FLUIGENT

ALGINATE MICROBEADS PRODUCTION

INTRODUCTION

Microencapsulation is one of the most interesting fields in the area of pharmaceutical technology since its inception many years ago.

Microencapsulation products (microparticles, microbeads) can be defined as small entities that contain an active agent or core material surrounded by a shell or embedded into a matrix structure.[1]

Among all these materials, alginate appears as a good candidate for many applications. Alginate spheres are one of the most widely investigated cell encapsulation materials as they are biocompatible, nontoxic, biodegradable, and relatively cheap.[2]

Their structural similarity to the extracellular matrices of living tissues allows for applications in wound healing, delivery of bioactive agents such as small chemical drugs and proteins, and cell transplantation.[3]

Alginate microbeads are widely used in pharmaceutical research, tissue engineering, and regenerative medicine.[4]

Although the production of alginate microbeads appears to be a successful drug delivery system, it is often hindered by technical challenges, mainly associated with high viscosity of polymer fluids.[3]

Many methods are used for encapsulation of agents (bacteria, pancreatic islets, API) in microbeads, such as extrusion, batch method, spray drying...

The most widespread method for producing alginate microbeads is extrusion, where a solution of alginate is injected through a needle into a calcium bath to produce ionically crosslinked beads.

This easy to use method allows one to produce alginate microbeads which have a wide size distribution, without possible automation and no control over the bead size. This is a big limitation because in microbead production, particle size is key parameter.

Size is directly correlated to the application, and differs depending on the product requirements. For instance, in food, microgels with micrometer sizes are preferred in order to minimize the sensory perception of powdery or graininess in food such as yoghurt and ice cream [5]

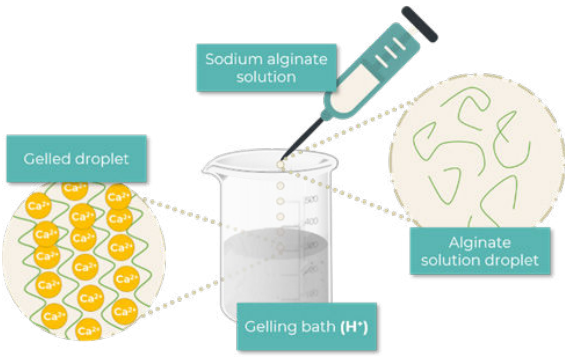

Figure 1: Extrusion method for Alginate microbeads production

In biomedical applications, drug delivery through submicron nanogels allows drugs to be targeted at specific sites in the body. Nanogels (100–200 nm) used in cancer therapy experiments tend to preferentially accumulate in a number of cancerous tumors [6] [7]

As the reproducibility and throughput of such methods is low, there is demand for better techniques and control of the process to produce alginate microbeads.

Droplet-based microfluidics, offers an efficient method for improvement of this process. It is a powerful tool which enables the production of micrometric monodispersed droplets.

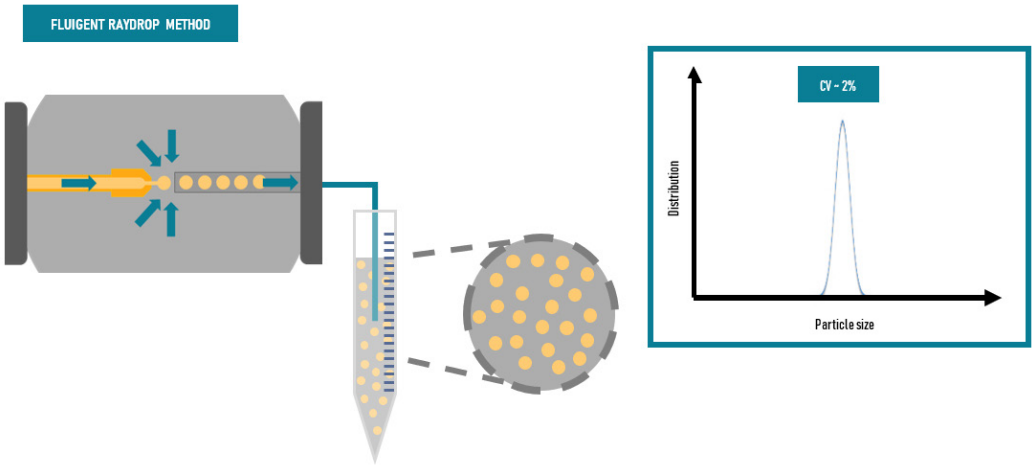

Figure 2: Fluigent microfluidic method for emulsion production

Following is a method for encapsulation of reagent into alginate microbeads with total control of bead formation.

Droplet-based microfluidic method is used to precisely control the production of microbeads without the drawbacks of large size distribution that present other methods.

Microfluidics allows one to have better control over the process of production and encapsulation and reach higher monodispersity than other methods such as extrusion methods, batch...

	EXTRUSION METHOD	FLUIGENT MICROFLUIDIC METHOD
Particle size distribution	Up to 50%	~2%
Reproducibility	Low	High
Live particle size control	No	Precise
Continuous (in line) production	No	Yes

Figure 3: Comparison between extrusion method and Fluigent microfluidic method

MATERIALS AND METHODS

Materials

Reagents:

- **Dispersed phase:** Alginate (Sigma Aldrich) and Calcium EDTA disodium solution (Sigma Aldrich). The solution is composed of 1% Alginate, 100mM solution of calcium EDTA together dissolved in Water and then filtered using syringe filter at 0.2µm.
- **Priming and cleaning phase:** Distilled water filtered using syringe filter at 0.2µm.
- **Continuous phase:** 2% dSURF solution. dSurf solution is composed of Fluigent surfactant at 2% dissolved in HFE 7500 fluorinated oil filtered using syringe filter at 0.2µm.
- **Collection sample:** 2% dSURF (Fluigent) with 0.05% v/v acetic acid (Sigma Aldrich)
- **Emulsion breaker:** Perfluoroctanol (PFO) solution (Sigma Aldrich) (20% v/v in HFE 7500 fluorinated oil)

Products/Instrument:

- **Microfluidic flow controller:** The Flow EZ is the most advanced flow controller for pressure-based fluid control. It can be combined with a Flow Unit to control pressure or flow rate. It can be used without a PC. Two Flow EZ with 2 bar of full scale pressure are used in the setup presented here.
- **Flow sensor:** The Flow Unit is a flow sensor that allows real time flow rate measurement. By combining a Flow Unit with the Flow EZ, it is possible to switch from pressure control to flow rate control, allowing for the generation of highly monodispersed droplets over a long period of time. Two Flow Units M are used here to monitor and control the flow rates of the dispersed and continuous phase during the run.
- **2-Switch:** Easy to use 3-port/2-way microfluidic valve is used to switch between water and alginate solution. This allows to have better reproducibility by priming the system and add cleaning step during the experiment.
- **Droplet generator:** The RayDrop droplet and emulsion chip is used to control the generation of alginate droplet. The RayDrop is based on the alignment of two capillaries immersed in a pressurized chamber containing the continuous phase. The dispersed phase exits one of the capillaries through a 3D-printed nozzle, placed in front of the extraction capillary for collecting the droplets. This non-embedded implementation of an axisymmetric flow-focusing is referred to co-flow-focusing. The advantage lies in his geometry which is leading the droplet formation and then remove all wettability issues that could appear in other microfluidic chip. This allows to generate highly monodispersed emulsions with any kind of fluids including alginate.

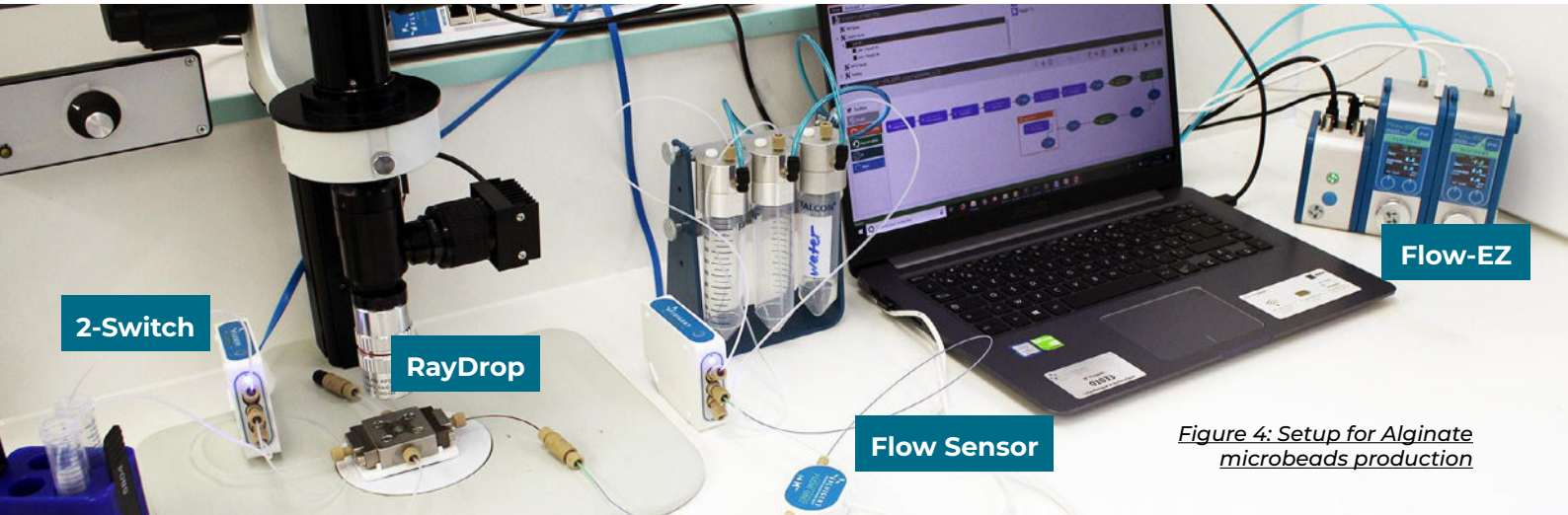

Figure 4: Setup for Alginate microbeads production

Methods

Solution preparation:

- **Dispersed phase:** Make a fresh solution of 1% alginate and 0.1 M Calcium EDTA dissolved in distilled water:
  1. Alginate powder was dissolved in distilled water in order to make a solution of 2% (w/v) alginate.
  2. Calcium EDTA was dissolved in distilled water at 0.1M.
  3. The two solution were then mixed together to make a fresh solution of 1% alginate and 0.05M calcium EDTA.
  4. To allow good reproducibility during droplet generation process, the solution has been filtered with using syringe filter at 0.2µm.
- **Continuous phase:** Use the 2% dSURF solution. Again, make sure to well filter the solution to avoid any issues during droplet generation process.
- **Collection solution:** The collection solution is composed of 0.05 % (v/v) acetic acid in the continuous phase (2% dSURF)
- **Emulsion breaker:** The emulsion breaker solution is composed of Perfluoroctanol (PFO) solution (Sigma Aldrich) dissolved at 20% v/v in dSURF or HFE 7500 fluorinated oil

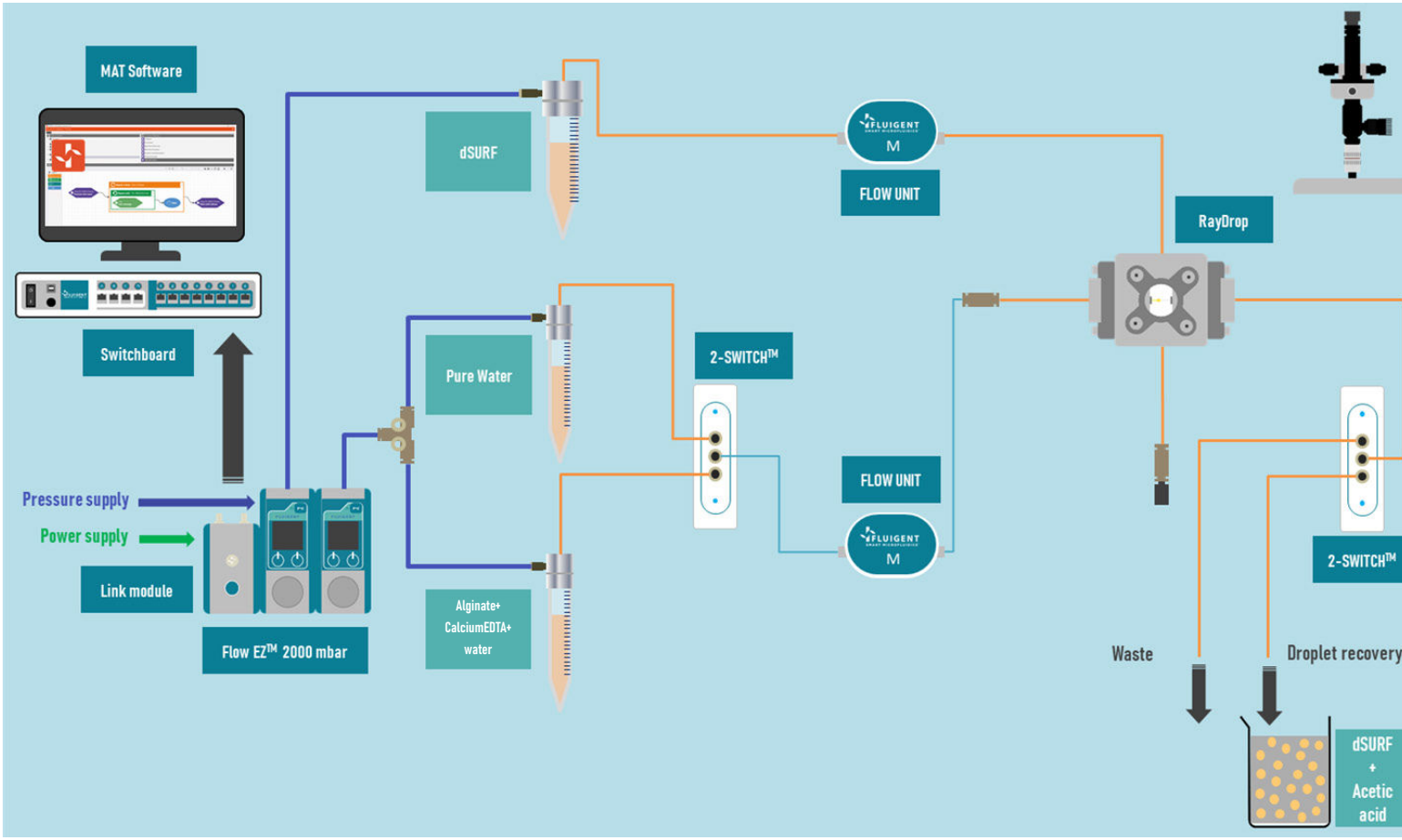

Figure 5: Scheme of the setup for Alginate microbeads production

Droplet generation process:

The droplet generation set-up is illustrated in the previous picture. For droplet generation process we used the RayDrop droplet generator. 2 Flow EZ were connected to alginate solution, pure water as dispersed phase and dSurf as continuous phase.

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1. First, water is injected as dispersed phase and dSurf as continuous phase to prime the system and generate water in oil droplets. This step allows for the removal of all air bubbles present in the system (tubing, connectors...) until a steady state is reached.
2. As soon as a steady state of droplet generation is reached, Alginate solution can be easily injected by switching the first 2-switch to the 2nd position. The pressure may be adjusted to maintain the proper flow rate with the alginate solution.
3. Alginate droplet might appear after less than 1 min.
4. Pressure and/ or Flow rate can be then adjusted to target the desired droplet size

For more information on droplet generation check the Good practice guide for more detail on droplet generation process with the Raydrop.

Microbeads Precipitation:
Once droplets are generated, they are recovered and collected into a reservoir.

This step will generate polymerization process and beads will be recovered. By decreasing the pH of the alginate droplet using acetic acid in the collection reservoir, the calcium-EDTA dissociates and releases CA2+ ions which allows for crosslinking of alginate chains.

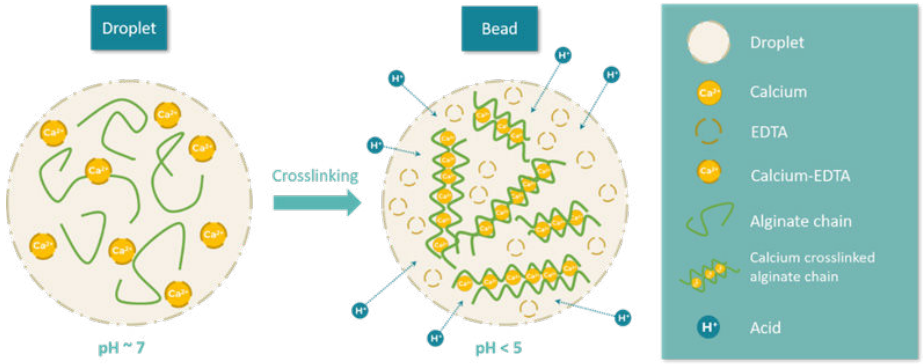


Figure 6: Description of alginate crosslinking mechanism

Droplet monitoring and Analysis:
To observe droplet generation an Inverted microscope (Leica DM IRB) coupled with an IDS UI-3070CP camera have been used. For continuous droplet visualization and measurement, the software uEye Cockpit from IDS has been used. To estimate droplet frequency the following formula has been used.

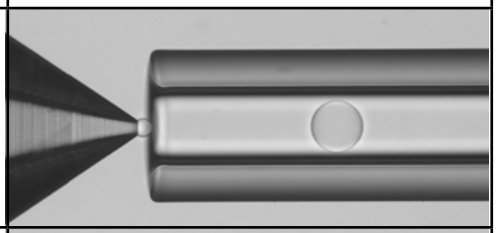
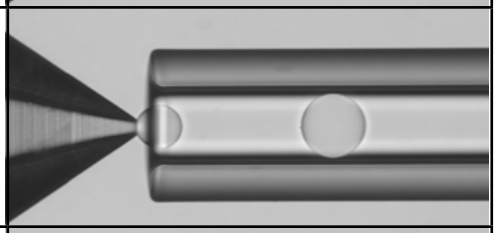
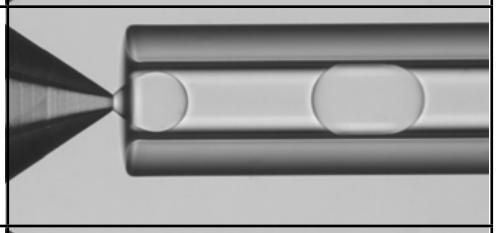
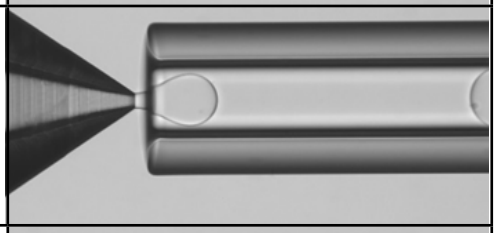
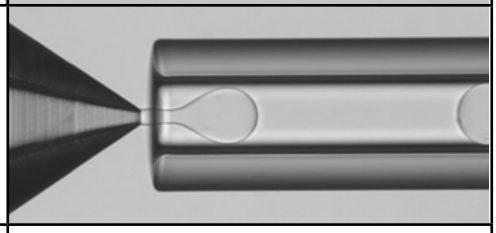
Droplet rate (Hz) = Droplet phase flow rate (µL/s) / Droplet volume (µL).

Bead recovery:
After precipitation, the beads are in suspension in the collection vial (dSurf + acetic acid). To break the emulsion and recover the beads some steps must be followed:

- Addition of PFO diluted at 20% in HFE to break the emulsion (approximately the same volume as the recovered emulsion volume)
- After this operation, which aims to remove the surfactant, all the drops collect in a sort of clump which at first gives the impression that everything has coalesced into a single
- large drop. On closer inspection we can see that it is a clump but that there is no coalescence
- Then remove the remaining oil with a pipette. There is just the cluster of beads in the bottom of the Falcon
- Add PBS buffer to resuspend the beads (a dye in the buffer can be used at this step to have better contrast between these two aqueous phases)

RESULTS

Configuration 1: RayDrop: inlet nozzle = 30µm, outlet capillary= 150µm

Dispersed phase / Droplet phase		Continuous phase		Droplets		Picture
P (mbar)	Q (µL/min)	P mbar)	Q (µL/min)	Diameter (µm)	Rate (Hz)	
312	0.5	1816	100	95	19	
319.5	1	1134.5	100	106	25	
776	5	309	25	155	42	
1303	10	344	25	plug	25	
1303	10	100	1150	127	166	
1699	15	100	1183	133	217	
1699	15	160	1810	115	320	

ALGINATE MICROBEADS PRODUCTION

After generation, droplets are recovered in the collection reservoir where the crosslinking mechanism appears as described in the method section. Microbeads were then recovered and analyzed for monodispersity measure.

Using this setup, we were able to produce alginate beads with a dispersion in beads size lower than 5%. (around 50 microbeads have been analysed for a CV of about 2%).

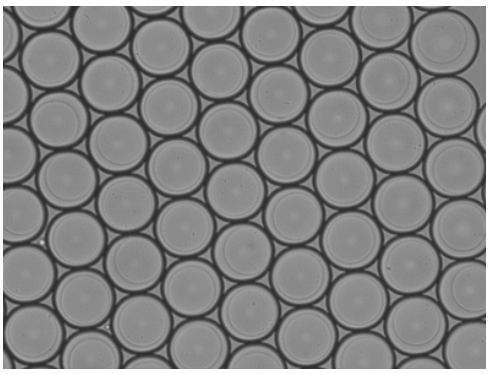


Figure 8: Pictures of Alginate beads in suspension in dSurf after crosslinking

Bead recovery:
To recover only the beads and put them in suspension into aqueous solution, the PFO solution was added. After breaking the emulsion, beads are resuspended in PBS solution. To have better contrast and be able to observe alginate beads, we have added here blue food dye in the PBS.

Figure 9: Alginate beads in suspension into PBS

In this configuration beads from 90µm to 150µm can be made.

However, RayDrop versatility and flexibility allows one to easily change configuration and change capillary size. This allow to target other droplet/beads size using the RayDrop.

Configuration 2: RayDrop: Inlet nozzle = 30µm, outlet nozzle= 60µm

In this case we have changed the outlet capillary to another nozzle of about 60µm. This allows to increase the shear stress to the dispersed phase and then produce smaller alginate droplet with higher generation frequency.

With this specific configuration 70µm and even lower alginate droplets size were easily and successfully generated. Flexibility in term of droplet size is however limited (dripping regime range is reduced and jetting comes quickly with this specific configuration).

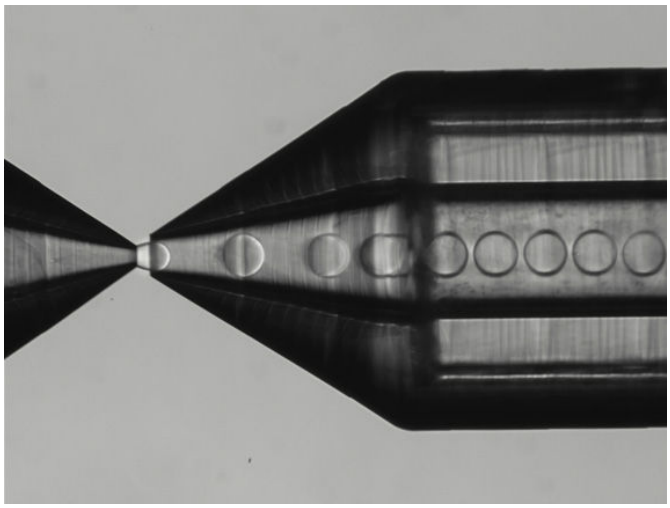


Figure 10: Alginate beads formation with nozzle of 60µm in outlet

CONCLUSION

In this application note we have demonstrated that alginate beads can be successfully produced using the RayDrop with precise control of droplet size.

Microbeads of 95-160µm diameter were generated with standard RayDrop configuration (Nozzle of 30µm and outlet capillary 150µm) using alginate solution at 1% in water. Other concentrations of alginate such as 2%, which is widely used in biological application, have also successfully tested by following the same process. In this last case, higher internal diameter tubing has to be used to deal with a more viscous solution.

As a consequence, this setup and protocol can be used for encapsulation of mammalian cells, bacteria and other reagents into alginate beads.

We have successfully demonstrated that using Fluigent product allow to control precisely alginate beads formation with high monodispersity.

As presented, using the RayDrop with different nozzle size allow to have better flexibility. Changing capillary size can be easily done by the user to target different droplet sizes.

REFERENCES

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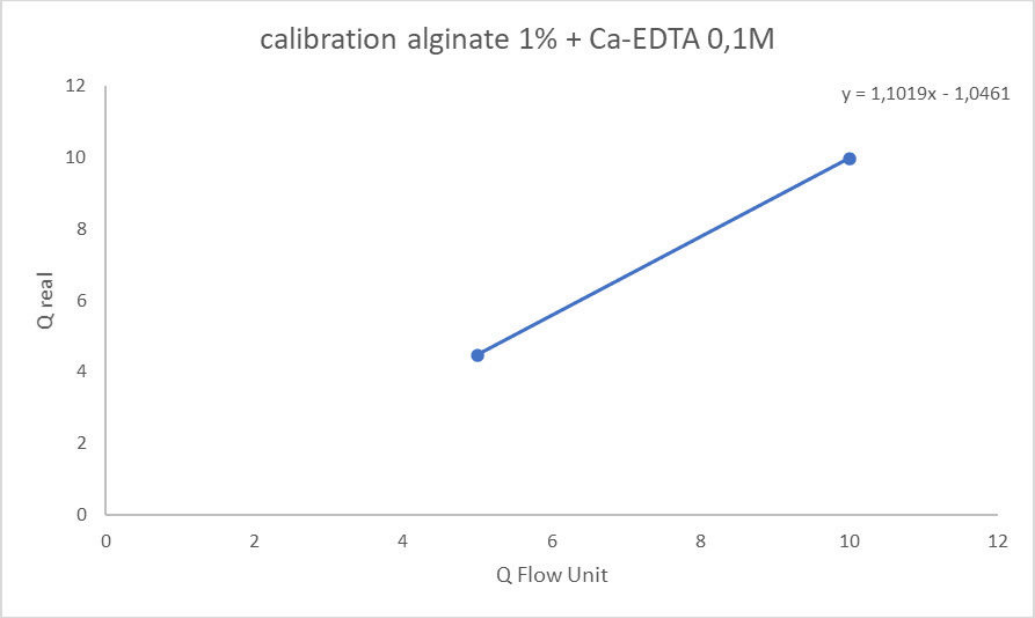
APPENDIX

Alginate 1% flow sensor calibration



For better precision one flow unit M has been calibrated to have the exact flow rate of Alginate solution during all experiment.

We calibrated the Alginate by weighting the volµme during a specific time. This experiment gave us the following equation:

$$Q_{real} = 1{,}1019*QFU-1{,}0461$$

The Flow unit have been placed in water calibration mode and thanks to the previous equation we enter this equation in the AIO software to have the real Alginate flow rate.

Full Alginate phase diagram