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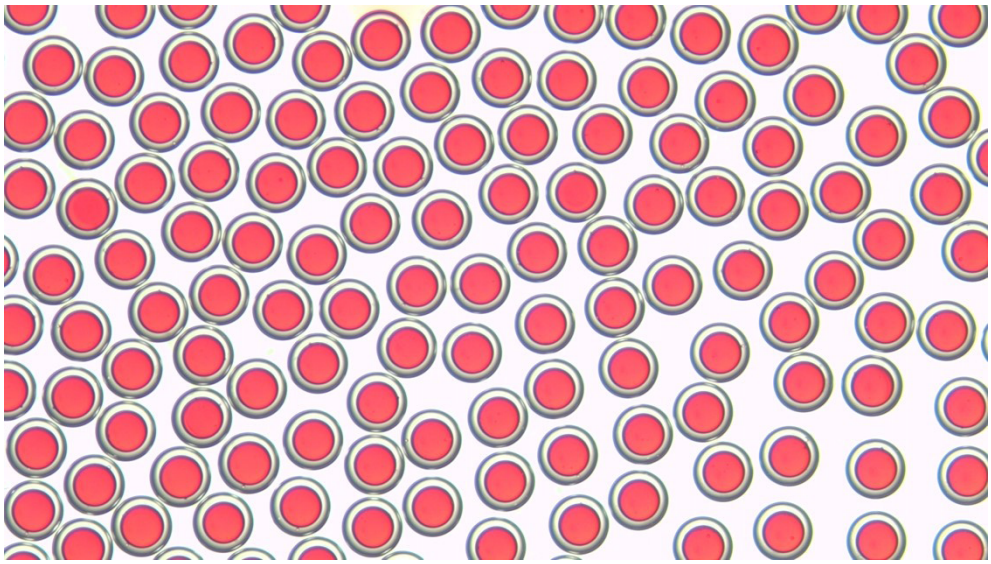
CHITOSAN MICROCAPSULES SYNTHESIS

APPLICATION NOTE



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INTRODUCTION

Over the past few decades, core-shell microcapsules have been extensively used for the delivery and release of materials in the pharmaceutical, cosmetic, and food industries Microencapsulation of lipophilic drugs has attracted special attention because highly effective, but poorly water-soluble drug candidates are common outcomes of drug discovery programs. In this context, microcapsules consisting of a chitosan shell and an oily core have been extensively studied as chitosan, a cationic polysaccharide, exhibits numerous benefits.
These include: excellent biological activity, good biocompatibility and biodegradability and pH sensitivity for acid-triggered oral delivery. Traditional microencapsulation methods such as coacervation, spray drying, and solvent evaporation, require complex processes and equipment and are difficult to control the size and load of the microcapsules. Microfluidics allows for the production of monodisperse double emulsions with a high level of control over both the size and the structure.

In this Application Note, chitosan-shell/oily-core microcapsules are generated using the Raydrop double emulsion chips, a capillary based microfluidic device and Fluigent pressure-based flow controllers. This allows for the generation of double emulsions in a user friendly manner. The influence of the fluidic parameters on the size and the release from the oil across the shell are studied and presented.

MATERIALS

1. Setup

The production of droplets has been performed with the Raydrop Platform, a lab system integrating all the components needed to produce simple and double emulsions. This platform is divided into three parts: mechanical, fluidic, and optical.

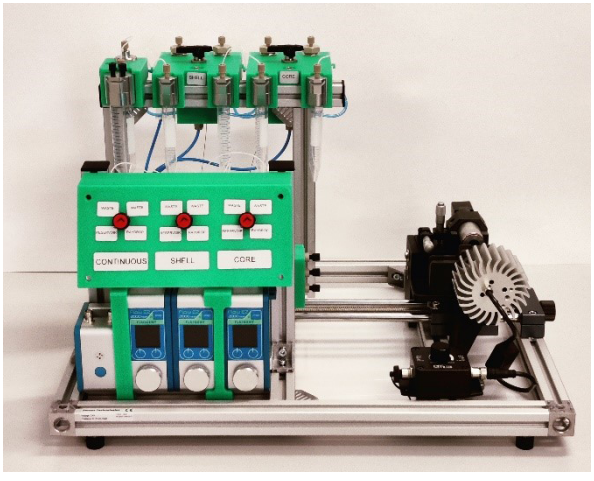


Figure 2: *Raydrop Platform*

- **Mechanics:** The mechanical assembly includes x-y-z displacement plates that allow to adjust the focus and the observation window in the Raydrop.
- **Fluidics:** The fluidic section includes the Fluigent Flow EZ controllers and the required tubing and valves which permit automated fluidic injection. A pressure is set on each reservoir, and fluids are delivered to the microfluidic chip. It also includes Falcon reservoirs and the Raydrop, in which double emulsions are generated.
- **Optics:** The optical assembly contains an LED light source and a color USB 3.0 camera. This camera is connected to a computer to observe the droplet formation in real time, to control the stability of the emulsion and measure the size of interest (core, shell).

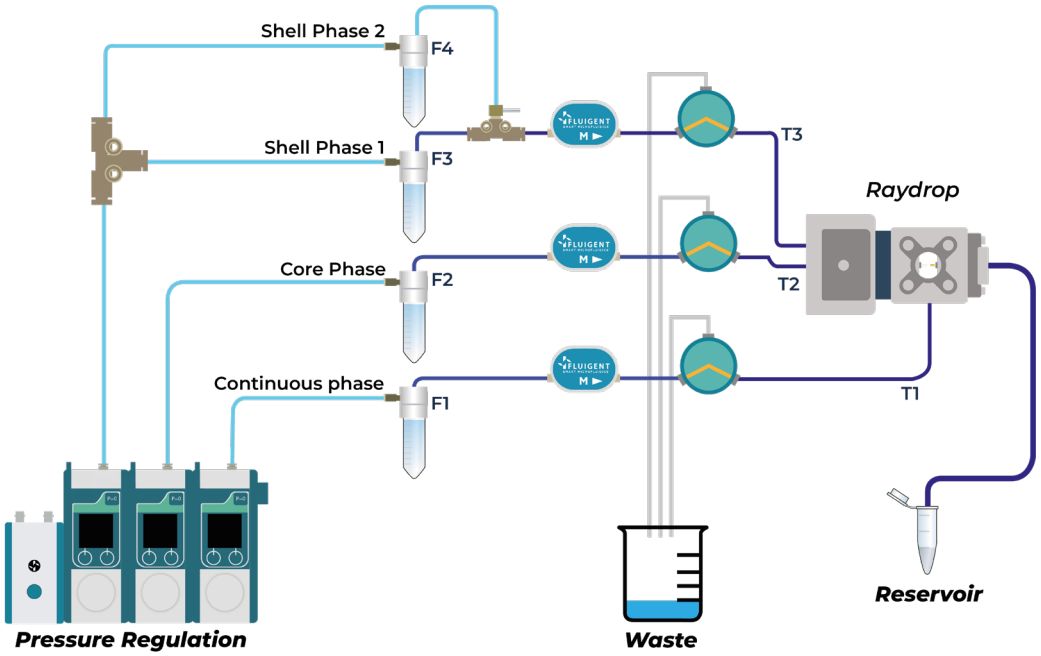


Figure 3: *Experimental set-up to produce double emulsion*

Fluid reservoirs

Falcon identification	F1	F2	F3	F4
Volume (mL)	50	50	50	15
Phase*	Continuous	Core	Shell (priming and cleaning)	Shell
Composition	1-octanol + 2% Span80	Soybean oil + Sudan IV	Water + 2% acetic acid	Water + 2% chitosan 30-100 cps + 1% PLURONIC® F-127 + 2% acetic acid

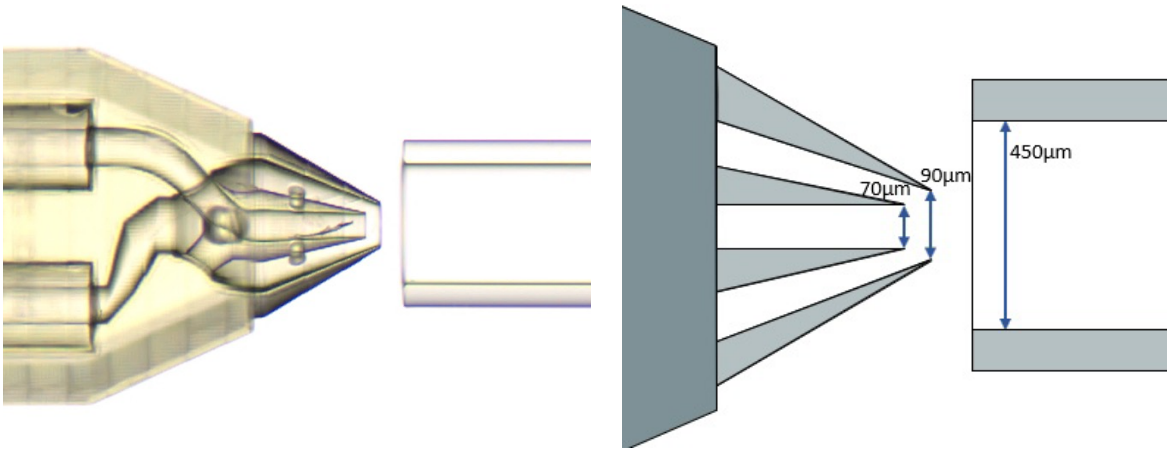
*Each phase must be filtered to avoid clogging the tubing or the nozzle of the Raydrop. Therefore, there is a filter after each Falcon. In this case, the Falcons' filters F1 and F4 have a 10 µm filter pore size and the Falcons' filters F2 and F3 have a 2 µm filter pore size.

2. System components

Microfluidic system:

- Pressure controller: Fluigent LineUp™ Flow EZ 7 bar[1]*
- Flow rate sensor: Fluigent Flow Unit M (x2) and L (x1)
- Microfluidic droplet generator: Raydrop double emulsion*

**Raydrop™ is a registered trademark of Secoya Technologies*



Picture of the Raydrop **Scheme of the Raydrop**

Figure 4: *Microfluidic droplet generator*

Nozzle information

Part	Core nozzle	Size-shell nozzle	Size-extraction capillary
Inside diameter (µm)	70	90	450

Software information

Fluigent OxyGEN software (formerly AiO software)

[1] The pressure controllers used are 7 bar full scale. The maximum pressure used for the generation of double emulsions with large shells is 2440 mbar (corresponding to a shell phase flow rate of 24.3 µL/min). Except from this scenario, maximum working pressure is 1650 mbar. Priming and cleaning steps can require a pressure higher than 2 bar.

3. Reagents

Core phase:

Soybean oil (8001-22-7, Sigma-Aldrich) containing red dye Sudan IV (Sigma-Aldrich)

Shell phase:

Water containing 2% chitosan (viscosity 30-100 mPa·s, Glentham Life Sciences UK), 2% acetic acid (Sigma-Aldrich), 1% Pluronic® F-127 (Sigma-Aldrich).

Note that the chitosan is very viscous. Therefore, the preparation of this solution should be done in the following order: add Pluronic® F-127 to the water while stirring with a bar magnet and, once the PLURONIC® F-127 is dissolved, gradually

Continuous phase:

1-octanol (Glentham Life Sciences UK) containing 2% Span 80 (Sigma-Aldrich)

Collection phase:

Heptane (VWR) containing 2% Span 80 (Sigma-Aldrich) and 0,3 wt% glutaraldehyde (50% in H2O, Glentham Life Sciences UK)

METHOD: SYNTHESIS OF CHITOSAN CAPSULES

Monodisperse chitosan particle synthesis is performed in 2 main steps:

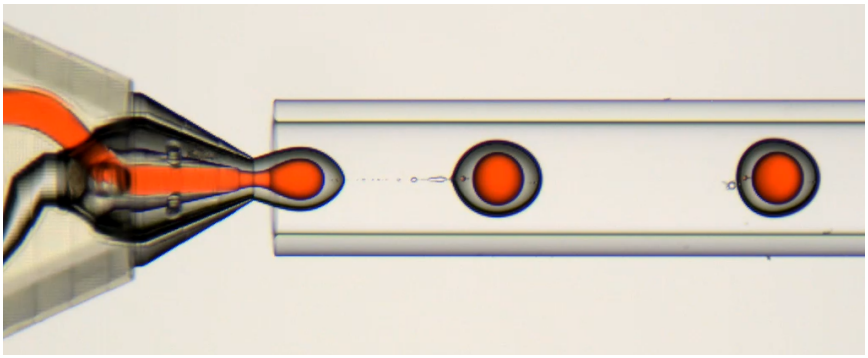
- Generation of monodisperse double emulsion in the Raydrop
- Capsule formation by reticulation of the chitosan shell in the collection bath

1. Double emulsion generation

To generate droplets, the system must first be primed with pure solvent in the shell phase (here water + 2% acetic acid). Once droplet formation is stable, the shell phase is switched to the chitosan-based solution. This avoids clogging issues during the transient phase. The user should follow the steps below, using the typical flow rates shown in the table below:

1. Set the valve on the Falcon F3 (priming solution)
2. Fill the Raydrop with the continuous phase (refer to the user guide for more information)
3. Set the continuous phase (F1) to the desired flow rate
4. Set the shell phase (F3) to the desired flow rate. At this point, a co-flow of 1-octanol and water is generated.
5. Set the core phase (F2) to the desired flow rate to generate double emulsions
6. When the double emulsion is stabilized, switch the valve to the chitosan-based shell solution (F4)
7. Wait** until the chitosan solution crosses the tubing and reaches the Raydrop to form a double emulsion with a chitosan solution shell and a soybean oil in the 1-octanol continuous phase

	Continuous phase	**Shell**	**Core**
Composition	1-octanol + 2% Span 80	Water + 2% chitosan 30-100cps + 1% PF127 + 2% AcOH	Soybean oil
Flow rate (µL/min)	130	15	12



[2]

Figure 5: *Generation of double emulsion droplets in the Raydrop. Red dye Sudan IV is added in the core phase to increase the contrast.*

8. If necessary, stabilize the double emulsion by varying the flow rates
9. Adjust the flow rates to obtain the desired droplet diameter and shell thickness
10. Collect the droplets at the outlet of the Raydrop in a plastic container e.g. polypropylene (droplets will tend to wet a glass container).

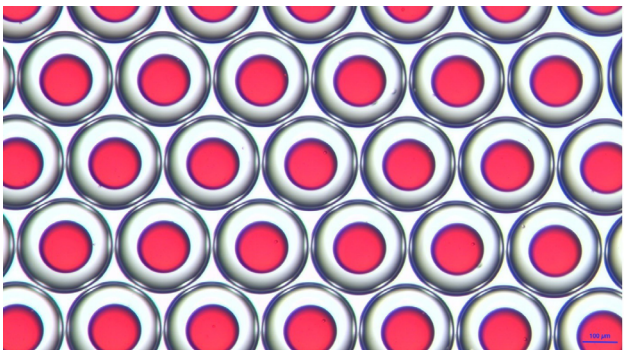


Figure 6: *chitosan-shell/oil-core double emulsion collected in the 1-octanol continuous phase*

Before stopping the experiment, flush the shell tubing (T3) and the nozzle of the Raydrop with the priming solution (F3). This priming/cleaning solution only contains water and acetic acid, which allows the evacuation of the chitosan. This cleans the tubing clogging is avoided.

11. To flush the chitosan out of tubing and Raydrop, switch the valve on the priming solution (F3)
12. Wait** until the cleaning solution crosses the tubing and reaches the Raydrop to form a double emulsion with a water solution shell and a soybean oil in the 1-octanol continuous phase
13. Stop the flow of the core phase
14. Then, stop the flow of the shell phase
15. Finally, stop the flow of the continuous phase

** *This can take 10 to 15 minutes, depending on the flow rate of the chitosan phase and the diameters and length of the tubing.*

[2] *DCA-MAM-019-video1-frame-rate-4.75*

2. Capsule formation

After generation, the droplets are collected in a cross-linking solution of 0.3% glutaraldehyde in hexane. The chitosan reacts with glutaraldehyde by solvent extraction and chemical cross-linking based on the Schiff base reaction. The droplets are solidified and become glutaraldehyde cross-linked chitosan microcapsules.

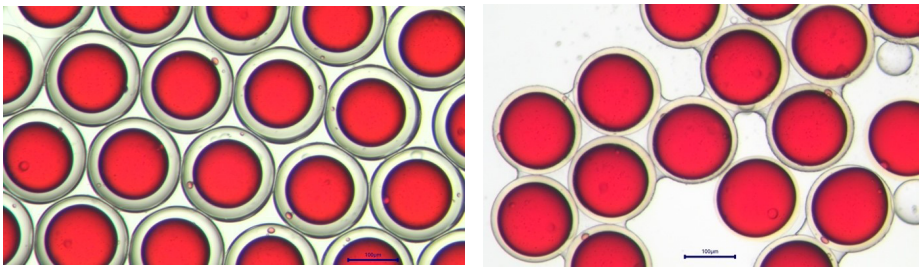


Figure 7: *Glutaraldehyde cross-linked chitosan microcapsules on the cross-linking bath[3]. On the left, after 4 minutes in the cross-linking bath. On the right, after 1h in the cross-linking bath. The shell thickness decreases and becomes progressively yellow, as a part of its water content diffuses in the continuous phase. Expelled water is clearly visible wetting the capsules.*

RESULTS

In this Application Note, different parameters were studied. First, the evolution of the droplets over time was observed. Then, the influence of the middle and the outer phase flow rates were studied.

1. Evolution of the droplet diameter during the cross-linking process

After generation, the double emulsion droplets are collected into the collection solution. An analysis of the size of the capsules is performed using a microscope and measurement software (ToupView). For a given sample, several measurements of the capsule diameter are done at different times. The evolution of the diameter is highlighted in Figure 8 and the operating conditions are shown in Table 1.

[2] *DCA-MAM-019-video1-frame-rate-4.75*

Table 1: *Operating conditions for the evaluation of the diameter of capsules*

	Continuous phase	**Shell**	**Core**
Composition	1-octanol + 2% Span 80	Water + 2% chitosan 30-100cps + 1% PF127 + 2% AcOH	Soybean oil + Sudan IV
Pressure (mbar)	164	1650	1384
Flow rate (µL/min)	162	16,4	13,6

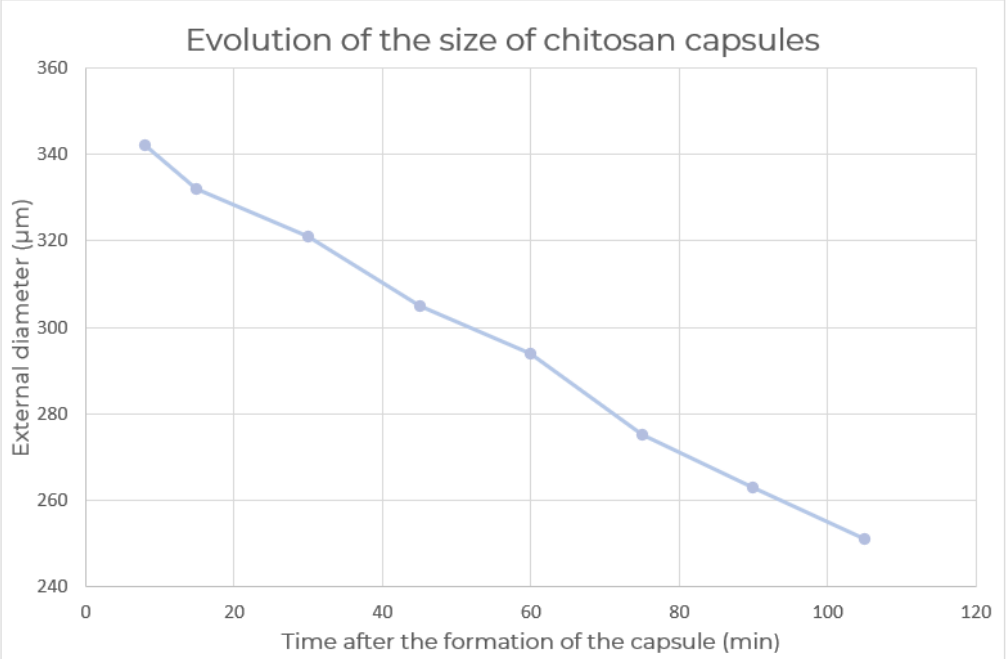


Figure 8: *Size of chitosan capsules as a function of time*

The capsules have a diameter ranging from 342 µm (7 min) to 202 µm (>1 000 min, not displayed in the graph). We observe that during the cross-linking, the diameter of the capsules decreases. In fact, a diameter of 332 µm is obtained 15 minutes after droplet formation, while a diameter of 263 µm is obtained 60 minutes after droplet formation. This result agrees with the publication of Du et al. [3] and can be explained by the fact that during cross-linking, water in the shell phase is extracted.

2. Influence of the middle phase flow rate

After analyzing the size of the capsules over time, the influence of the middle phase flow rate is observed. We varied the shell flow rate at fixed continuous and core phase flow rates. For each shell phase flow rate, a video was recorded and the diameter of the shell and the core were measured using software. The evolution of these two diameters is underlined in Figure 9 and the operating conditions are shown in Table 2. Figure 10 shows the evolution of the thickness of the droplet with the evolution of the shell flow rate.

Table 2: *Operating conditions for the evaluation of the size of capsules for a middle flow rate variation*

	Continuous phase	**Shell**	**Core**
Composition	1-octanol + 2% Span 80	Water + 2% chitosan 30-100cps + 1% PF127 + 2% AcOH	Soybean oil + Sudan IV
Pressure (mbar)	174	variable	1378
Flow rate (µL/min)	180	2 – 27 µL/min	13,6

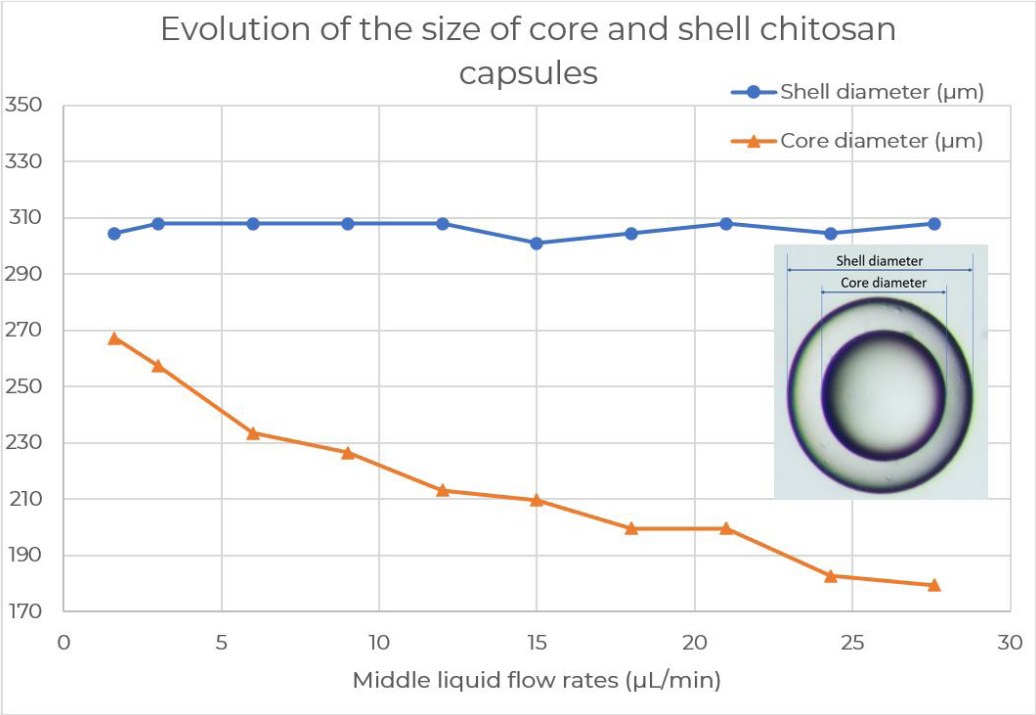


Figure 9: *Core size and shell size as a function of the shell liquid flow rate*

We observe that the shell diameter remains relatively constant when varying the shell liquid flow rate. A shell diameter of 308 µm is obtained using a shell flow rate of 3 µL/min, while a similar shell diameter of 305 µm is obtained using a shell flow rate of 24 µL/min. However, we observe that when the flow rate of the shell phase increases, the diameter of the core decreases. Indeed, a core diameter of 257 µm is obtained using a shell flow rate of 3 µL/min, while a core diameter of 183 µm is obtained using a shell flow rate of 24 µL/min. This phenomenon can be explained by the fact that the size of the shell is constrained in a limited range by the geometry of the system when the continuous phase flow is constant.

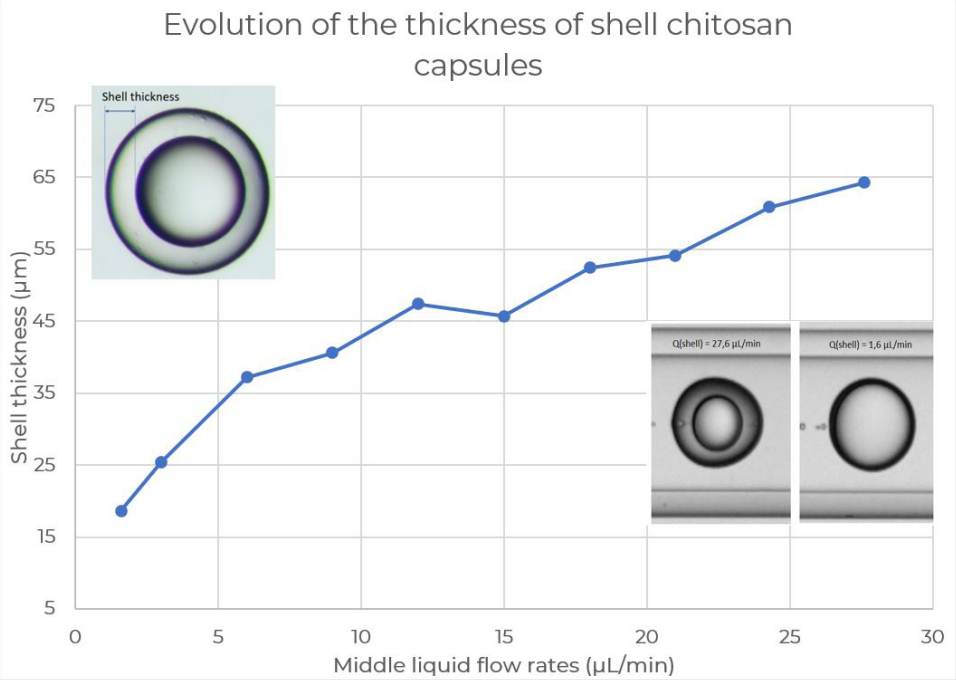


Figure 10: *Thickness of the shell as a function of the shell liquid flow rate*

Figure 10 underscores the increase of the thickness of the shell when the shell liquid flow rate increases. The shell thickness increases with the shell liquid flow rate. A shell thickness of 25 µm is obtained when the shell liquid flow rate is 3 µL/min, while a shell thickness of 61 µm is obtained when the shell liquid flow rate is 24 µL/min. As the diameter of the droplet remains almost constant (the diameter being fixed by the continuous phase flow rate and by the geometry of the system) but the flow rate of the shell phase increases, the droplet generation frequency increases and the core size decreases.

3. Influence of the outer phase flow rate

Previously, the continuous phase flow rate and core flow rate were fixed and the shell flow rate was varying. In this part, the shell flow rate and core flow rate are fixed but the continuous phase flow rate is varying. Once again, for each continuous phase flow rate, a video was recorded and the diameter of the shell and the core were measured. The change in diameter as a function of flow rate is shown in Figure 11 and the operating conditions are shown in Table 3.

Table 3: *Operating conditions for the evaluation of the size of capsules for a continuous flow rate variation*

	Continuous phase	**Shell**	**Core**
Composition	1-octanol + 2% Span 80	Water + 2% chitosan 30-100cps + 1% PF127 + 2% AcOH	Soybean oil + Sudan IV
Pressure (mbar)	variable	1553	1378
Flow rate (µL/min)	variable	16,4	13,6

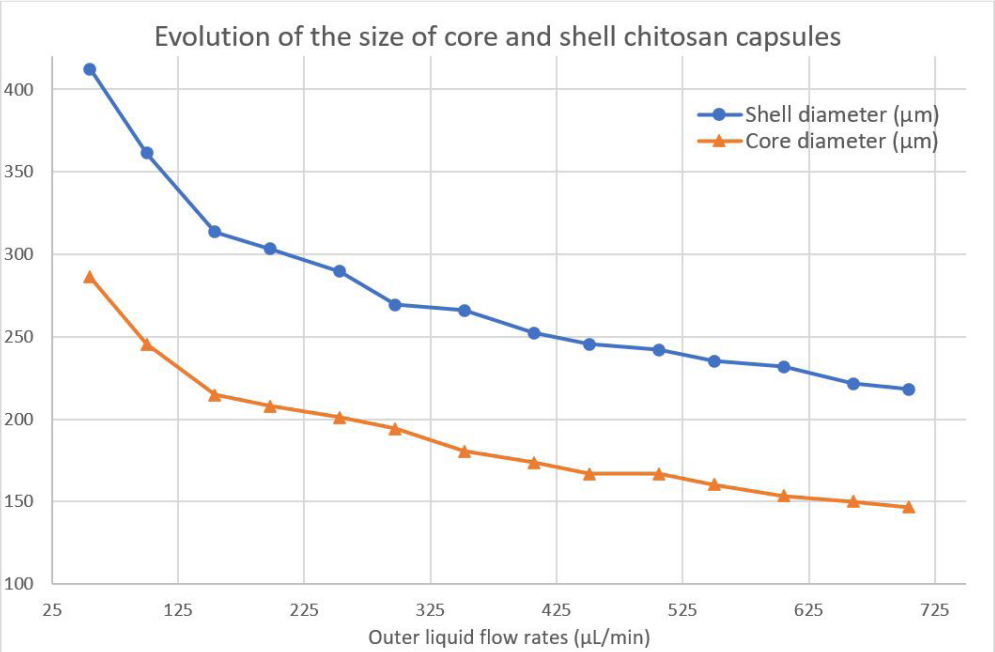


Figure 11: *Core size and shell size as a function of the outer liquid flow rate*

We observe that the shell diameter remains relatively constant when varying the shell liquid flow rate. A shell diameter of 308 µm is obtained using a shell flow rate of 3 µL/min, while a similar shell diameter of 305 µm is obtained using a shell flow rate of 24 µL/min. However, we observe that when the flow rate of the shell phase increases, the diameter of the core decreases. Indeed, a core diameter of 257 µm is obtained using a shell flow rate of 3 µL/min, while a core diameter of 183 µm is obtained using a shell flow rate of 24 µL/min. This phenomenon can be explained by the fact that the size of the shell is constrained in a limited range by the geometry of the system when the continuous phase flow is constant.

4. Capsule release analysis

Collected droplets are separated in two samples with different cross-linking times. The first sample contains droplets that have cross-linked for 17 minutes and the second contains droplets that have cross-linked for 45 minutes. Each sample is then washed with a buffer solution at pH 7.3 and then stored in the buffer.
Analysis of the release of the dye contained by the capsules is performed using a spectrophotometer. This allows one to determine the absorbance of the Sudan IV dye of the solution containing the capsules according to a specific wavelength.
For each sample, 15 measurements were made over a period of 24 hours. The results are presented in Table 4. No significative difference was observed between the two samples. In both cases about 95% of the encapsulated oil remained after 24h when stored in a water solution, demonstrating that a compact shell structure preventing oil leakage is formed in less than 20 minutes of cross-linking.

Table 4: *Release rate of capsules*

	Capsules cross-linked for 17 minutes	**Capsules cross-linked for 45 minutes**
Release rate of the red dye in the buffer solution containing the capsules	< 5%	< 5%

5. Conclusion

The production of stable, monodispersed microcapsules with a solid chitosan shell and a liquid oil, non-polar core using a microfluidic system has been successfully achieved. The Fluigent microfluidic platform also allows one to tune the core diameter and the shell thickness by adjusting the flow rates of the different fluids. Due to excellent oil encapsulation properties and a very limited leakage over time, these microcapsules can be used in a wide range of applications, including the encapsulation of volatile products like mint oil [3] as well as specific drugs, which will be delivered according to the pH acidity [2].

6. References

[1] KILDEEVA, N. R., PERMINOV, P. A., VLADIMIROV, L. V., NOVIKOV, V. V. and MIKHAILOV, S. N., 2009. About mechanism of chitosan cross-linking with glutaraldehyde. Russian Journal of Bioorganic Chemistry. 1 May 2009. Vol. 35, no. 3, p. 360–369. DOI 10.1134/S106816200903011X.

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[3] DU, Yuhan, MO, Liangji, WANG, Xiaoda, WANG, Hongxing, GE, Xue-hui and QIU, Ting, 2020. Preparation of mint oil microcapsules by microfluidics with high efficiency and controllability in release properties. Microfluidics and Nanofluidics. June 2020. Vol. 24, no. 6, p. 42. DOI 10.1007/s10404-020-02346-2.