

Effects of Process Parameters in Water-in-Oil Emulsion Method on Diameters of Chitosan Microspheres

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Authors' Summary: Control over the diameters of chitosan microspheres is necessary for the delivery of chemotherapeutic drugs and/or radiation to specific blood vessels at the sight of cancerous lesions. A common method for producing microspheres is the water-in-oil emulsion method. The purpose of this study was to investigate how different process parameters in the water-in-oil emulsion method would affect chitosan microsphere diameters. Physical parameters such as reaction chamber size and stir bar length considerably affected microsphere diameters. The results of this study indicate that the successful synthesis of chitosan microspheres using the water-in-oil emulsion method depends on multiple process parameters which influence size and size distribution.

Abstract

Chitosan microspheres are used for targeted drug delivery and radiomicrosphere therapy; however, control over microsphere diameters is necessary for these treatment modalities to be effective. In this study, the effects of parameters in the water-in-oil emulsion method on the diameters of chitosan microspheres were investigated. Decreasing the volume of glutaraldehyde (crosslinking agent) caused the microspheres to swell more in 200 proof ethanol, whereas increasing the volume inhibited the formation of microspheres. The volume of chitosan solution (2% w/v solution in 2% v/v acetic acid) as well as the volume of tween 80 (surfactant) appeared to have no effect on microsphere diameters. Increasing the size of the round-bottom flask that held the emulsion resulted in the formation of microspheres likely over 1 mm in diameter. In contrast, increasing stir bar length appeared to decrease microsphere diameters. No clear correlation was observed between microsphere diameters and emulsion volume. Despite following the water-in-oil emulsion method of a previous study which had achieved an average microsphere diameter of 30 μm , such an average diameter was not achieved in this study. In conclusion, the results of this study indicate that physical parameters such as round-bottom flask size and stir bar length have a considerable effect on the diameters of chitosan microspheres and that the success of the synthesis is operator dependent such that some, as yet, unidentified factor must have a considerable influence on size and size distribution.

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Introduction

Chitosan, a cationic polysaccharide obtained from the deacetylation of chitin, is used extensively in the formation of microspheres for drug delivery because of its ability to release drugs at a steady and consistent rate, stay at the target site due to its mucoadhesive properties, and degrade into non-toxic products [1,2,3]. Chitosan microspheres have also shown potential in delivering radiation to unresectable liver tumors in radiomicrosphere therapy (RMT). For instance, in a lung perfusion study by Amor-Coarasa *et al.*, chitosan microspheres containing encapsulated ^{90}Y accumulated in target lung tissues. Almost all radiation released from the lungs was excreted to the urine; as a result, no significant dose of radiation was delivered to other organs [4].

The effectiveness of both targeted drug delivery and RMT is dependent on microsphere diameters. As the diameters of microspheres decrease, their surface area-to-volume ratios increase resulting in higher rates of drug release [5]. In RMT the optimal microsphere diameter ($\approx 30\ \mu\text{m}$) is three times larger than the smallest blood vessel diameter ($\approx 10\ \mu\text{m}$) [4]. This ensures that the microspheres are deposited in capillaries and do not remain in circulation. In addition, the microvascular density of liver tumors is 3 – 200 times greater than the surrounding liver tissue making the allocation of $30\ \mu\text{m}$ microspheres preferential to tumors in comparison to normal tissue [4].

Due to the importance of chitosan microsphere diameters on the effectiveness of targeted drug delivery and RMT, it is necessary to control the process parameters that affect the diameters of chitosan microspheres. A common method of synthesizing chitosan microspheres is the water-in-oil (W/O) emulsion method. Chitosan, which is only soluble in water under acidic pH conditions, is dissolved in 1-3% aqueous acetic acid solution [1]. The chitosan solution is added drop-wise into an oil phase under continuous stirring. The mixing of the water and oil phase results in the formation of water-polymer droplets which are stabilized and hardened through the addition of a surfactant and cross-linking agent, respectively. The disadvantage of this method is that control over microsphere diameters between batches is complicated [6]. Therefore, multiple studies have been conducted to investigate the effects of various process parameters in the W/O emulsion method on the diameters of chitosan microspheres.

In the W/O emulsion method the diameters of chitosan microspheres can be controlled by controlling the size of the aqueous droplets added to the oil phase [7]. However, the diameters of the final product depend upon several factors including stirring speed, chitosan molecular weight, chitosan concentration, and the amount of cross-linking agent and surfactant used during the formation of the emulsion. Studies have shown that increasing the stirring speed and thus increasing shear forces within the emulsion leads to a reduction in microsphere diameters [8,9,10]. In contrast, increasing chitosan molecular weight and/or concentration leads to more viscous water-polymer droplets thus resulting in the formation of larger microspheres [10,11]. Increasing the amount of cross-linking agent added to the emulsion results in the formation of denser microspheres that swell less in liquid medium [12]. Multiple studies have reported a reduction in the diameters of microspheres following an increase in surfactant concentration within the emulsion [13,14,15]. Manipulations to the volume ratio between the dispersed and continuous phase (DP/CP ratio) of the emulsion have shown conflicting results [10]. In addition, there have been observations in the literature in which manipulations to the

DP/CP ratio led to the formation of aggregates rather than discrete microspheres [11,16,17].

In this study, multiple parameters in the W/O emulsion method were tested to determine their effects on the diameters of chitosan microspheres. These parameters included the volume of cross-linking agent (glutaraldehyde), volume of surfactant (tween 80), volume of chitosan solution, and the intensity of shear forces within the emulsion. The volume of chitosan solution (dispersed aqueous phase) was manipulated while keeping the volume of toluene solution (continuous oil phase) constant to observe how changing the DP/CP ratio of the emulsion would influence microsphere diameters. The shear forces within the emulsion were not altered by changing the stirring speed; instead, reaction chamber (round-bottom flask) size, stir bar length, and emulsion volume were altered to influence the shear forces within the emulsion.

Materials and Methods

Materials

Chitosan with a molecular weight of 15 kDa and an 85% degree of deacetylation (Polysciences, Inc., USA) was used in this investigation. However, when experimenting with the volume of glutaraldehyde, chitosan with a molecular weight of 100 – 300 kDa (Acros Organics, USA) was used instead. Chemicals employed in this study included toluene, tween 80, glutaraldehyde, acetic acid, petroleum ether, and 200 proof ethanol (Sigma-Aldrich, USA).

Preparation of Chitosan Microspheres

Chitosan microspheres were prepared using the W/O emulsion method described by Amor-Coarasa *et al.* [18]. A 2% (w/v) chitosan solution was prepared by dissolving 0.08 g of chitosan in 3.92 mL of 2% aqueous acetic acid solution. A toluene solution was prepared by mixing 20 mL of toluene and 100 μL of tween 80 for 10 minutes at 1150 rpm using a 32 mm egg-shaped magnetic stir bar in a 50 mL round-bottom flask. 1 mL of chitosan solution was added drop-wise at a rate of 1 drop per 2 seconds into the stirring toluene solution using a plastic 1 mL syringe with a 27 gauge tip. Following 15 minutes of stirring, 100 μL of glutaraldehyde were added as a cross-linker at a rate of 1 drop per 2 seconds using a 250 μL glass syringe with a 27 gauge tip. The emulsion was left to stir for another 90 minutes. The contents of the round-bottom flask were washed three times with petroleum ether and twice with 200 proof ethanol. 200 proof ethanol was added a third time to the round-bottom flask in order to suction the particles out using a plastic pipet and transfer them to a centrifuge tube.

Sizing of Chitosan Microspheres

Using a transfer pipette, a sample of chitosan microspheres was taken from a centrifuge tube and placed on a glass slide. Microspheres were viewed and measured using a compound light microscope and an attached camera equipped with measuring software (AmScope, USA). Average diameters \pm SD were calculated from the measurements of at least 50 microspheres. Diameter ranges were determined by recording diameters of the smallest and largest microspheres observed. No statistical analysis was performed; therefore, both the average diameters \pm SD and diameter ranges represent the results of single experiments.

Tested Parameters of W/O Emulsion Method

The following parameters in the W/O emulsion method were tested: volume of glutaraldehyde, volume of tween 80, volume of chitosan solution, volume of emulsion, size of round-bottom flask and stir bar length. The volumes of tween 80, glutaraldehyde, toluene, and chitosan solution used when preparing the chitosan microspheres yielded an emulsion volume of 21.2 mL. The volumes of these emulsion components were changed simultaneously to observe the effect of different emulsion volumes on the diameters of chitosan microspheres (Table 1). With regard to syringe tip size, in the experiments on the volume of chitosan solution and round-bottom flask size, 20 gauge instead of 27 gauge syringe tips were used to add the chitosan solution and glutaraldehyde. In addition, in the experiment on the volume

of the glutaraldehyde micropipettes were used rather than syringes.

Results

The effect of the volume of glutaraldehyde on the physical properties of chitosan microspheres was explored. Reducing the volume of glutaraldehyde from 100 to 50 μL appeared to increase the diameters of the microspheres (Table 2). The microspheres made with 50 μL of glutaraldehyde lacked surface smoothness and were often found in aggregates (Figure 1ab). In contrast, the

Table 1. Volumes of emulsion and emulsion components. The tested volumes of emulsion were half (10.6 mL) and three-quarters (15.9 mL) the original emulsion volume (21.2 mL).

Emulsion (mL)	Tween 80 (μL)	Glutaraldehyde (μL)	Toluene (mL)	Chitosan Solution (mL)
21.2	100	100	20	1
15.9	75	75	15	0.75
10.6	50	50	10	0.5

Table 2. Tested parameters and resulting diameter ranges of chitosan microspheres.

Tested Parameter	Diameter Range (μm)	
Glutaraldehyde (μL)	50	300 - 800
	100	300 - 500
	150	—
Trial 1 - Chitosan Solution (mL)	1	200 - 400
	0.5	100 - 300
Trial 2 - Chitosan Solution (mL)	1	100 - 200
	0.5	30 - 200
Flask Size (mL)	50	100 - 200
	100	—

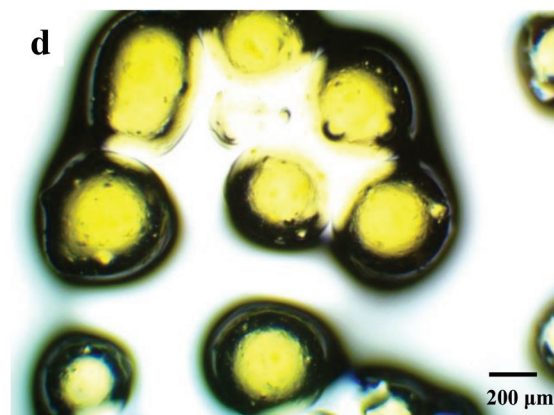
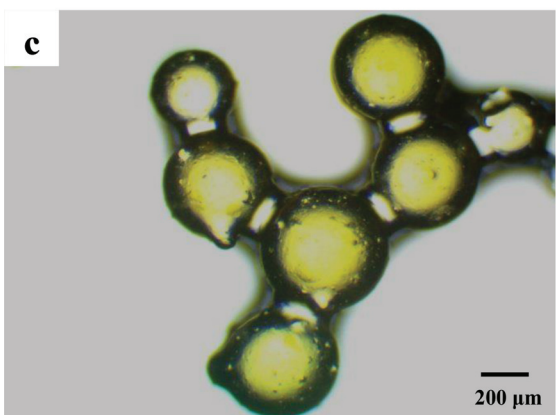
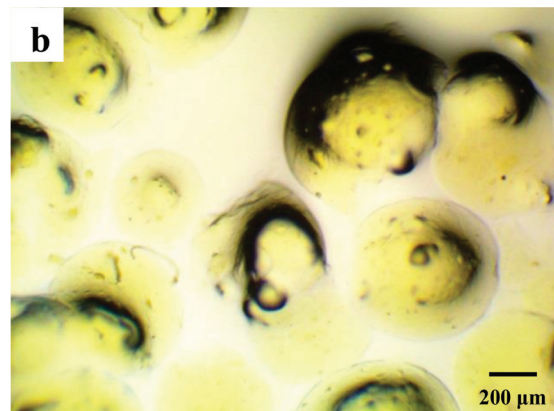
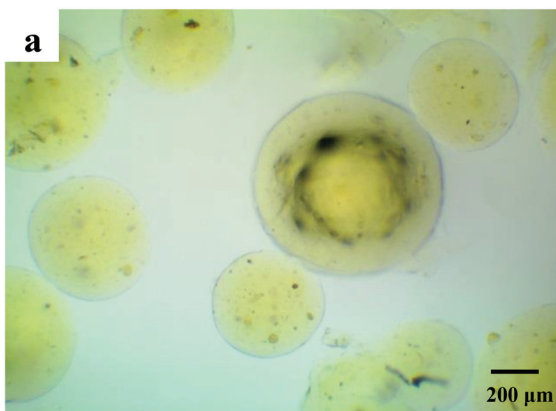


Figure 1. (4X) Chitosan microspheres made with 50 (ab) and 100 μL (cd) of glutaraldehyde.

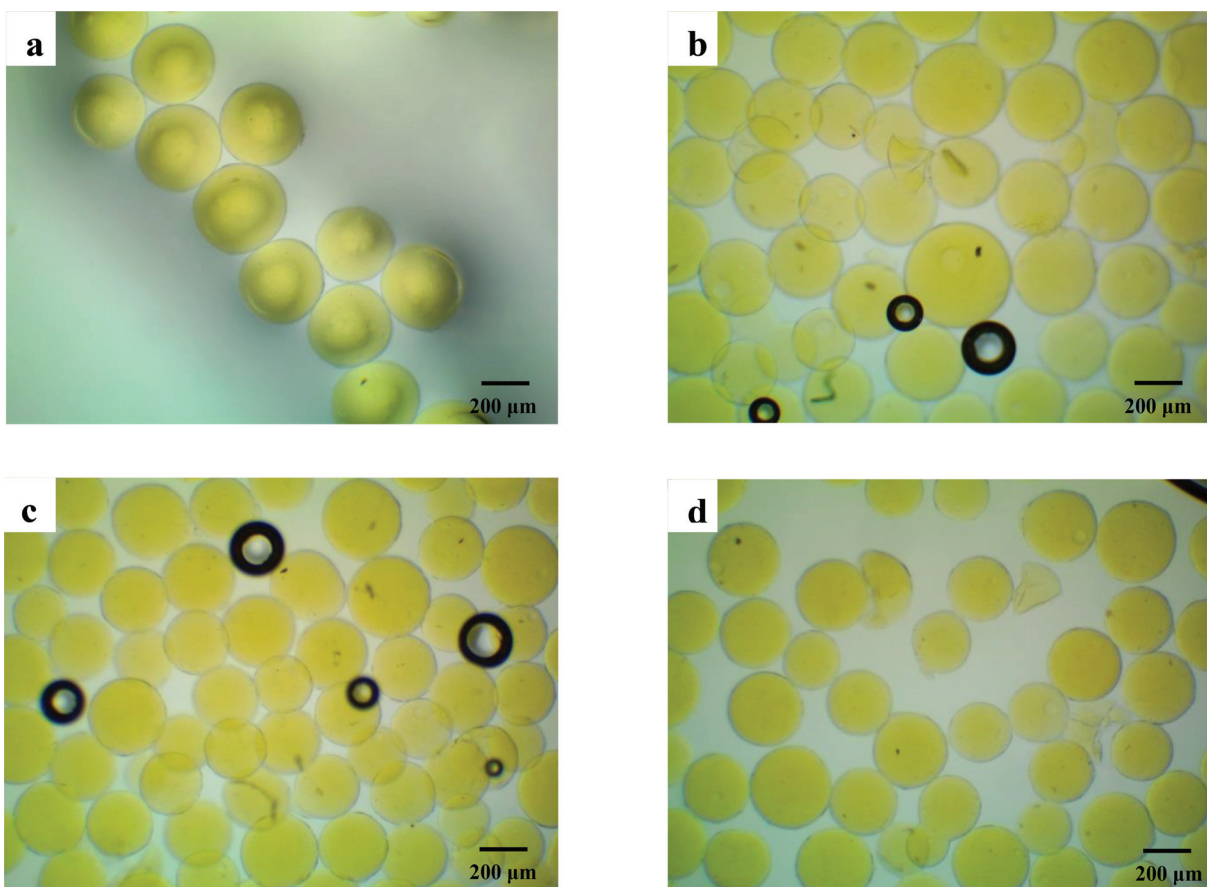


Figure 2. (4X) Chitosan microspheres made with 1 (ab) and 0.5 mL (cd) of chitosan solution from Trial 1.

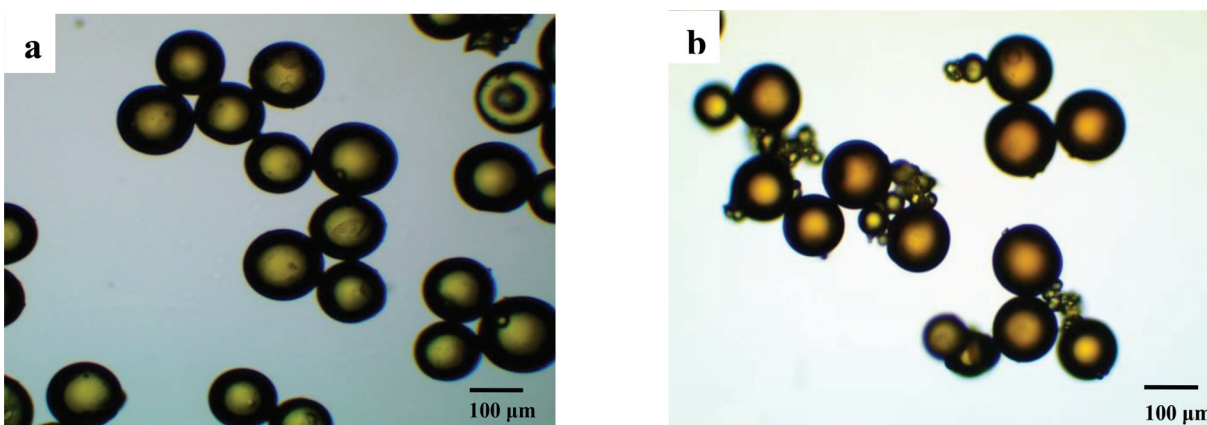


Figure 3. (10X) Chitosan microspheres made with 1 (a) and 0.5 mL (b) of chitosan solution from Trial 2. These microspheres were sized and photographed after the 200 proof ethanol had evaporated off the microscope slide leading to an overall reduction in size and more visible spherical appearance.

microspheres made with 100 μL of glutaraldehyde showed clear spherical geometry and few aggregates were present (Figure 1cd). Increasing the volume of glutaraldehyde from 100 to 150 μL resulted in large aggregates of chitosan thus inhibiting the formation of microspheres.

Reducing the volume of chitosan solution and thereby changing the DP/CP ratio of the emulsion appeared to have no effect on microsphere diameters. In Trial 1, the sample made with 1 mL of

chitosan solution contained microspheres that were 300 - 400 μm in diameter. Microspheres of this size were not observed in the sample made with 0.5 mL of chitosan solution (Table 2). However, a majority of the microspheres made with 1 and 0.5 mL of chitosan solution overlapped in size exhibiting diameters between 200 and 300 μm (Figure 2). In Trial 2, the sample made with 0.5 mL of chitosan solution contained microspheres with diameters below 100 μm . Microspheres of this

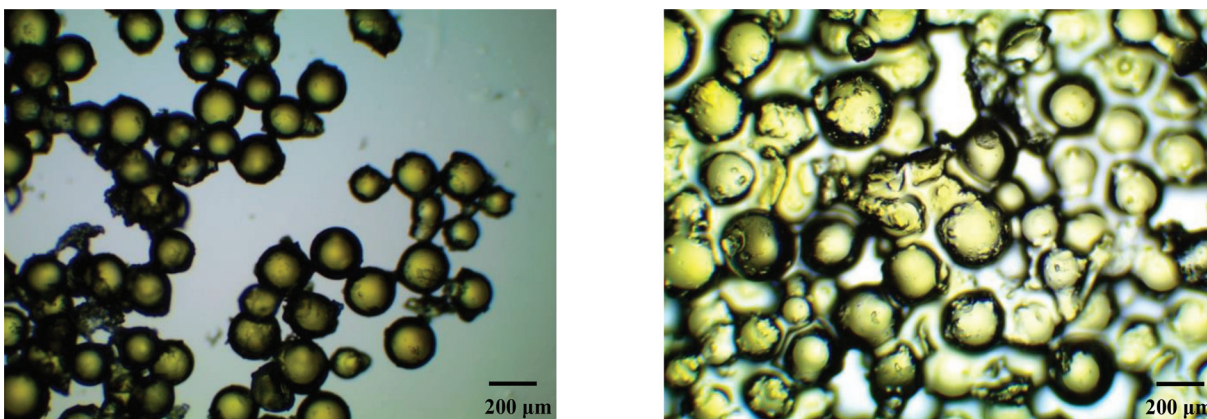


Figure 4. (4X) Chitosan microspheres made in a 50 mL round-bottom flask.

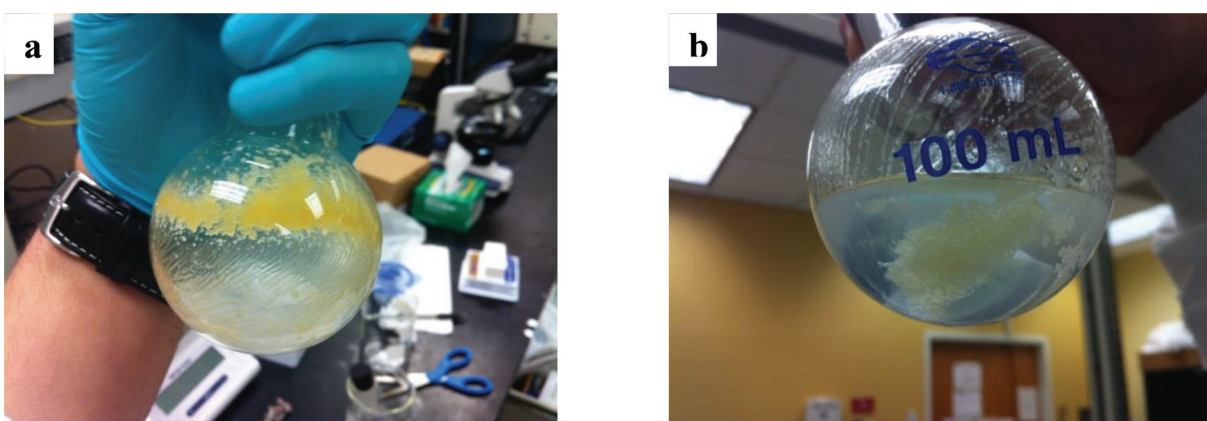


Figure 5. (a) 100 mL round-bottom flask in which turbulent stirring caused the emulsion to adhere to the sides of the flask. (b) 100 mL round-bottom flask containing microspheres clearly visible with the naked eye and likely over 1 mm in diameter.

size were not observed in the sample made with 1 mL of chitosan solution (Table 2). However, a majority of the microspheres made with 1 and 0.5 mL of chitosan solution overlapped in size exhibiting diameters between 100 and 200 μm (Figure 3).

Increasing the size of the round-bottom flask that held the emulsion from 50 to 100 mL led to several complications in the formation of microspheres. The microspheres made in the 50 mL round-bottom flask lacked surface smoothness and were often found in aggregates (Figure 4). At first, when switching to a 100 mL flask the stir bar would spin in a wobbly, erratic motion causing most of the emulsion to adhere to the sides of the flask (Figure 5a). The larger size of the round-bottom flask gave the stir bar more room to spin off its axis of rotation resulting in turbulent stirring. Following careful positioning of the round-bottom flask on the magnetic stir plate, the stir bar returned to more consistent, steady stirring. The resulting microspheres could be clearly seen with the naked eye and were likely over 1 mm in diameter (Figure 5b). As a result, the microspheres were not viewed under the microscope and no diameter range was recorded.

Increasing the length of the magnetic stir bar from 32 to 38 mm slightly decreased the average diameter of the microspheres (Figure 6). However, the microspheres made using a 38 mm stir bar exhibited poor surface smoothness and were found in aggregates (Figure 7). The volume of tween 80 had no effect on the average diameter of the microspheres (Table 3). In contrast to

the microspheres made with 100 μL of tween 80, the microspheres made with 75 and 125 μL of tween 80 exhibited poor surface smoothness (Figure 8). Although reducing the volume of emulsion decreased the average diameter of the microspheres, there was significant overlap in the diameters of microspheres made with different volumes of emulsion (Figure 9). The microspheres made with 21.2 and 10.6 mL of emulsion had smooth surfaces while the microspheres made with 15.9 mL of emulsion appeared unsmooth and were consistently found in aggregates (Figure 10).

Discussion

The aim of this study was to investigate the effects of multiple parameters in the W/O emulsion method on the diameters of chitosan microspheres. The increase in microsphere diameters observed following a reduction in the volume of glutaraldehyde was likely a result of more swelling in the 200 proof ethanol. Reducing the volume of glutaraldehyde decreased polymer crosslinking within the microspheres thus increasing the uptake of liquid by the polymer mesh [12]. In contrast, increasing the volume of glutaraldehyde resulted in excess polymer crosslinking thus inhibiting the formation of microspheres.

Although reducing the volume of chitosan solution (dispersed phase) and thereby manipulating the DP/CP ratio of the emulsion appeared to have no effect on microsphere diameters, there have been multiple observations in the literature in which polymer

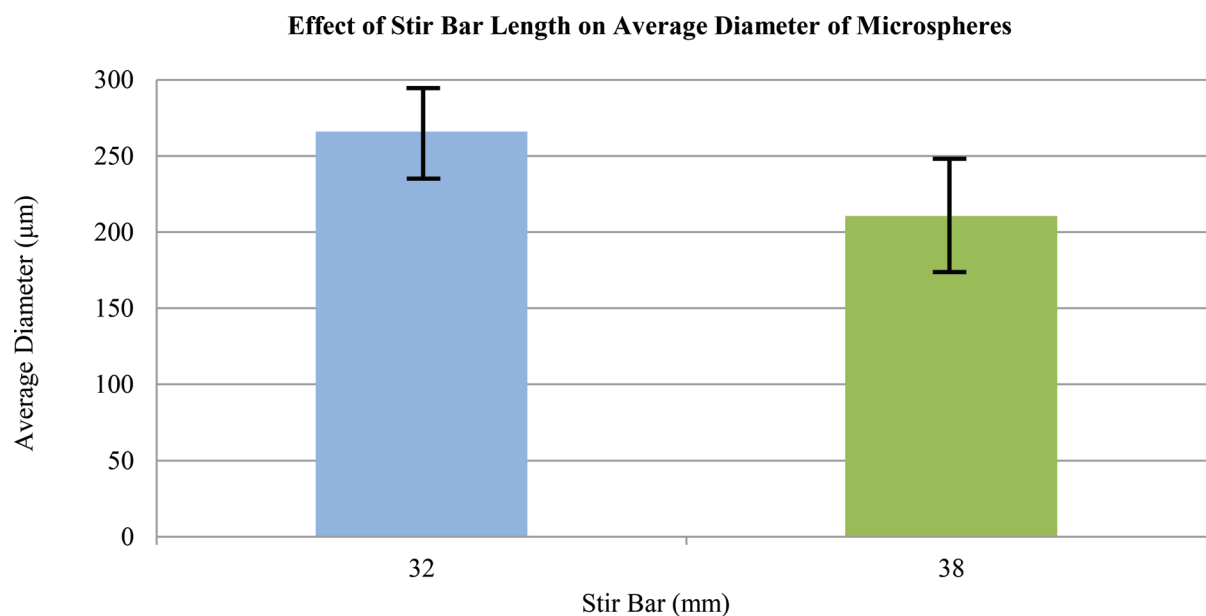


Figure 6. Average diameters \pm SD (black bars) of microspheres made with 32 and 38 mm stir bars. Average diameters \pm SD were calculated from the measurements of at least 50 microspheres and represent the results of single experiments.

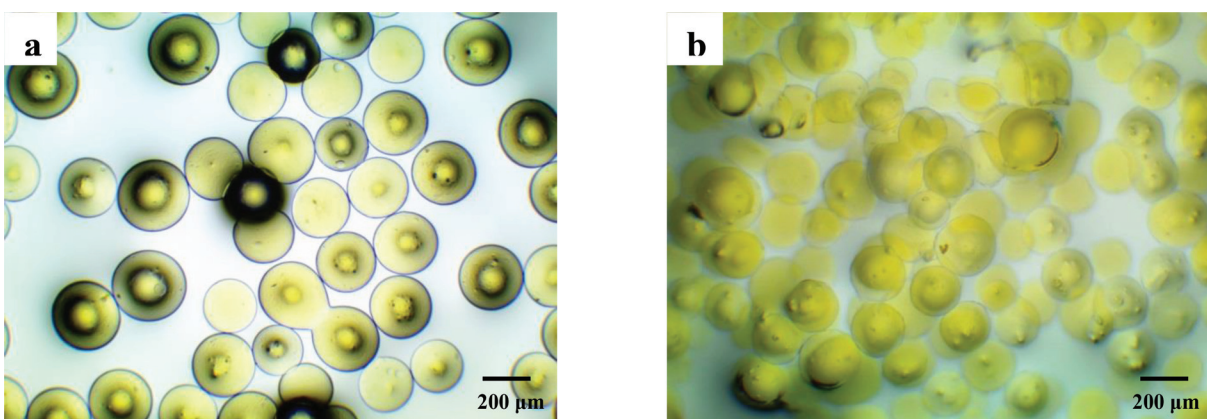


Figure 7. (4X) Chitosan microspheres made using 32 (a) and 38 mm (b) magnetic stir bars.

Table 3. Tested parameters and resulting average diameters \pm SD of chitosan microspheres. The control refers to microspheres made with no manipulations to parameters in the water-in-oil emulsion method.

Tested Parameter		Number of Microspheres Measured	Average Diameter \pm SD (μm)
Control	—	81	266 \pm 30
Stir Bar Length (mm)	38	64	211 \pm 38
Tween 80 (μL)	75	57	258 \pm 44
	125	52	255 \pm 25
Emulsion (mL)	10.6	72	155 \pm 58
	15.9	69	204 \pm 62

aggregates rather than discrete microspheres formed when manipulating the DP/CP ratio. In a preliminary study by Baimark *et al.*, increasing the volume of the dispersed phase reduced microsphere yields and led to the formation of particle aggregates [11]. In a study by Sah, poly (lactic-co-glycolic acid) (PLGA) microspheres were made using an oil-in-water emulsion method. When 8 mL of PLGA solution were added to 20 or 50 mL of water phase, distinct, spherical droplets formed. In contrast, when the volume of the water phase was increased to 80 mL or more, the microspheres formed quickly into large, irregular precipitates [16,17]. Collectively these studies show that manipulations to the DP/CP ratio influence the formation of polymer aggregates in emulsification methods.

Studies have shown that increasing the volume of tween 80 significantly decreases the average diameters of chitosan microspheres [13]. Similar results have been reported with surfactants other than tween 80. For instance, Roy *et al.* reported a reduction in the average diameter of chitosan microspheres loaded with mefenamic acid when increasing the concentration (% w/v) of span

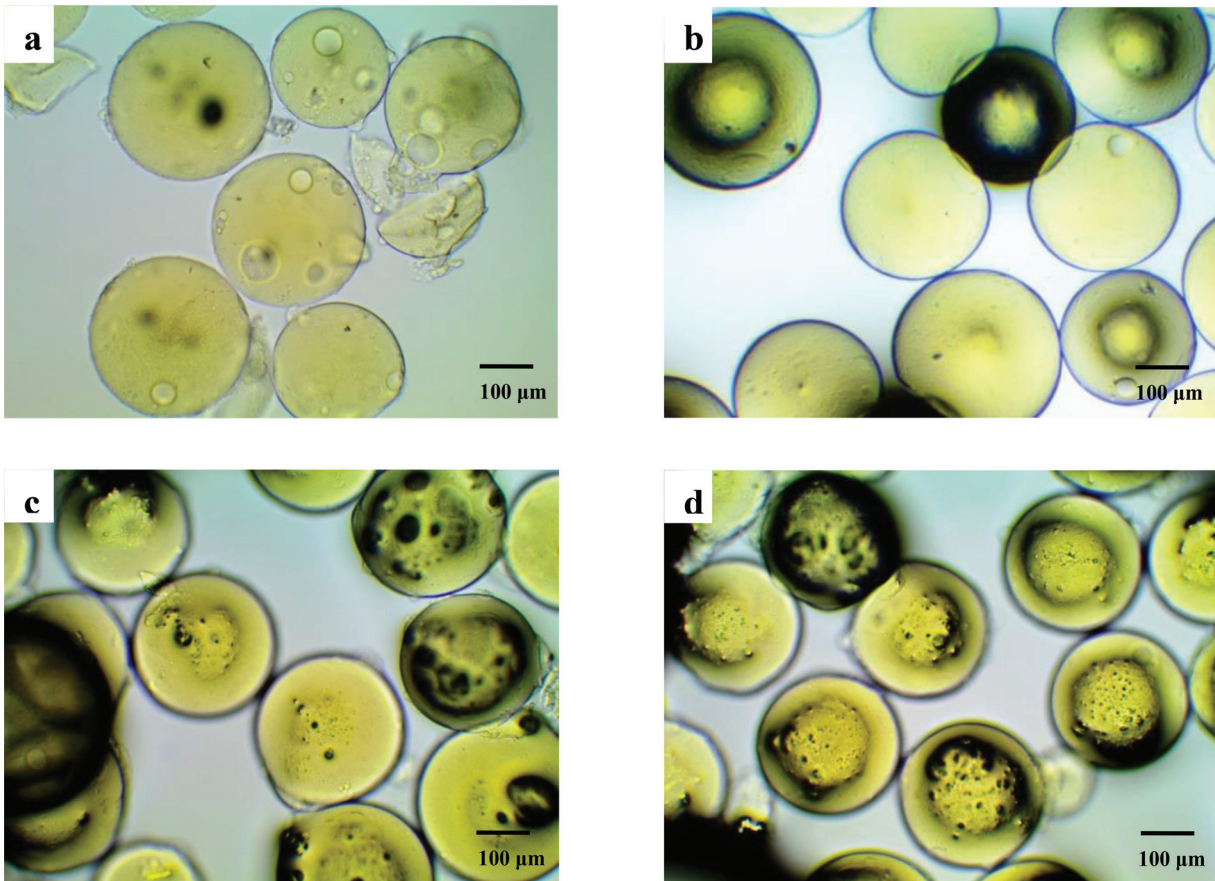


Figure 8. (10X) Chitosan microspheres made with 75 (a), 100 (b), and 125 µL (cd) of tween 80.

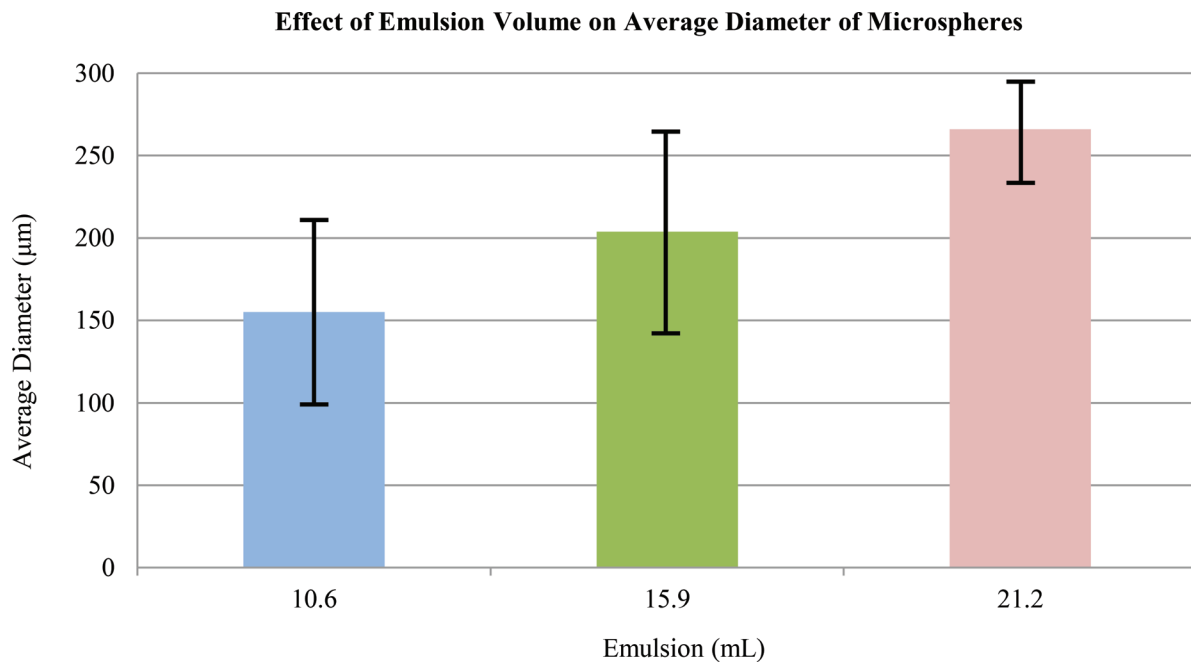


Figure 9. Average diameters \pm SD (black bars) of microspheres made with 10.6, 15.9, and 21.2 mL of emulsion. Average diameters \pm SD were calculated from the measurements of at least 50 microspheres and represent the results of single experiments.

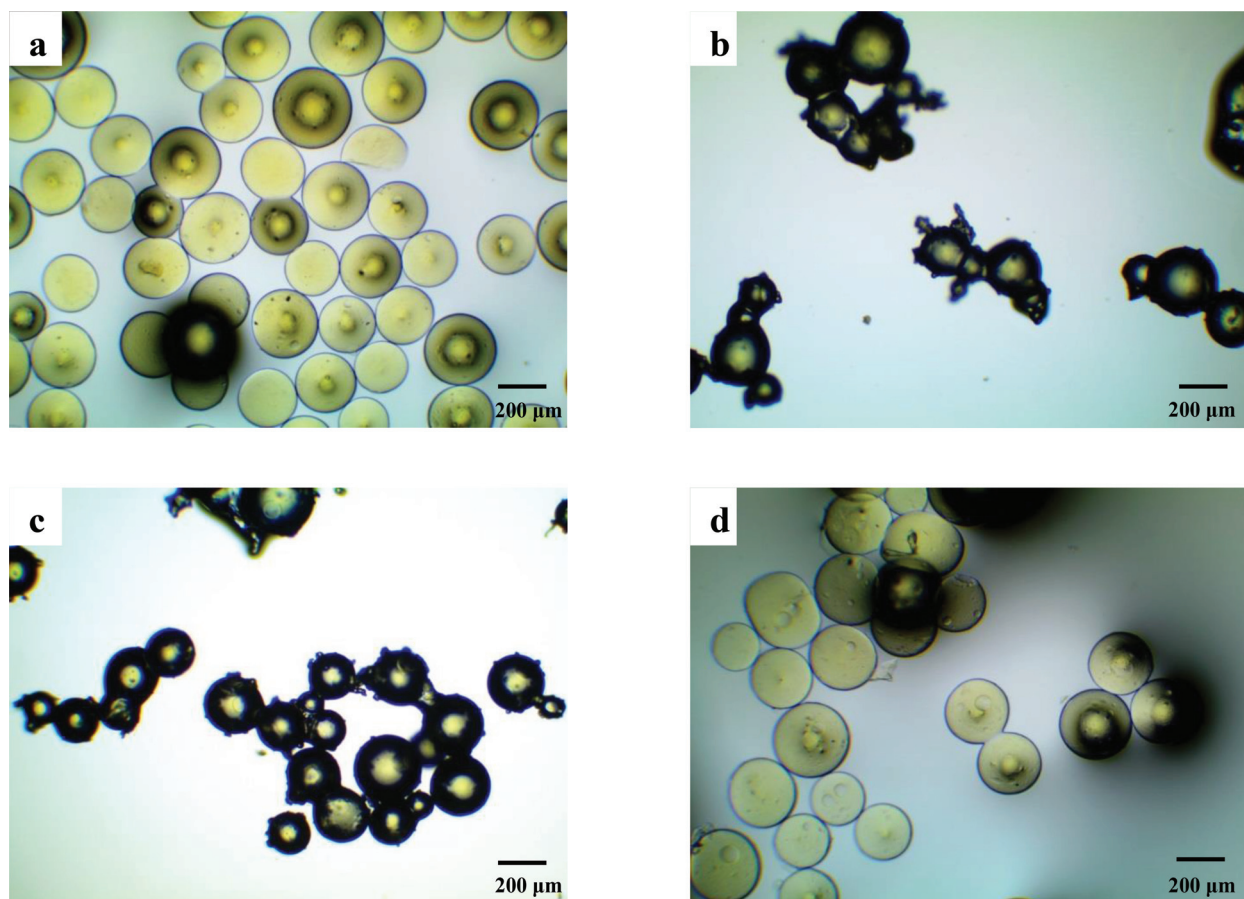


Figure 10. (4X) Chitosan microspheres made with an emulsion volume of 21.2 (a), 15.9 (bc), and 10.6 mL (d).

85 in the oil phase [14]. Therefore, the volumes of tween 80 investigated in this study likely needed to be changed more significantly to affect the average diameter of the chitosan microspheres. Although the purpose of tween 80 as a surfactant was to stabilize the aqueous droplets in the oil phase, increasing the volume of tween 80 caused the microspheres to have unsmooth, irregular surface geometry. Similar results were reported in a study by Chen *et al.* in which increasing the ratio of tween 80 to span 80 lead to more rough and irregular surface geometry in multi-(amino acid) copolymer microspheres [15].

Increased stirring rates intensify shear forces within the emulsion leading to a reduction in average microsphere diameter [8,9]. Although stirring rate (1150 rpm) was kept constant throughout this study, the shear forces within the emulsion were certainly influenced by round-bottom flask size and stir bar length. Increasing round-bottom flask size from 50 to 100 mL caused the height of the emulsion in the round-bottom flask to decrease. As a result, a less structured vortex with weaker shear forces developed above the stir bar thus hindering the dispersal of aqueous droplets and leading to the production of larger microspheres. In contrast, increasing the stir bar length from 32 to 38 mm caused more movement within the emulsion and greater dispersal of aqueous droplets resulting in the formation of smaller microspheres. The results of this study in collection with the expanding literature indicate that increased stirring rates decrease average microsphere diameter however the extent of this reduction depends on multiple factors including the size ratio of the stirring apparatus and mixing vessel [10].

Due to significant overlap in the diameters of microspheres made with different emulsion volumes (10.6, 15.9, and 21.2 mL), no clear correlation was observed between average microsphere diameter and emulsion volume. Reducing emulsion volume appeared to influence shear forces in two ways. First, the ratio between the volume of fluid and the surface area of the stir bar decreased resulting in greater movement throughout the emulsion. Second, the height of the emulsion within the round-bottom flask decreased resulting in a less structured vortex with weaker shear forces. These conflicting influences indicate that there is an optimum emulsion volume at which vigorous shear forces are attained without negatively affecting the integrity of the stirring vortex.

Amor-Coarasa *et al.* obtained chitosan microspheres with an average diameter of $30 \pm 5 \mu\text{m}$ that swelled 20 – 25% when placed in contact with water [18]. Even when exactly following the parameters outlined in the W/O emulsion method of Amor-Coarasa *et al.* microspheres within this size range were not observed. Two parameters not detailed in the W/O emulsion method of Amor-Coarasa *et al.* were round-bottom flask size and stir bar length. These two parameters determine the consistency of stirring and strength of shear forces within the emulsion. In addition, there was no mention of what instrument (syringe, micropipette, etc.) or rate of drop-wise addition should be employed when adding the chitosan solution and glutaraldehyde to the toluene solution. The literature indicates that the diameters of microspheres can be manipulated by controlling the size of the aqueous droplets added to the oil phase [1]. For instance, in a membrane emulsification study by Wang *et al.* a linear relationship

was found between pore size and the diameters of chitosan microspheres [7].

In conclusion, this study illustrated how physical parameters in the W/O emulsion method such as round-bottom flask size and stir bar length have a considerable effect on the stirring dynamics of the emulsion and the resulting diameters of chitosan microspheres. Chitosan microspheres continue to be instrumental in targeted drug delivery as well as RMT. However, the optimal microsphere diameter for RMT ($\approx 30 \mu\text{m}$) was not achieved indicating that the success of the synthesis is operator dependent such that some, as yet, unidentified factor must have a considerable influence on size and size distribution.

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