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Development of bacteriophage-loaded microstructures using Microfluidics

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E co-orientação
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RESUMO

Ao longo dos anos, nos países desenvolvidos, tem-se verificado um aumento da preocupação, por parte dos consumidores, relativamente à segurança dos alimentos que ingerem. O estilo de vida tem levado a uma crescente procura por produtos de conveniência, seguido de fruta e vegetais minimamente processados, referidos como produtos "fresh-cut". Muitos dos surtos causados pela ingestão de alimentos, estão associados à contaminação destes por parte de microrganismos patogénicos, como *Salmonella* e *Listeria monocytogenes*. O uso de bacteriófagos emerge como uma possível solução para reduzir e eliminar a carga destes microrganismos nos alimentos, uma vez que são seguros para consumo e específicos para determinados microrganismos, o que os torna excelentes ferramentas para fins de segurança alimentar.

Contudo, sabe-se que os bacteriófagos perdem viabilidade ao longo do armazenamento e após aplicação devido a condições ambientais, sendo essencial encontrar metodologias de proteção para que os mesmos mantenham total atividade quando adicionados diretamente nos produtos alimentares. A microencapsulação surge como uma solução, no entanto, de um ponto de vista económico, grande parte dos métodos de encapsulação atuais são caros e alguns dos métodos usam temperatura durante o processo o que inviabiliza o seu uso para a encapsulação dos bacteriófagos. Há assim uma necessidade de encontrar uma tecnologia de microencapsulação economicamente viável para produção de microcápsulas carregadas com bacteriófagos, sendo que a microfluídica pode ser uma solução viável.

O principal objetivo deste trabalho consiste no uso da microfluídica para gerar estruturas micrométricas, com biopolímeros de grau alimentar (agarose), carregadas com um agente antibacteriano natural, os bacteriófagos. O processo de formação de capsulas recorrendo á microfluidica, foi otimizado, e as técnicas de encapsulamento "On-Chip" e "Off-Chip" desenvolvidas, procedendo-se á caracterização das capsulas obtidas. Um tamanho de 85 µm foi alcançado, recorrendo á técnica "On-Chip". Verificou-se também uma alta taxa de produção de capsulas, assim como um elevado nível de monodispersividade, sendo todo o processo de produção flexível, recorrendo á referida técnica desenvolvida. Obteve-se uma libertação de fagos de cerca de 60% nos primeiros 5 minutos, sem a adição de um agente externo. Em conclusão, esta tecnologia abre caminho para a implementação de produtos eficazes para controle de segurança alimentar.

PALAVRAS-CHAVE: BACTERIÓFAGO, ENCAPSULAÇÃO, MICROFLUIDICA

ABSTRACT

Over time, in developed countries, people have become more concerned about the safety of the food they eat, and so, there has been a growing demand for convenience products, followed by fresh-cut fruits and vegetables. Unhygienic practices in preparation of fresh-cut products may pose risks to public health by causing foodborne illnesses, due to microbial contamination. Many of the outbreaks associated with foodborne diseases, are related to contamination of the food products by pathogenic microorganisms, such as *Salmonella* and *Listeria monocytogenes*. The use of bacteriophages emerges as a possible solution to reduce and eliminate the bacterial load of these microorganisms present in foods, since these kind of viruses, are safe for human consumption and specific for certain microorganisms, which makes them excellent tools for food safety purposes.

However, it is known that bacteriophages lose viability throughout storage and after application, due to environmental conditions such as pH, temperature, ions, salinity, osmotic pressure and exposure to organic solvents, and as such it is essential to find protection methodologies so that they maintain full activity when added directly to food products. Microencapsulation, arises as a solution, yet, in an economic point of view, some of the actual encapsulation methods are expensive, and others use temperature during the process which unviable their use for bacteriophages encapsulation. So, there's a need to find a new cost-effective microencapsulation technology, for production of bacteriophage-loaded microcapsules, being that microfluidics, emerge as a viable solution.

The main objective of this work, consist in the use of microfluidics to generate microstructures with food grade biopolymers (agarose), loaded with a natural antibacterial agent, the bacteriophages. The droplet formation process was optimized and the "On-Chip" and "Off-Chip mixing" techniques developed, followed by the characterization of the obtained droplets, where it was verified that a size of 85 μm was achieved using the On-Chip technique. A high-throughput formation of monodisperse agarose beads, in a very controlled and automated way, was accomplished, being that the entire production process malleable. A phage release of about 60% was obtained in the first 5 minutes, without the need of any external triggers. In conclusion, the developed technology opens a new path for the deployment of new products for food safety purposes.

KEYWORDS: BACTERIOPHAGE, ENCAPSULATION, MICROFLUIDICS

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LIST OF ABBREVIATIONS AND ACRONYMS

aw	Water activity
DNA	Deoxyribonucleic acid
GRAS	Generally regarded as safe
Re	Reynolds number
We	Weber number
Pe	Péclet number
Ca	Capillary number
FRR	Flow rate ratio
PEG	Polyethylene glycol
CAP	Cellulose acetate phthalate
DS	Degree of substitution
PDMS	Poly(dimethylsiloxane)
PFU	Plaque-forming unit
Q_C	Flow rate of the continuous phase
Q_D	Flow rate of the disperse phase
DSC	Differential scanning calorimeter

1. INTRODUCTION

1.1. Food industry concerns

Over the years, in most developed countries, people have become more concerned about the safety of the food they eat, which is related with a growing education level among consumers (Da Costa, 2000). Fruits and vegetables are one of the major dietary sources of nutrients and are of great importance to the human diet. Advances in the food processing chain, have enabled the food industry to supply nearly all types of high-quality fresh fruits and vegetables to those who desire and are willing to purchase them all year around (Artés and Allende, 2005). In developed countries, there has been a growing demand for convenience products and the same trend is followed by fresh-cut fruits and vegetables. However, microbial contamination of fresh-cut products can cause threats to human health causing various symptoms like diarrhoea, abdominal cramps and vomiting, and in some cases it may lead to death. In the last decades, there has been a dramatic increase in the outbreaks of foodborne diseases caused mainly by *Escherichia coli* and *Salmonella* spp. by consumption of fresh and minimally processed fruits (Callejón *et al.*, 2015). Food safety is thus a growing concern in the food industry (Bhagwat, Saftner and Abbott, 2004).

An increase in the market of minimally processed fresh products, has occurred due to its convenience and health benefits to consumers. As consumption of fresh-cut products goes up, an important concern to consumers arises, which is the safety of such products. Fresh-cut fruits and vegetables offer an array of advantages but are highly perishable. Unhygienic and ignorant practices in preparation of fresh-cut products, may pose risks to public health by causing foodborne illnesses (Qadri, Yousuf and Srivastava, 2015).

1.2. Fresh-cut produce

Fresh-cut produce is defined as “any fruit or vegetable or combination thereof that has been physically altered from its original form, but remains in a fresh state” (International Fresh-cut Produce Association). In the last few years there has been a fast growth of fresh-cut produce industry worldwide, mainly because of the increasing consumer demand for healthy, freshly prepared, convenient fruits and vegetables. Fresh-cut produce may

consist of peeled, sliced, shredded, trimmed and/or washed fruits and vegetables (Francis *et al.*, 2012).

Fresh-cut produce available in the market can include a wide range of products, being possible to obtain practically all the vegetables and fruits in the form of fresh-cut (James, Ngarmsak and Rolle, 2011). Despite the benefits derived from eating raw fruits and vegetables, the quality and safety are still an issue of concern as these foods have long been known to be vehicles for transmitting infectious diseases (Beuchat, 1995; Allende *et al.*, 2002).

The destruction of surface cells during processing (such as peeling, slicing and shredding) of fresh-cut produce exposes the cytoplasm and provides microorganisms with a richer source of nutrients as compared to intact produce (Barry-Ryan, Pacussi and O'Beirne, 2000). Besides, the high water activity and approximately neutral (vegetables) or low acidic (many fruits) tissue pH facilitate rapid microbial growth. These conditions provide a perfect platform for many important human pathogens and spoilage microorganisms to contaminate fresh-cut produce, which results in a faster deterioration of fresh-cut produce compared to whole fruits or vegetables. There are several physical and physiological facts that affects the viability and quality of the fresh-cut products which are a direct result of the wounding associated with processing. Flaccidity due to loss of water, changes in colour (especially browning at the cut surfaces) and microbial contamination deteriorate the appearance of fresh-cut produce (Qadri, Yousuf and Srivastava, 2015).

1.2.1. Factors that affect the microflora present in fresh-cut products

Fresh and minimally processed fresh-cut products are naturally contaminated by microorganisms because of several sources, including the farm environment, post-harvest handling and processing. Hence, the microflora linked with fresh-cut produce is diverse. Also, contamination with pathogens can occur at various points during growing, harvesting, processing and handling of the produce. The microflora associated with most vegetables is dominated by gram-negative bacteria, while dominant microflora associated with raw fruits mostly includes yeasts and moulds (Burnett and Beuchat, 2001). The factors that influence the survival and/or growth of the microorganisms on the fresh produce may include the type of organism, the commodity and environmental conditions in the field and thereafter, including storage conditions (Qadri, Yousuf and Srivastava, 2015).

Fruits and vegetables may be contaminated with pathogenic microorganisms at any time from farm to fork frequently, due to the fact they are in contact with soil, insects, animals or humans, and this contamination may be through stool, human handling, harvesting equipment, processing, transportation and distribution (Sothornvit and Kiatchanapaibul, 2009).

Before processing, the protective character of the vegetables and fruits natural barriers (cell walls and outer covering) may prevent the growth of microorganisms on the uninjured outer surface of fruits or vegetables. The physical environment surrounding the produce surface in the field is considered to be inhospitable for the growth and survival of bacteria due to lack of nutrients and free moisture, temperature and humidity fluctuations and ultraviolet light. However, environmental conditions can have great influence on bacterial populations (Qadri, Yousuf and Srivastava, 2015). Survival and growth of bacterial populations may be promoted by the presence of free moisture on produce surface (Beattie and Lindow, 1999).

In fresh-cut produce production chain, there are several processing steps and in each one of these steps, may occur microbial contamination. The protective barrier provided by the epidermis against development of microbes on the fruit surface is removed during processing (Qadri, Yousuf and Srivastava, 2015). Thus, processing and storage lead to enhanced decay and loss of quality of fresh-cut fruits and vegetables (Busta *et al.*, 2003). The damage of the tissues during processing operations such as cutting, shredding and slicing not only makes the fresh-cut produce more susceptible to microbial attack compared to intact produce, but also causes damage to fruit and vegetable tissues and cellular structure, leading to leakage of nutrients and cellular fluids (Heard, 2002). Therefore, minimal processing may increase microbial decomposition of fresh-cut products due to transfer of microflora from surface to the fruit flesh, which acts as a complete medium for growth.

Packaging is one of the important factors influencing the microbial quality of fresh-cut products. Fresh-cut products are mostly packaged under modified atmospheric conditions, being usually sealed within semipermeable packages, in which the respiratory activity of these vegetables modifies the gas atmosphere inside the package, decreasing and increasing, oxygen and carbon dioxide levels, respectively, and, are also stored under refrigeration which gives rise to a favourable environment and time for proliferation of spoilage microorganisms and microorganisms of public health significance (Francis, Thomas and O'Beirne, 1999; Qadri, Yousuf and Srivastava, 2015)

The economic value of fresh-cut products is impaired by microorganism's proliferation because it may lead to decrease in product shelf life, through spoilage, and also pose a risk to public health by causing foodborne illnesses. Gaseous composition of the atmosphere surrounding the fresh-cut produce has a profound effect on the microbial quality and shelf life. Low oxygen-modified atmospheres may inhibit the growth of spoilage microorganisms and increase the shelf life of packaged produce (Qadri, Yousuf and Srivastava, 2015).

1.3. Main bacteria present in food industry

Within all the existing microorganisms, bacteria are those that present major obstacles in the preservation of food. Most bacteria are relatively harmless, but they can excrete enzymes that can alter the composition of food, and in some cases, can produce toxic substances (Heard, 2002). Table 1 and 2 present the bacteria reported in literature and the respective vegetable or fruit, in which they can be found.

Table 1 – Examples of bacteria that can be found in vegetables and fruits

Organism	Vegetables and Fruits	Reference
Pseudomonas	Citrus, Broccoli, Cabbage, Carrots, Lettuce head, Lettuce leaf, Mushrooms, Potatoes, Tomatoes	(Barth *et al.*, 2009)
Erwinia	Apples, Pears, Broccoli, Cabbage, Carrots, Cucumbers, Lettuce head, Lettuce leaf, Onions, Potatoes, Tomatoes	
Bacillus	Carrots, Cucumbers, Onions, Potatoes, Tomatoes	
Clostridium	Potatoes	
Lactic acid bacteria	Tomatoes	

Table 2 – Examples of the occurrence of foodborne pathogens in fresh-cut salad products

Pathogenic Species	Food Product	Country	Reference
Shigella	Salad vegetables	Egypt	(Satchell *et al.*, 1990);
Listeria monocytogenes	Prepacked salad Prepared vegetables Coleslaw	Ireland U.K. U.K.	- (Harvey and Gilmour, 1993; MacGowan *et al.*, 1994)
Staphylococcus spp.	Salad vegetables	Egypt	(Satchell *et al.*, 1990)
Salmonella	Mixed salad vegetables	U.S.	(Lin, Fernando and Wei, 1996)
Yersinia enterocolitica	Packaged vegetable products	France	(Manzano *et al.*, 1995)

Within the approximately 30 bacteria found in food products, the most problematic are *Escherichia, Salmonella, Pseudomonas, Bacillus, Clostridium, Lactobacillus* and *Staphylococcus.* Some species such as *Salmonella typhi*, *Staphylococcus aureus* and *Clostridium botulinum* are pathogenic. As presented in table 2, many pathogenic species can contaminate fresh-cut produce. In the past few years, we have witnessed an increase in the number of foodborne outbreaks associated with fresh and fresh-cut products. Pathogenic microorganisms do not necessarily cause spoilage of produce and in absence of any signs of spoilage, the produce may be consumed leading to the development of foodborne illness (Qadri, Yousuf and Srivastava, 2015).

The psychrotrophic pathogen *Listeria monocytogenes* and themesophilic pathogen *Salmonella* are among the most important pathogens involved in human illness associated with consumption of fresh products. Indeed, outbreaks of salmonellosis and listeriosis, due to consumption of vegetables, have been reported (Rangel *et al.*, 2005).

1.4. *Salmonella*

Bacterial intoxication in food industry is the major cause of foodborne infections. They're mainly due to species such as *Listeria monocytogenes, Yersinia enterocolitica*,

species of *Salmonella*, *Staphylococcus aureus* and *Clostridium* spp (Sillankorva, Oliveira and Azeredo, 2012).

Salmonella is a ubiquitous bacterium, being widely distributed in nature. It can be found in the intestinal tract of humans, birds and mammals, due to the consumption of contaminated food, which may occur through various means, such as insects, birds, rodents, pets, people and water (Lin, Fernando and Wei, 1996; Shinohara *et al.*, 2008; Pui *et al.*, 2011).

1.4.1. Pathogenicity

Salmonellosis, the infection caused by *Salmonella* is mainly derived from the consumption of contaminated foods. However, it can also be transmitted by simple contact with an infected animal or from person to person (Shinohara *et al.*, 2008; Pui *et al.*, 2011; Foley *et al.*, 2013).

The pathogenicity of this species is due to its ability to invade intestinal cells, in which the release of endotoxin occurs during cell lysis (Pui *et al.*, 2011; Foley *et al.*, 2013). In rare cases, the infection caused by this bacterium can lead to death of the host, in individuals with immune deficiency, children and elderly. It has a high morbidity rate, and a relatively low mortality rate (Pui *et al.*, 2011; Foley *et al.*, 2013).

1.4.2. Characteristics

This bacterium can multiply at temperatures from 5 to 47 °C with an optimum temperature for growth between 35 and 37 °C. For temperatures below 10 °C, the metabolism is significantly reduced. Although, freezing does not completely inhibit the activity of *Salmonella*, it does eliminate part of the population. Surviving bacteria can easily multiply when the temperature becomes favourable again (Pui *et al.*, 2011).

The optimum pH for growth of this bacterium is usually between 6.5 and 7.5, with its growth generally being inhibited below 4.5 and above 9 (Pui *et al.*, 2011).

Below a water activity of 0.93, the growth of *Salmonella* is interrupted. It is also inhibited by the presence of salts higher than 5.8% (Pui *et al.*, 2011).

1.4.3. Food products that can be contaminated by Salmonella

The most frequently contaminated foods are meat and meat products, some poultry meats, eggs and egg products, milk powder or liquid. Also, vegetable products, can serve as vectors for *Salmonella* (Shinohara *et al.*, 2008).

1.5. Bacteriophages

Bacteriophages (phages), viruses that infect bacteria, and represent one of the most abundant biological entities in nature (Brüssow and Hendrix, 2002; Karimi *et al.*, 2016). They can often be found in the same environments where their bacterial host(s) inhabit, or once were present (Sillankorva, Oliveira and Azeredo, 2012). Until 2006, over 5500 different bacteriophages, had been discovered, each of which being able to infect one or several types of bacteria (Ackermann, 2007). It has been calculated that there are 10^{31} different bacteriophages on the planet Earth (Karimi *et al.*, 2016). Since their discovery, they have been used to treat and prevent bacterial infections, but their popularity decreased with the development of antibiotic industry, which led to their marginalization (Choińska-Pulit *et al.*, 2015).

The penetration of the phage into the bacterial cell happens through the recognition and attachment of phages to specific receptors that are expressed on the surface of the host cell, which properties affect this phenomenon (Karimi *et al.*, 2016). Being obligatory parasites, upon multiplication by taking over host protein machinery, they can either cause cell lysis to release the newly formed virus particles (lytic cycle) or lead to integration of the genetic information into the bacterial chromosome without cell death (lysogenic cycle) (Sulakvelidze, 2011; Sillankorva, Oliveira and Azeredo, 2012). Phages that replicate only via lytic cycle are known as virulent phages, while phages that replicate using both lytic and lysogenic cycles, are known as temperate phages (Barksdale and Arden, 1974).

There are a variety of phages with a different range of hosts and mechanisms of production, so they can be divided into two main classes based on their production and generation (lytic and temperate phages). Lytic phages such as T4, of which bacterial cells are lysed and disrupted by replication of the virions inside the cell and the infection is rapidly transferred to new hosts (Hashida *et al.*, 2014). And temperate phages such as lambda (λ) phage, that allows it to either reside within the genome of its host through lysogeny, converting the host cell into a generation factory without lytic disruption, being

able to cross the bacterial membrane to escape by leaking out without disruption of the cell wall and membrane, or enter into a lytic phase, in which it kills and lyses the cell (Fortier and Moineau, 2009). Towards a food safety perspective, lytic phages are possibly one of the most harmless antibacterial approaches available (Sillankorva, Oliveira and Azeredo, 2012).

In order to choose which phage to use, one must proceed to the selection of some of the most potent and effective phages present in an available collection. It should be verified if there are a large variety of phage, since the abundance of different phages gives us an opportunity to isolate and compose a phage preparation against all kinds of current bacterial pathogens, regardless of resistance development. In situations, where no active phages are found for a specific pathogen, their isolation directly from the environment, needs to be attempted and if successful, can be produced for application (Gill and Hyman, 2010).

The majority of bacteriophages do not tolerate well some physical and chemical characteristics such as high and low temperatures, ions, pH, and salinity but a few phages have been isolated from extremely harsh conditions, such as Sahara deserts, hot springs, the North Sea, and polar inland waters (Wichels *et al.*, 1998; Breitbart *et al.*, 2004; Säwström *et al.*, 2008; Lin *et al.*, 2010; Jończyk *et al.*, 2011; Prigent *et al.*, 2015). One of the main advantages of phage therapy is that they can "auto dose", which means that they sequentially infect bacterial cells as the phage population grows (Loc-Carrillo and Abedon, 2011). Other advantages include their relatively inexpensive and simple production technology, their easy isolation, including from food products, and simple purification (Sillankorva, Oliveira and Azeredo, 2012; Karimi *et al.*, 2016). Besides their efficiency even in low doses, they are also able to eradicate biofilm populations (Loc-Carrillo and Abedon, 2011).

Phages are present in almost all foods we eat and daily consumed by humans through ingestion of water and food, and as such, are presumed to be safe, which, with their specificity, makes them excellent tools for food safety purposes, and can bring benefits to the entire food chain, reducing pathogen colonization in livestock (phage therapy), in the decontamination of raw meats, fresh products or even raw milk (biocontrol), in the disinfection of equipment and contact surfaces (biosanitation) and expanding shelf life of ready-to-eat products (biopreservation) (Mahony *et al.*, 2011; Sillankorva, Oliveira and Azeredo, 2012)

Phage therapy has however some disadvantages such as: the identity of the pathogen strain needs to be known (Drulis-Kawa *et al.*, 2012); the possible emergence of phage-resistant derivatives is a reality, but can be circumvented using a cocktail of different phages (Mahony *et al.*, 2011); their sensitivity to extreme heat, pH, salinity, osmotic pressure, ions and exposure to organic solvents (Jończyk *et al.*, 2011; Choińska-Pulit *et al.*, 2015). In terms of phenotypes resistant to phages, the most common resistance mechanism to phage infection is the loss of receptors by the host bacteria, which consequently disables phage adsorption (Drulis-Kawa *et al.*, 2012).

1.6. Approaches to reduce microbial risks using encapsulated bacteriophages

Bacteriophages emerge as a solution to face microbial contamination of "fresh-cut" products. However, it is essential to find systems where phages can be safely added directly to food products (Anany *et al.*, 2011).

Phages, normally, can be added by dipping, spraying or as a liquid food products. However some of them can be inactivated during the washing of the food products. Adjacent to this, there are two more problems that may occur, the dilution of phages and the potential development of bacterial resistance (Anany *et al.*, 2011). Thus, there is a need to protect food added phages, in which encapsulation technologies appears to be the best strategy available (Wall *et al.*, 2010).

Encapsulation can be defined as a technology of packaging solids, liquids, or gaseous materials in miniature structures that can release their contents at controlled rates under the influence of specific conditions (Anal and Singh, 2007). In this specific case, encapsulation can be described as a process whereby phages are retained within a wall material to reduce phage injury or loss, so they can survive the processing and storage conditions of food products (Krasaekoopt, Bhandari and Deeth, 2003).

There are some methods that have been used to encapsulate phages, such as extrusion, emulsions with solvent evaporation, electrospinning, spray drying, and using whey protein films (Salalha *et al.*, 2006; Puapermpoonsiri, Spencer and van der Walle, 2009; Vonasek, Le and Nitin, 2014; Colom *et al.*, 2017). However, manufacturing and application strategies should be optimized to achieve the desired microcapsule from an

economic point of view, making them effective for application (Salalha *et al.*, 2006; Yongsheng *et al.*, 2008; Vandenheuvel *et al.*, 2013).

As all methodologies, microencapsulation face specific challenges, such as the preservation and maintenance of phage activity during long-term storage and after the liberation process, respectively. Moreover, phages are sensitive to the solvents and reagents used in the food products processing, which should be non-toxic, according to the "Generally Regarded as Safe" (GRAS) (Puapermpoonsiri, Spencer and van der Walle, 2009; Jończyk *et al.*, 2011; Drulis-Kawa *et al.*, 2012).

In an economic point of view, the cost of microcapsule production, with the actual encapsulation methods is too high, which may be its biggest disadvantage. Thus, there is a need to find novel cost-effective microencapsulation technologies (Ghosh, 2006). Within this context, the development of bacteriophage-loaded microstructures using microfluidics, emerges as a possible solution.

1.6.1. Nano/micro fabrication using microfluidics

Microfluidics studies the behavior of fluids geometrically constrained to the microscale, and the properties that apply under these conditions. Microfluidics, being as well an important method for nano/micro fabrication, is defined as the field of science for designing, manufacturing and operating processes and devices with dimensions ranging from a few millimeters to micrometers, and which are characterized by displaying at least one channel with dimension smaller than 1 mm (Orlu-Gul *et al.*, 2014).

The use of this technology allows to reduce reaction volumes and the associated costs of chemical and biological experimentations by several orders of magnitude, while at the same time, increasing performance (Mcdonald *et al.*, 2000; Gibson *et al.*, 2011). Briefly, microfluidic techniques allow much faster reactions, formation and control of a defined interface between two phases due to laminar flow conditions, which is characterized by parallel streams flowing linearly without mixing, low consumption and power dissipation and automation. From an economic point of view, the cost of production of microfluidic devices is relatively low, therefore with an inferior financial demand and which may increase production in terms of amplification process, reducing costs and time for the implementation from the laboratory to the industrial process (Chang *et al.*, 2008; Skurtys and Aguilera, 2008; Van Gerven and Stankiewicz, 2009; Bettinger and Borenstein, 2010; Marre and Jensen, 2010).

Recently, the application of this technology has drawn attention to the use of micro-droplets for chemical and biological reactions in controlled environments, being each droplet analogous to the traditional chemist's flask, with the added physical advantages of reduced reagent consumption, automated handling, rapid mixing, and continuous rather than batch processing, providing a unique reaction environment (Rhutesh K Shah *et al.*, 2008; Theberge *et al.*, 2010).

1.6.2. Properties to consider in droplet formation

Normally, the processes that involve fluid mechanics can be described by many variables and properties that reveal the dominating acting forces. Although, in the microscale, the number of variables are reduced, since the viscosity and interfacial tension are the prevailing effects (Baroud and Willaime, 2004; Squires, 2005). Therefore, the relevant variables are the Reynolds number (Re), that expresses the ratio of inertial to viscous forces in fluid flow, the Weber number (We), which indicates the relative importance of inertial effects when compared to the interfacial tension in a multiphase system, the Péclet number (Pe), that expresses the relative importance between diffusion and convection, the capillary number (Ca), that gives the ratio of viscosity to interfacial tension, and the flow rate ratio (FRR), which can be calculated by the relation between the flow rate of the continuous and the dispersed phase, for droplet microfluidics. (Baroud and Willaime, 2004; Atencia and Beebe, 2005; Baroud, Gallaire and Dangla, 2010; Jahn *et al.*, 2010; Balbino *et al.*, 2013; Mijajlovic *et al.*, 2013). The most important variable to take into account is the Reynolds Number (Re), which is used in many fluid flow correlations and to describe the boundaries of fluid flow regimes (laminar, transitional and turbulent). The viscous force of the material is what tends to keep the layers moving smoothly, and when it is sufficiently high, it removes any disturbances from the flow and laminar flow is obtained. However, as velocity increases, the inertia forces increase and particles are pushed out of the smoother path, causing disturbances within the flow, which will lead to a turbulent flow (Wang, Yang and Zhao, 2014).

Eq. 2 $$\mathrm{Re} = \frac{\rho v L}{\mu}$$

Reynolds number is where can be calculated by the equation 2, where ρ represents the density of the fluid (kg/m^3), v the velocity (m/s), L corresponds to the characteristic length of the fluid (m) and μ the dynamic viscosity of the fluid (Ns/m^2). The flow is consider to be laminar when Re smaller than 2300, transient when is between 2300 and 4000, and turbulent if it higher than 4000 (Wang, Yang and Zhao, 2014).

Another parameter that should be consider is the microdevice geometry, such as the capillary and planar microfluidic devices, and the terrace geometry.

The capillary devices, are assemblies of coaxial capillary tubes, where one is placed inside another with a bigger diameter, being widely used for droplets, vesicles and microparticles generation. There are two types of capillary devices, the one where the fluids move in the same direction, and the one, where fluids are introduced from the two ends of the external capillary in opposite directions (Rhutesh K. Shah *et al.*, 2008). The biggest advantage of the use of these structures, is the fact that the droplets are surrounded completely by the outer phase, which increases the effects of interfacial forces between the two phases. Still, is difficult to scale up because of manufacturing challenges (Duncanson *et al.*, 2012).

On the other hand, the planar microfluidic devices are channels with rectangular cross section, which differ from each other according to the type of junction between the channels, being that the most commonly used for droplets generation are T-, Y- and cross-junctions. The droplet formation, in this kind of devices, occurs as a result of the interaction of two immiscible phases: the disperse phase (organic) and the continuous phase (aqueous), being an effect of the interfacial tension and shear forces at the fluid-fluid interface. (Garstecki *et al.*, 2006; Christopher *et al.*, 2008; Abate *et al.*, 2009; Steegmans, Schroën and Boom, 2009; Liu and Zhang, 2011; Ushikubo, Birribilli, *et al.*, 2014).

In the case of T-junction devices, the fluids are inserted in two perpendicular tubes, being widely used due to the facility in the generation of monodisperse droplets, as a result of the shear forces and interfacial tension at the fluid-fluid interface (Garstecki *et al.*, 2006; Christopher *et al.*, 2008; Gupta, Murshed and Kumar, 2009; Ushikubo, Birribilli, *et al.*, 2014). In the case of cross-junction (flow focusing), the device has four crossed perpendicular or oblique tubes, where usually the flow of the lateral channels constricts the main channel fluid flow, in a technique known as hydrodynamic flow focusing, being typically used for droplets formation and self-assembled structures. (Jahn *et al.*, 2004; Àlvarez-Puebla *et al.*, 2005; Liu and Zhang, 2011).

The biggest advantage of the planar microfluidic devices is the fact that they can be easily designed and produced, allowing the rapid prototyping of more complex structures. Yet, differently from the capillary devices, the fluids are limited by the device walls at the top and bottom surfaces of the chip, which decreases the interfacial area between the two phases (Ushikubo, Oliveira, *et al.*, 2014).

Microdroplets in microfluidics offers a pathway for the generation of three types of biomaterials, being microparticles, microcapsules and micro-gels (Orlu-Gul *et al.*, 2014). Following, a table with some examples of these types of biomaterials reported in the literature.

Table 3 – Examples of classes of materials available for fabrication using microdroplet devices (adapted from Orlu-Gul *et al.* 2014)

Microcapsules	Vesicles	Liposomes
	Bio-polymers	Chitosan, Alginate
	Polymeric	PLA, PLLA, EC
Microparticles	Bio-polymers	Chitosan, Alginate, Gelatin, Pectin
	Inorganic	Silica
	Polymeric	PEG, PCL, PLA. PGA, PLGA

Microcapsules, benefit from narrow size distribution, strict control over size and low amounts of reagents used (of the order of microliters) (Kim *et al.*, 2011). Polymeric microcapsules, such as, hydrophobic PLLA microcapsules, produced using a microfluidic chip, are indicative of higher drug-loading encapsulation efficiency and sustained release behaviour (He *et al.*, 2011).

Vesicles, microscopic subdivisions surrounded by a thin membrane, are generally self-assembled, which contain an inner aqueous core for encapsulation of a hydrophilic drug while a hydrophobic drug can be incorporated into the lipid bilayer (Jaafar-Maalej *et al.*, 2010).

1.6.3. Biomaterials involved in encapsulation

Since the droplets will be applied in the food industry, in some cases, after the microencapsulation process, it may enter in contact with the digestive tract of the host, after eating the food products, so there are some factors that must be considered in the choice of the biomaterial that will be used. Physicochemical properties, the toxicity; and the manufacturing and sterilization processes are the most important parameters to take into account when choosing the encapsulation material (Gbassi and Vandamme, 2012).

Bio-polymers, such as alginate, pectin and chitosan, are inherently water soluble, non-toxic, biodegradable and biocompatible. Monodisperse alginate hydrogel microbeads can be produced with high uniformity and control over size, shape in a T-junction microfluidic device (Tan and Takeuchi, 2007). The higher degree of control with a narrow size distribution is decisive on the clearance rate of the Ca-alginate-drug complex from the body and specific drug dosage (Huang *et al.*, 2011).

There are some variables to take into account in the choice of the material, which are, the degree of substitution (DS) of a polymer, which is the average number of substituent groups attached per base unit (in the case of condensation polymers) or per monomeric unit (in the case of addition polymers), the intrinsic viscosity, that provides insight to molecular structure and interactions in solution, and the partial specific volume, which express the variation of the extensive volume of a mixture in respect to composition of the masses (Blaga, 1970; Lee and Tripathi, 2005).

In general, biopolymers are described as organic or inorganic macromolecules, consisting of repeated chains of monomers linked by covalent bonds, which conformation and chemical structure provide specific functionality such as the ability to form gels (Gbassi and Vandamme, 2012). Some of the materials most often used in encapsulation may be, for example, food-grade polymers such as chitosan, alginate, cellulose acetate phthalate (CAP), carrageenan, whey proteins, gelatin, pectin, carob bean gum, starches, polyesters and agarose (Anal and Singh, 2007; Jyothi *et al.*, 2010; Gbassi and Vandamme, 2012; Colom *et al.*, 2017). Following a table with different biocompatible materials and some of their respective characteristics.

Table 4 – Materials that can be used in encapsulation and some of their respective characteristics

Material	Characteristics	Reference
Alginate	° Provides basic protection against acidity ° High efficiency in protein delivery as control of the amount of protein released ° Enhanced protein stability ° Temperatures in the range of 60 °C to 80 °C are needed to dissolve alginate in water. ° Insoluble in acidic media	(Lee, 2000; Kailasapathy, 2002; Harnsilawat, Pongsawatmanit and McClements, 2006; Gbassi *et al.*, 2009)
Gelatin	° Frequently applied in food and pharmaceutical industries ° Forms a solution of high viscosity in water at high temperatures, which sets to a gel during cooling ° Gelation occurs below 35ºC ° Has a very special structure and versatile functional properties	(Rokka and Rantamäki, 2010; Gbassi and Vandamme, 2012)
Whey proteins	° Usually used due to their amphoteric character ° Can be easily mixed with negatively charged polysaccharides such as alginate, carrageenan or pectin ° When the pH is adjusted below their isoelectric point, allows for interaction with negatively charged polysaccharides	(Doleyres, Fliss and Lacroix, 2002; Harnsilawat, Pongsawatmanit and McClements, 2006)
Carrageenan	° A rise in temperature (60 to 80 °C) is required to dissolve it ° Gelation occurs by cooling to room temperature	(Yuguchi *et al.*, 2002; Mangione *et al.*, 2003)
Chitosan	° It is water insoluble at a pH higher than 5.4. ° Can form a semipermeable membrane around a negatively charged polymer	(Huguet, Neufeld and Dellacherie, 1996; Rokka and Rantamäki, 2010)
CAP	° Is a polymer insoluble at a pH below 5 ° Soluble when the pH is greater than 6	(Krasaekoopt, Bhandari and Deeth, 2003; Anal and Singh, 2007)
Agarose	° Dissolves in near-boiling water, and forms a gel when it cools ° In gel forms a meshwork that contains pores ° Can have high gel strength at low concentration ° Frequently used in molecular biology	(Lootens, Amici and Plucknett, 2000)

Herein, we propose the use of microfluidic devices for the controlled encapsulation of phages in agarose for food control. The use of microfluidics allowed an improvement in the reproducibility and monodispersity of bacteriophage-loaded microcapsules their application for food safety purposes.

2. MATERIALS AND METHODS

2.1. Bacterial strains, bacteriophage and media used

The bacteria *Salmonella Enteritidis* (SE) 821, used in this project was isolated by the Ricardo Jorge Nacional Institute, and used to propagate the previously isolated and characterized bacteriophage PVP-SE2 (Sillankorva *et al.*, 2010). SE 821 was grown in LB medium or in solid LB medium (LB + 1.2 % (w/v) agar). For phage propagation LB top-agar (LB + 0.6 % (w/v) agar) was used.

2.1.1. Bacteriophage propagation

Bacteriophage PVP-SE2 was propagated using previously optimized protocols using the plate lysis and elution method (Sambrook, J. and Russell, 2001). In brief, overnight grown (37 ºC) SE 821 culture was poured (100 µL) on a Petri dish containing solid LB medium and 3 mL of LB top-agar (0.6% (w/v) agar) was posteriorly added, and stirred gently to distribute the SE 821 and agar uniformly. After drying, 10 µL of the bacteriophage stock solution (diluted 1:100) were added using the paper spread technique, and stored at 37 ºC (Cormier and Janes, 2014). After, 3 to 5 mL of SM Buffer (5.8 g NaCl + 2g $MgSO_4.7H_20$ + 50 mL 1M Tris-HCl (pH 7.5) per 1 L) were added to the plate and incubated overnight at 4 ºC and 80 rpm.

2.1.2. Bacteriophage purification and concentration

Phage-containing SM buffer was collected, mixed with NaCl (0.584 g per 10 mL), and incubated during one hour on ice, with gentle shaking (80 rpm). The solution was transferred to 50 mL tubes, and centrifuged for 15 min at 8300 rpm. The resulting supernatant, was transferred to an Erlenmeyer, where was posteriorly added polyethylene glycol 8000 (1g per 10 mL), dissolving with gentle shaking, and incubated at 4 ºC and 20 rpm, for 16 hours (Yamamoto *et al.*, 1970). The solution was centrifuged for 15 min at 8300 rpm and the supernatant discarded. The resulting pellet was ressuspended in SM buffer, and chloroform was added (2.5 mL per 10 mL of resuspended solution) and vortexed for 30 seconds (Sambrook, J. and Russell, 2001).

Lastly, the solution was centrifuged for 20 min at 4300 rpm, and the upper aqueous phase was collected, filtered (0.2 µm pore size) to a 15 mL tube, and stored at 4 ºC until further use.

2.1.3. Bacteriophage quantification

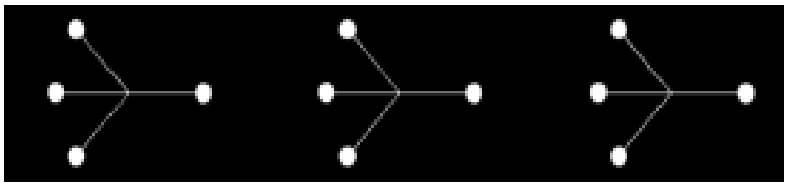
Bacteriophage quantification was performed according to the drop technique (Kropinski *et al.*, 2009). SE821 culture (100 µL) was added to solid LB Petri dishes (1.2% (w/v) agar), and immediately covered with LB top-agar (0.6% (w/v) agar) softly homogenizing. Increased 10-fold dilutions of the bacteriophage solution were performed sequential, in a 96-well microplate, in duplicate, and 10 µL droplets of each of these dilutions, were added to the Petri Dishes, and incubated at 37 ºC for 10 hours (Gallet, Kannoly and Wang, 2011; Kauffman and Polz, 2018). The phage plaques were counted, and the plaque forming units (PFU) per millilitre calculated by the following formula:

Eq. 2 $$\text{PFU/mL} = \frac{Number\ of\ phage\ plaques \times 10^{Dilution} \times 1000}{Amount\ of\ sample\ plated}$$

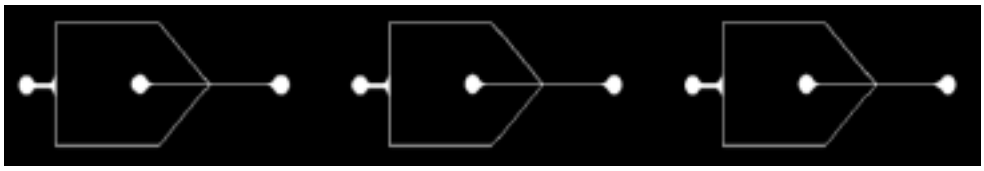
2.2. Microfluidics device design

The first step was to design the microfluidic devices, using Autocad 2017 software, with different geometries, as seen in Figure 1, in order to identify the optimum configuration that would allow the fabrication of droplets. Two design types were designed, the Y-junctions and flow focusing chips.

a)



b)



c)

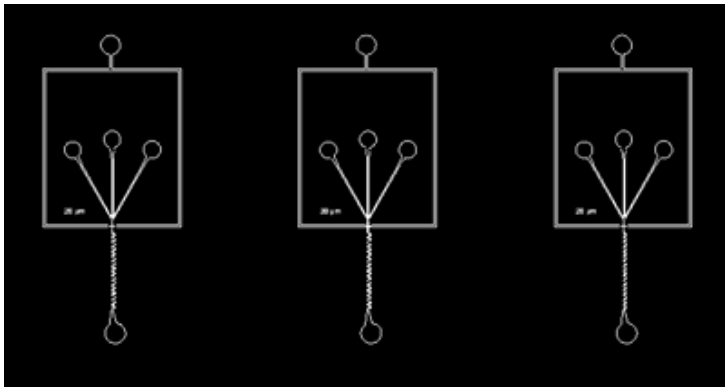


Figure 1 – Microfluidic designs in AutoCAD 2017 software **a)** Y-junction chip, with three inlets, and one outlet **b)** Flow focusing chip, with one inlet for the continuous phase, and another for the dispersed phase **c)** Flow focusing chip, with one inlet for the continuous phase, and three for the dispersed phase

Every device has a width of 100 µm and a depth of 80 µm, denominated 80 µm junction. The flow focusing design with three inlets, represented in figure 1, has also been design with different size junctions (10, 20, 40 and 60 µm).

2.3. Master fabrication

The master (mould) for the microfluidic devices was fabricated in a clean room environment by photo-lithography. SU-8 2025 photoresist (Micro-Chem) was spin-coated onto a silicon wafer (diameter: 3-inch, Compart Technology Ltd.) at 500 rpm for 5 s and then ramped to 1000 rpm at an acceleration of 300 rpm s^{-1} for 33 s, to achieve a final thickness of 80 µm of the SU-8 photoresist layer. After spinning, the wafer was prebaked for 1 min at 65 °C and then 7 min at 95 °C. The master was then, aligned with a lithography mask (designed in AutoCad), and exposed to UV light on a mask aligner

(MJB4, Suss Microtech). After post-exposure baking and development, the master was heated for at 150 ºC for 10 min, in order to remove the stress of the material (Abalde-Cela *et al.*, 2011; Zartman and Hoelzle, 2015).

2.4. PDMS replicas fabrication

The fabrication of the PDMS replicas was achieved by soft-lithography, where polydimethylsiloxane (PDMS, Sylgard 184) and a cross-linking agent (Sylgard®, 184 Silicone elastomer) were mixed in a proportion of 10:1 in order to form a solid, transparent and flexible elastomer, being posteriorly added on top of the master previously fabricated, before it became solid . The remaining air bubbles, were removed by a desiccator. The mixture was cured at 65ºC for 1h and, after cooling, the PDMS was peeled-off from the master and all the inlets and outlets were punched, in order to be able to connect the tubing with the device. Then, each replica was bonded to a glass slide, through a plasma-activation of both surfaces, performed in a Harrick Plasma PDC-002-CE® plasma cleaner, where the PDMS is oxidized in air oxygen plasma. The oxygen plasma removes biological, hydrocarbon material through a chemical reaction with highly sensitive oxygen radicals and ablation by energetic oxygen ions. This leaves behind silanol (SiOH) groups on the surface, which make the surface more hydrophilic and increasing the surface wettability. After plasma-activation of the surfaces, the PDMS is positioned to touch the glass surface straight away, to form bridging Si-O-Si bond at the interface, producing a permanent seal. (Abalde-Cela *et al.*, 2011; Zartman and Hoelzle, 2015).

2.5. Device functionalization

Since the fluids are in contact with the walls of the channels, the device must have high affinity for them. In order to further increase the hydrophobicity of the channels, the device was cleaned with isopropanol, and then functionalized with HFE-7500 fluorocarbon oil 3M (Fluorochem, Belgium) and a commercial surface coating agent (Aquapel™, Pittsburg Glass Works, US), in order to increase their hydrophobicity and allow the formation of water-in-oil microdroplets. Finally, every device was flushed with pressurized nitrogen for drying (Abalde-Cela *et al.*, 2011).

2.6. Microfluidic experiments

The continuous and dispersed flows were driven with Norleq syringe pumps, and plastic syringes of 5 mL (Terumo) and 1 mL (Terumo), respectively. These syringes were connected via polyethylene tubing (internal diameter of 0.38 mm, Portex, Smithsmedical) to the microfluidic device. A mixture of Fluorous oil (HFE-7500 3M Fluorochem) and surfactant, Pico-Surf$^{(TM)}$ 1 (5% in Novec 7500, Sphere Fluidics Ltd), was used as the continuous phase.

Initially the dispersed phase was formed by a solution of ultra-low gelling temperature agarose (A5030, Sigma-Aldrich), which has a gelling temperature between 8 and 17 °C, in a 0.8% gel, and a melting temperature below 50 °C. In further experiments agarose was used with the addition of an external agent such as Riboflavin (Vitamin B2, Sigma-Aldrich), fluorescent nanoparticles of 50 nm (Fluoresbrite® Yellow Green Microspheres 0.05µm, Polysciences, Inc.) and 200 nm (Fluoresbrite® Yellow Green Microspheres 0.20µm, Polysciences, Inc.), and bacteriophage PVP-SE2.

All experiments were carried out, on a heat plate, at approximately 35 °C to avoid the gelation of the agarose solution. The formed droplets were collected on ice, in order to gelate the agarose once the beads were produced. Milli-Q water (Millipore®) was used throughout all of the experiments, to prepare solutions and dilute the biomaterial used. After each experiment, the droplets were collected and stored at 4 °C. In order to evaluate the best microfluidic conditions for the droplet formation, an optimization of the flow rate, agarose and surfactant concentration was performed (Table 5).

Table 5 – Evaluated conditions for droplet formation optimization

Continuous phase Flow rate (µL h^{-1})	500	1000	1500	2000
Agarose Concentration (% w/v)	1	2	3	5
Surfactant Concentration (% v/v)	2		2.5	
Dispersed phase Flow rate (µL h^{-1})	100			

For the analysis of the obtained droplets, an inverted microscope (Nikon Eclipse MA200) with the NIS-Elements F3.22 software was used. Images were observed using an objective of 10×, and their size was determined using ImageJ software.

Different device designs were used, in order to observe how these would affect the droplet formation and stability of the process.

2.7. Loading distribution characterization

In order to simulate phage distribution inside the agarose droplet/bead, an experiment with fluorescent nanoparticles was performed, using two types of nanoparticles, 50 nm (Fluoresbrite® Yellow Green Microspheres 0.05µm, Polysciences, Inc.) and 200 nm (Fluoresbrite® Yellow Green Microspheres 0.20µm, Polysciences, Inc.), which corresponds to the size of the capsid and full size of the bacteriophage PVP-SE2, respectively (Sillankorva *et al.*, 2010). These particles were encapsulated using two different geometry devices. A fluorescence microscope (Olympus BX51 Fluorescence Microscope) was used and the particle distribution visualized by the Olympus CellSens Standard software.

Since the used surfactant, is not food-grade, a transfer from the oil to water phase, was necessary. For this purpose, different trials using 1H,1H,2H,2H-perfluoro-1-octanol 97% (Sigma-Aldrich), were performed, in order to find a method in which the droplets maintained their structure without collapsing (Abalde-Cela *et al.*, 2011).

After the transfer to a water phase was accomplished, a lyophilisation at -50 ºC (LyoQuest, Telstar) of the hydrated droplets was made for 48 hours, to evaluate their possible storage in different conditions (Abdelwahed *et al.*, 2006).

The shelf life of the hydrated and lyophilized beads was measured, at room temperature and at 4 ºC.

DSC (Differential scanning calorimetry) analysis, was performed in order to measure the glass transition temperature of the samples. For this purpose a DSC analyser 6000 (Perkin Elmer), was used, where approximately 20 mg of sample were placed into aluminium pans, and sealed with the aluminium covers (Perkin-Elmer, DSC, B0143016/B0143003, respectively). The heating and cooling steps were performed between the temperatures of -25 and 90 °C at a scanning speed of 10 ° C min^{-1}, being the finally results obtained in Pyris Series software (version 13) (Watase, Nishinari and Hatakeyama, 1988).

2.8. Release assays

After the optimization of the microfluidic conditions (agarose concentration, flow-rate, surfactant concentration and device geometry) in order to obtain the smaller droplets, the bacteriophage-loaded agarose droplets were produced, and the release of bacteriophage was assessed.

For this, 50 µL of droplets, which corresponds to approximately 55000 and 155700 droplets, in the case of the size of the bead of 120 and 85 µm respectively, were added to 150 µL of SM buffer in a 1.5mL tube, at room temperature (23.5 °C), with no external triggers agents added. 10 µL samples were taken every minute till 5 minutes, and then every 5 minutes, until 20 minutes, always replenishing the amount of taken sample with SM buffer (Peschka, Dennehy and Jr, 1998; Malik *et al.*, 2017). Samples were immediately diluted and plated as described before (2.1.3). These assays were performed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Bacteriophage production

The two small-scale bacteriophage propagations and purifications resulted in 20 mL and 15 mL stock solutions with approximately 8.5×10^{11} PFU mL^{-1} and $3{,}5 \times 10^{10}$ PFU mL^{-1}, respectively. After 8 months of storage at 4 ºC, a 2 to 3-log decrease in the phage concentration was noticed, reaching the values of 4.5×10^{9} PFU mL^{-1} and 4×10^{7} PFU mL^{-1}. Some phages are more tolerant to storage than others, for many years at this temperature, however many authors have described loss in phage viability after days or even a few weeks (Clark, 1962; Clark and Klein, 1966; Olofsson, Ankarloo and Nicholls, 1998; Olson, Axler and Hicks, 2004; Jończyk *et al.*, 2011; Cooper, Denyer and Maillard, 2013). This loss in viability of PVP-SE2 could have been prevented for instance if agarose (2% (w/v)) had been used in the preparation of SM buffer (Nobrega *et al.*, 2016; Gonzá Lez-Menéndez *et al.*, 2018; Manohar *et al.*, 2018).

3.2. Microfluidics device fabrication

Masters (mould) were fabricated by photo-lithography. Once the mould was obtained, conventional soft-lithography methods were performed to fabricate microfluidic PDMS devices, being posteriorly functionalized, since the fluids are in contact with the walls of the channels, and so the device must have high affinity for them. In order to further increase the hydrophobicity of the channels Aquapel™ (Pittsburg Glass Works, US), a hydrophobic solution normally used in the car windshield washer systems to repel rain drops, was used (Abalde-Cela *et al.*, 2011).

3.3. Droplet generation optimization

Considering that the dispersed phase is composed by an agarose solution (Agarose ultra-low gelling temperature, Sigma Aldrich), which is the material chosen for phage encapsulation, and the continuous phase by a mixture between oil (HFE 7500, Fluoro Chem) and surfactant (Pico-Surf™ 1, Sphere Fluidics), a bead formation trial was performed. For that purpose a flow focusing device was selected, as shown in Figure 2, in which there are two inlets, one for the oil and surfactant (inlet A) and another one for

the agarose (inlet B). These inlets, connect to their corresponding channels, converge into a single outlet channel leading to the outlet reservoir.

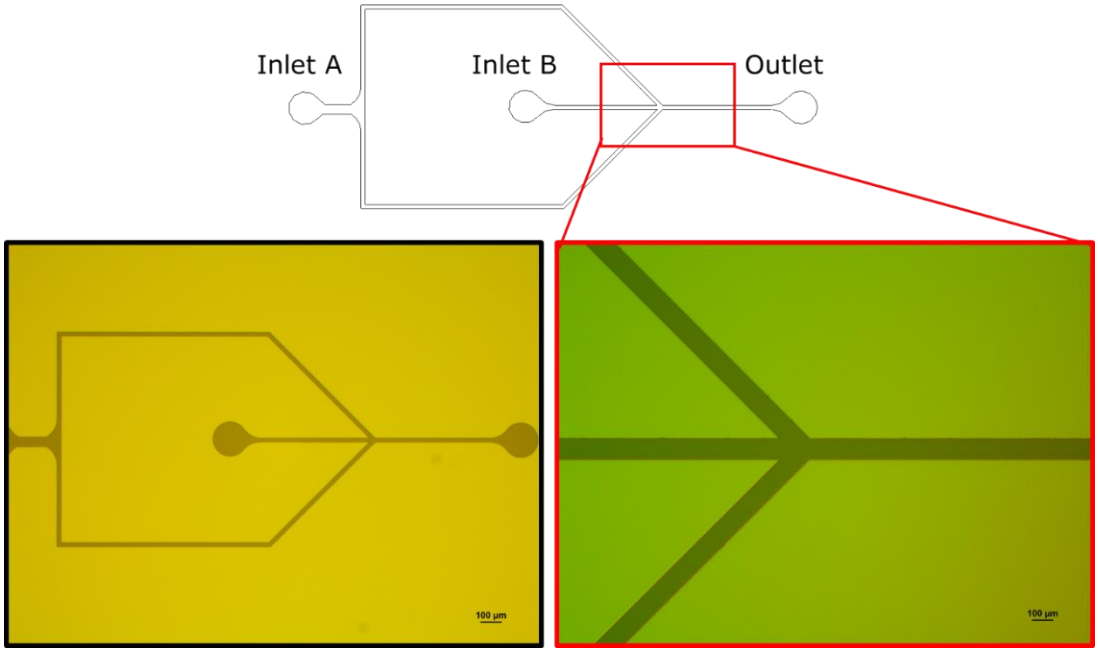


Figure 2 – Scheme and optical image of a flow focusing device, with an 80 µm junction; Inlet A: continuous phase input; Inlet B: dispersed phase input; Outlet: dispersed phase beads output (Abalde-Cela, 2017)

Droplet formation occurs when the agarose stream (dispersed phase) and the oil stream (continuous phase) meet at the junction, where the local flow field, determined by the geometry of the junction, and the flow rates of the two fluids create a free surface instability, since they are not miscible, deforming the interface. In this way, the droplets/beads are formed in a monodisperse way by controlled flow rates.

The main goal of this optimization task would be to obtain the largest amount of beads, with the smallest size possible in order to increase the surface area available, decreasing their volume, and consequently increasing their release efficiency.

In order to evaluate the best conditions for bead generation and stable formation, the agarose and surfactant concentration, and flow rates, were optimised. The obtained single droplets (agarose beads in an oil phase) were taken and immediately analysed using an inverted microscope. The formed droplets were very unstable, once they dried quickly at room temperature, which decreased their surface tension, and accelerated their

degradation. The images obtained, were measured using ImageJ software. The images taken had differences in scale, due to the small adjusts in the microscope to better focus the beads, so the scale of all images was adapted to 1250 × 937 pixels in height, in order to achieve the correct values of the beads size. It needs to be emphasized that obtained samples only lasted about 1 to 2 minutes until completely collapsing.

To evaluate how agarose concentrations would interfere in the bead production process, a fixed dispersed and continuous phase flow rates, of 100 µL h^{-1} and 1500 µL h^{-1}, respectively, and a surfactant in oil concentration of 2% (v/v) (continuous phase) were used. Agarose concentrations of 5% (w/v), 3% (w/v), 2% (w/v) and 1% (w/v) were tested, being the images of the obtained beads, displayed in the following figures.

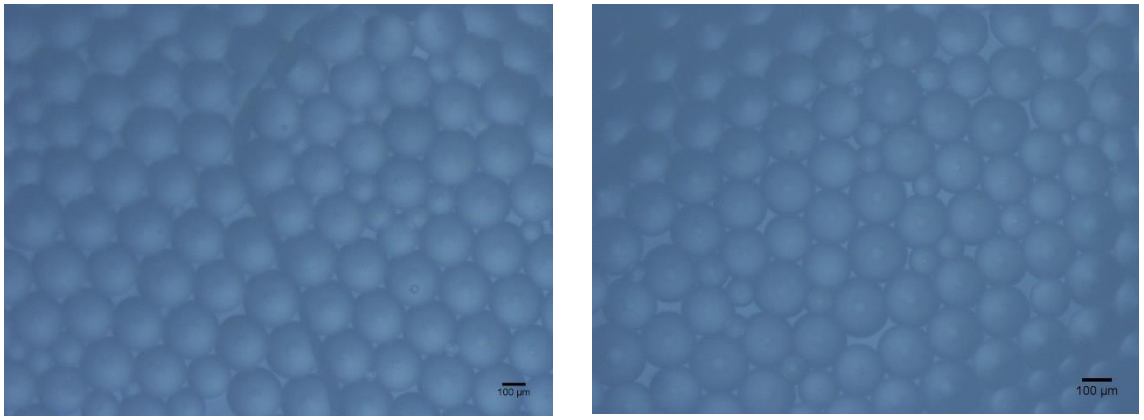


Figure 3 – Obtained 5% agarose beads, with the corresponding parameters of $Q_{Continuous}$= 1500 µL h^{-1}; $Q_{Disperesed}$= 100 µL h^{-1}; [continuous phase] = 2%, [disperse phase] = 5% (Optical image)

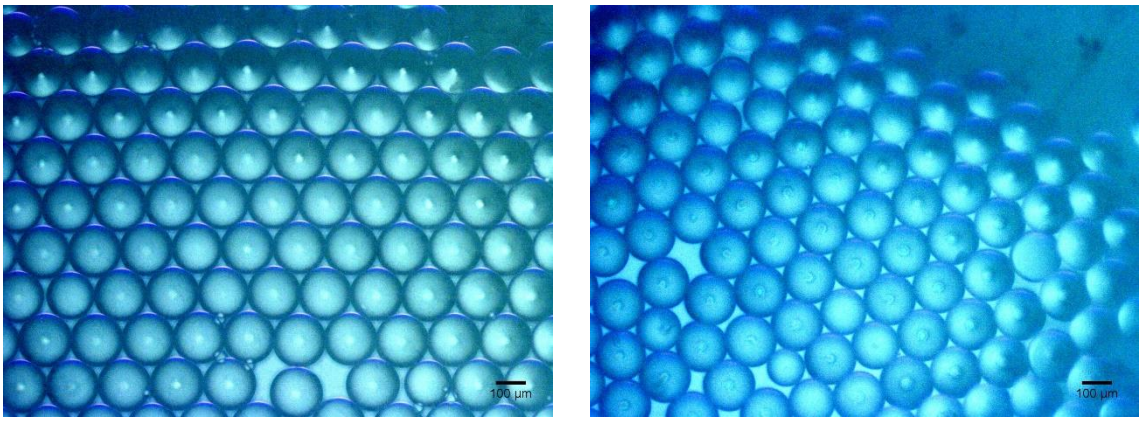


Figure 4 – Obtained 3% agarose beads, with the corresponding parameters of $Q_{Continuous}$= 1500 µL h^{-1}; $Q_{Disperesed}$= 100 µL h^{-1}; [continuous phase] = 2%, [disperse phase] = 3% (Optical image, with an increase of 0.25% of contrast, applied in ImageJ)

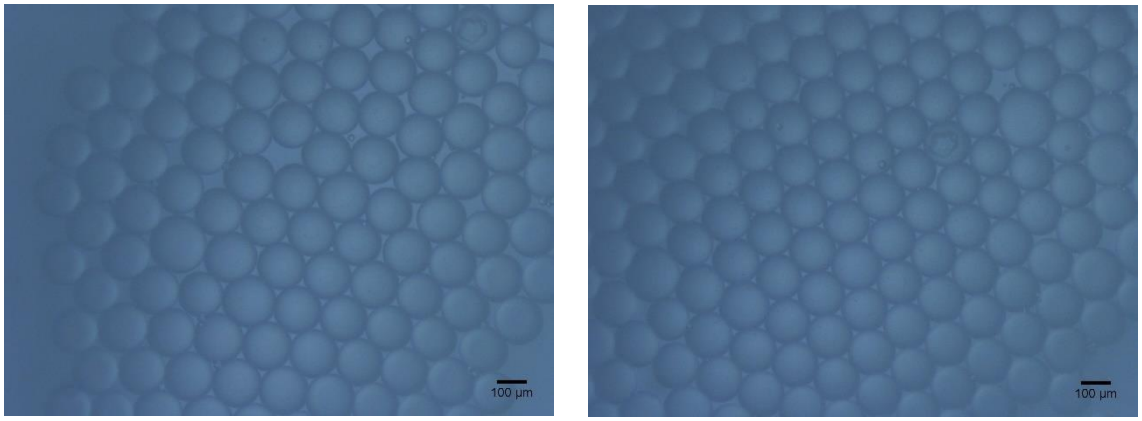


Figure 5 – Obtained 2% agarose beads, with the corresponding parameters of $Q_{Continuous}$= 1500 µL h^{-1}; $Q_{Disperesed}$= 100 µL h^{-1}; [continuous phase] = 2%, [disperse phase] = 2% (Optical image)

The 1% (w/v) agarose beads produced were not stable, most likely because the concentration was too low to keep the bead structure intact and that caused their almost immediate collapse, after the samples were taken, and therefore the referred concentration of agarose was excluded from further experiments.

On the other hand, a concentration of 5% (w/v) resulted in beads of heterogeneous size. Additionally, the process took 5 to 10 minutes to stabilize, and the droplets were only stable for 10 to 15 minutes which was not sufficiently efficient in terms of durability, particularly due to the premature gelation of the agarose inside the syringe resulting in deformed beads as shown in figure 6.

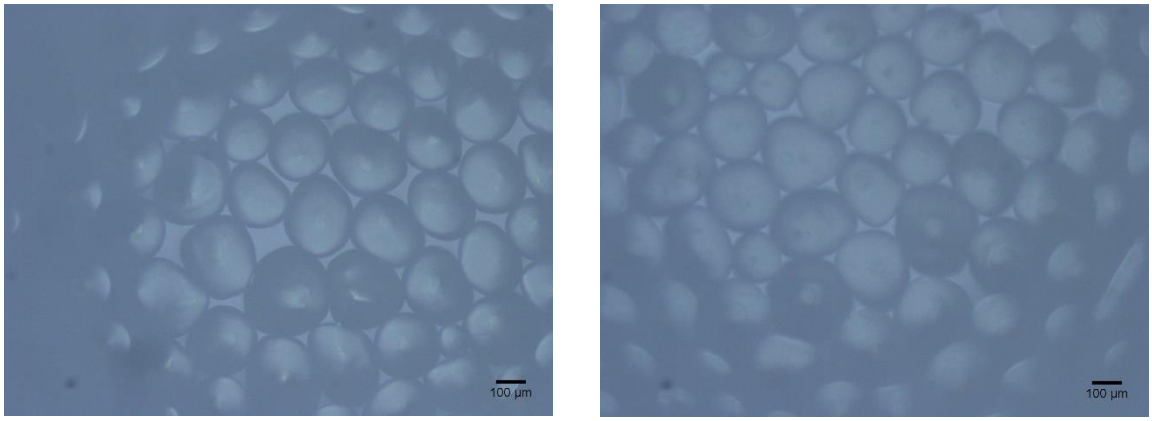


Figure 6 – Agarose 5 % beads deformation due to the material gelation, with the corresponding parameters of $Q_{Continuous}$= 1500 µL h^{-1}; $Q_{Dispersed}$= 100 µL h^{-1}; [continuous phase] = 2% , [disperse phase] = 4% (Optical image)

Relatively to the agarose 2 and 3% (w/v), an average size of 145 and 155 µm, respectively was obtained. Both concentrations showed to form stable droplets, for a significant time. Since the size difference between both concentrations used was close, different trials changing the flow rate of the continuous phase were performed, in order to understand how the flow rate would interfere in the bead formation, and clarify the main difference between both agarose concentrations. To achieve this, different continuous phase flow rates ($Q_{Continuous}$) were used (Table 5), fixing the dispersed phase flow rate ($Q_{Dispersed}$) at 100 µL h^{-1}, altering this way the ratio between these two flow rates (Q_C/Q_D). The assays using these two concentrations were repeated five times, in order to acquire reliable average sizes of the beads (see Annex 1), and microscopy images acquired (Figure 7 and 8).

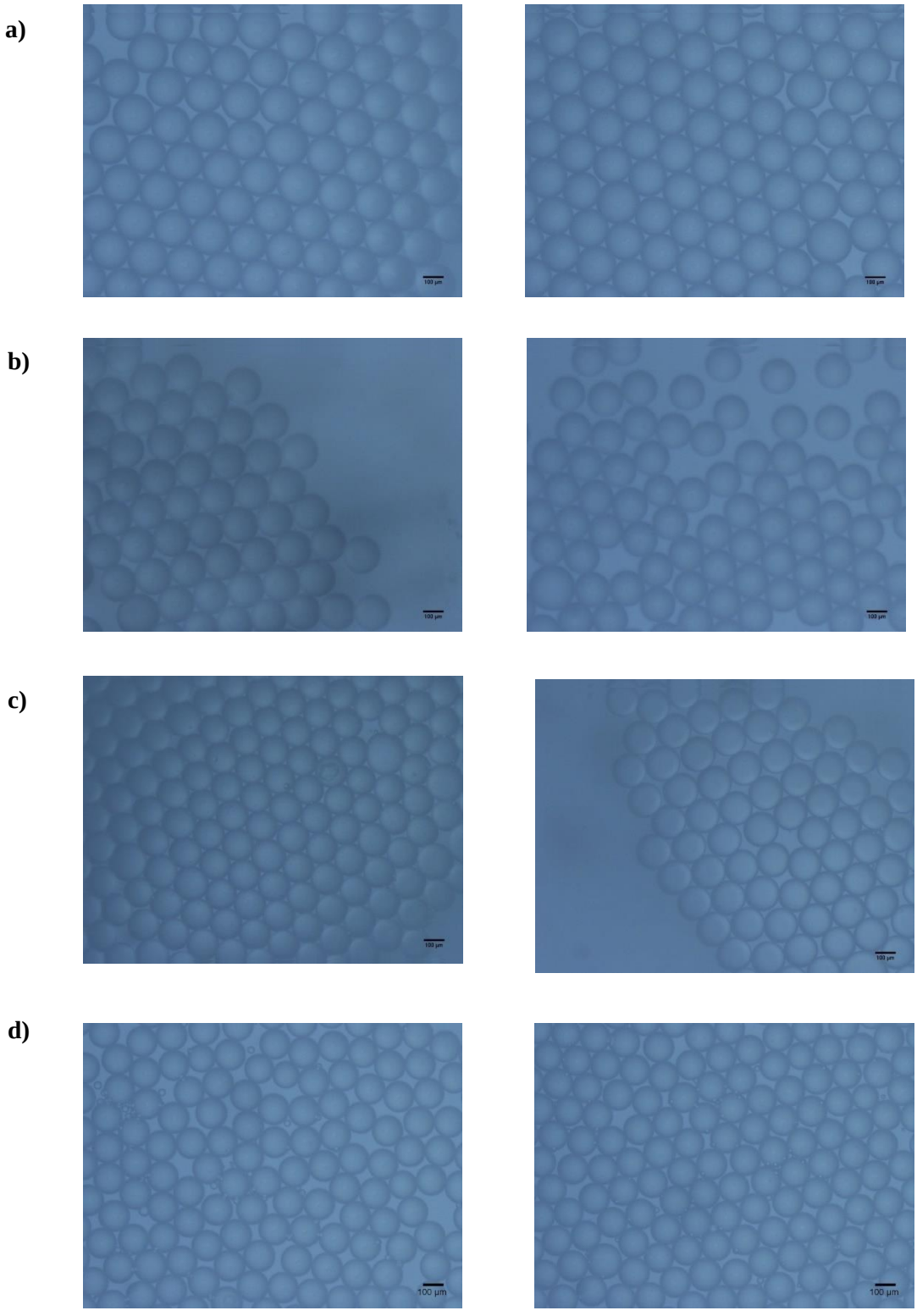


Figure 7 – Obtained agarose 2% (w/v) beads at different flow-rate ratios (Optical image); **a)** $Q_C/Q_D = 5$; **b)** $Q_C/Q_D = 10$; **c)** $Q_C/Q_D = 15$; **d)** $Q_C/Q_D = 20$;

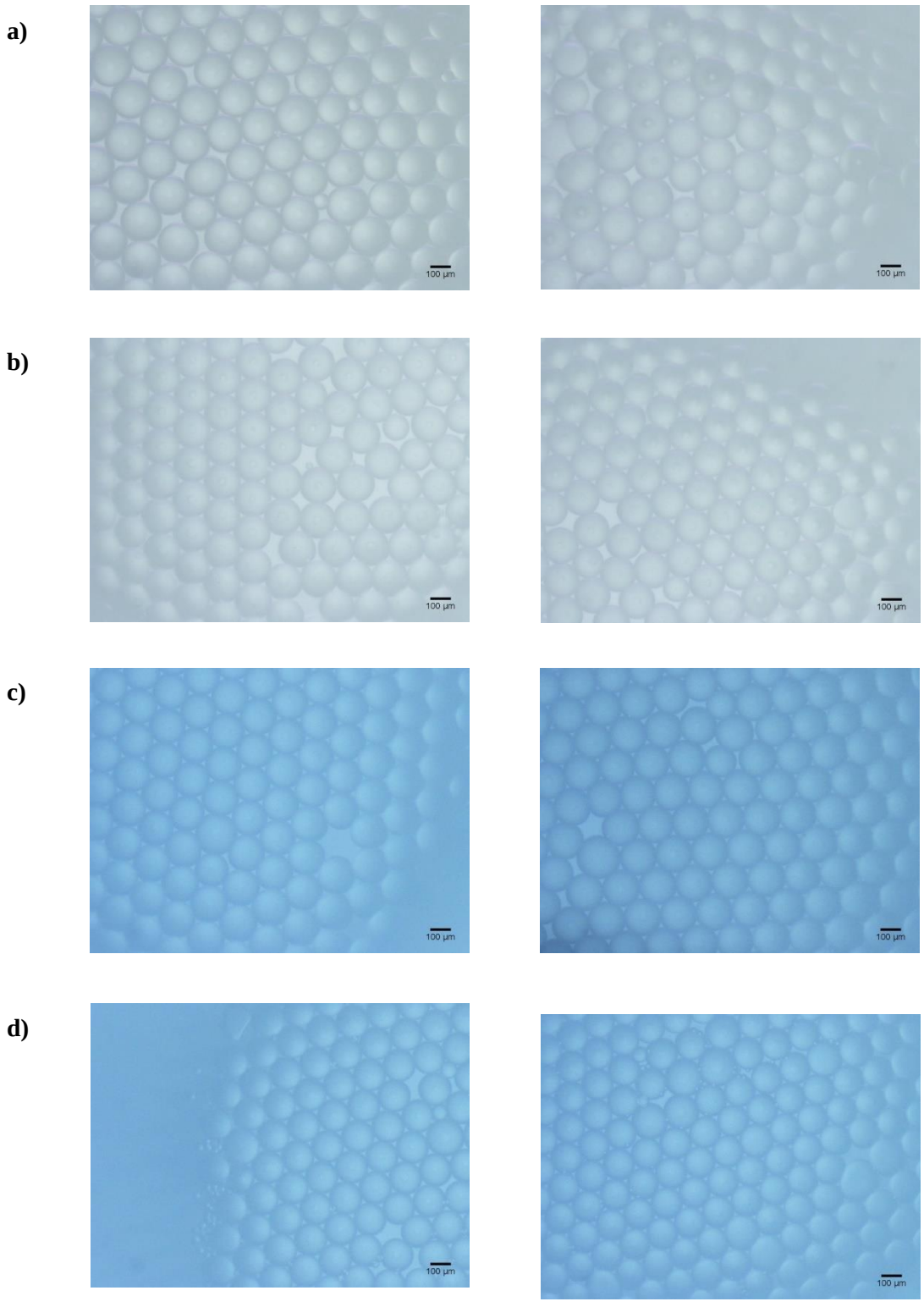


Figure 8 – Obtained agarose 3% (w/v) beads at different flow-rate ratios (Optical image); **a)** $Q_C/Q_D = 5$; **b)** $Q_C/Q_D = 10$; **c)** $Q_C/Q_D = 15$; **d)** $Q_C/Q_D = 20$;

After the bead measurements on ImageJ, it was verified that the ratios (Q_C/Q_D) of 5, 10, 15 and 20 resulted in droplets of 162 ± 3, 145 ± 2, 131 ± 3 and 122 ± 3 µm, for the agarose 2% (w/v), and 183 ± 5, 166 ± 4, 147 ± 6, 130 ± 6 µm for the agarose 3% (w/v), respectively (figure 9).

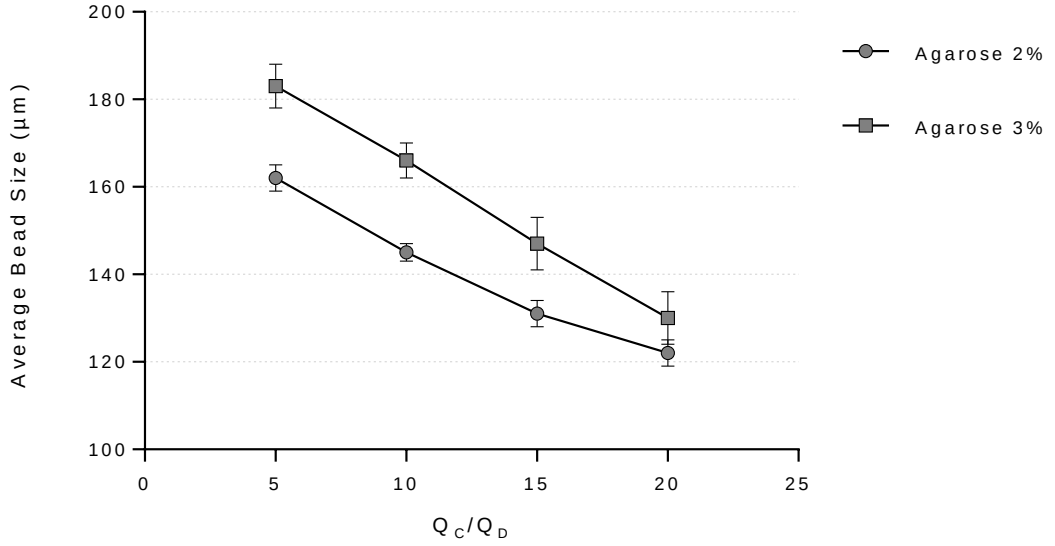


Figure 9 – Representative relation between the ratio of the flow rates (Q_C/Q_D) and the obtained size of the agarose beads of 2 and 3% (GraphPad Prism 7.05)

The difference in the beads size, changing the agarose concentration is not as significant as the increasing of the ratio between the flow rates. The lowest bead size achieved (120 µm) were accomplished at a ratio (Q_C/Q_D) of 20, and an agarose concentration of 2% (w/v). Overall this experiment showed that the flow rate was the most significant parameter in the optimization of the bead generation process.

To understand how the increased agarose concentration behaved with increased flow rate ratios (Q_C/Q_D), the same trial was implemented with the agarose 5% (w/v) (Figure 10, and Annex 1.3).

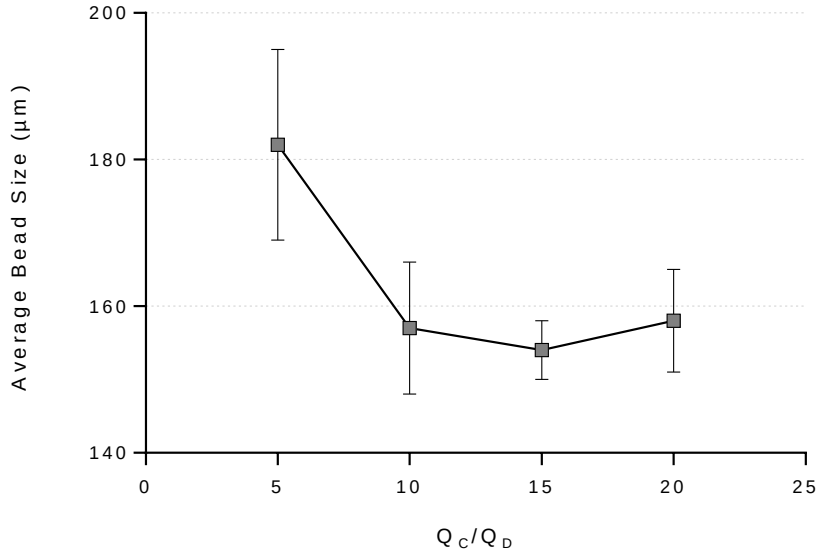
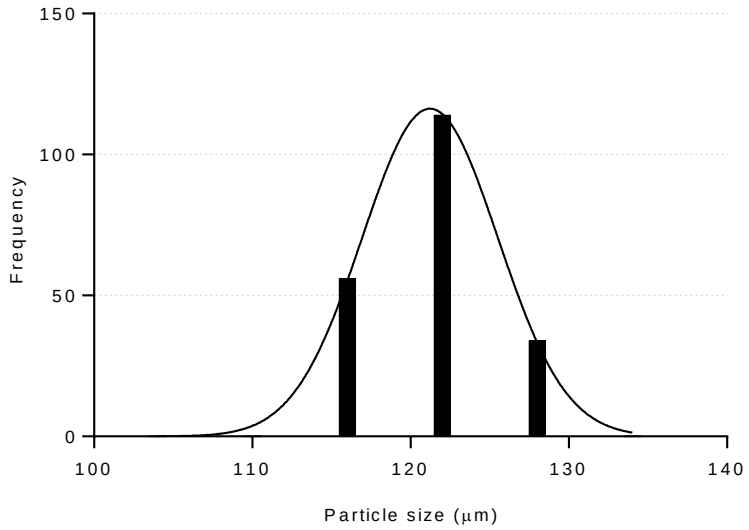


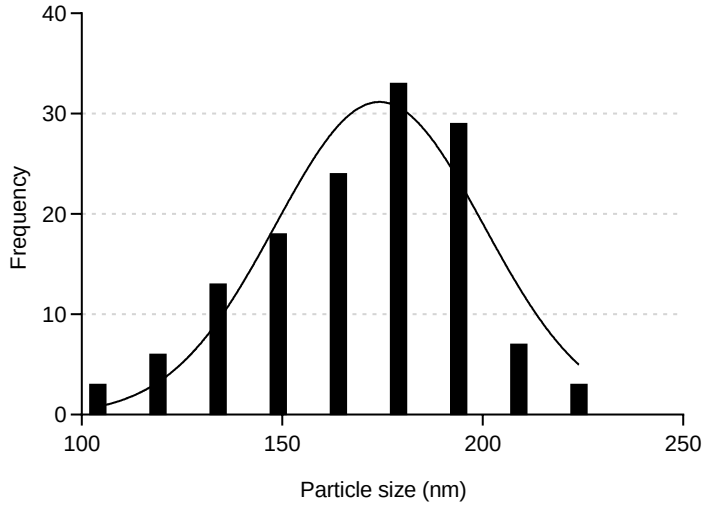
Figure 10 – Representative relation between the ratio of the flow rates (Q_C/Q_D) and the obtained size of the 5% agarose beads (GraphPad Prism 7.05)

In order to evaluate the dispersity of the agarose beads produced, for each one of the three concentrations used, a graphic analysis was made considering a fix Q_C/Q_D value of 20.

a)



b)



c)

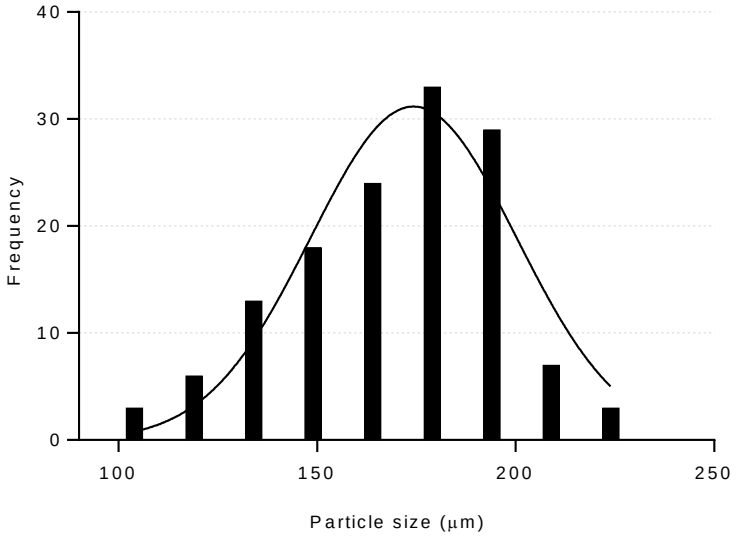


Figure 11 – Size distribution graph, of the obtained size for a Q_C/Q_D value of 20 for agarose concentrations of **a)** 2% (w/v); **b)** 3% (w/v); and **c)** 5% (w/v) (GraphPad Prism 7.05)

It was concluded that the agarose 2% (w/v) beads were monodisperse, since it had a low variation of the bead size, which can be explained by the fact that the gelation occurs later since the concentration is lower, that the others tested, and so the viscosity will to be lower, turning the process more stable, as posteriorly verified. However, in the 3% (w/v) and 5% (w/v), the samples were not as monodisperse, once there are a significant variation of the droplet size, being even more evident in the 5% (w/v) agarose measurements.

Besides, when using 5% (w/v) agarose, it was also observed that increasing the flow rate ratio didn't have any additional effect in the diminution of the beads size, since the agarose stream was too dense, and thus the continuous phase stream didn't have enough power to cross the dispersed phase stream.

The volume of each bead in the 120 µm droplets was calculated, obtaining the value of 9.05×10^{-4} µL. Applying a flow rate of the dispersed phase of 100 µL h^{-1}, there were generated approximately 1841 beads per minute (31 beads/s). Overall, the use of this microfluidic methodology for generation of droplets reflected to be very effective, resulting in a high quantity of agarose beads produced per second.

After these optimizations, an assay to only evaluate the production time was completed using the following optimized conditions: $Q_{Continuous}$= 2000 µL h^{-1}; $Q_{Disperesed}$= 100 µL h^{-1}; agarose concentration of 2% (w/v) (dispersed phase); and a surfactant concentration in oil of 2% (v/v) (continuos phase).

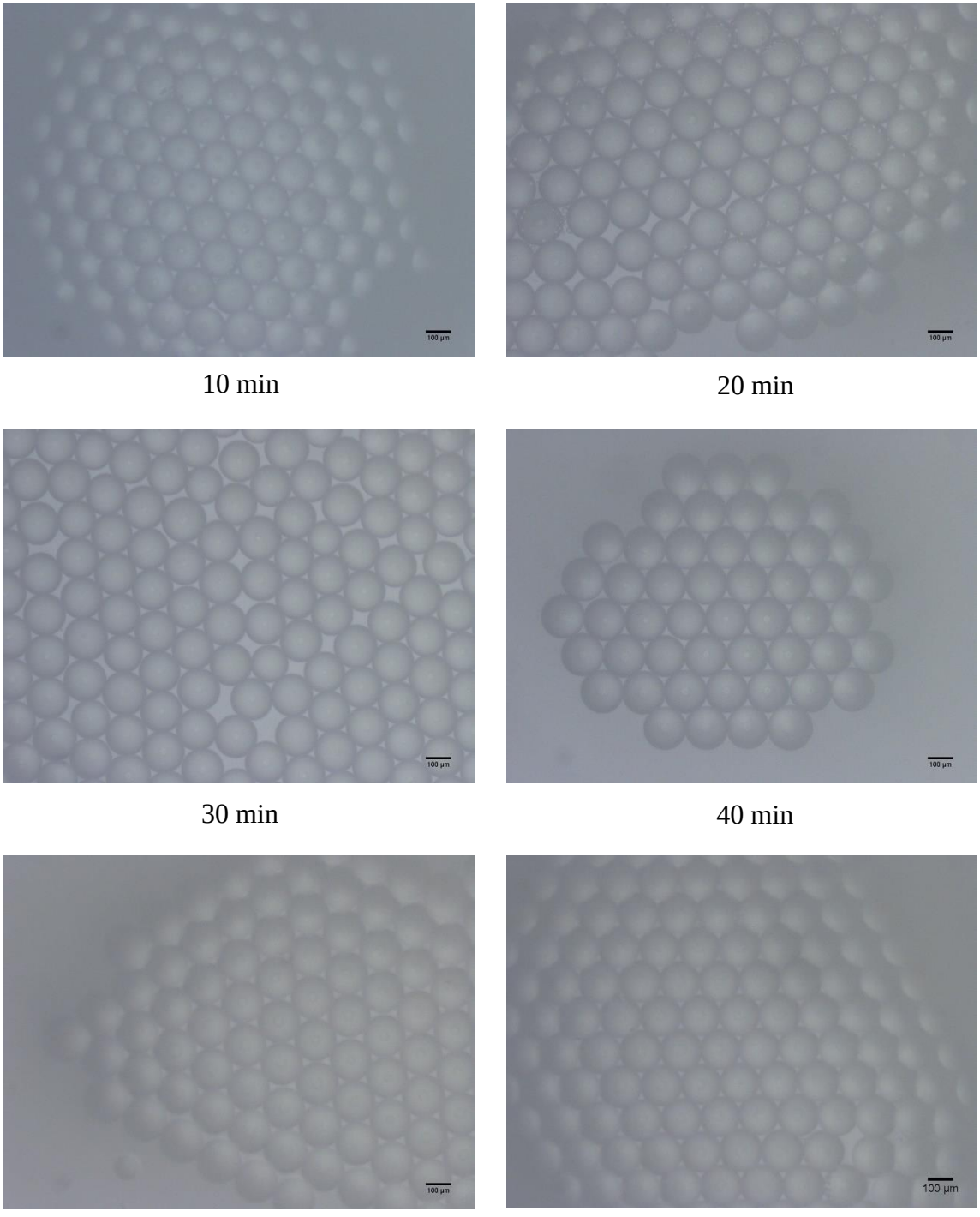


Figure 12 – Process stability, with the corresponding parameters of $Q_{Continuous}$= 2000 µL h^{-1}; $Q_{Dispersed}$= 100 µL h^{-1}; [continuous phase] = 2%, [disperse phase] = 2% (Optical image)

As shown in figure 12, the process was only stable for about one hour, occurring bead degradation after this time (figure 6). In order to achieve a higher stable production time,

the surfactant concentration was increased from 2 to 2.5% in oil, keeping the remaining parameters used previously.

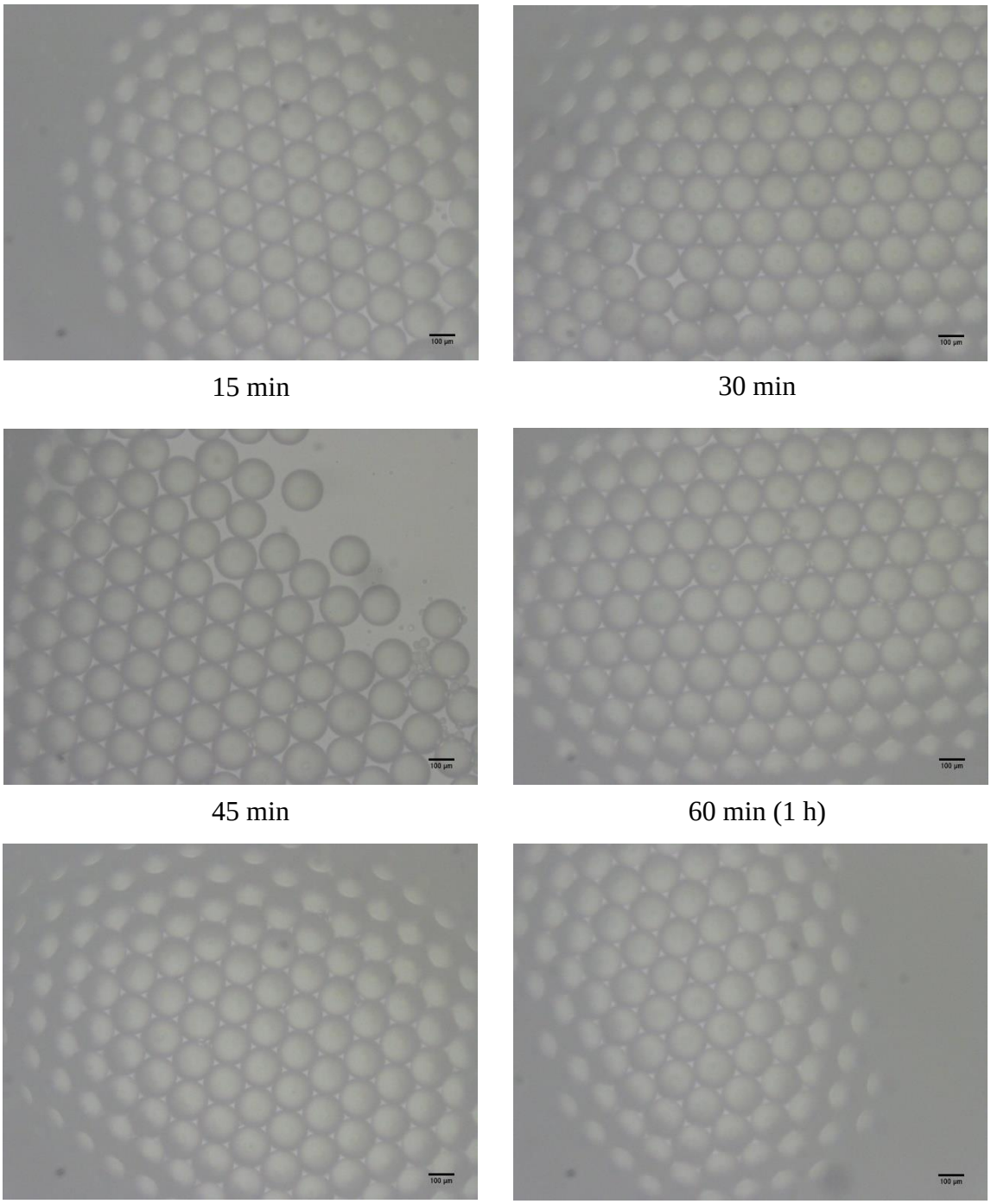


Figure 13 – Process stability, with the corresponding parameters of $Q_{Continuous}$= 2000 µL h^{-1}; $Q_{Disperesed}$= 100 µL h^{-1}; [continuous phase] = 2.5%, [disperse phase] = 2% (Optical image)

Through the analysis of figure 13, it was verified that increasing the surfactant concentration, an increase in time will be achieved, in which the process is stable. Another way, that might increase the duration of the process, would be finding a strategy in which the agarose could be maintained warm for longer periods of time, since when it cools down, a deformation starts to occur due to the gelation of the agarose, during the droplet fabrication process.

In other attempt to decrease the size of the agarose beads, a "split" technique was tested, using the previously optimized conditions. For that, a Y-junction device (Figure 1) was placed in series with the flow focusing one, which in theory should divide the droplets to half in the device junction. The bead formation process occurred the same way as described above, yet instead of collecting the sample after their formation, the tubing was connected to the other device "outlet", blocking its middle "inlet", obtaining in this way a two device system in series (Figure 14).

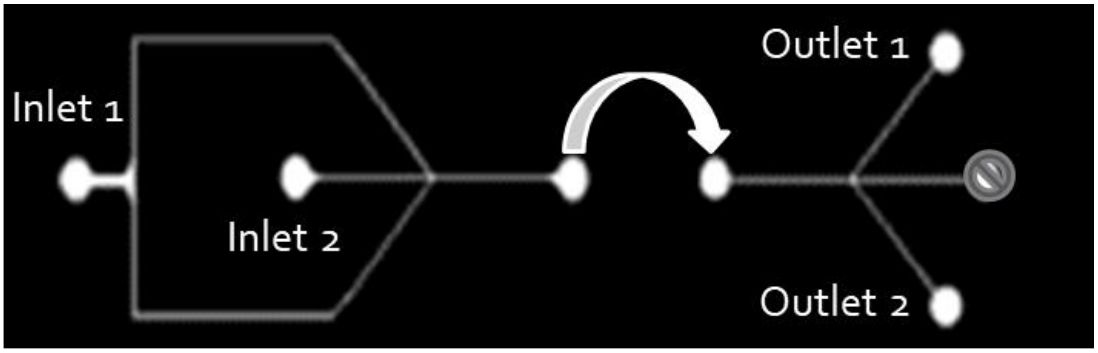


Figure 14 – Scheme of the split technique; Inlet 1: continuous phase input; Inlet 2: dispersed phase input; Outlet 1 and 2: dispersed phase beads output

As visualized in figure 15, a decrease in the bead size was achieved. However, the droplets become more polydispersed, which in practical terms, excluded this methodology.

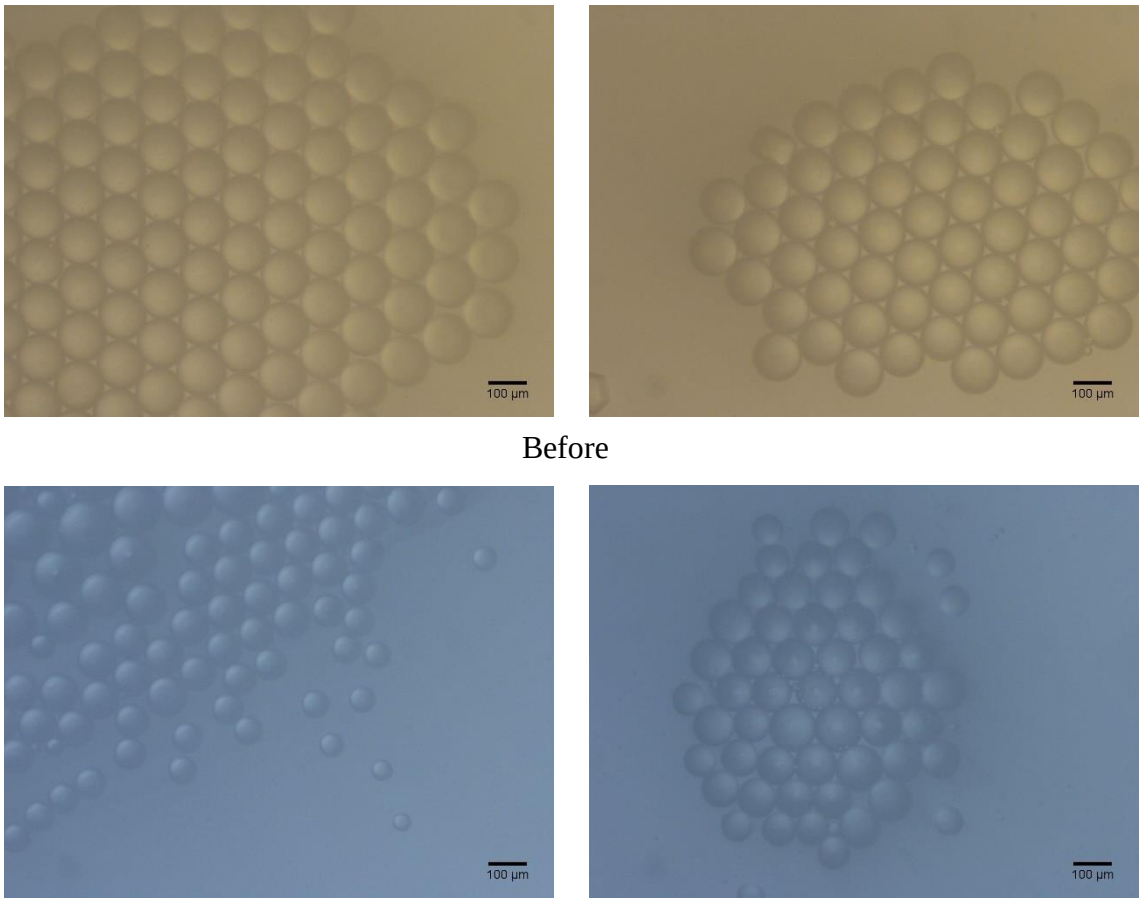


Figure 15 – Obtained agarose 2% beads, before and after "split" technique (Optical image)

In conclusion the optimized conditions for a stable bead formation, with the smallest size reachable (≈120 µm), were setting the flow rates of the continuous phase (surfactant in oil) and dispersed phase (agarose), at 2000 and 100 µL h^{-1}, respectively, and their correspondent concentrations (surfactant and agarose) at 2.5 and 2%.

3.4. Riboflavin encapsulation – *Off-Chip Mixing*

Riboflavin (Vitamin B2) was used to verify whether the addition of one more agent would affect the process stability and the beads monodispersity. The concentration of riboflavin used was 0.065 mg/mL, since it was previously reported that this concentration allowed an efficient encapsulation in bio-based structures (Azevedo, Cerqueira and Vicente, 2013).

For this analysis, the method described in the droplet generation optimization (3.3) and the two inlet flow focusing device (Figure 2) were used. However, since an extra agent would be encapsulated, this encapsulation technique was denominated “Off-Chip Mixing”. This technique consists in the preparation of the mixture of the dispersed phase, outside the device, which in this study will be riboflavin (0.065 mg/mL) and agarose (2% w/v), proceeding thereafter to the loaded-bead generation, using the same method described previously (3.3).

As seen in figure 16, adding an extra agent did not affect the bead generation process, and the beads remained monodispersed, and the process was stable for 1 hour and 30 minutes.

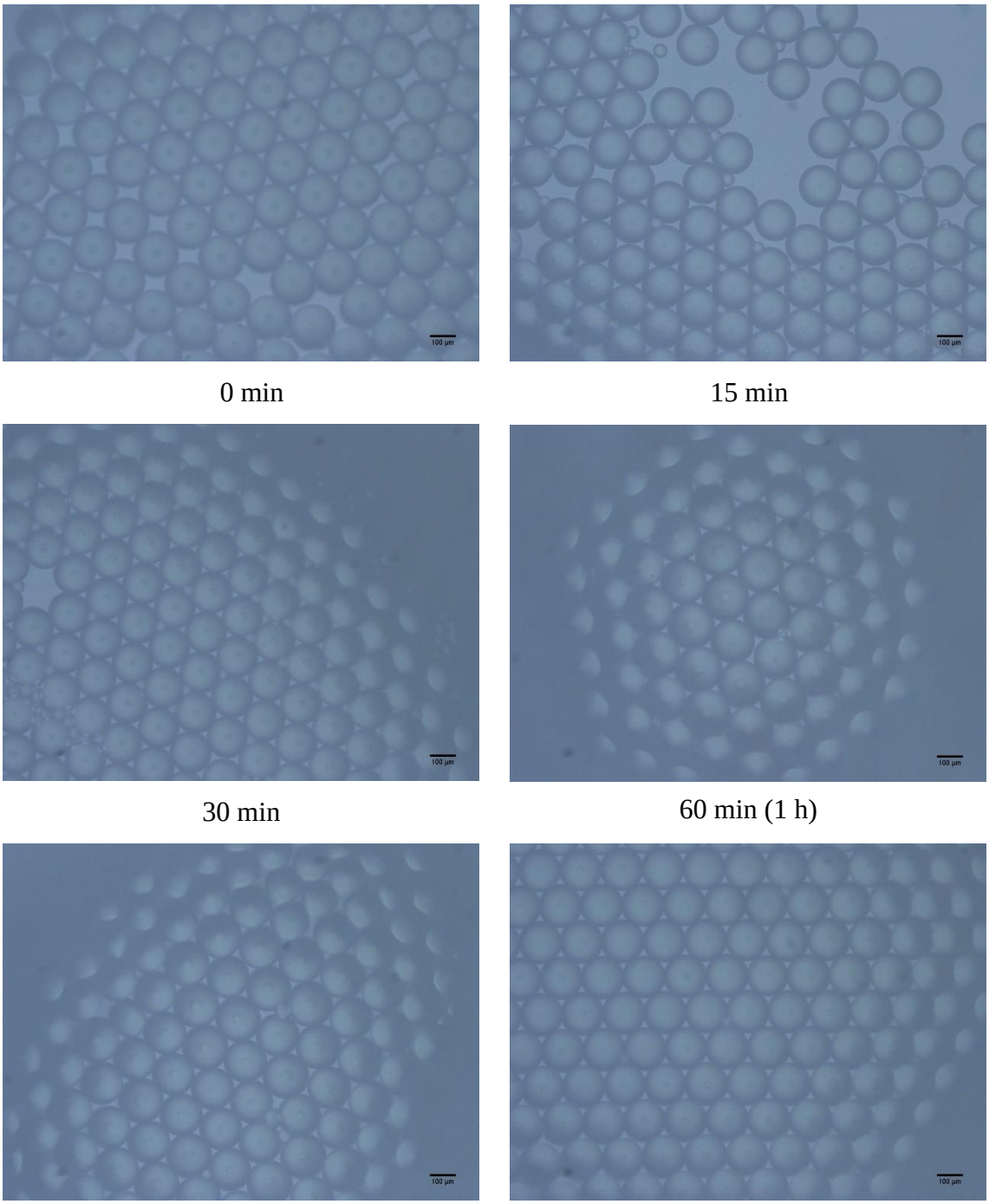


Figure 16 – Riboflavin encapsulation - Off-Chip Mixing - Process stability (Experimental parameters: $Q_{Continuous}$= 2000 µL/h; $Q_{Disperesed}$= 100 µL/h; [continuous phase] = 2.5%, [disperse phase] = 2% (Optical image)

3.5. Fluorescent particles encapsulation - *On-Chip Mixing*

After verifying that adding an external agent did not affect the process stability, an experiment using fluorescent nanoparticles of 50 nm and 200 nm (Fluoresbrite® Yellow Green Microsphere, Polysciences, Inc.), was made in order to simulate and evaluate the phage distribution inside the agarose bead. The sizes of the fluorescent particles were chosen to replicate the dimension of the bacteriophage PVP-SE2 capsid (57 nm), and the full size of the bacteriophage (57 nm capsid + 125 nm tail) (Sillankorva *et al.*, 2010).

In order to achieve a more efficient bead formation, and consequent encapsulation, a different geometry device, flow focusing with 3 disperse phase inlets (Figure 1), was used. This device allows the substitution of the dispersed phase, encapsulation material or encapsulated agent, during the process, without the need for preparation of a premix, as seen on the "Off-Chip Mixing" method, used previously.

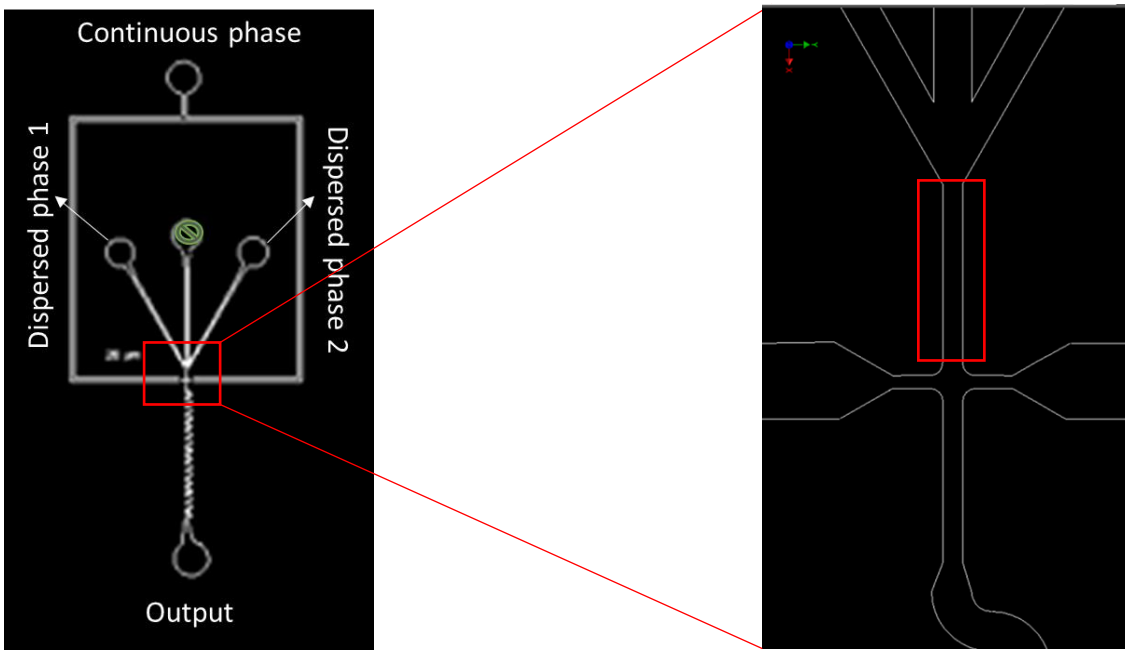


Figure 17 – Scheme of the microfluidic device used for On-Chip Mixing, and zoomed image of the junction and the portion of the channel where the laminar flow occurs

In this method, the mixing occurs inside the device. So, instead of one dispersed phase there are two, which in this case, are the agarose and the fluorescent nanoparticles phases. This methodology has one particularity, which is the fact that the concentration of the dispersed phase decreases to half during the bead formation, due to the joining of the streams of the dispersed phases before the junction (see Figure 17, zoomed image), before

the junction the stream is divided in two equal parts, half of agarose and half fluorescent nanoparticle suspension.

The bead formation occurred as mentioned previously, as the streams of the continuous and dispersed phase, meet at the device junction. However, the beads formed are not homogenous, being composed by half of each of the dispersed phase. The homogenization of the bead only occurred after its formation, due to the chaotic advection, caused by an "S" shape channel, between the junction and the outlet, which was responsible for promoting the mixing of the two components inside the bead.

Taking into account that the ideal concentration for phage encapsulation is reported to be of 1×10^9 PFU mL^{-1} (Malik *et al.*, 2017), a similar concentration was used for the encapsulation of the fluorescent nanoparticles (4.64×10^9 particles mL^{-1} and 2×10^9 particles mL^{-1}, for 50 nm and 200 nm particles, respectively). It should to be noted that, the concentration will drop to half and that the same applies to the agarose solution that needs to be added at 4% so that the final concentration after the two flows join is 2% (w/v).

The experimental conditions were: continuous phase flow rate (surfactant in oil) = 2000 µL h^{-1}; dispersed phase flow rates (for both agarose and fluorescent nanoparticles) = 100 µL h^{-1}; surfactant concentration (continuous phase) = 2.5% (v/v); agarose concentration (disperse phase 1) = 4% (w/v); 200 nm fluorescent nanoparticles concentration (disperse phase 2) = 2×10^9 particles mL^{-1}. The same conditions were applied to the 50 nm fluorescent nanoparticles encapsulation (4.64×10^9 particles mL^{-1}).

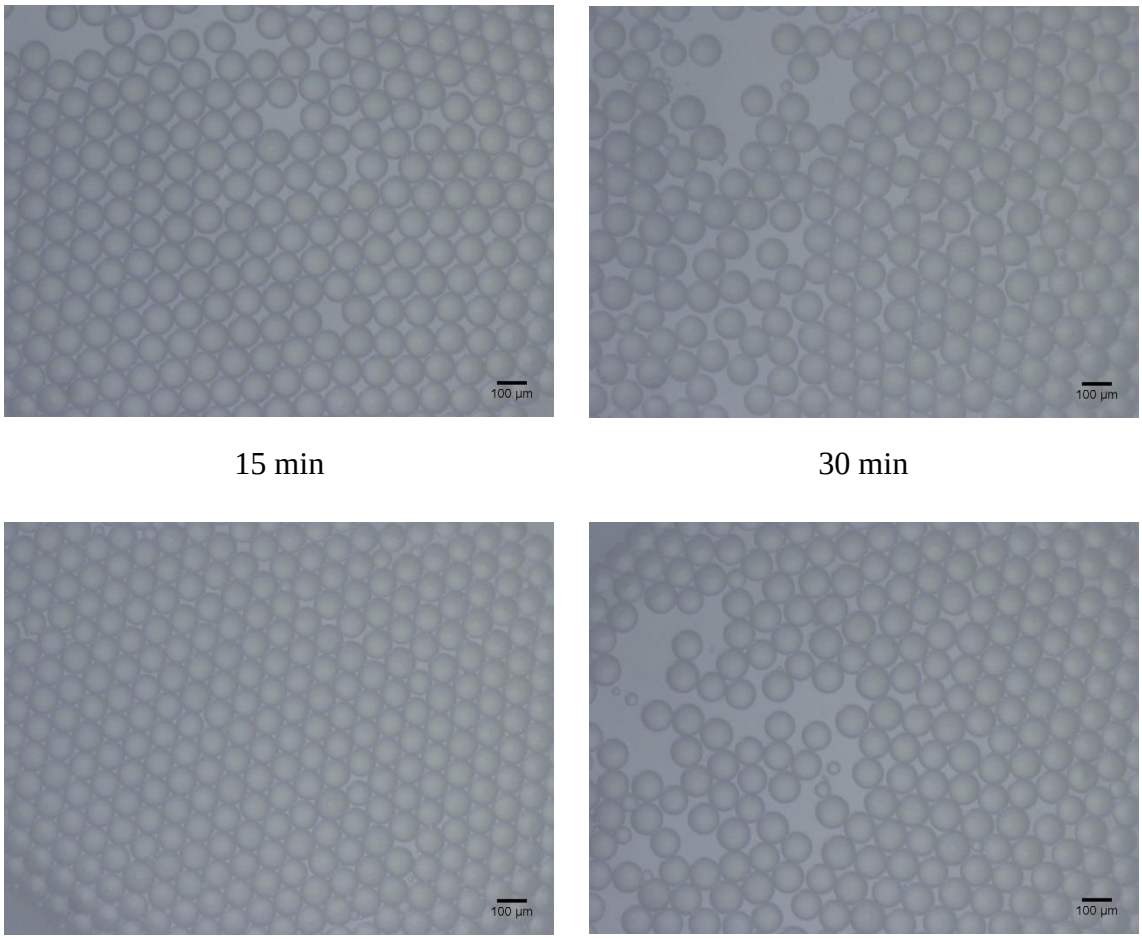


Figure 18 – 200 nm fluorescent nanoparticles encapsulation - On-Chip Mixing - Process stability (Optical image); Experimental parameters: $Q_{Continuous}$= 2000 µL h^{-1}; $Q_{Disperesed}$= 100 µL h^{-1}; [continuous phase] = 2.5%, [disperse phase 1] = 4%, [disperse phase 2] = 2×10^9 particles mL^{-1})

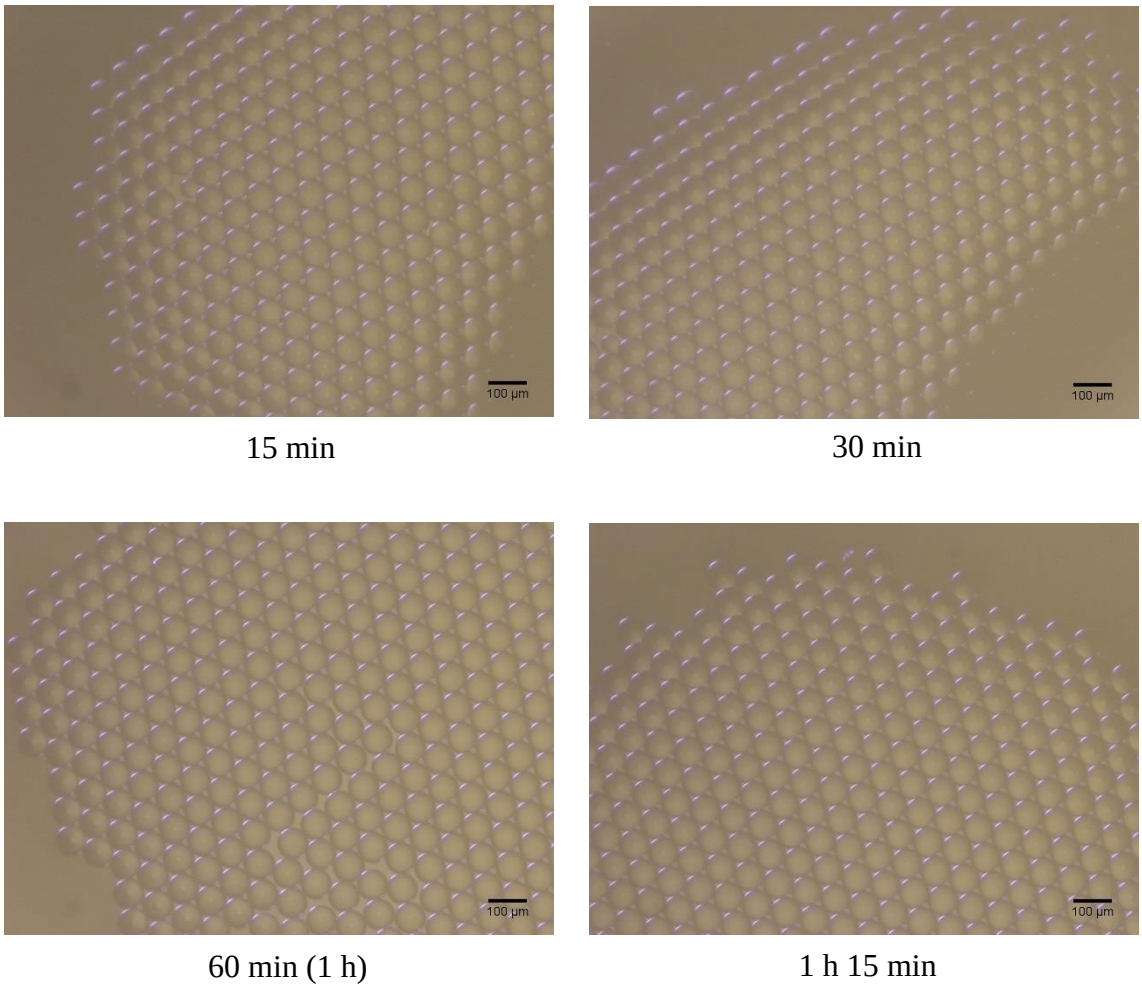


Figure 19 – 50 nm fluorescent nanoparticles encapsulation - On-Chip Mixing - Process stability (Optical image); Experimental parameters: $Q_{Continuous}$= 2000 µL h^{-1}; $Q_{Dispersed}$= 100 µL h^{-1}; [continuous phase] = 2.5%, [disperse phase 1] = 4%, [disperse phase 2] = 4.64 × 10^9 particles mL^{-1}

Through the analysis of the obtained images, it was verified that the process was still stable for a significant period of time (about 1 hour and 15 minutes), considering it was used an ultra-low gelling temperature agarose, and the beads were still monodisperse. Still, a main difference from the previous method was evident. The size of the beads was significantly smaller, reaching an average size of 85 ± 3 µm, measured in ImageJ. "On-Chip Mixing" appears to be more efficient for bead generation and encapsulation, than initially expected.

In order to understand why this decreased of the bead size occurred, a "Off-Chip Mixing" trial was done with same device, blocking the dispersed phase inlets, and

opening the middle inlet that was previously blocked (see figure 17), for both 50 and 200 nm fluorescent particles formation.

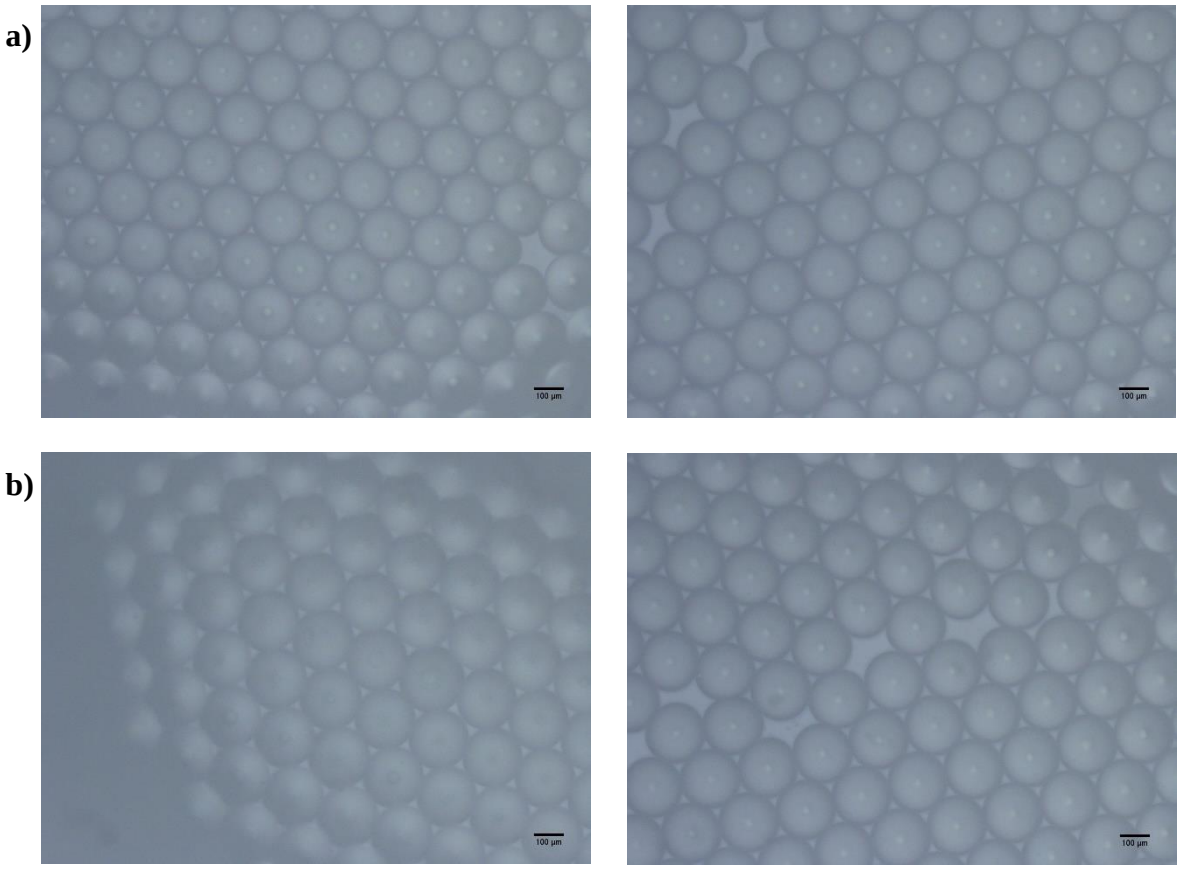


Figure 20 – Off-Chip Mixing - **a)** 50 nm fluorescent nanoparticles encapsulation, **b)** 200 nm fluorescent nanoparticles encapsulation (Optical image)

With the "Off-Chip Mixing", an average size of about 180 µm was obtained. So comparing this with the previous data, it can be settled that the used device had influence on the beads size. However, it was not clear why there was a decrease of the size of the beads, which possibly may be explained by the use of an agarose concentration of 4% (w/v) in the "On-Chip Mixing". This higher concentration of agarose will eventually starts to gel outside the device, however the concentration will drop to half once it gets in contact with the fluorescent particles, making it more fluid at the time of the bead generation.

Overall, it can also be concluded that the "Off-Chip Mixing" is more efficient in the flow focusing device with 2 inlets and one outlet (Figure 2), and that the "On-Chip

Mixing" is more efficient on the device with two inlets for the disperse phase (Figure 14). The best average droplet size (85 ± 3 µm) was obtained by the "On-Chip Mixing" on a flow focusing device. Taking this data into account, the "On-Chip Mixing" was selected as the method for bacteriophage encapsulation.

To understand how was the distribution of fluorescent particles encapsulated per bead, a Poisson distribution was made, considering a droplet size of 85 µm, and a final concentration of 2×10^9 particles mL^{-1} of the 200 nm fluorescent nanoparticles, through the following equation.

Eq. 3

$$f(X, \lambda) = \frac{e^{-\lambda}\,\lambda^{k}}{k!}$$

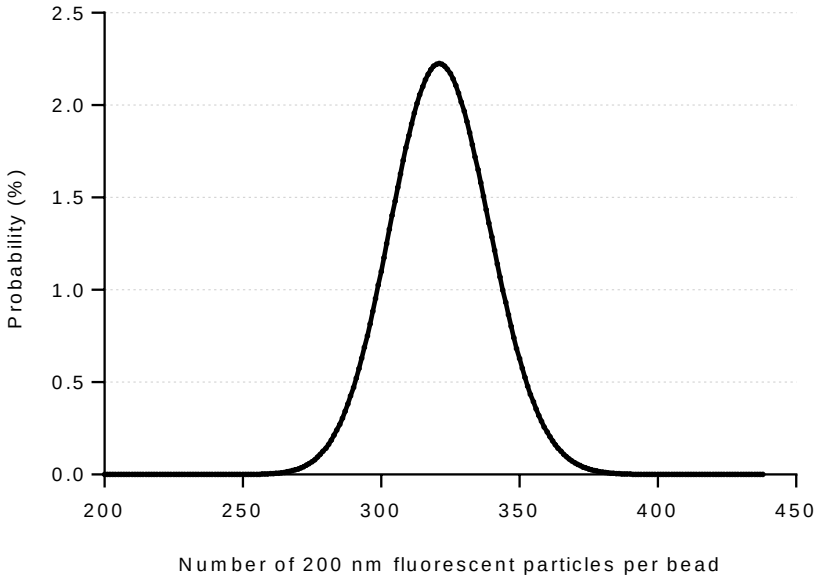
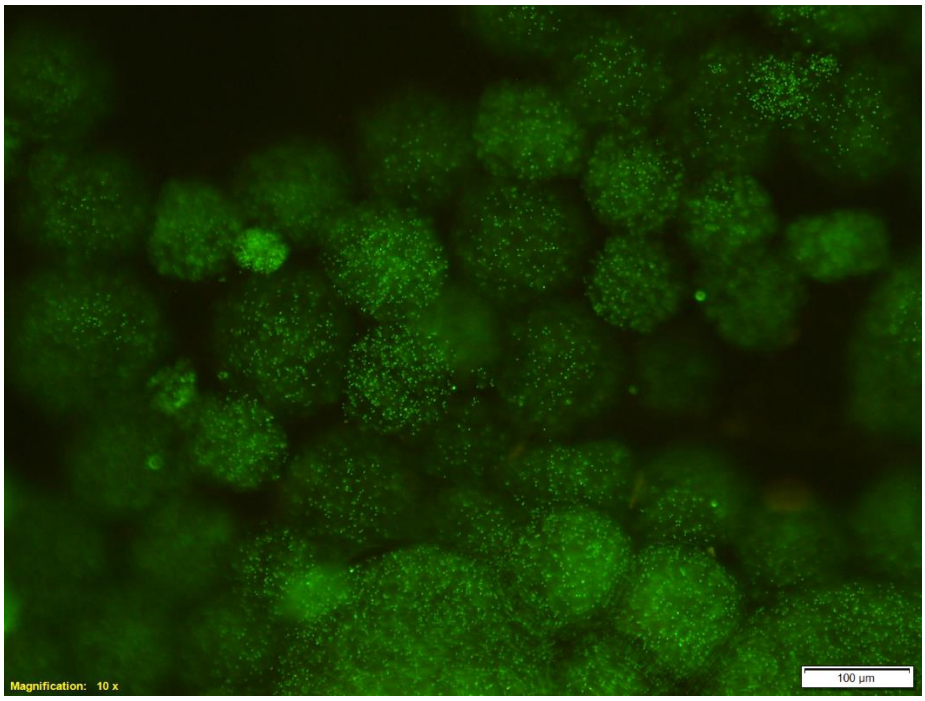


Figure 21 – Poisson distribution analysis of the number of fluorescent particles per bead (GraphPad Prism 7.05)

After proceeding to the graph analysis, it was estimated that about 2% of the total agarose bead population was loaded with 331 fluorescent particles per bead, and the probability of having non-loaded beads is zero, so it has not be considered, which demonstrates the efficiency of the method used.

After estimating the encapsulation efficiency per bead, an analysis using a fluorescence microscope (Olympus BX51) was performed, in order to observe the particle distribution inside the bead.

a)



b)

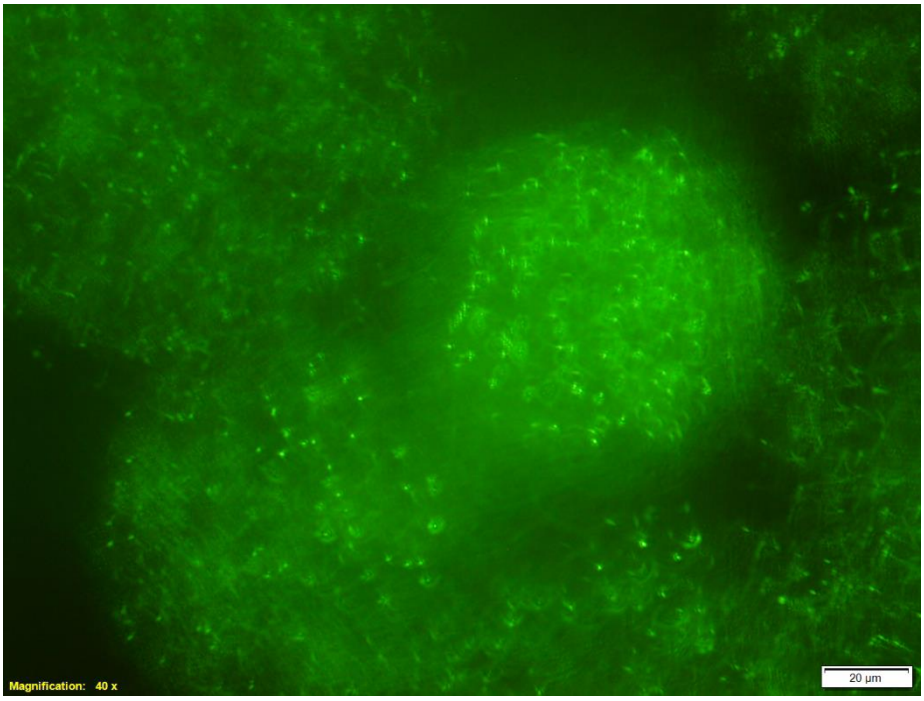
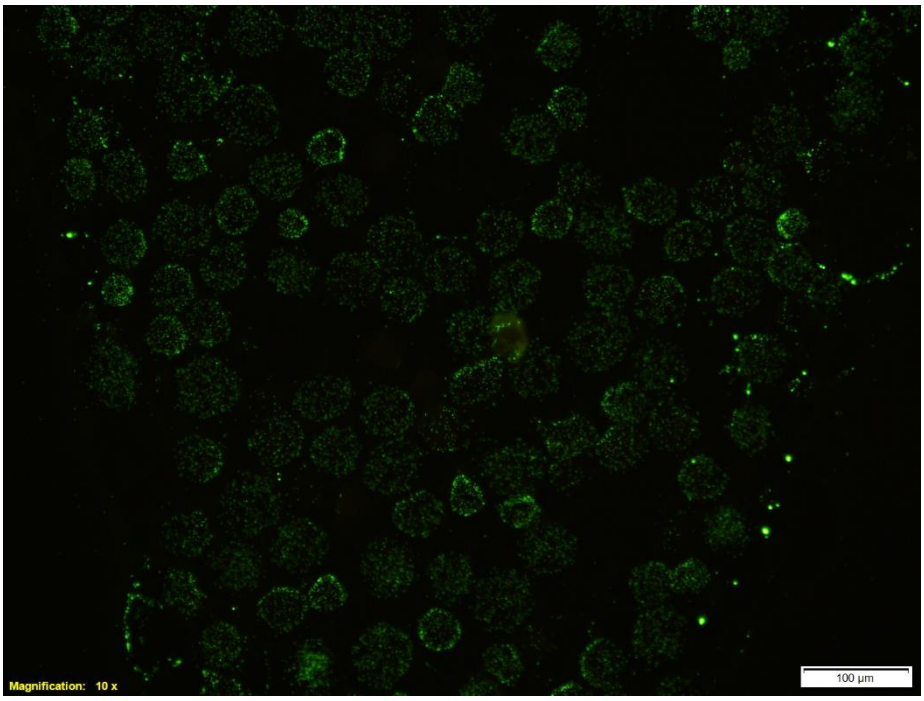


Figure 22 – Off-Chip Mixing – Encapsulated 200 nm fluorescent nanoparticles **a)** 10× Objective; **b)** 40× Objective (Fluorescence optical image)

a)



b)

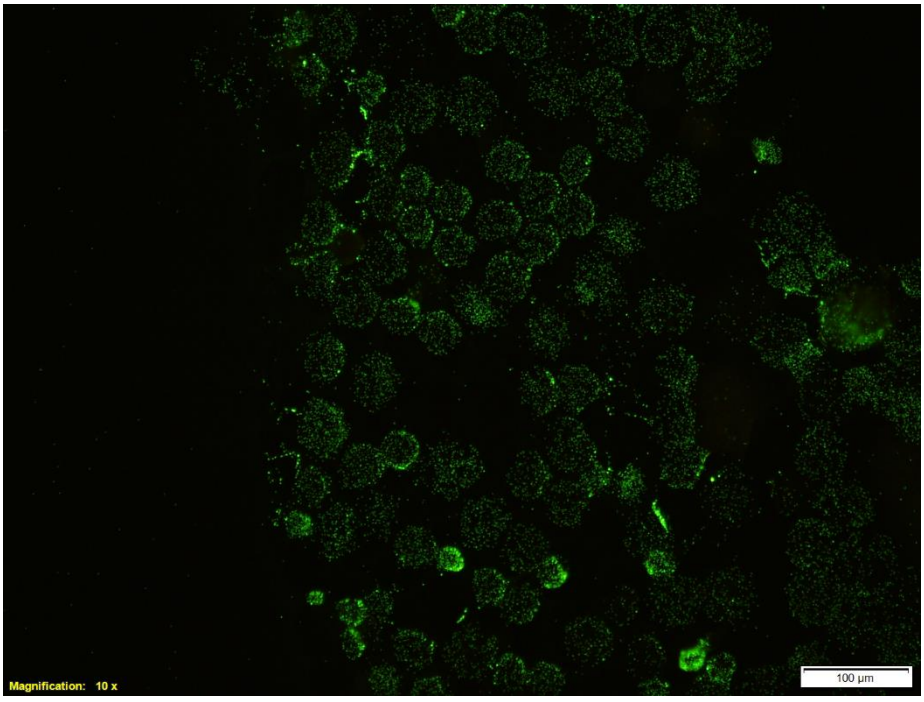


Figure 23 – On-Chip Mixing – Encapsulated 200 nm fluorescent nanoparticles, bead degradation in the glass slide after **a)** 2 minutes and **b)** 5 minutes (Fluorescence optical image)

It can be concluded that the encapsulated distribution inside the bead seems to be homogenous, which in practical terms, will be useful for liberation of the loaded material.

3.6. Evaluation of the material thermal behaviour

Since the used surfactant (Pico-Surf$^{(TM)}$ 1) and oil (HFE-7500) are not food-grade there was a need to transfer the beads from the oil to a water phase. For that purpose 1H,1H,2H,2H-Perfluoro-1-Octanol, was used. Since it is a "bad surfactant" it will destabilize the droplets in the oil phase, making them immigrate to the water phase safely.

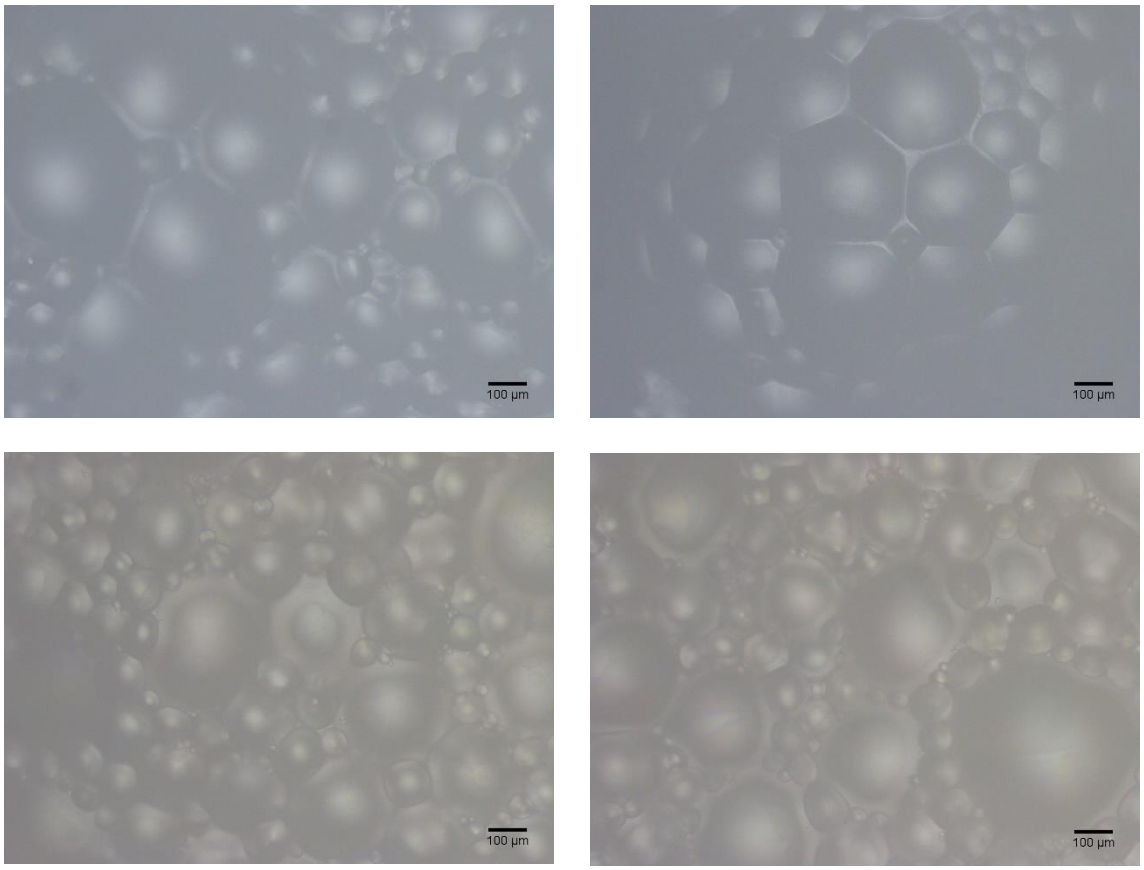


Figure 24 – Oil to water phase (Optical image) - Bead degradation, **a)** assay performed at room temperature; **b)** assay performed on ice, without pre-cooling 1H,1H,2H,2H-Perfluoro-1-Octanol, and no surfactant 2.5% (v/v) added

Even using this chemical, as can be observed on figure 24, the agarose beads collapsed and deformed. Therefore, a new methodology was tested to transfer the beads safety to a water phase. This methodology consisted in adding 100 µL of beads and the same volume of surfactant 2.5% (v/v) in oil, on a 2 mL tube, and in parallel prepare a solution, on ice, of 1H,1H,2H,2H-Perfluoro-1-Octanol (150 µL + 850 µL MilliQ water). It was of extreme importance to maintain this mixture cold during the entire process, if no degradation will occur (figure 24). The mixture was added slowly to the 2 mL tube with sample, 1:1 v/v,

stirring gently, and finally the tube was put to rest on ice for 5 minutes till the two phases had separated.

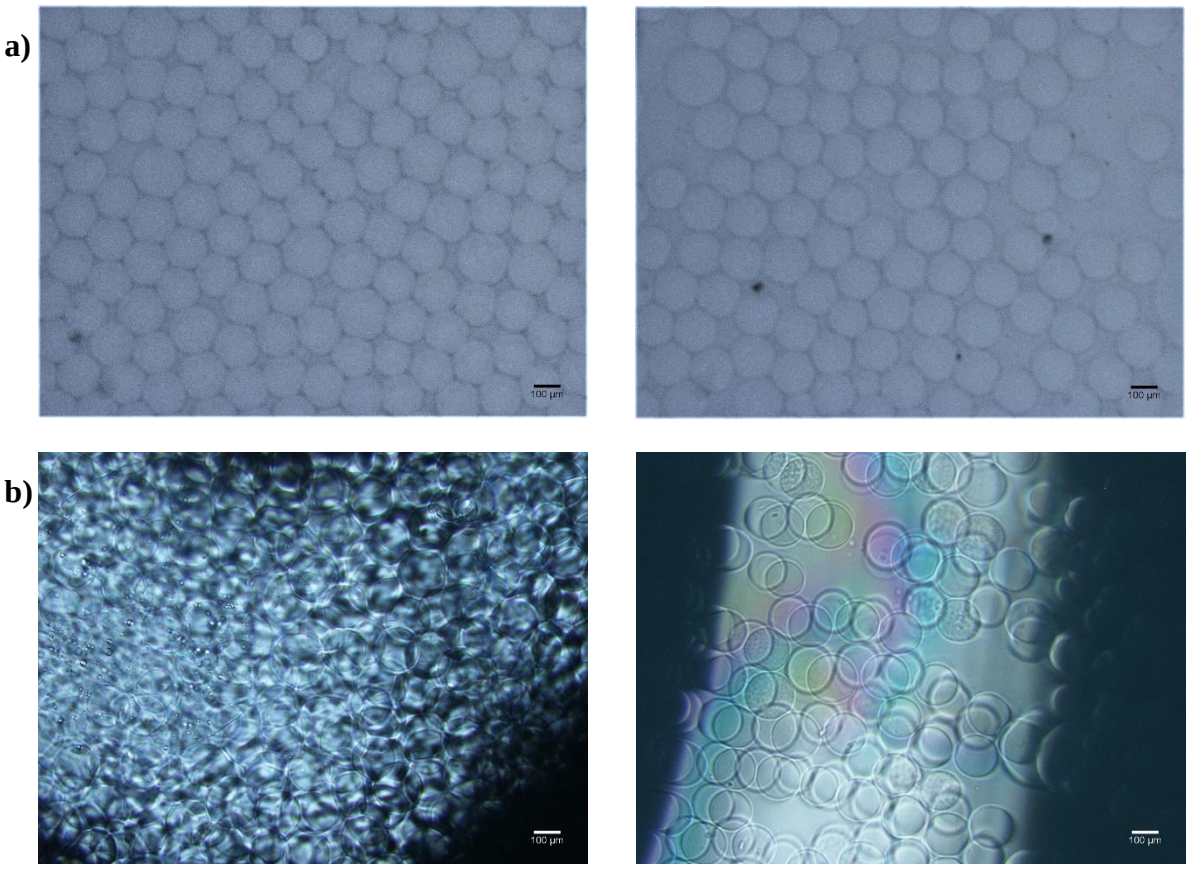


Figure 25 – Oil to water phase (Optical image); **a)** Increased contrast on ImageJ 0.25% **b)** Dark field mode

When transferring the beads to the water phase, the obtained image contrast of agarose to water was worse than agarose to oil, not being possible to draw conclusions. Therefore, images were acquired using the dark field mode (figure 25), obtaining a more visible result, which confirmed that the beads kept their structure after the transfer, and did not collapse.

To ass whether the agarose beads in water could be stored as a powder, the bead suspension was subjected to lyophilisation (LyoQuest, Telstar).

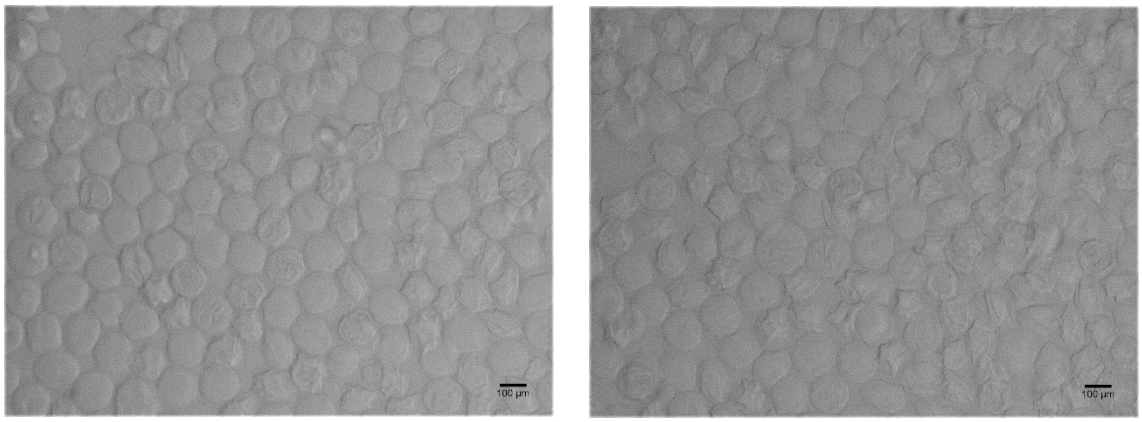


Figure 26 – Rehydration of the lyophilised agarose beads (Optical image)

After dehydration, it was noticed that the beads could be rehydrated several times, and in about 5 minutes, after rehydration, they had recovered their original geometry, making this a more practical form for their storage.

After, a trial to measure the hydrated (no lyophilised) and lyophilised beads shelf-life was made in order to understand how long they lasted at different temperatures, to better understand at which conditions they could be stored. Two different conditions were tested: 4 ºC, and room temperature of 19.5 ºC ± 3.5 ºC, which corresponded to the temperature variation in the laboratory, during the trial.

The lyophilized and hydrated beads lasted at least one month at 4 ºC, being samples taken every few a few days. On the other side, at room temperature, the lyophilized lasted approximately 72 hours, while the hydrated droplets lasted less than 24 hours. So, in terms of commercial application it is definitely better to use the lyophilized form for storage, and then rehydrate them before use.

Differential scanning calorimeter (DSC) analysis was performed to understand the thermal behaviour of the material, once it measures the heat capacity of it.

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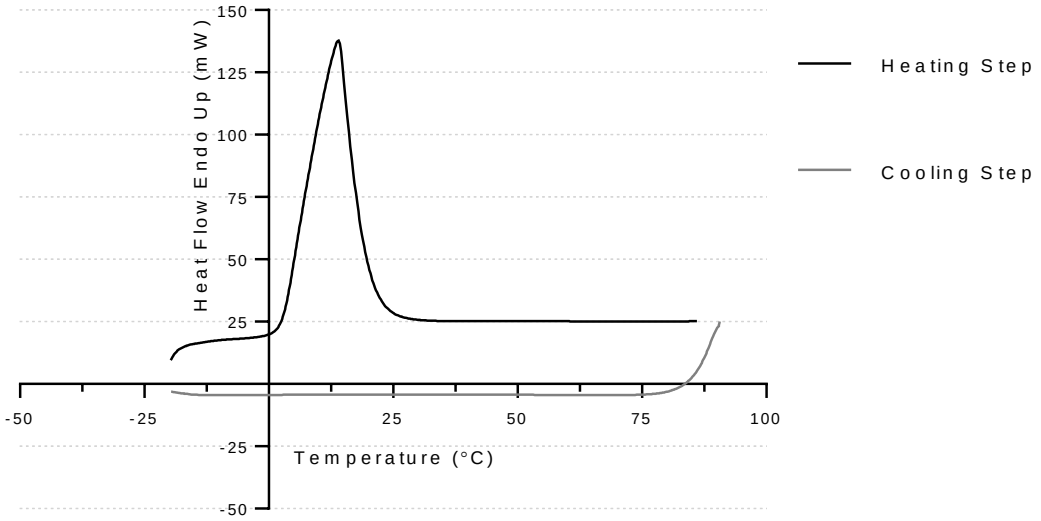

Figure 27 – Agarose 2%, DSC analysis in triplicate: Endothermic peak = 14 ºC; ΔH= 345 J g^{-1} (GraphPad Prism 7.05)

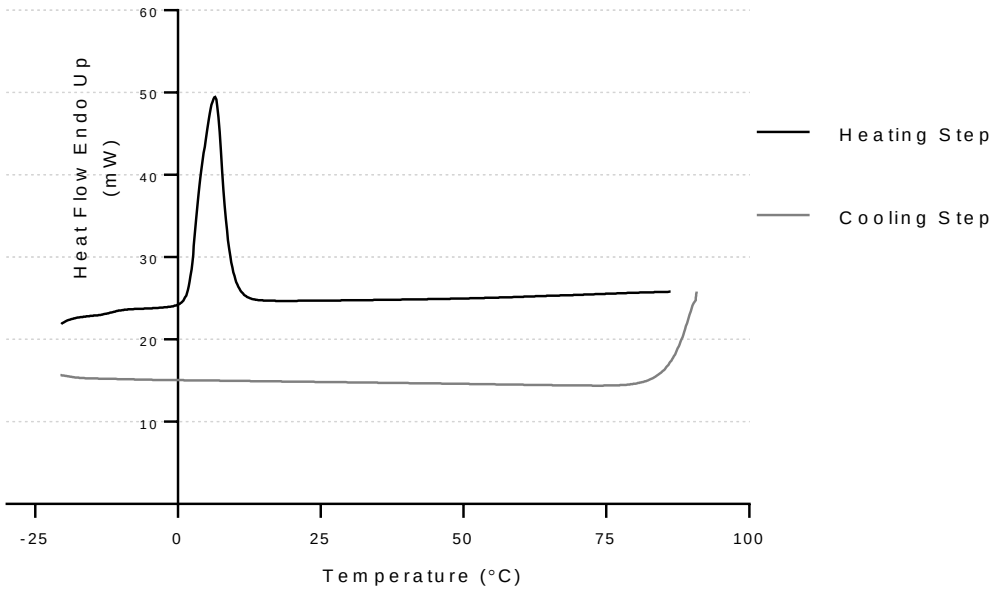

Figure 28 – Agarose 2% beads, DSC analysis in duplicate: Endothermic peak = 6 ºC; ΔH= 32 J g^{-1} (GraphPad Prism 7.05)

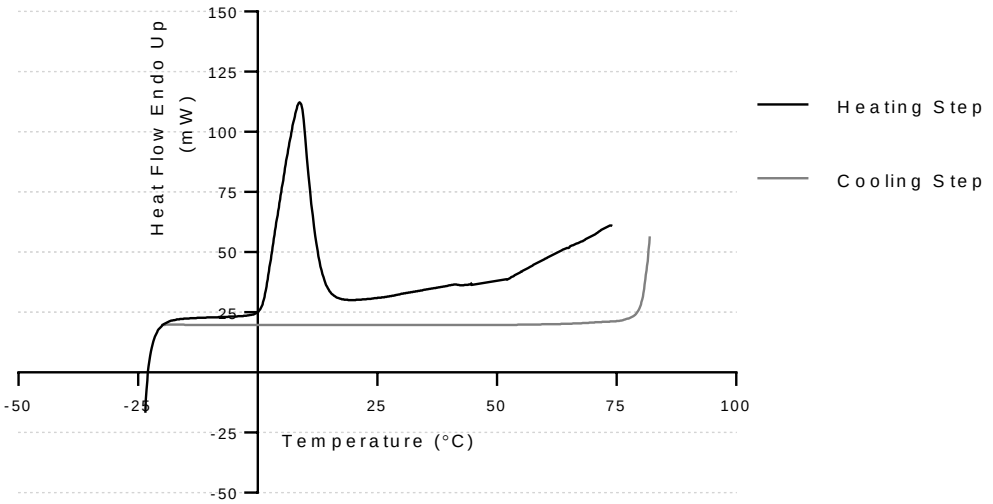

Figure 29 – Encapsulated 200 nm fluorescent nanoparticles in agarose 2%, DSC analysis: Endothermic peak = 9 ºC; ΔH= 187 J g^{-1} (GraphPad Prism 7.05)

DSC analysis revealed that the agarose beads had a lower endothermic temperature peak, compared to pure agarose, which in practical terms for the industrial field, will be more suitable for releasing purposes, since it will need less energy (heat), to make the bead collapse. In the case of the encapsulated fluorescent nanoparticles, we can conclude that adding an extra agent to the bead, only increases the endothermic peak about 3 ºC.

3.7. Bacteriophage encapsulation and release assays

After the encapsulation process was optimized, bacteriophage encapsulation was performed using the "On-Chip Mixing" method, where the 85 μm droplets were obtained, using the following conditions: continuous phase flow rate (surfactant in oil) = 2000 μL h^{-1}; dispersed phase flow rates (for both agarose and bacteriophages) = 100 μL h^{-1}; surfactant concentration (continuous phase) = 2.5%; agarose concentration (disperse phase 1) = 4%; bacteriophage concentration (disperse phase 2) = 2.25×10^9 PFU mL^{-1}.

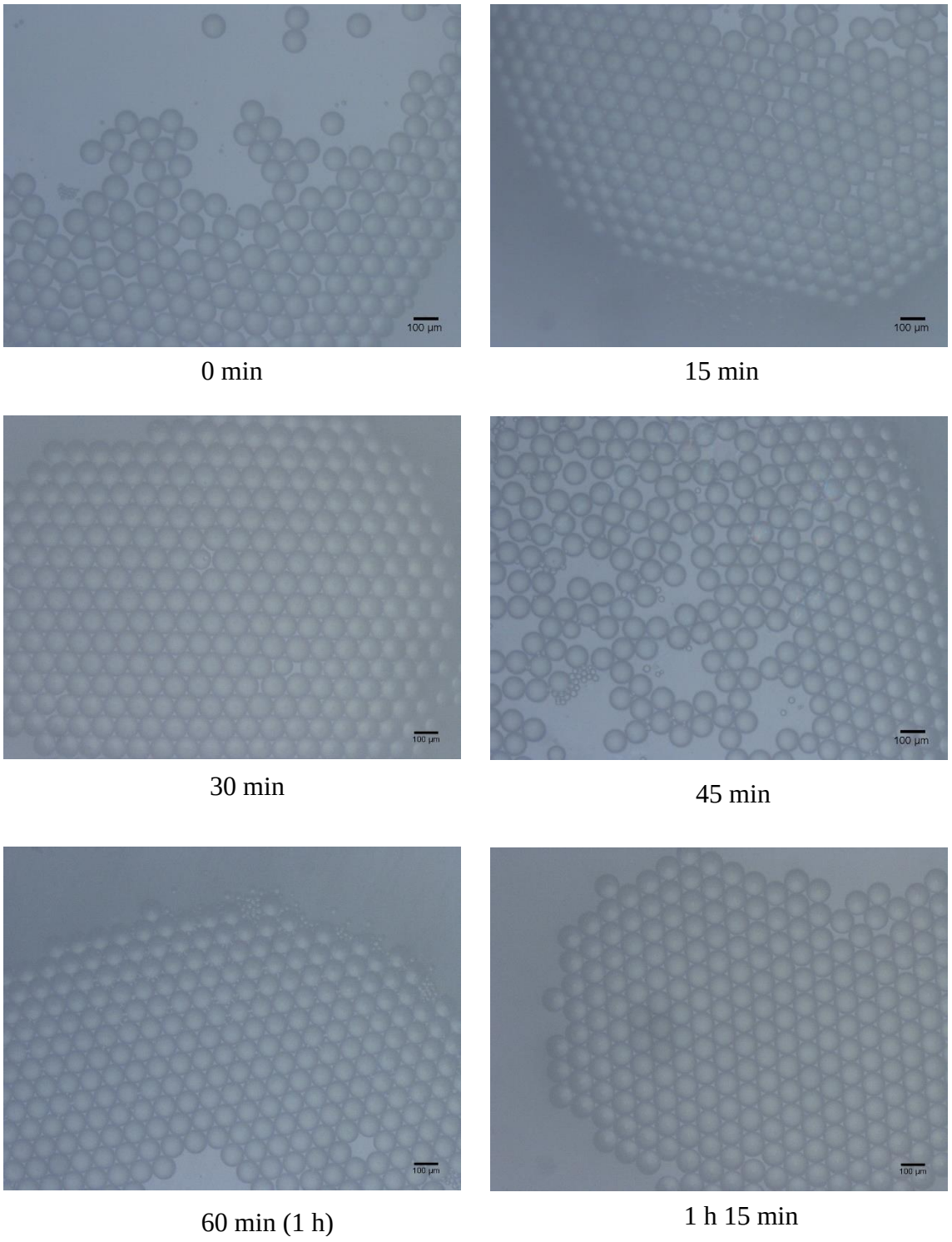

Figure 30 – Bacteriophage encapsulation - On-Chip Mixing - Process stability (Optical image); Experimental parameters: $Q_{Continuous}$= 2000 µL h^{-1}; $Q_{Disperesed}$= 100 µL h^{-1}; [continuous phase] = 2.5%, [disperse phase 1] = 4%, [disperse phase 2] = 2.25×10^9 PFU mL^{-1})

As confirmed by microscopy imaging (figure 30), the process was stable for an hour and fifteen minutes, as previously obtained with the fluorescent particles. Bacteriophage release was tested at 23.5 ºC, and consisted in diluting the droplets loaded with bacteriophages in SM buffer (1:4 v/v), removing samples at different time points and plating them, using the drop technique, on *Salmonella Enteritidis* (SE) 821 bacterial lawns, to quantify the concentration of bacteriophages released (figure 31).

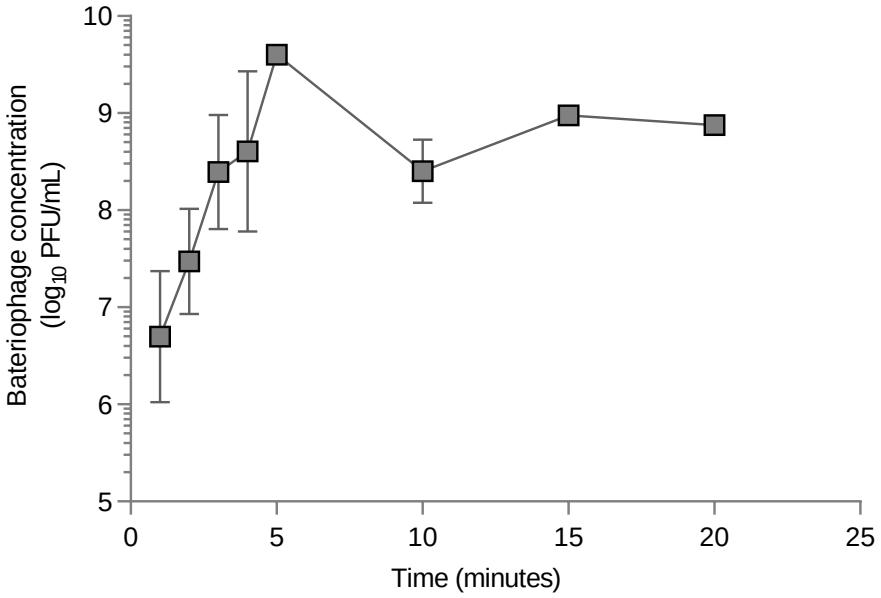

Figure 31 – Bacteriophage release per minute (N=3). Y axis is expressed in logarithmic (GraphPad Prism 7.05)

Bacteriophages were released in the first 5 minutes of the experiment, achieving a release value of approximately 60 %, with no external trigger agents added. Other method has been reported in the literature, where a release of 90% of bacteriophages was achieved after 2 hours, but in their experiments the release was triggered by pH and temperature, which is also an interesting approach if phages are desired to be released only under these conditions (Boggione *et al.*, 2017; Vinner and Malik, 2018).

4. CONCLUSION

In this work, a controlled production of highly monodisperse agarose droplets, using microfluidic devices was optimized and its use validated for the successful formations of droplets containing fluorescent particles and bacteriophage PVP-SE2. The lowest mean droplet size obtained was 85 µm, achieved after optimization of different geometry devices, flow-rates and concentrations of both continuous and disperse phases.

The "On-Chip Mixing" methodology developed in this project is novel and showed to be more efficient in terms of process malleability than the "Off-Chip Mixing" methodologies described to date. In addition, as the experimental conditions, as well as the encapsulated agent, can be changed in the middle of the process, without the need to restart the process in between which is of great advantage.

Microscopy imaging showed that fluorescent particles are uniformly dispersed in the droplets. Furthermore, the obtained bacteriophage-loaded agarose droplets, released more than half of their phage cargo within the first 5 minutes, without the need of any external triggers. It was also demonstrated that these droplets could be stored in liquid or in powder forms without causing any significant damage, being that for a long-term storage powder form prove to be a better option.

Overall, the method developed using microfluidic devices allows a high-throughput formation of monodisperse agarose beads, in a very controlled and automated way. Can be used with bacteriophages and fluorescent particles, as demonstrated in this work, being able to be easily adapted for the efficient formation of loaded-microdroplets using other potentially interesting agents.

For future work, trigger agents, such as temperature or pH, should be tested, in order to evaluate whether they affect the release of encapsulated bacteriophages. Also, droplet formation with other biomaterials, using the referred methodology should be attempted to find, for instance, less expensive encapsulation materials. Furthermore, tests with fresh-cut products should be carried out to evaluate the capacity of the encapsulated bacteriophages to reduce *Salmonella* contamination. Additionally, droplets formed using the "On-Chip Mixing" could be loaded with bacteriophages relevant for other pathogens involved in foodborne outbreaks (e.g. *E. coli, L. monocytogenes*).

In conclusion, this technology paves the way towards the implementation of effective products for food safety control.

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APPENDIX

Annex 1 – Results of the flow rate optimization for the agarose bead size

Annex 1.1 – Agarose 2% (w/v)

$Q_{continuous}$(µL/h) Oil + Surfactant	$Q_{dispersed}$(µL/h) Agarose	Bead Average size (µm)				
		1	**2**	**3**	**4**	**5**
500	100	162	162	161	161	162
1000		145	146	145	146	145
1500		132	132	129	132	132
2000		121	124	122	120	123

Annex 1.2 – Agarose 3% (w/v)

$Q_{continuous}$(µL/h) Oil + Surfactant	$Q_{dispersed}$(µL/h) Agarose	Bead Average size (µm)				
		1	**2**	**3**	**4**	**5**
500	100	195	198	171	173	175
1000		181	166	160	159	164
1500		146	144	146	154	145
2000		136	129	130	133	119

Annex 1.3 – Agarose 5% (w/v)

$Q_{continuous}$(µL/h) Oil + Surfactant	$Q_{dispersed}$(µL/h) Agarose	Bead Average size (µm)				
		1	**2**	**3**	**4**	**5**
500	100	181	184	175	182	189
1000		162	158	156	157	150
1500		153	147	153	163	155
2000		154	150	165	155	163