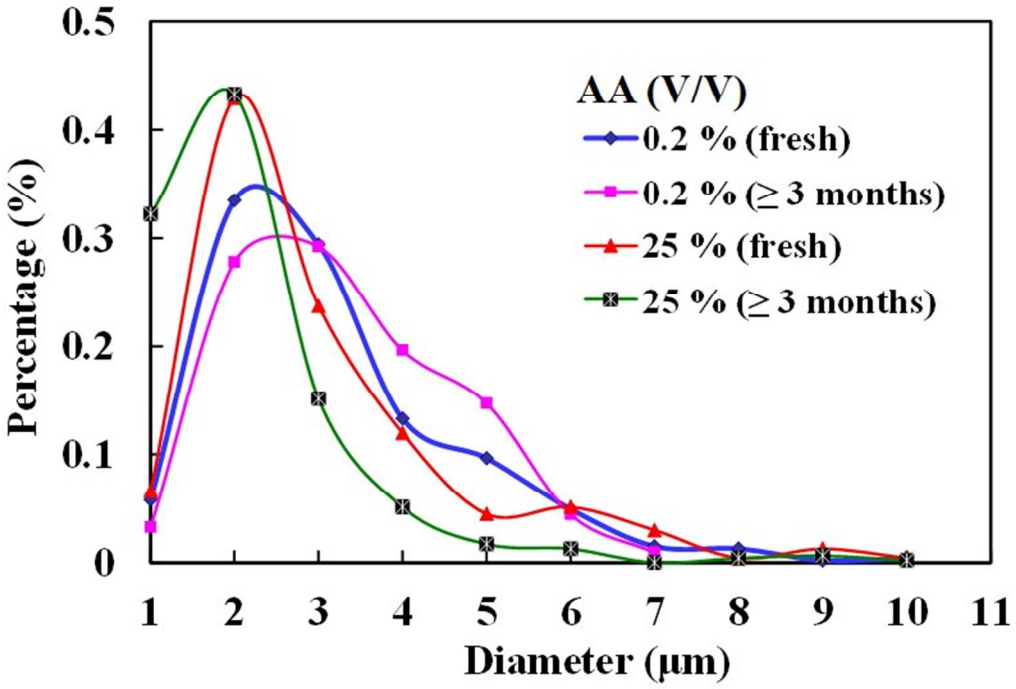

Figure 4. Cross linked chitosan microcapsule size variation as a function of storage time and acetic acid concentration.

2.3 Morphology of liquid-filled microcapsules.

In figure 5, four SEM images of the ultrasonically prepared tetradecane-filled chitosan microcapsules are presented in figure 5. Figure 5a and 5b display the microcapsules sonicated at 0.2 % and 25 % v/v acetic acid aqueous solutions, respectively. Since the chitosan shells were relatively soft, the microcapsules collapsed under the vacuum environment of SEM. The microcapsule size and size distribution based on SEM measurements are consistent with that of microscopic images. Inserts show that the microcapsules which were heated at 50°C for 5 min before SEM sample preparation have spherical morphology and smooth surface structure. After

heating, the chitosan shells became stiffer and enabled to maintain the microcapsule structures under vacuum. The shell thickness of microcapsules was measured by mechanically breaking the microcapsules before sample preparation (figure 6). With the variation of acetic acid concentrations, the shell thickness increased rapidly from around 134 nm at 0.2 % v/v to 511 nm at 2 % v/v and then gradually decreased to 150 nm at 25 % v/v. This trend can be related to the change in relative viscosity and the folding/unfolding transition due to the degree of protonation of the chitosan amino groups at different acetic acid concentrations.

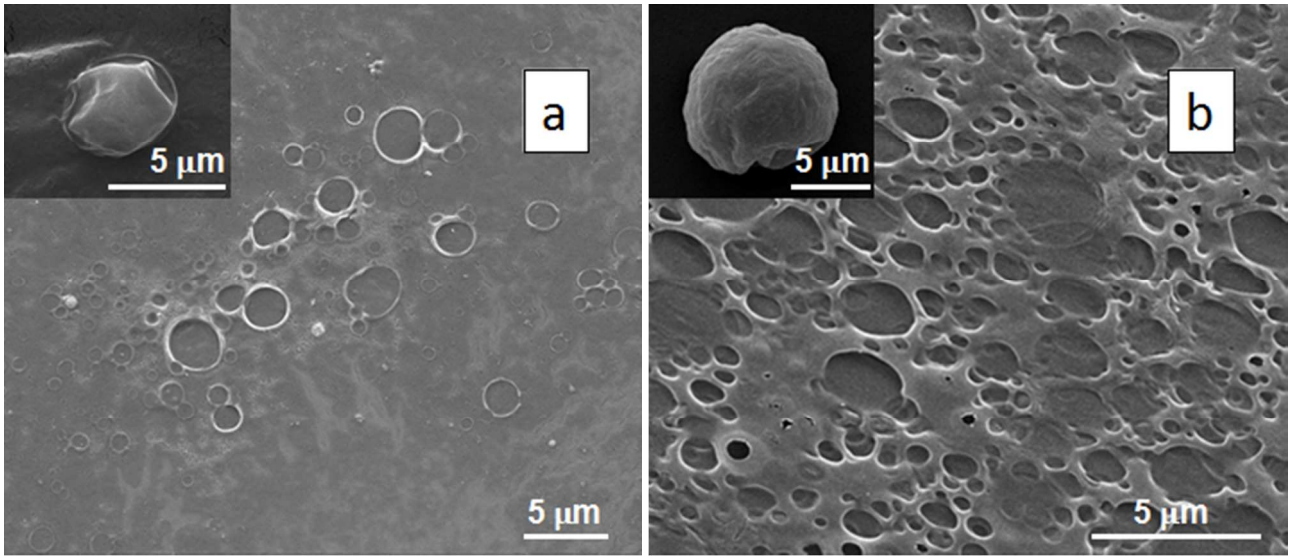

Figure 5. SEM images of tetradecane-filled chitosan microcapsules (a. 0.2 %, b. 25 % at room temperature; inserts for heated microcapsules at 2% and 25%, respectively).

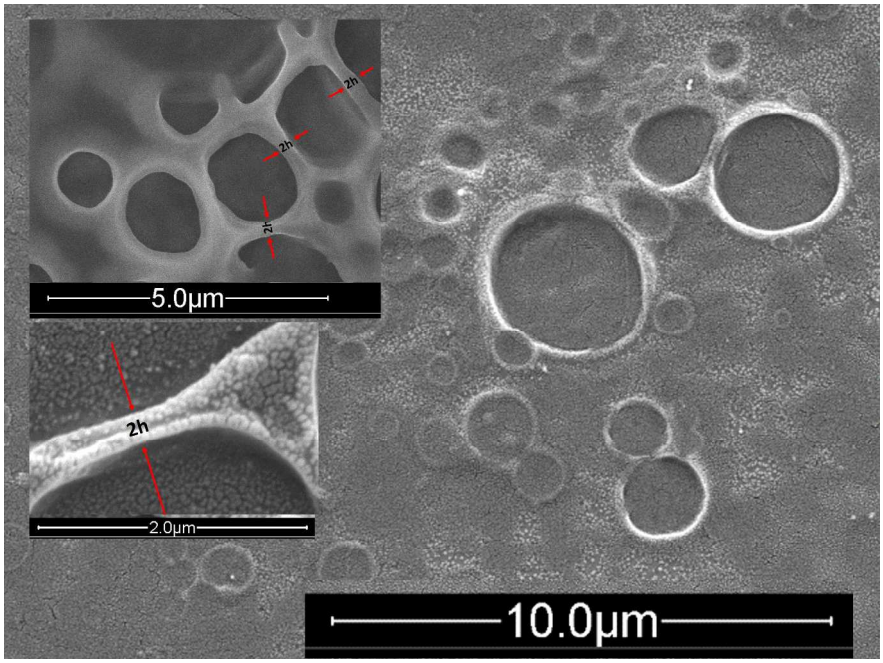

Figure 6. SEM images of chitosan microcapsules used for measuring the thickness of the shell.

Table 1. Shell thickness of capsules as a function of acetic acid concentration.

Sample Number	Acetic Acid Concentration (% V/V)	Average Size Diameter (μm)	Average Shell thickness h(nm)
2	0.2	3.7±1.5	134±10
20	2	4.9±2.0	511±73
160	16	2.9±1.3	235±35
200	20	3.6±1.5	207±94
250	25	3.5±1.6	150±24

2.4 Mechanical strength of chitosan microcapsules.

We carried out mechanical compression experiments on individual chitosan microcapsules using Atomic Force Microscope (Asylum Research MFP-3D AFM) as described in detail in part 2 of this paper. We measured the stiffness and Young's modulus of individual capsules as a function of their size. Along with strength of the capsules we also measured magnitude of forces and deformations required to rupture them. The stiffness of a microcapsule is equivalent to interfacial tension in the case of a liquid drop coated with surfactants in an emulsion system, except that the stiffness is a function of elasticity and the radius of the shell whereas the surface tension is independent of size of emulsion drops. The measured average stiffness of microcapsules varied from 82 to 111 mN/m and average Young's modulus ranging from 0.4 to 6.5 MPa. We found that when incorporated into a hot liquid fat and cooled back to room temperature the capsules survived without breaking (data not shown).

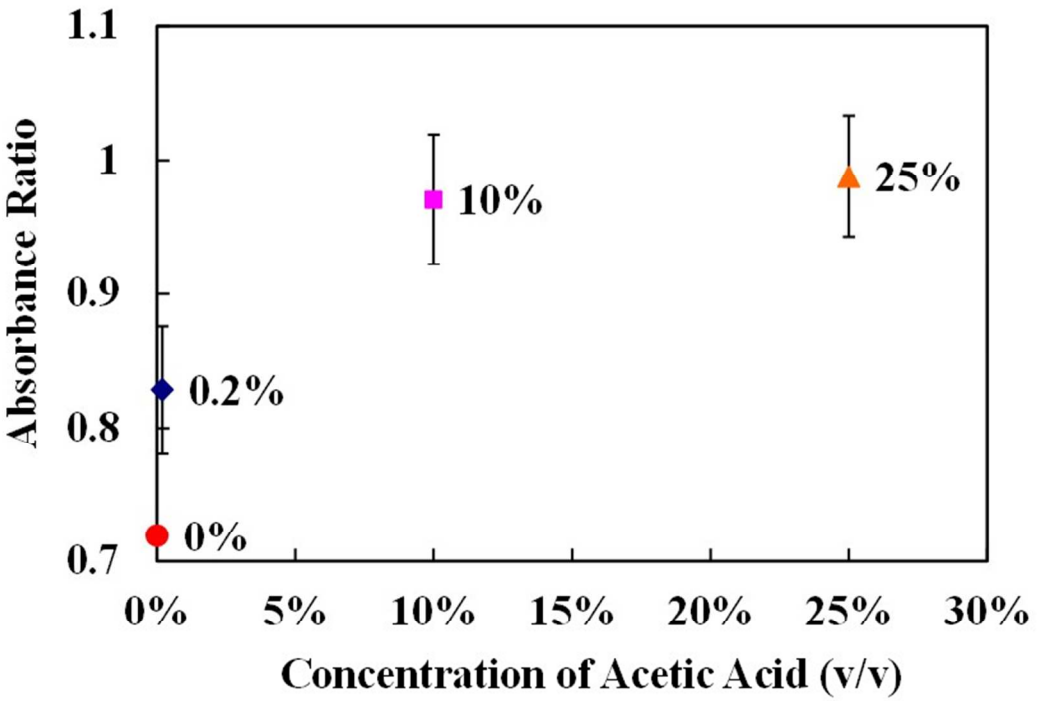

Figure 7. Absorbance ratio of C=O stretching to -CH2- vibration in FTIR measurement (the experiments at each acid concentration were repeated at least 4 times).

2.5 Mechanism of the formation of stable chitosan microcapsules.

FTIR measurement was carried out to see if there were any changes between commercial chitosan sample and ultrasonically-generated chitosan shell used to form microcapsules. In figure 7, it is observed that an increase of acetic acid concentration resulted in an increase in absorbance ratio of C=O stretching of amide groups at 1650 cm^{-1} in acetylated units to -CH_2- vibration at 2920 cm^{-1} in each units. It demonstrates that an increasing amount of amino groups reacted with acetic acid and converted into amide groups during sonication due to physical and chemical effects. The exploration of the detailed mechanism is still in progress but similar spectra could be obtained when chitosan solutions were sonicated with or without core materials. It implies that the core materials exerted no effect on the formation of the possible structure. This result indicates that deacetylation degree of chitosan reduced at high concentration of acetic acid, which represents that the characteristics of this shell material switched slightly from chitosan to chitin. Intractable molecular structure [30, 31] and strong intra-intermolecular hydrogen bonds [32]of chitin have a positive effect on its deprotonation degree, hydrophobicity and stiffness. Reversely, it weakens the amphiphilic property and capacity of this polymer to stabilize oil droplets inside. To some extent, it influences the encapsulation behavior of the chitosan shell in the system at high acid amounts, resulting in a decrease of microcapsule size.

Apart from the enhancement of intra-intermolecular hydrogen bonds in the partially acetylated chitosan shell, another two mechanisms can be proposed to explain the preparation of microcapsules. The first mechanism is due to the amphiphilic property of chitosan itself, which has been reported by Colombo et al. [24]. The amino groups in chitosan chains can be protonated in acetic acid solutions and become positively charged ammonium ions. CH_3COO- serves as the counterion of the ammonium ion to assist the electrostatic repulsive force and facilitate the stabilization of microcapsules in aqueous solutions. The second mechanism is due to the formation of covalent cross links which is probed using stress relaxation experiments as described below.

2.6 Stress Relaxation of the formation of stable chitosan microcapsules.

The formation of the microcapsules also involves the generation of crosslinking bonds. Zhao et al. [33-35] reported that the cross-linked network formed by long-chained polymers can be comprised of ionic and covalent crosslinks. For example, alginates and chitosan can form either ionic or covalently cross linked polymer network depending on the type or amount of cross linkers used[33], either of as an added cross linker or by the interaction or reaction of functional groups on the chain. In the case of hydrated networks, the response of the network to a perturbation in compression via an applied force can lead to both a short time scale elastic response of the shell material coupled with a longer time scale response associated with the drainage of the liquid from the shell. As the shell is porous, this drainage does not occur instantly, hence the response of the microcapsule to the applied force relaxes slowly as the liquid in the network escapes and as the shell approaches an equilibrium strain and a final force value which is less than the initial force. We carried out this type of force relaxation measurement on the chitosan microcapsules by suddenly (Velocity ~10 μm/s) applying a set force (~10 nN) on an individual capsule by colloidal probe in AFM, see the inset in figure 8(a). Once a set force is applied on the capsule, the position of probe is kept constant using constant Z-sensor in a dwell mode available in Asylum Research MFP-3D AFM and the relaxation of the force is monitored over time, as shown in figure 8 (a) and (b) for 0.2 and 25% v/v acetic acid concentrations, respectively. The force behavior follows an exponential force relaxation behavior with time, limiting to a constant force final value. This behavior is attributed to the covalent cross linking mechanism in the network [33]. However, in the case of an ionic cross linked network, with sudden application of force, the relaxation behavior is linear hence it decrease with time as a result of constant breaking and reforming of ionic cross links [33]. Zhao et al [33] showed that the linear force relaxation observed from alginate hydrogels made with ionic cross links ultimately resulted in the complete

destruction of gels with no elastic recovery. Based on the exponential relaxation behavior observed in our experiments, the possibility of the formation of covalent bonds, which hold chitosan molecules together, is assumed. A similar mechanism was observed in the creation of DNA nanospheres under ultrasonic irradiation by Shimanovich et al. [36]. As indicated by Hu et al[34] and observed by Zhao et al [33], the force relaxation behavior on cross linked shells depends on the permeability and porosity of the network. The relaxation behavior and time required to saturate to the final force value depends on the diffusivity of liquid through the pores hence the porosity of the network. Faster relaxation indicates smaller porosity and permeability of the network. As shown in figures 8 (a) and (b) for 0.2 and 25% v/v acetic acid concentrations, chitosan microcapsules made with 0.2% acetic acid has smaller porosity compared to capsules made with 25% acetic acid. These differences in porosity also correlated well with stability as discussed earlier. The capsules made with 0.2% acetic acid were more stable compared to capsules made with 25% acetic acid which showed leakage of encapsulated oil after 3 months of storage. As presented in part 2 of this paper, due to low porosity, the chitosan shell of capsules made with 0.2% acetic acid ruptured at 62% deformation relative to their radius whereas due to high porosity, the capsules made with 25% acetic acid ruptured at 110% deformation.

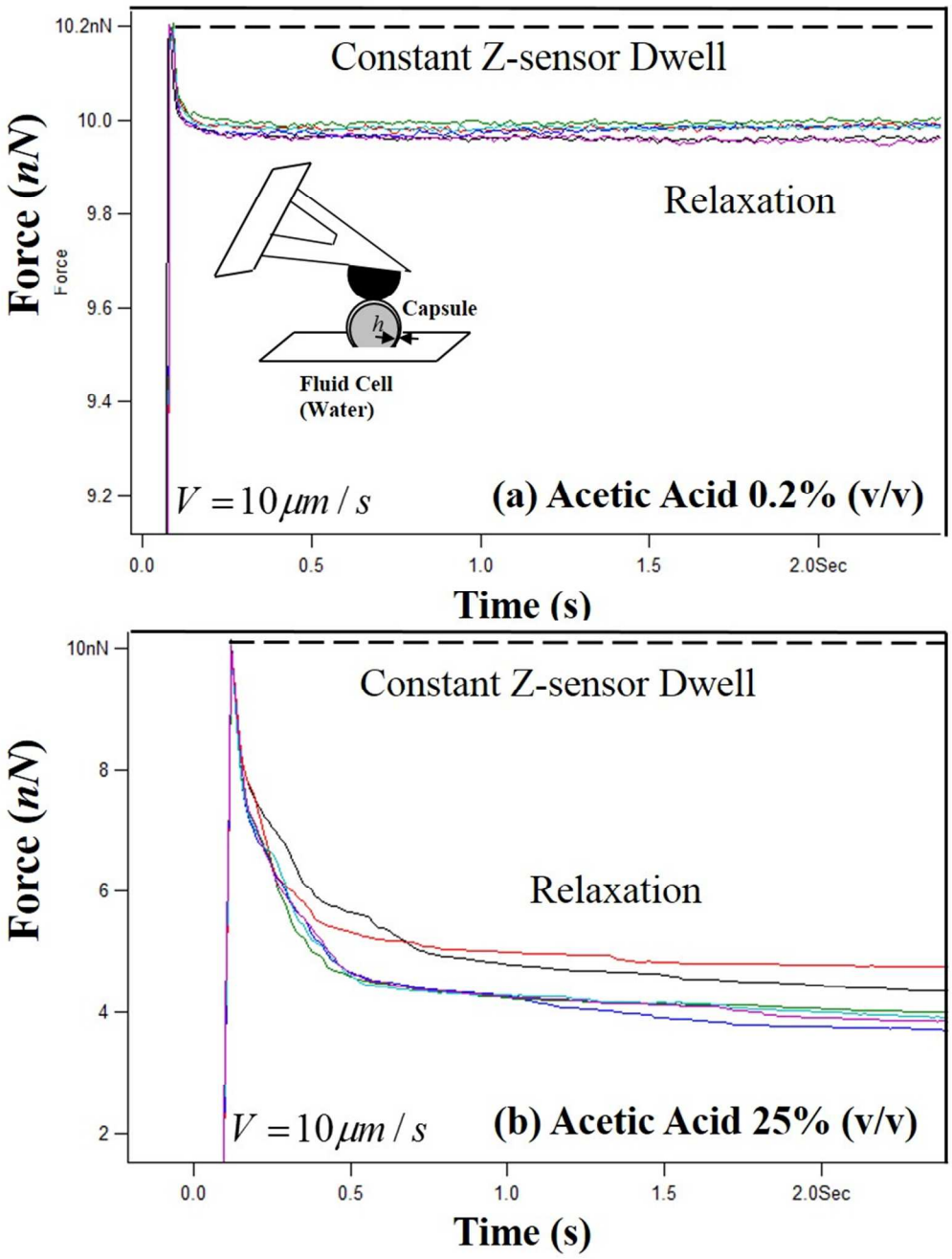

Figure 8. Relaxation experiments on individual capsule to show that the cross-linking is covalent in nature, 0.2% v/v acetic acid (a), 25%(b). Multiple experimental runs on same individual capsule are shown for each sample. The exponential relaxation as observed here is due to covalent cross-linking. If the cross-linking is ionic, the network should continuously break with applied force and the relaxation behavior should be linear as a function of time. The relaxation behavior and time required for saturation to final force depends on permeability and porosity of the cross-linked network of chitosan shell. Hence capsules made with 0.2% v/v acetic acid less porous.

4. Conclusions

Stable tetradecane-filled chitosan microcapsules were ultrasonically synthesized in aqueous solutions at different acetic acid concentrations. The microcapsules have a spherical morphology and smooth surface structure. Not only can the size, size distribution and stability of microcapsules be controlled by the variation of acetic acid amounts, but also the shell thickness and mechanical strength. The proposed mechanisms involve 3 parts: the improved intra-intermolecular hydrogen bonds in the partially acetylated chitosan shell, the amphiphilic property of chitosan itself, and the formation of covalent bond crosslinks. Since acetic acid is commonly used to solubilize chitosan, this one-step method can be applied to prepare chitosan microcapsules with desired size, stability and mechanical strength at an appropriate acetic acid concentration. The simplicity of this method and versatility of chitosan make it a promising way to encapsulate aromas and oils in products and drugs and therapeutic agents in pharmaceutical products.

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Conflicts of Interest:

There are no conflicts to declare.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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