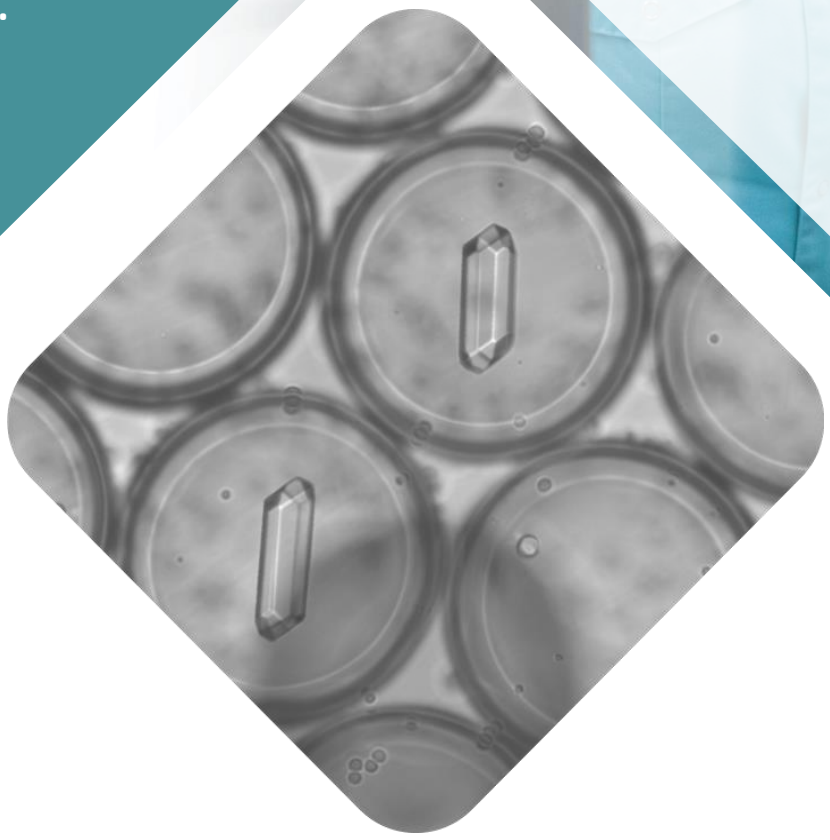
Secoya FLUIDIFY PHARMA

FLUIGENT SMART MICROFLUIDICS™

UV-Crosslinked Microcapsule Production Protocol

Adapted for single crystal formation in core-shell capsules.



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*Based on the publication *Single crystal formation in core-shell capsules*, Mettler et al., Chemical communications 2023 (Open source)

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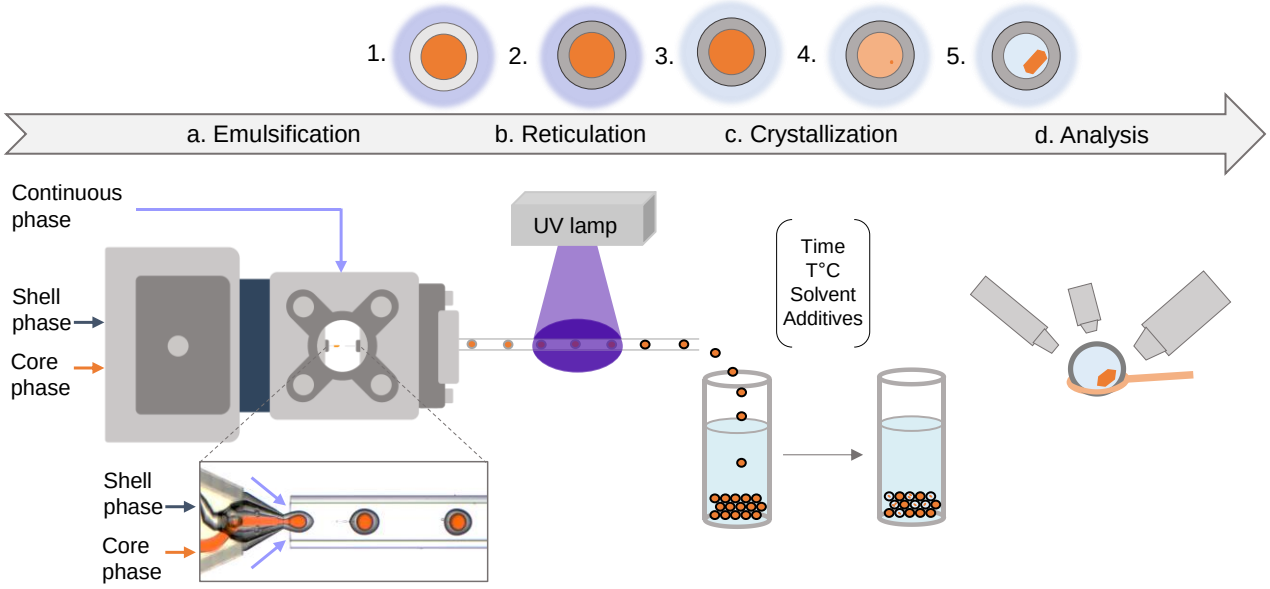
1 Abstract

In this document, we extended the scope of microfluidic-based crystallization methods by introducing solid microcapsules. Hundreds of identical microcapsules were generated per second by the UV-crosslinked microcapsule production platform. This allows for fast screening of crystallization conditions. XRD analyses were performed directly on encapsulated single crystals, demonstrating the potential of this process for the identification of compounds.

2 Graphical abstract



Schematic representation of the process from the double emulsion generation to the crystal formation and analysis in microcapsule [1].

Steps a. to d. highlight the production path

- Step a. production of the double emulsion
- Step b. shell reticulation by irradiation of the polymer
- Step c. storage of the capsules into the desired solvent or air, leading to crystallization
- Step d. in situ X-Ray diffraction analysis of selected single crystals

Icons 1. to 5. depict the evolution of double emulsions along the production path

- Double emulsion after emulsification
- Microcapsule after the reticulation of the shell
- Microcapsule placed in the collection medium

- Nucleation event within the microcapsule upon exchange through the shell
- Encapsulated crystal

3 Introduction

The production of solid core-shell microcapsules can be achieved through various methods of shell curing [2] depending on the nature of the shell: solvent evaporation or precipitation (for PLGA capsules [3][4]), ionic crosslinking or gelation (for alginate capsules [5]), chemical cross-linking (for chitosan capsules [6]), UV light cross-linking (for PEGDA capsules [7]). This last method is frequently used to produce polymeric capsules. It has the advantage of being solvent-free and fast compared to other curing methods.
This work presents the production of core-shell methacrylate capsules with the RayDrop® microfluidic device [8] and its platform for the screening of crystallization conditions. Various solubilized molecules are encapsulated and, small crystals are visible through the transparent shell.

4 Materials and Methods

Creating single crystal formations in microcapsules produced using the RayDrop is a three-step process. First, a double emulsion composed of an aqueous core surrounded by a methacrylate resin is generated in the microfluidic device (RayDrop, Secoya Technologies). It is then transported outside the device in a glass capillary. This capillary is then irradiated by a UV lamp to solidify the shell and form solid capsules. These are collected in a bath containing solvent. Diffusion occurs through the porous shell and nucleation occurs leading to to crystal formation. Once the crystals are formed, they are analyzed by XRD.

4.1 Microfluidic system

The production of droplets is performed with the UV-crosslinked Microcapsule Production Platform: A system integrating the components needed to produce single and double emulsions using the RayDrop device. This platform is divided into four parts: mechanics, fluidics, optics, and a UV module. More information about this platform can be found on the webpage.

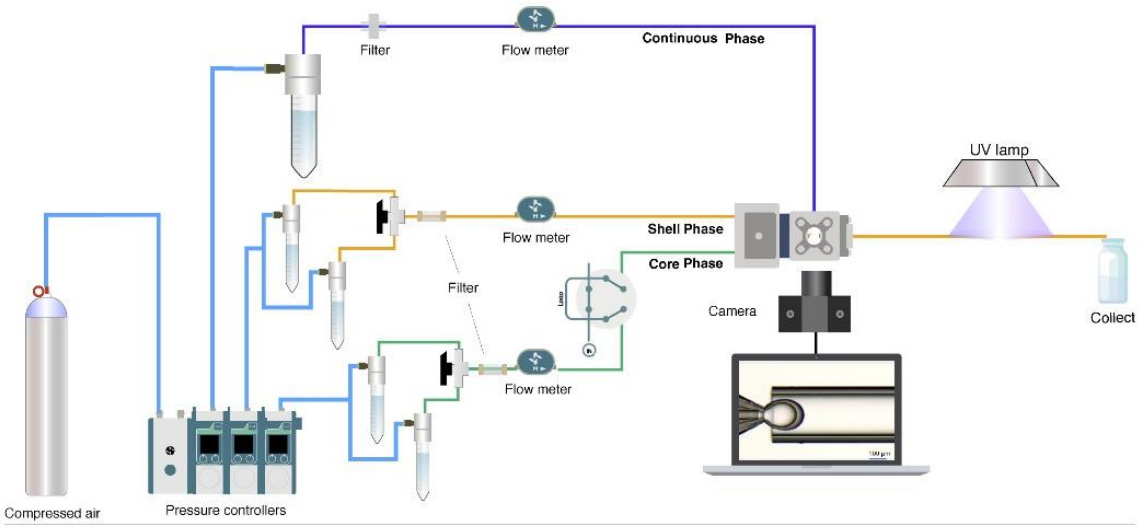


Figure 1: Experimental set-up to produce double emulsion. It includes double reservoirs for the shell and core phases and an injection loop.

Fluid reservoirs

Falcon identification	F1	F2	F3	F4	F5
Volume (mL)	50	50	50	50	15
Phase[1]	Continuous	Core (priming and cleaning)	Core	Shell (priming and cleaning)	Shell
Composition	Water + 2% PVA	Water	Not used	EtOAc	Allnex methacrylate-based resin + 20% EtOAc + 0.1% wt TPO

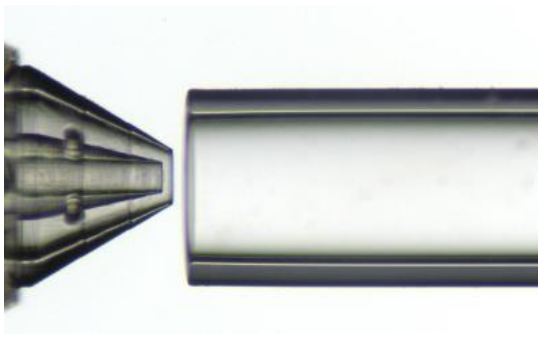
[1] *Each phase is filtered to avoid clogging the tubing or the nozzle of the RayDrop. Therefore, there is an integrated filter after each Falcon on the platform. In this case, the continuous phase filter has a 10 µm filter pore size and the shell and core filters have a 2 µm filter pore size.*

Figure 2: RayDrop Platform

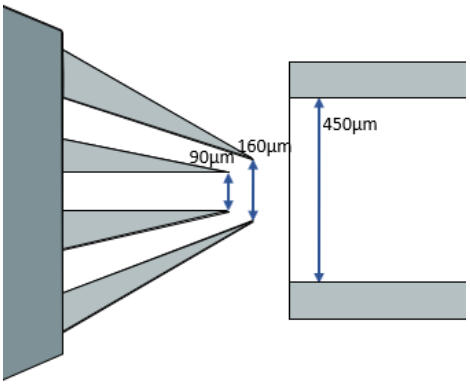
- Mechanics: The mechanical section includes an x-y-z mechanism to allow users to adjust the focus and the observation window in the RayDrop.
- Fluidics: This consists of flowrate controllers with the required tubing and valves, allowing for automated fluidic delivery. It includes a comprehensive flow path developed by Fluigent, with high pressure controllers (LineUp FlowEZ), filters, flowmeters (Flow Units), and valves to simplify the start-up, shutdown, and cleaning of the system. An injection loop is connected to the core phase to produce samples of double emulsions containing the compound to encapsulate. A pressure is set on each reservoir, and fluids are delivered to the microfluidic chip. It also includes FalconTM tube reservoirs and the RayDrop, to generate the double emulsions. After each reservoir, a filter is included that eliminates impurities. The core phase flows through an injection loop to deliver the solution to be encapsulated (for instance distilled water with small amounts of API).
- Optics: The optical part of the platform 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, control the stability of the emulsion, and measure the size of interest (core, shell).
- UV module: The UV module consists of a UV LED head 365 (Wavelength: 365 nm, Peak Irradiance: 1000 mW/cm^2 at 10mm (with focus lens), a UV control

Unit (Input voltage 84 - 264V AC, Max power delivered: 50W (24 VDC - 2,1 A), a RayDrop holder, a UV protective box and a glass capillary.

The RayDrop is thedevice used to produce controlled double emulsions. Double emulsions can be produced without surface treatment surfactants are usually added to the different phases to maintain the emulsion.



Picture of the Raydrop



Scheme of the Raydrop

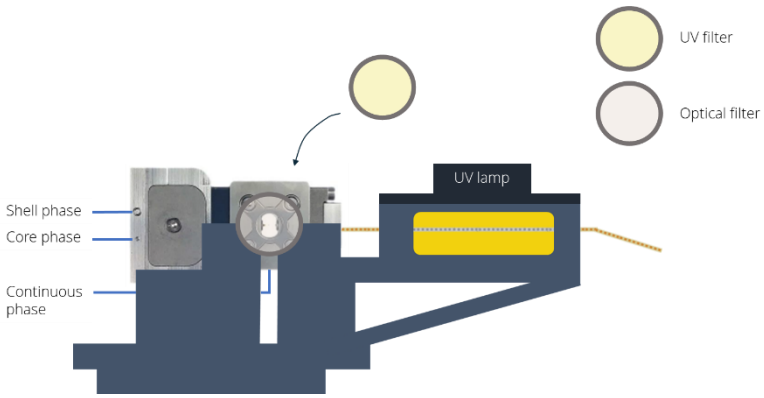
Figure 3: Insert and extraction capillary.

Nozzle information

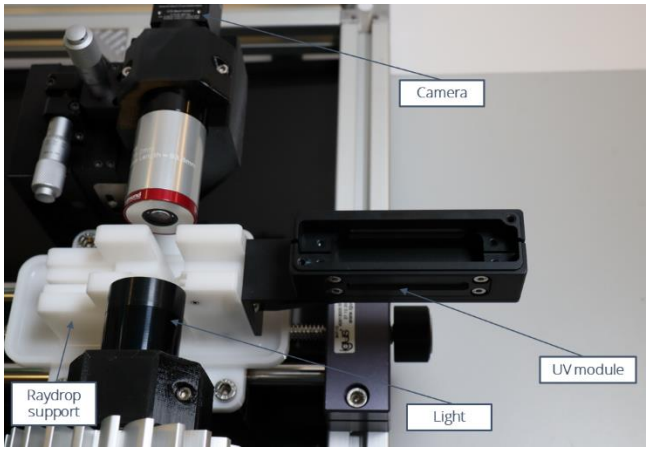
Part	Core nozzle	Size-shell nozzle	Size-extraction capillary
Inside diameter (µm)	90	160	450

Double emulsions are formed by pumping the three fluids through the Raydrop® using a pressure controller. The flowrates are controlled using flowmeters.

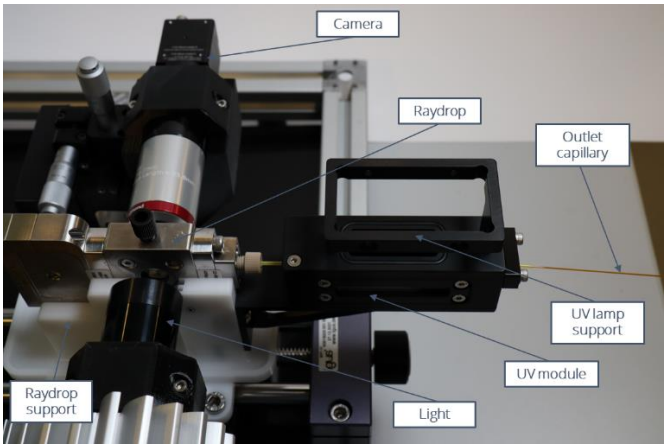
The UV module controls the cross-linking of the resin shell, as shown in Figure 4.



Scheme of the module



Module integrated into the platform.



RayDrop and outlet tubing placed in the module. A UV lightbulb should be placed in the lamp holder.

Figure 4: The UV module integrated to the RayDrop Platform

This module is movable allows users to observe not only the formation of the emulsion in the RayDrop but also to check that the emulsion remains in the outlet tubing. The observation is made with the camera of the platform, by moving the module (see Figure 5) so that the camera can follow the emulsion progression in the outlet tubing.

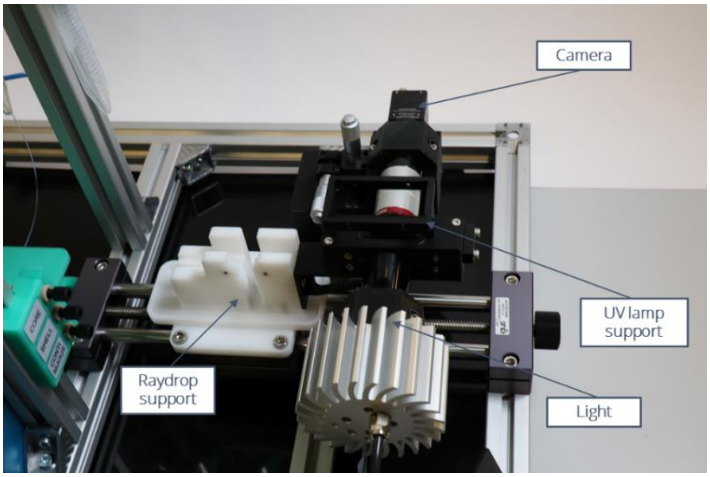


Figure 5: Module in observation position in the outlet tubing.

Two windows with an anti-UV glass filter guarantee safe observation for the user.
The UV light source irradiates at a wavelength of 365nm to initiate the cross-linking of the resin. Hard shell capsules come out at the end of the tubing, for collection in a vial.

The in-situ cross-linking of the emulsion (see Figure 6) is useful: it avoids coalescence and deformation of the droplets that can arise in an ex-situ process where the droplets are cross-linked after collection.

A detailed user guide can be found on our website to operate the UV module.

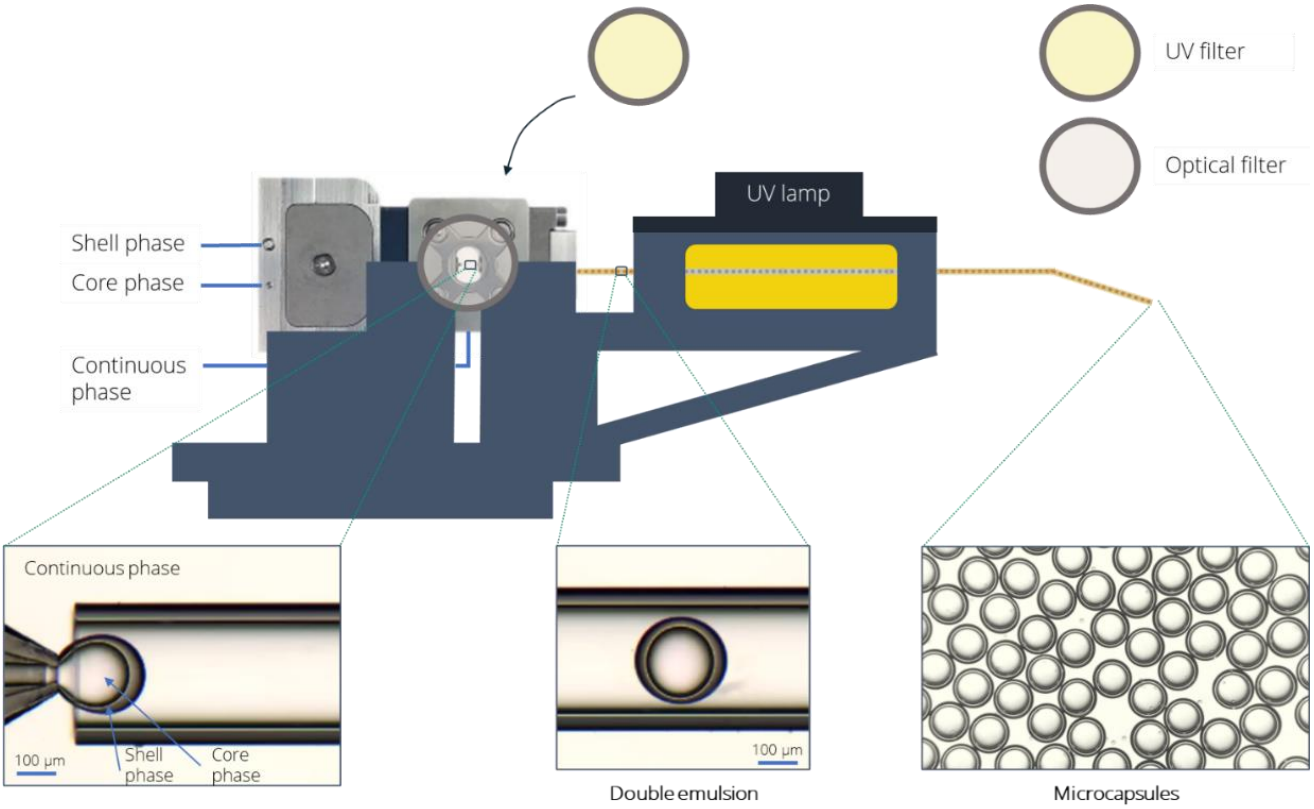


Figure 6: In-situ cross-linking process leads to the formation of monodispersed microcapsules.

4.2 Reagents

Continuous phase

Distillated water containing 2% Poly (vinyl alcohol) (PVA, Sigma-Aldrich)

Shell phase

Commercial Allnex methacrylate-based resin containing 20% of ethyl acetate (EtOAc, Merck) and 0.1% wt of photoinitiator Diphenyl (2,4,6-trimethylbenzoyl) phosphine oxide (TPO, Sigma-Aldrich)

The choice of a photoinitiator depends on his properties (biocompatibility, etc.) but also on its irradiation wavelength and on his solubility in the solvent used.

Core phase
Distilled water with the molecule to crystallize (ammonium sulfate (≥99.0%), copper (II) sulfate (≥99%), glycerol (≥99.5%), glycine (≥99.0%), sodium chloride (≥99.0%), proteinase K from Tritirachium album (≥30 units/mg), Lysozyme from chicken egg white (≥40,000 units/mg))

Priming and cleaning phase
Ethyl acetate (Merck)

4.3 Production process

4.3.1 Emulsion formation

To generate a double emulsion, the system must first be primed with pure solvent in the shell phase. Once droplet formation is stable, the shell phase is switched to the methacrylate solution. This avoids clogging issues during the transition phase. Follow the steps below:

1. First, the RayDrop is filled with the continuous phase, and a flow rate is maintained at approx. 250 µL/min.
2. The shell phase valve is switched to pure ethyl acetate and the flow rate increased on the Falcon tube to reach 50 µL/min to purge air bubbles.
3. In the same manner, the core phase is introduced to the RayDrop by applying pressure in the reservoir.
4. As soon as a steady emulsion formation regime is reached, the solution of methacrylate resin can be selected by switching the droplet phase valve to the second position.
5. It then takes a few minutes or less, to go from priming pure solvent to the resin at the nozzle. During this transition time, the flow rate will decrease at constant pressure as the viscosity of the resin is greater than that of pure ethyl acetate. The pressure can be gradually increased to maintain the target flow rate. Once the flow rate is observed to be stable at constant pressure and the visual observation of the droplet generation is stable, adjust the flow rates to control the dimensions of your double emulsion.

4.3.2 Capsules formation

To form solid particles, the emulsion is irradiated by a UV light. The photoinitiator reacts to the light and initiates cross-linking with the methacrylate. A chain-growth mechanism is enabled, leading to a three-dimensional network.

1. Adjust the UV module so that the camera shows the double emulsion in the outlet tubing (the glass capillary)
2. Verify that droplets are of the same size and that they are being produced at a constant rate. If not it is not regular, adjust the flow rates to stabilize production.
3. When the drop train is stable, switch on the UV light and collect it in a vial.
4. When enough capsules have been produced, switch off the UV lamp.
5. To stop production, switch back from resin to ethyl acetate and wait a few minutes to be sure that all the resin is removed from the tubing and nozzle. Stop the droplet phase switch off the valve and stop the continuous phase.

4.3.3 Droplet collection

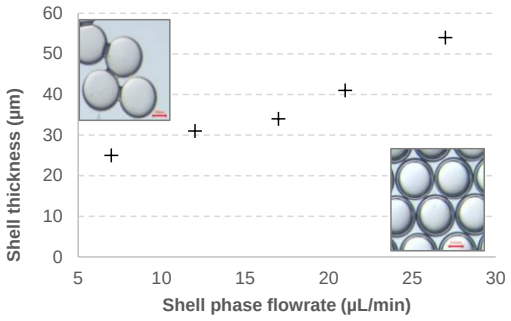
The droplets are collected in a vial usually containing water with a salt or an organic solvent. The composition of the collection solution depends on the compound's ability to crystallize. This aspect is further developed in the rest of this document.

5 Results

The generated solid microcapsules contain various compounds to crystallize and are therefore collected in specific collection baths. The crystallization takes place via diffusion through the shell, meaning it's imperative to control the shell thickness.

5.1 Shell thickness control

The size of the double emulsion and the shell thickness are controlled by the flow rates of the continuous, shell, and core phases (see Figure 7). Figure 7 shows the influence of the shell flow rate on the shell thickness, with fixed core and continuous phase flow rates (respectively 17 and 160 µL/min).



5.2 Crystallization of sodium chloride

Figure 7: Shell thickness evolution.

The crystallization of a common salt was tested. Sodium chloride (200 g/L) was used as a solute in the core phase. Capsules were produced, collected in a watch glass, and slowly dried out at room temperature. The water contained in capsules evaporated out of the porous shell. The salt crystallized and formed mostly one crystal in each capsule, as shown in Figure 8. The capsules are independent nanoliter-scale crystallization vessels.

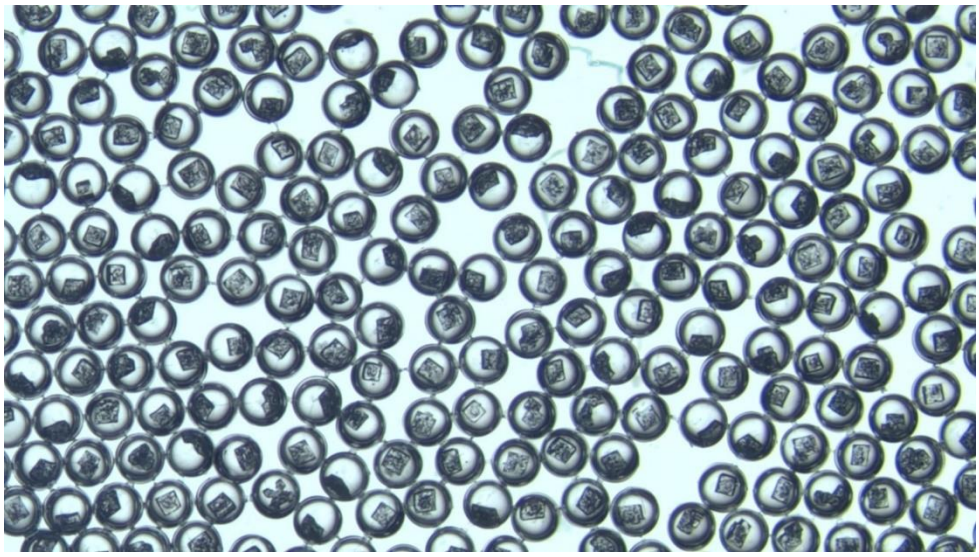


Figure 8: NaCl crystals in microcapsules.

5.3 Crystallization of copper (II) sulphate

Copper (II) sulfate was evaluated at 200g/L. Capsules were collected in various collection baths (air, water, acetone, methanol, ethanol, 2-propanol, ethyl acetate) and stored at different temperatures (ambient, 4.5°C, and -18°C) for screening. In water, $CuSO_4$ was retained in capsule cores and did not lead to crystal formation. In ethanol, 2-propanol, or acetone, though these solvents diffused through the shell and some capsules crystalline aggregates were observed, but not as single crystals.

Single crystal formation in core-shell capsules

Best results (see Figure 9) were obtained when the collection bath used was ethyl acetate as it ethyl acetate diffused into capsules, decreasing the solubility of $CuSO_4$ at room temperature. After two days, capsules contained $CuSO_4 \cdot 5H_2O$ single crystals. It is likely that the equilibrium state remained in the $CuSO_4 \cdot 5H_2O$ metastable zone, as only a few capsules produced crystals, but the crystals observed were well formed (see Figure 10).

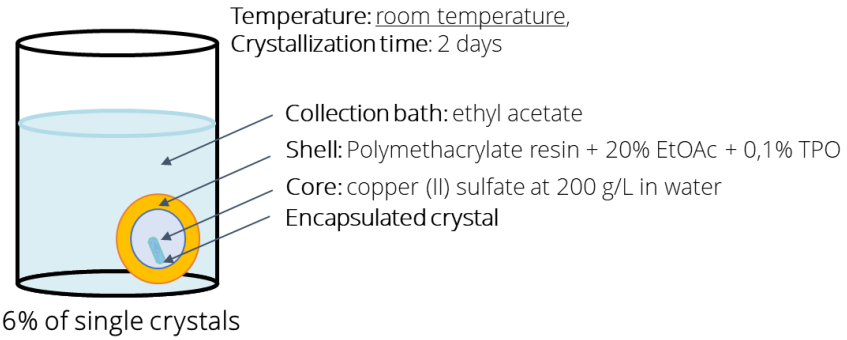


Figure 9: Best conditions for copper (II) sulfate single crystal formation.

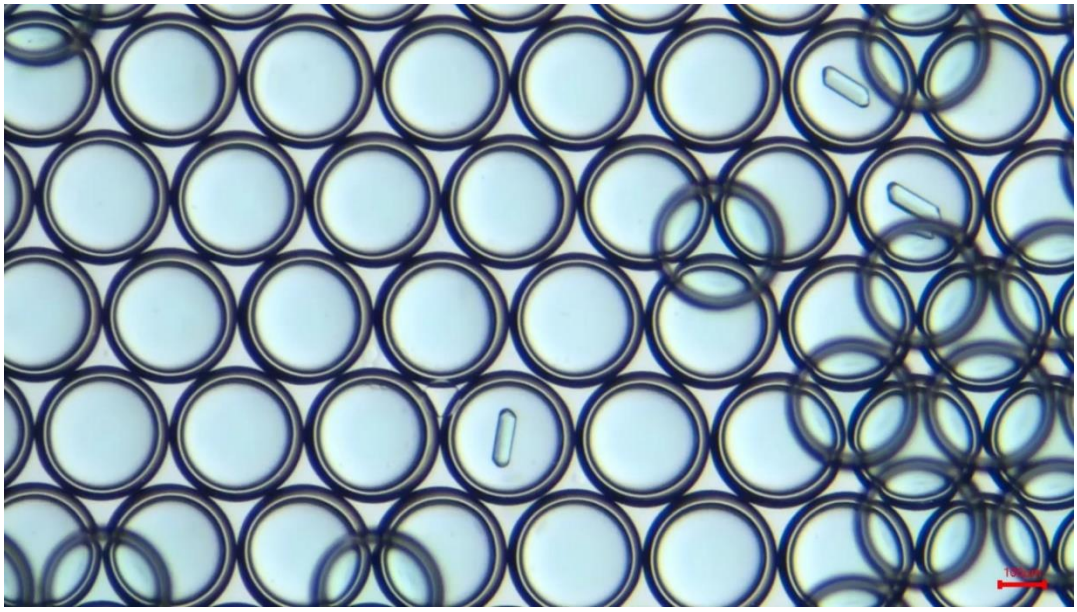


Figure 10: Copper sulfate blue crystals in capsules. No clear effect of the collection temperature was observed.

5.4 Crystallization of glycine

A study was done onglycine molecules, which have several possible crystal structures. A solution of 190 g/L of glycine was encapsulated. Screening of collection solvents was performed, with most forming crystalline aggregates.

After 1 day of drying in air at 4.5°C (see Figure 11), 3% of capsules contained single crystals. Among these crystals, polymorphs, α-glycine, and γ-glycine were observed (see Figure 12) and confirmed by single-crystal X-Ray diffraction analysis. As a conclusion, this method is useful for screening polymorphs by producing hundreds of capsules.

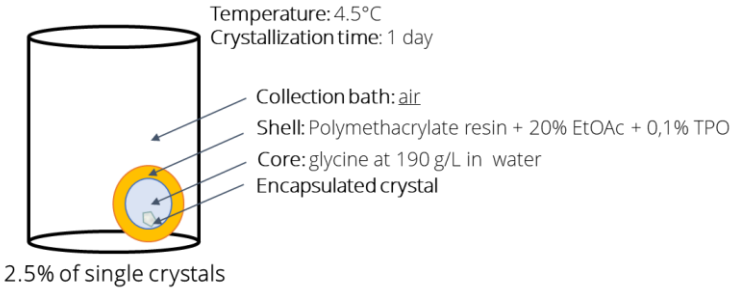
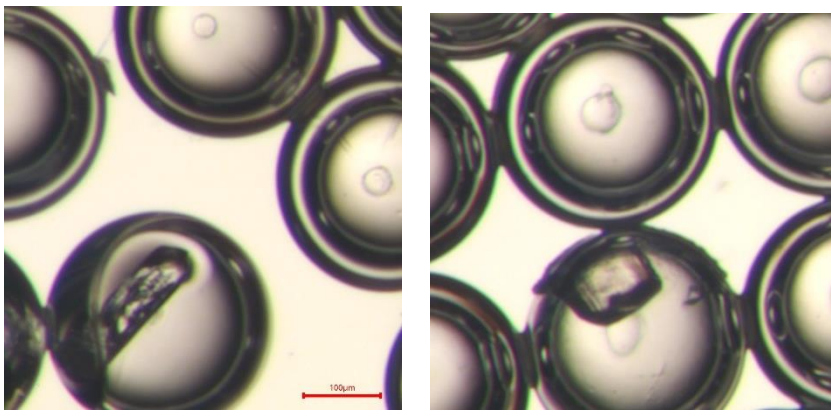


Figure 11: Best conditions for glycine polymorphs formation.



α-glycine γ-glycine

Figure 12: Two polymorphs of glycine.

5.5 Crystallization of proteins: lysozyme and proteinase K

The last type of molecules studied were macromolecules. The crystallized proteins were lysozyme and proteinase K. The crystallization method was adapted from conventional protein crystallization procedures. Both proteins, were dissolved in saline solutions: lysozyme 20 mg/mL is dissolved in a NaCl 5% solution at pH = 5 (see Figure 13) and proteinase K 20 mg/mL was dissolved in an ammonium sulphate 1.1 M solution at pH = 7.

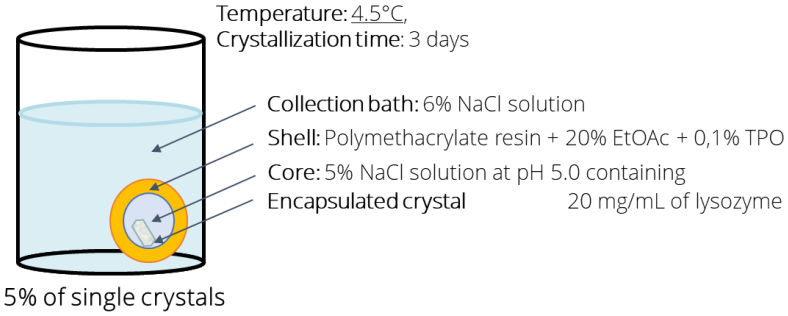


Figure 13: Conditions for lysozyme crystals formation.

Capsules produced were collected in collection baths composed of the same salt (NaCl or ammonium sulfate), but with a slightly higher concentration (6 wt% NaCl) to induce a transfer through the shell. The differences in water activity between the inside and outside of the capsules would likely induce slow water diffusion from the inside by osmosis. After three days at 4.5 °C, crystals were formed in almost every capsule. In some capsules, a single crystal was observed. When the salinity of the inner phase of capsules is increased, an increase of single crystal formation is observed, as shown in Figure 14.

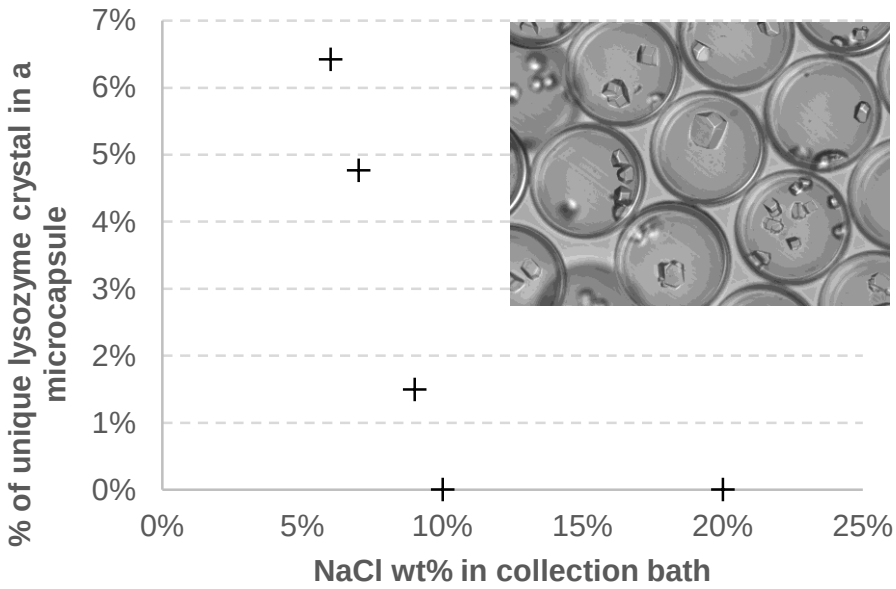


Figure 14: Percentage of microcapsule containing one single crystal as a function of the NaCl concentration in the collection bath.

The same crystallization process by water diffusion was used on the proteinase K under the conditions of Figure 15. The sample was microscopically examined, and two populations of capsules are observed: capsules broken by the osmotic pressure that do not contain proteinase K, and intact capsules containing one or more bipyramidal crystals (see Figure 16).

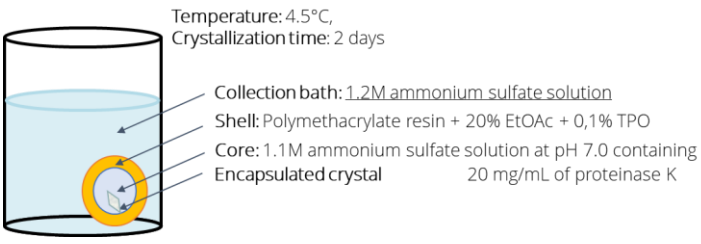


Figure 15: Conditions for proteinase K crystals formation.

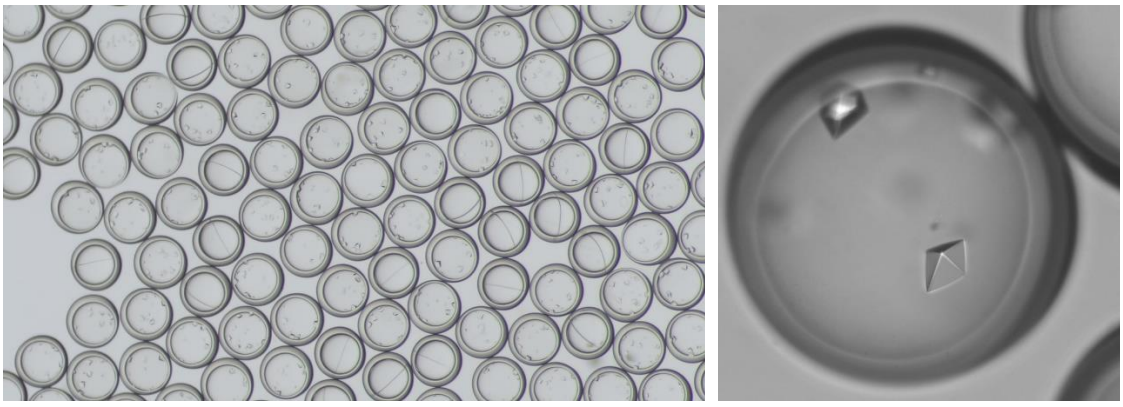


Figure 16: Overview of capsules containing proteinase K crystals.

6 Conclusion

The UV-crosslinked microcapsule production platform is an effective tool to produce highly monodisperse microcapsules of polymethacrylate containing various solutes to crystallize. The combination of a microfluidic platform containing the RayDrop and a UV module including a UV lamp simplifies the production of many samples. The resulting capsules are mechanically stable and allow for easy handling and transport.

This platform is ideal for the screening of different conditions, as it enables the production of thousands of capsules per minute, with each capsule acting as an independent crystallization vessel for both small molecules and macromolecules. The injection loop has an adaptable volume, with 20µL as the smallest, so only a very small amount of solute to crystallize is needed for one experiment (for instance, less than 1 mg for the use of 20µL of lysozyme at 20 mg/mL).

By changing the configuration of the RayDrop, the size of the capsules produced can be optimized.

The method can be extended to other polymers that allow diffusion to take place through the shell and to other compounds to be encapsulated for crystallization in capsules or other applications such as drug delivery.

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