Microparticles for oral delivery and cell encapsulation using membrane emulsification

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MICROPARTICLES FOR ORAL DELIVERY AND CELL ENCAPSULATION USING MEMBRANE EMULSIFICATION

Doctoral Thesis

Submitted By

Serena Morelli

Supervised by

Dr. Marijana M. Dragosavac
Professor Richard G. Holdich

Submitted in partial fulfilment of the requirements for the award in Doctor of Philosophy in Chemical Engineering

Loughborough University

3rd April 2017
GUTTA CAVAT LAPIDEM
(Dripping water carves a stone)

To my outstanding family
ACKNOWLEDGEMENTS

I wish to start saying that these three years completely changed my life. And, although it might not be significant for many of the readers, I am happy, happy and strong as I was never before.

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ABSTRACT

Microparticles have been extensively investigated for pharmaceutical applications, more specifically they have been widely applied as carrier of drug molecules, proteins, enzymes, DNA and cells. In this work, the Membrane Emulsification (ME) technique was employed for the manufacturing of uniform sized emulsions with predictable drop size. The liquid drops produced were, in a second step, converted to microparticles. ME is a process which consists in pressing the dispersed phase through the micropores of a membrane into the emulsion continuous phase. The investigation of the best operating conditions of emulsion production, the emulsion composition and the optimization of the formulations for the production of microparticles using the technique of ME were the aim of this thesis work. For the preliminary tests the Dispersion Cell system (lab-scale device) was used with nickel or stainless steel microengineered disk membranes with cylindrical pores. W/O emulsions were mainly produced. During the process of emulsion production the main parameters studied were the shear stress applied, the dispersed phase flux, the membrane type and the emulsifier type.

The use of a simplified model based on a force balance was used for the drop size prediction. The maximum shear stress reached is used for the calculations. It was shown that the model gave a more accurate drop diameter prediction when the flux of the dispersed phase is low.

For the solidification of the polymeric drops a reaction of (chemical or ionic) crosslinking or a physical method (thermal gelation) was employed. Influence of the solidification process was evaluated on the final product. The most important factors affecting the process of particles formation studied were the type of crosslinker used together with its concentration and the reaction time. It was shown that the crosslinking reaction is directly affecting the properties of the final product: the capability of the particles to swell in an aqueous media, the pH sensitivity of the material, the release rate of an encapsulated model compound.

The microparticles produced were loaded with copper, sodium salicylate and Blue Dextran as model molecules and yeast as probiotic cells. The release was assayed with time and, accordingly with the application, in acidic (pH= 1.2- 3), neutral (pH= 7- 7.3) or basic (pH= 8) environment. Formulations were optimized to achieve a sustained release (using Poly (Vinyl) alcohol (PVA) as polymeric material), a release into the stomach acidic environment (using PVA blended with chitosan (CS)), a release (of probiotics) in the intestine- colon area having
a neutral- basic environment (using gelatine coated microparticles) or for cells immobilization (using alginate as polymeric matrix).

The technique of ME was evaluated as a novel method for cell encapsulation, the use of membranes with the appropriate pore size led to the generation of drops containing cells and possible occurrence of cell filtration by the membrane was prevented. In order to demonstrate the cell survival to the encapsulation process, tests of the released yeast capability to metabolize glucose with time and cells dyeing with fluorescent probes were performed.

For an industrial application of the process of microparticles production using ME, the Dispersion Cell was substituted by the Pulsed (Oscillatory) Flow system. The use of this device presents some advantages for the process scale up as the manufacturing of emulsions with a high dispersed phase concentration (emulsions concentrated up to 30% were successfully produced) with a single pass through the membrane module and possibility to solidify the drops right after their production. The operating parameters of the process were investigated for the production of W/O emulsions using this system, furthermore the possible application of a differently coated (FAS coated) membrane (instead of the classical PTFE coated) was evaluated for multiple uses.
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Figure 47 pH dependent Cu²⁺ Release of for microparticles made of either the polymeric blends of PVA and chitosan or pure chitosan. All the samples were crosslinked using 10 vol.% GA. The size of the dried microparticles used was approximately 30 µm ± 2 µm and CV%= 20 ± 3%. Error bars represent the standard deviation of the measurements.

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<th>Unit</th>
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<tbody>
<tr>
<td>( a )</td>
<td>Amplitude of the oscillation</td>
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</tr>
<tr>
<td>( A )</td>
<td>Interfacial area</td>
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</tr>
<tr>
<td>( A_{hexagon} )</td>
<td>Area of the hexagon</td>
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</tr>
<tr>
<td>( A_{pore} )</td>
<td>Area of a membrane pore</td>
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<td>( b_h )</td>
<td>Blade height</td>
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<td>( CV% )</td>
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<tr>
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<td>Volume of the drop predicted by a force balance</td>
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<tr>
<td>$V_t$</td>
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<tr>
<td>$x$</td>
<td>Formed (predicted) drop diameter</td>
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**Greek symbols**

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<tr>
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<tr>
<td>$\delta$</td>
<td>Boundary layer thickness</td>
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<td>$\varepsilon$</td>
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<tr>
<td>$\eta$</td>
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<tr>
<td>$\tau$</td>
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<tr>
<td>$\tau_{max}$</td>
<td>Maximum shear stress</td>
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</table>
\[ \omega \quad \text{Angular velocity} \quad \text{Rad s}^{-1} \]

\[ \omega_f \quad \text{Angular frequency} \quad \text{Rad s}^{-1} \]
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAS</td>
<td>Atomic Absorbance Spectrophotometer</td>
</tr>
<tr>
<td>ABIL EM 90</td>
<td>Modified polyether–polysiloxane</td>
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<td>BD</td>
<td>Blue dextran</td>
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<td>CMC</td>
<td>Critical Micelle Concentration</td>
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<td>Continuous phase</td>
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<td>Chitosan</td>
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<td>D&lt;sub&gt;av&lt;/sub&gt;</td>
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<td>Nickel membrane</td>
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<td>O/W emulsion</td>
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</tr>
<tr>
<td>O/W/O</td>
<td>Oil in water in oil emulsion</td>
</tr>
<tr>
<td>O&lt;sub&gt;1&lt;/sub&gt;/O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Oil in oil</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PGPR</td>
<td>Polyglycerol polyricinoleate</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly (Vinyl Alcohol)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>PVAc</td>
<td>Poly Vinyl Acetate</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SHMP</td>
<td>Sodium hexametaphosphate</td>
</tr>
<tr>
<td>SPAN 80</td>
<td>Sorbitan monooleate 80</td>
</tr>
<tr>
<td>SPG membrane</td>
<td>Shirasu-porous-glass membrane</td>
</tr>
<tr>
<td>SS</td>
<td>Sodium salicylate</td>
</tr>
<tr>
<td>SSm</td>
<td>Stainless steel membrane</td>
</tr>
<tr>
<td>STPP</td>
<td>Sodium tripolyphosphate</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyethylene glycol sorbitan monolaurate</td>
</tr>
<tr>
<td>W/O emulsion</td>
<td>Water in oil emulsion</td>
</tr>
<tr>
<td>W/O/W</td>
<td>Water in oil in water emulsion</td>
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</tbody>
</table>
1. INTRODUCTION

1.1 AIMS AND OBJECTIVES

When a drug is administered, the therapeutic effects are determined by the pharmacological properties of the pharmaceutically active compound; however independent to the effectiveness of the drug molecule the need to reach the target with the proper concentration that both minimizes the side effects and maximizes the therapeutic effects are crucial for the success of the therapy. In some cases, the existence of physiological barriers could preclude the use of an otherwise effective drug molecule. Specific drug carriers (e.g. drug delivery systems) have the purpose of enhancing and facilitating the action of a therapeutic molecule to provide the correct amount of drug at the specific site of action for the required time, or in response to specific environmental stimuli.

Between the several possible routes for drug administration the most convenient, and well accepted, is oral which could provide systemic or topic effects. It affords the compliance of patients and it is generally less expensive than the parenteral routes as it does not require the presence of specialist medical staff and can be well controlled by the patient. There are several advantages related to the oral delivery of therapeutics, such as the large surface area available for the absorption of the drug (200 m² in the small intestine) (Hillery et al., 2002). The complexity of the gastro-intestinal tract represents the main issue related to oral drug administration. Hence, common problems related to the oral administration of therapeutics are the low solubility, the high potency, and the poor chemical stability of the active substances. The therapeutic must be in a formulation that helps it resist the environmental conditions in which it would be after the ingestion (pH, enzymes, presence of mucus and food…) and reach in an unmodified state the site of action (Hillery et al., 2002).

Within the variety of devices developed for drug delivery (polymer-based disks, rods, capsules, pellets…) biocompatible polymeric microparticles are the most common as they present many advantages: the possibility to encapsulate a wide range of materials of pharmaceutic interest such as small molecules, nucleic acids, proteins, enzymes and cells. Most common disadvantages associated with the use of microparticles in drug delivery are: difficulty of large scale industrial manufacturing, inactivation of the drug/cell death during the process of production, insufficient control over the drug release rate (Kim and Pack, 2006).
Aim of this work was to propose an alternative technique for microparticles production using membranes for the encapsulation of ions, small molecules and probiotic cells for pharmaceutical advanced applications.

Microparticles were obtained by the solidification of liquid drops produced using the Membrane Emulsification technique, hence aim of the first part of the research project was the production of uniform-sized W/O emulsion optimizing the process operating parameters and the phases composition. Membrane Emulsification has been extensively used for the production of W/O or O/W emulsions, as well as for the production of more complex microstructures such as multiple emulsions, Pickering emulsions, foams, micro-nanoparticles (Spyropoulos et al., 2014a). The technique presents many advantages over conventional methods of emulsion production (e.g. rotor-stator systems, high pressure homogenizers…) especially for the encapsulation of shear and temperature sensitive materials (e.g. cells, drugs). With this method, the emulsion drops are generated by pressing the dispersed phase through the pores of a membrane into the continuous phase, and the shear generated by the movement of the continuous phase produces the detachment of the drops from the membrane surface. This technique uses much lower shear compared to the classical methods of emulsion production and it is particularly useful for the encapsulation of temperature or shear sensitive compounds. A further advantage is the possibility to have fine control over the size of the drop produced by regulating some operating parameters of the process. Many devices for Membrane Emulsification have been proposed for process scale up, production of a high quantity of emulsion (Piacentini, Dragosavac, et al., 2017) or the production of highly concentrated emulsions (up to 50 vol.% of dispersed phase in the emulsion) and processes operating continuously are possible.

In this thesis, a thorough investigation of emulsion composition was conducted using a lab-scale device for Membrane Emulsification: the Dispersion Cell (Kosvintsev et al., 2005) system in association with disk hydrophobic membranes with a range of pore sizes between 10 and 30 μm. With this device the process operating parameters were optimized for the production of uniform drops, hence parameters such as dispersed phase flux, shear stress, membrane type and membrane coating material were investigated. W/O emulsions were mainly produced and the influence of the oil type, emulsifier type, dispersed and continuous phase properties were evaluated. Microparticles were obtained by solidification of the liquid
drops, the process of solidification was varied accordingly with the type of polymer used in the formulation which was selected with the final use of the microparticles in mind:

- Poly (Vinyl) Alcohol microparticles were produced by chemical crosslinking using glutaraldehyde for the sustained release of Cu$^{2+}$ used as model very low molecular weight compound (Chapter 4.1);
- Blended Poly (Vinyl) Alcohol and chitosan microparticles were obtained with chemical crosslinking using glutaraldehyde for the pH controlled release of Cu$^{2+}$ and Sodium salicylate used as model drug (please refer to Chapter 4.2);
- Blended chitosan and gelatine or pure gelatine microparticles were obtained by the thermal gelation and ionic crosslinking (using sodium hexametaphosphate). Particles were used to encapsulate yeast cells. pH controlled release suitable for intestine-colon area delivery was achieved using Eudragit S100 as a gastro-resistant coating material (please refer to Chapter 4.3); and
- Alginate microparticles containing viable yeast cells were produced by solidification of the alginate drops by ionic crosslinking using Ca$^{2+}$ or Mg$^{2+}$ (please refer to Chapter 4.4).

In the last part of the research project the process scale up was addressed using the Pulsed (Oscillatory) Flow Membrane Emulsification (Holdich et al., 2012) device. The use of the Pulsed system was advantageous for the possibility to produce highly concentrated emulsions with a single pass of emulsion over the membrane surface. In addition it would be possible to connect in series to the membrane module (where the emulsion is produced) a plug-flow reactor for simultaneous and continuous drop solidification.

The project methodology can be summarised within three main steps:

Step 1. **Droplet production**: initially the composition of the emulsion was optimised and the process operating parameters investigated in order to obtain stable and uniform sized emulsions. In this step the material to be encapsulated was directly added to the emulsion dispersed phase in the appropriate concentration, thus Cu$^{2+}$, Blue Dextran, sodium salicylate, or yeast cells were mixed with the polymeric phase.

Step 2. **Droplet solidification into solid particles**: once the emulsion with the desired characteristics was obtained, the process of solidification of the liquid drops was investigated. A crosslinking chemical reaction: ionic crosslinking reaction, or thermal gelation, were
exploited. Time of reaction, presence of a catalyst and amount of crosslinker were investigated. Subsequently the process of recovery, washing and drying the produced solid polymeric microparticles was optimized accordingly with the formulation and compatibility with the encapsulated material.

Step 3. **Release studies and cell viability**; the release of the encapsulated material inside the microparticles was investigated with time and using solutions at different pH (from 1.2 to 8) in order to mimic the variation of pH through the gastro-intestinal tract. Viability of the yeast cells encapsulated within the microparticles was confirmed using fluorescent probes and analysing the yeast cell glucose metabolism with time.

### 1.2 RESEARCH QUESTIONS

This doctoral thesis aims to address several “research questions” in the research field of the production of Micro-engineered particles for Oral Drug Delivery:

1. Is it possible to produce highly uniform microcarriers for small molecules and cells of desired sizes?
2. Can these microcarriers be used for the release of the encapsulated material (unmodified) into a specific section of the human gastro-intestinal tract after the oral administration exploiting the difference in pH?
3. How suitable is the Membrane Emulsification technique for encapsulation of small molecules and cells?
4. What would be the best method to scale up the particle production process?

Possible answers to these questions are provided within this thesis and a summary of the results gathered is reported in the “Conclusions” chapter.

All the experimental work presented in this thesis was done by the author, the only exception are the experiments about the production of the alginate microparticles which were performed by a visiting student, Clementine Sivelle, during her project (from April to June 2016) in which she was supervised weekly by Professor Holdich, and the thesis author on a daily basis.

Some of the work presented here has been published in peer-reviewed journals and presented at international conferences.
1.3 OUTLINE OF THE THESIS STRUCTURE

The literature review is presented in **Chapter 2**: an initial overview of the most common routes of drug administration is reported with a main focus on the oral way (Chapter 2.1). In Chapter 2.2 a review on the use of microparticles for pharmaceutical applications is provided, focusing the discussion on the encapsulation of therapeutic materials and cells. A classification of the most common polymeric materials (natural and synthetic) used for the production of microparticles intended for oral drug delivery is reported. In Chapter 2.3 is presented an overview of the conventional methods of microparticles production as well as the production of microparticles by emulsification process. Most used techniques of emulsions production will be compared with the Membrane Emulsification investigated in this thesis. A description of the available devices for Membrane Emulsification is provided, a description of the Shear-Capillary model for droplet size prediction is reported.

The experimental section is provided in **Chapter 3**. The lab-scale device, Dispersion Cell System, used for formulations and process parameters optimization is illustrated and described in Chapter 3.1. In Chapter 3.2 is discussed the method of emulsions characterization, the analysis of the phases physical properties and the contact angle measurements. In Chapter 3.3 are reported the materials and methods used of the microparticles production accordingly with the type of formulation developed. In this chapter are described the methods of investigation of microparticles swelling and release, as well as the cell viability tests performed. Chapter 3.4 reports the method of microparticles characterization in terms of morphology and elemental composition. The scale-up system is discussed in Chapter 3.5 with a detailed description of the Pulsed (Oscillatory) Flow device (including membranes used and cleaning procedure) with an introductive section reporting the preliminary investigation on membrane coating type and optimization of the emulsion formulation.

All the experimental results and discussions are reported in **Chapter 4**. Chapter 4.1 reports the experimental data regarding the PVA microparticles production process (optimization of the emulsification process and post-emulsification treatments) and time-related release studies. In Chapter 4.2 are displayed the data regarding the production of the PVA-Chitosan microparticles as well as the release tests performed with time and at different pH conditions. Resulting data on Gelatine-Chitosan microparticles production are reported in Chapter 4.3.
with the yeast release studies performed at different pHs and the yeast cell viability tests. In Chapter 4.4 the results of the Alginate microparticles production process for cell immobilization are shown, encapsulated yeast viability tests are displayed. In Chapter 4.5 data on the comparison between the performance of PTFE and FAS coated membrane are reported; for multiple production of W/O emulsions. The tests run using the Pulsed (Oscillatory) Flow system are shown in Chapter 4.6. Preliminary tests performed using the Dispersion Cell for the emulsion composition optimization and membrane type are also reported in this chapter. A summary of the main results is provided in Chapter 4.7.

Chapter 5 reports the conclusions gathered from the experimental data obtained, a section of proposed future works is also provided. The conclusion chapter is followed by a list of all the papers referenced in this thesis (Bibliography) and by the Appendix where further information on the instruments, instruments calibrations and software used in this work are provided.
2. LITERATURE REVIEW

2.1 THE ORAL DELIVERY OF PHARMACEUTICS

2.1.1 Overview of the routes of drug delivery

Drugs and active compounds may be introduced into the human body by various anatomical routes. The choice of the appropriate delivery route depends on many factors such as the type of disease, the therapeutic effect to achieve, the possible side effects, and the formulation type available (Jain, 2008). Another distinction is based on the needed of a systemic effect, or a targeted action of the drug to a diseased organ. A classification of the drug delivery routes can be done on the anatomical site of administration (Jain, 2008):

- Oral Drug Delivery
- Parenteral Drug Delivery
- Transmucosal Drug Delivery
- Nasal Drug Delivery
- Colorectal Drug Delivery
- Pulmonary Drug Delivery
- Cardiovascular Drug Delivery
- Drug Delivery to the Central Nervous System

All previously mentioned routes will have a certain systemic effect, although, if a systemic delivery of the drug is required the most effective routes of administrations are the oral, parenteral and pulmonary. Within the evaluation of the several routes of administration available, there are some general requirements to be taken into account:

1. **Large surface area** for drug adsorption; the small intestine represents a fundamental region of drug absorption (surface area ~200 m²) (Levine, 1973; Hillery et al., 2002). The lungs surface area is also extremely large (~ 100 m²) (Pilcer and Amighi, 2010) and it represents an alternative to the parenteral an oral routes.

2. **Presence of degradative enzymes** could deactivate the drug before the adsorption, this represents one of the main disadvantages of oral administration.

3. **Time of contact** between the drug and the absorbing tissue is also extremely important and can affect the amount of drug that will actually cross the mucosa.

4. **Blood supply** is required to ensure the reaching of the “post-absorption” site of action of the drug and the “sink conditions”(Hillery et al., 2002).
5. **Reduced variablity** of a certain site of absorption (physiological conditions) essential to ensure the reproducibility of the drug action. This is particularly true of high potency drugs with a narrow therapeutic window.

### 2.1.2. The oral route of administration

The oral administration route is certainly considered the most convenient and well-accepted. It helps with the compliance of patients due to its non-invasive nature, pain avoidance and possibility to formulate different types of drugs (Sastry et al., 2000). Oral delivery formulations are less expensive than other types because generally they do not require highly sterile conditions of manufacturing (Sastry et al., 2000) and specialized personnel for administration. The oral way is usually preferred for the achievement of a systemic effect of the drug, but with the appropriate formulation it is possible to obtain also local effects. Cathartics, antacids, and drugs used for the treatment of bacterial infections are some examples of active compounds with a local effect on a specific site of the GI (Levine, 1973). For that purpose, the formulation more than the drug molecule itself, plays a fundamental role on the achievement of the desired therapeutic effect and the reduction of the side reactions. Due to the importance of oral way of administration, over the past decades several drug delivery formulations have been developed; such as mucoadhesive, matrix systems, reservoir systems, microparticles, pH sensitive delivery systems (Hillery et al., 2002; Wen and Park, 2011). The complexity of the GI has to be considered when designing an oral drug delivery system; factors such as pH variations, motility, presence of mucus or food, individual variations, and drug metabolism should be taken into account for the production of a successful formulation. In this chapter the structure and the physiology of the GI tract including the most important factors affecting oral bioavailability will be discussed.

### 2.1.3 Structure of the gastrointestinal tract

The gastrointestinal tract is formed of four main regions: oral cavity, stomach, small intestine, large intestine (Figure 1). The function of this system is digestion which involves the breaking down of complex molecules (deriving from the food) into simpler molecules to be absorbed into the blood and the lymph systems (Hillery et al., 2002). Each compartment of the GI in designed for a specific function; accordingly the physiology of each region is different. The epithelium, pH, motility and residence time are the main difference between...
the four regions and they are also essential parameters affecting the behaviour of drugs and drug delivery devices.

Figure 1  Structure of the human gastrointestinal tract and relative physicochemical conditions; image modified from http://en.wikipedia.org/wiki/Digestive_tract (Copyright free).

Oral Cavity (Mouth)  
The initial food fragmentation occurs in the oral cavity; it is covered by a smooth and thin epithelium composed by several layers of cells. To promote the moisturising and initial digestion of dry food, the mouth epithelium secretes saliva making a slightly acidic environment (Levine, 1973).

Stomach  
The stomach acts as food reservoir, completing the fragmentation started in the oral cavity and is the start of proper digestion. To accomplish this function, the stomach secretes hydrochloric acid and enzyme (pepsin) necessary for the breaking of proteins (Levine, 1973). Subsequently the gastric content is very acidic: pH of the stomach in a fasting state is ~1.5 to 2.0 and in fed state is 2.0 to 6.0 (Hillery et al., 2002; Arora et al., 2005). The stomach pH is subject to individual variations and pathologic conditions. Although the stomach is not the
principal site of drug absorption, the rate in which the stomach empties (stomach emptying time) (Singh and Kim, 2000) is fundamental for the rate and the extent of drug adsorption in the small intestine (Hillery et al., 2002). The normal retention time in the stomach is 2-3h after a normal meal, or a maximum of 30 minutes after a glass of water (Khurana, 2006). Viscosity, volume and caloric content of the meal can affect the gastric emptying time as well as the age, gender and presence of pathological conditions (diabetes, Chron’s disease); stress usually increases the gastric emptying rate while depression produces a reduction of it (Arora et al., 2005). If a topic action of the drug is required in the stomach, formulations able to increase the stomach retention time can be produced increasing the bioavailability of the drug, the drug solubility in acidic conditions improving the overall therapeutic effect. For controlling the gastric retention many mechanism can be exploited such as muchoadesion (Shah et al., 1999; Beri et al., 2013), flotation (Dave et al., 2004; Arora et al., 2005), sedimentation, expansion, or the simultaneous administration of substances to delay the gastric emptying time (Arora et al., 2005).

Small intestine
The main function of the small intestine is the absorption of the end-product of the digestion and their preparation to be available in a useable form by the organism (drug actions and interactions). As a consequence the small intestine also represents the main site of drug absorption. It consists of the duodenum, jejunum and ileum. The absorbing epithelium of the small intestine provides the structure to increase the absorbing surface area (Levine, 1973). All these microstructures increase the available area of absorption of the small intestine (approximately 2.3 m long with a 4 cm diameter) to 200 m² (Levine, 1973; Hillery et al., 2002). The environment through the length of the small intestine changes from slightly acidic (pH=4-5) in the initial part (duodenum) due to the content of the stomach emptied in this tract. Alkaline secretions from the pancreas and bile neutralize the pH rapidly alongside the rest of the small intestine to a pH of 6-7 (Levine, 1973). In normal conditions, 3-5h are required to pass from the one end, to the other, of the small intestine (Dressman and Reppas, 2010). In contrast to the stomach, the residence time in the intestine is longer to enhance the sorption of the nutrients since mass transfer takes place along the entire length (Levine, 1973).
Large intestine (Colon)

The large intestine, or colon, is approximately 1.5 m long with a 6 cm diameter (Hillery et al., 2002). In this area of the GI the pH is between 6 and 8 (Ashford and Fell, 1994). The terminal segment of the colon (rectum) may be used as site for drug administration when the oral route is not usable. This route prevents the inactivation of the drug operated by the liver, avoiding the so called “first pass” metabolism (Levine, 1973). Contributing to the morphology and functions of the gastrointestinal tract is the intestinal microflora, which has significant metabolic activity, and participates in the metabolism (Friend, 1992). The human gastrointestinal microflora are very complex, it was estimated that the human GI tract contains more the 100 trillion of microbial microorganisms collectively called microbiota (Davis and Milner, 2009). A small portion of bacteria is present also in this region attached on the epithelium, or present in the mucus, as for example the Helicobacter pylori. This microorganism is responsible for 90% of stomach ulcers and it is implicated in gastric cancer (Tlaskalová-Hogenová et al., 2004). Colonic microflora contribute to the health of the bowel, and it has a role in reducing the cancer risk, production of essential nutrients, and stimulation of intestinal immunity (Davis and Milner, 2009).

2.1.4 Formulation properties influencing oral bioavailability

For oral drug delivery formulations (e.g. solutions, suspensions, emulsions, capsules and tablets) factors such as the particle size and type of additives have a great influence on formulation dissolution and drug absorption rate (Hillery et al., 2002).

2.1.4.1 Particles size

The size of particles generally accepted for oral drug delivery are in a range between 20-120 µm because this size range provides the retention of the particles by the intestinal tissue and acts as sustained release depot (Rathbone et al., 2002). In terms of drug release rate from the dosage form, a reduction in particle size results in an increase in the total surface area, increasing the dissolution rate and, as a consequence, increasing the rate of sorption. Controlling the particle size is possible to control the release rate obtaining with a formulation (Berkland et al., 2002; Siepmann et al., 2004). The reduction of the particle size can also be a strategy to increase the solubility of poor water soluble compounds that are marketed as micronized, or microcrystalline, forms (Hillery et al., 2002).
2.1.4.2 Additives

Additives are substances added to the pharmaceutical formulation to enhance the stability, the mechanical properties, or the dissolution of the formulation matrices. Wetting agents (surfactants) are commonly used to lower the interfacial tension and the contact angle between immiscible liquids, or between solid particles and liquid vehicles. The most commonly used in the formulation of oral delivery formulations are Polysorbate 80 and sodium dodecyl sulphate; these substances have been shown to increase the drug sorption across the human GI tract (Hillery et al., 2002). The chemical nature of the surfactant alters the dissolution rate of lipid materials, and membrane characteristics, increasing the sorption (Gad, 2009). Diluents are used as fillers, to bulk up the formulation. They are usually carbohydrates such as lactose, dextrose, sucrose or microcrystalline cellulose. They can promote the faster disintegration and release of the drug (Hillery et al., 2002). Substances such as starch, gelatine, polyvinylpyrrolidone (PVP) are used as adhesives, to bind powders together in the process of wet granulation. The adhesive can be hydrophobic (producing slow disintegration of the formulation and delayed drug release) or hydrophilic (increasing the disintegration rate of the formulation and producing an increase in the drug release rate) (Hillery et al., 2002). Disintegrants cause the formulation to disintegrate rapidly increasing the drug dissolution while the use of lubricants can enhance the flowing properties of granules; they are hydrophobic materials and can prevent the wetting of powders and retard the dissolution (Hillery et al., 2002).
2.2 POLYMERIC MICROPARTICLES FOR PHARMACEUTICAL APPLICATIONS

Encapsulation is a technique by which a solid or a liquid active ingredient is packed within a second material in order to shield it from the surrounding environment, but at the same time it allows for the active ingredient to reach the area of action unmodified. This technology is being adopted in a variety of different fields: pharmaceutical, biological, medical, food or chemical industries (Dubey et al., 2009). Reasons of encapsulation include: separation of incompatible components, increasing of the solubility of lipophilic compounds, increasing the stability of delicate compounds (protection from the oxidisation or deactivation caused by environmental conditions), odour and taste masking, controlled or targeted release of active molecules in a specific site of action, easier product recover (Nedovic, V., & Willaert, 2006; Dubey et al., 2009). If a specific particle size is required for the delivery of the active ingredient in a determined region, it might be advantageous to form small particles called microparticles/microcapsules and the process of encapsulating the material within the micron-sized particles is called microencapsulation (Gharsallaoui et al., 2007). It is possible to classify the type of microparticles depending on their morphology: as monocored, polycored or matrix type. Monocored particles are characterized by a single internal hollow structure while the polycored microparticles have a number of different sized chambers below the shell. Monocored and polycored microparticles are both called microcapsules. Matrix type are internally full and the active ingredient is integrated within the matrix, matrix type are also called microspheres (Yoshizawa, 2004; Dubey et al., 2009; Chetty et al., 2010). Microparticles are extensively used for pharmaceutical/biological purposes, for controlled/sustained release of molecules of pharmaceutical interest (Dubey et al., 2009). Polymeric microparticles were produced for the encapsulation and delivery of DNA, for non-viral gene therapy (Denis-Mize et al., 2000; Truong et al., 2000; Mathiowitz et al., 2001; Little et al., 2005; Guliyeva et al., 2006; Bhavsar and Amiji, 2007) or for vaccine therapy (O’Hagan et al., 1998; Goh et al., 2004; Jiang et al., 2005). Particles were used for cancer treatment using different strategies: the embolization technique for liver cancer therapy which consists in injecting particles (usually made of gelatine or Poly (Vinyl) Alcohol) to block or reduce blood supply to the cancer (Madoff et al., 2003; Marelli et al., 2007; Rajput and Agrawal, 2010; Bonomo et al., 2010), or the use of radioactive microspheres commonly made of resins, or glass, for the release of the radiation locally to the cancer cells (Setoyama et al., 2006; Rajput and Agrawal, 2010). Microparticles were also produced for controlled
and targeted release of anticancer drugs (Cummings, 1998; Lin et al., 2005; Rajput and Agrawal, 2010; Kettenbach et al., 2008; Cerroni et al., 2011; Kim et al., 2013; Shi et al., 2014; Rai et al., 2016). Microparticles for protein delivery (Yang et al., 2001; Yuan et al., 2007) such as insulin (Déat-Lainé et al., 2013; Shrestha et al., 2014), growth hormones (Patel et al., 2008; Formiga et al., 2014), erythropoietin (Zhang et al., 2015) have also been produced.

2.2.1 Polymeric microparticles for oral delivery

Formulation of polymeric particles for the purpose of drug release systems has received increased interest over recent years (Freiberg and Zhu, 2004). Conventional oral drug administration methods, such as solutions, suspensions, tablets, capsules (Hillery et al., 2002), often do not provide protection from the acidic environment present in the stomach (Hillery et al., 2002) and do not provide a constant therapeutic blood concentration of the drug over time (Shah et al., 1997; Hoffman, 2008). A re-administration of the drug is required and the possibility of the drug reaching toxic levels. Encapsulating the drug into a polymeric matrix makes it possible to tailor the release, obtaining a site-specific release, prolonged action and/or sustained release (W. M. Saltzman, 2001; Sagiri et al., 2015). The use of polymers enables the production of formulations able to respond to specific environmental stimuli such as pH (Park et al., 2011), temperature (Hanga and Holdich, 2014) and magnetic fields (Dobson, 2006). Polymers are the most versatile class of compounds and they have been extensively used in pharmaceutical and biomedical (De Vos et al., 2013) applications due to biocompatibility (Freiberg and Zhu, 2004). A variety of devices have been produced using polymers for oral drug administration, such as polymer-based disks, pellets, rods etc. but the most common and versatile are the polymeric microparticles. By varying the chemistry of the polymers, it is possible to obtain a sustained release (Kim and Pack, 2006) or a site-specific release, for example through the stomach (Risbud et al., 2000) or the intestine (Kurkuri and Aminabhavi, 2004).

The polymers used for pharmaceutical applications must be biocompatible, not toxic, and nor carcinogenic. In the class of polymeric systems for delivery of pharmaceutical it is possible to distinguish two categories: gels and hydrogels (Gupta et al., 2002). Commonly these two terms are used to describe the same type of polymeric network as gel and hydrogel can be chemically similar, but they are physically distinct. Gels are described as semi-solid systems
containing a small amount of solid, dispersed in a relatively large amount of liquid, but still characterized by a solid-like behaviour rather than liquid-like. Gels are polymeric networks already in the swollen state and the further addition of liquid only produces a dilution of the polymer. The hydrogels are crosslinked network of hydrophilic polymers, they are characterized by the ability to absorb more than 20% of their weight in water and swell maintaining their three-dimensional structure. According to the destruction profile of the polymer matrix, directly involved in the release mechanism of the encapsulated drug, polymers can be divided in to surface and bulk eroding polymers (Pillai and Panchagnula, 2001). The surface eroding polymers are mainly hydrophobic (commonly used are polyanhydrides and poly(ortho esters) and Poly[1,3-bis(p-carboxyphenoxy) propane-co-sebacic anhydride] P(CPP-SA) (Zhang et al., 2003)), and the monomers are linked with relatively labile bonds; they are able to resist the permeation of water into the bulk of the polymer, degrading quickly into oligomers and subsequently monomers via hydrolysis at the polymer-water interface (Kim and Pack, 2006; Saltzman, 2001). In the bulk eroding polymers (most common are poly- (L-lactic acid) and poly(lactic-co-glycolide) (Zhang et al., 2003)), the water can penetrate into the polymer matrix, dissolving the drug inside which is released in this way (Kim and Pack, 2006). The bulk eroding polymers are characterized by a initial burst release of the drug, this is a phenomena in which the drug is released immediately in a big amount (O’Donnell and McGinity, 1997) this process may cause a too high concentration of the drug inside the body, that could be toxic. On the contrary, the surface eroding allows a constant release of the drug, which is released primarily at the surface of the microparticles as quick as the polymer breaks down. Another classification of polymeric materials used for oral drug delivery, can be done depending on the nature of the polymeric material and the method of production, they can be classified as synthetic and natural polymers. (Pillai and Panchagnula, 2001). An overview of the synthetic and natural polymers used for the production of microparticles for oral delivery is provided below.

2.2.1.1 Synthetic polymers used for oral drug delivery

Synthetic polymers have the advantage of being available in a wide range of compositions with adjustable properties (Pillai and Panchagnula, 2001). With the possibility of processing, blending or copolymerization it is possible to modify the mechanical properties (Angelova and Hunkeler, 1999) and response to environmental stimuli. The most common synthetic
polymers used for the formulation of oral drug delivery systems and their main characteristics are summarized into the Table 1.

**Table 1** Most commonly used polymers for the preparation of microparticles for oral delivery of therapeutics.

<table>
<thead>
<tr>
<th>Synthetic polymers</th>
<th>Principal characteristic and comments</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Poly (lactic-acid), Poly(glycolic-acid) and their copolymers</td>
<td>Biodegradable polymer, the copolymer ratio could be adjusted to regulate the degradation time. Used for the oral drugs and vaccine delivery and production of magnetically responsive microparticles for oral delivery of proteins.</td>
<td>(Cheng et al., 2006; Wischke and Schwendeman, 2008; Jiang et al., 2014)</td>
</tr>
<tr>
<td>Poly (acrylic-acid) based</td>
<td>pH sensitive polymer, used for the production of sIPN (semi-interpenetrating network) for the oral administration of drugs and insulin.</td>
<td>(Kurkuri and Aminabhavi, 2004; Yan and Gemeinhart, 2005; Sajeesh et al., 2010)</td>
</tr>
<tr>
<td>Poly (hydroxy butyrate) Poly (ε-caprolacton) and copolymers</td>
<td>Biodegradable polymer, proprieties can be changed for chemical modifications of the material. Microparticles were produced for oral immunization, oral delivery of steroids, drugs and cell microencapsulation.</td>
<td>(Atala and Mooney, 1997; Sinha et al., 2004; Riekes et al., 2011)</td>
</tr>
<tr>
<td>Poly (vinyl alcohol) based</td>
<td>Biocompatible, non-toxic, hydrophilic polymer with bioadhesive properties. Ease of processing. Strength, pH and temperature stability. Often used in association with other polymers to improve the mechanical properties of the formulation. Used for the production of microparticles for the encapsulation and oral delivery of drugs, DNA and proteins</td>
<td>(Kurkuri and Aminabhavi, 2004; Agnihotri and Aminabhavi, 2005; Fundueanu et al., 2007; Mundargi et al., 2008; Ray et al., 2010; Kaity et al., 2013; Sullad et al., 2014; Kaity and Ghosh, 2016)</td>
</tr>
<tr>
<td>Ingredient</td>
<td>Description</td>
<td>Resources</td>
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<tr>
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</tr>
<tr>
<td>Methacrylic acid-methylmethacrylate copolymers (Eudragit® polymers)</td>
<td>pH sensitive polymers, used for the production of enteric coating, and gastro-resistant formulations, they dissolve at pH above 7. Microparticles were produced for intestinal-colonic release, enzyme encapsulation and as oral immune delivery system.</td>
<td>(Hori et al., 2005; Rawat et al., 2007; Badhana et al., 2013)</td>
</tr>
<tr>
<td>poly(N-isopropylacrylamide) based</td>
<td>Thermosensitive polymer, used in association with pH sensitive polymers for the production of microparticles for protein-peptide or drugs oral delivery</td>
<td>(Serres et al., 1996; Ramkissoon-Ganorkar et al., 1999; Guo and Gao, 2007; Gao et al., 2013)</td>
</tr>
</tbody>
</table>

### 2.2.1.1 Poly (Vinyl) Alcohol

Poly (Vinyl Alcohol) (PVA) is a biocompatible, not toxic and not carcinogenetic polymer included into the *Handbook of Pharmaceutical Excipients* (DeMerlis and Schoneker, 2003). PVA has a relatively simple-linear chemical structure with pendant hydroxyl groups as shown in the Figure 2 b. The production starts from the polymerization of the monomer acetate, to Poly (Vinyl Acetate) (PVAc), which is followed by the hydrolysis of the acetate groups to hydroxyl groups. The hydrolysis reaction that converts PVAc into PVA is not complete and resulting into the production of a polymer with a certain degree of hydrolysis that depends upon the protraction of the hydrolysis reaction. This means that PVA is always a co-polymer of PVA and PVAc (Hassan and Peppas, 2000) as visible in Figure 2 a. As a consequence, the degree of hydrolysis has a certain effect on the on the chemical properties of this material, crystallinity, the solubility in water (Mansur et al., 2008).
Figure 2 Chemical structure of (a) partially hydrolysed PVA and (b) fully hydrolysed PVA taken from the literature (DeMerlis and Schoneker, 2003).

PVA with a high degree of hydrolysis (DH= 98-99%) or partially hydrolysed (DH= 87-88% or DH= 80%) is commercially available. It has been shown that PVA with a high degree of hydrolysis (higher than 98%) is characterised by a reduced solubility in water; (Hassan and Peppas, 2000) reported that the presence of the remaining carboxylic groups help weaken the inter-intramolecular hydrogen bonds formed by the hydroxyl groups, so the temperature must be raised up to 70-80ºC for the dissolution to occur (Briscoe et al., 2000). Residual presence of acetate groups also influences the polymer crystallinity, as high hydrolysed PVA results in more difficult to crystallize by heat treatment form (Hassan and Peppas, 2000). PVA is a water soluble polymer and can form, after solidification, a hydrophilic network that swells in aqueous solutions, or biological fluids. This characteristic makes the polymer suitable for the production of microparticles for drug delivery; the water absorption produces the swelling of the material, which causes the release of the encapsulated molecule for diffusion through the polymeric matrix (Varshosaz and Koopaie, 2002). PVA can be chemically crosslinked using bifunctional crosslinking agents like: glutaraldehyde, acetaldehyde, formaldehyde, other monoaldehydes (Hassan and Peppas, 2000) and glyoxal (Zhang et al., 2009). The reaction needs the presence of acidic conditions to occur, strong acids such as sulphuric acid, acetic acid, hydrochloric acid are added as catalysts for the reaction. The chemical crosslinking reaction involves the formation of ether bonds between the hydroxyl groups of PVA and the carbonyl groups of the aldehydes (Mansur et al., 2008) forming acetal bridges as reported in Figure 3.
Figure 3 Poly (Vinyl) Alcohol chemical crosslinking reaction with glutaraldehyde.

Liquid PVA can solidified with physical methods (Bolto et al., 2009) such as freeze-thawing (Hassan and Peppas, 2000) or electron-beam irradiation (Peppas and Merrill, 1976, 1977) or by photocrosslinking (Schmedlen et al., 2002). PVA hydrogels have the capability to incorporate water and swell, the diffusion of the solvent through the polymer matrix produces the extension of the polymer segments. The penetration of water is the “rate-limiting step” for swelling and as a consequence, for the drug release. The degree of crosslinking plays a fundamental role on the swelling proprieties of the polymer and the release behaviour as well. It was seen that, for lower concentrations of crosslinker, the polymer can incorporate a higher amount of water and the degree of swelling increases, so modifying the crosslinking degree is possible to modulate the swelling of PVA and prolong the drug release (Bolto et al., 2009).

2.2.1.1.2 Methacrylic acid- methylmethacrylate copolymers (Eudragit polymers)

Of all the synthetic polymeric materials, those most commonly used as pH dependent polymers are methacrylic acid copolymers; known by the commercial name of Eudragit®. Eudragit® L100-55, Eudragit® L100 and Eudragit® S100 are the main ones used and they dissolve at pH above 5.5, 6.0 and 7.0 respectively. In the present work, the acrylic polymer Eudragit S100 was used as a gastro-resistant polymer, to produce a coating able to dissolve in neutral to basic fluids; typical of the small intestine and colon tract. Eudragit S100 (Figure 4) is composed by units of methacrylic acid, and methilmethacrylate in a ratio 1:2 (Yoo et al., 2011).
Figure 4 Chemical structure of Eudragit S100, ratio of carboxylic groups to ester groups is 1:2, image taken from literature (Yoo et al., 2011).

Due to its properties, Eudragit S100 is considered a material particularly suitable for the delivery to the distal region of the small intestine and to the colon (Mrsny, 2012). In some cases the Eudragit polymers were mixed in different ratios to manipulate the drug release within the desired range of pH (Khan et al., 1999; Zahirul et al., 2000). Other works report the use of Eudragit S100 for the coating of already produced microparticles for colonic release via oral delivery; for example coating of calcium pectinate beads encapsulating theophylline (Maestrelli et al., 2008) or the anticancer drug 5-fluorouracil (Jain et al., 2007), chitosan microparticles containing diclofenac sodium (Lorenzo-Lamosa et al., 1998), or mesalamine for the treatment of ulcerative colitis (Badhana et al., 2013), celecoxib loaded microparticles made of Poly ε-caprolactone (Ghorab et al., 2011), Poly Poly(lactide-co-glycolide) (PLGA) microparticles containing budesonide (glucocorticoid) (Krishnamachari et al., 2007). Eudragit® S100 coating was also used for the production of gastroretentive microparticles for stomach delivery (Jain et al., 2005, 2006) due to its capability to float over the stomach fluids and release the therapeutic over an extended period of time (Jain et al., 2006). In all the cases reported previously Eudragit® S100 was used as coating material, but microparticles were also entirely produced using Eudragit® S100: piroxicam loaded microparticles for enteric delivery were produced (Obeidat and Price, 2006) or microparticles for the oral delivery of enzymes (Rawat et al., 2007).

2.2.1.2 Natural polymer for drug delivery

Natural polymers are usually biocompatible and often have an outstanding biocompatibility (Pillai and Panchagnula, 2001), they are considered relatively safer than the synthetic polymers for pharmaceutical applications (Tiwari et al., 2014). A disadvantage is that they are affected by batch to batch variability due to the difficulty of the purification process
The sources of natural polymers are many, they can be extracted from plants, parts of animals, microorganisms as bacteria, or fungi (Tiwari et al., 2014). Table 2 reports the most common natural polymers used for the production of microparticles for the oral delivery of therapeutics with a summary of the main characteristics.

Table 2 Most commonly used natural polymers for the production of microparticles for oral release of therapeutics.

<table>
<thead>
<tr>
<th>Natural polymers</th>
<th>Principal uses and comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plant origin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>Biodegradable, non-toxic materials.</td>
<td>(Cellulose: Patel et al., 2006; Mastiholimath et al., 2008;</td>
</tr>
<tr>
<td>Pectin</td>
<td>Used in association with other polymers for the production of microparticles for oral vaccine and drugs delivery, oral anticancer treatment. Cellulose microparticles were used as floating delivery systems for the release in the stomach. Pectin, guar and acacia gum were used for the release in the colorectal area. Starch microparticles were used as oral adjuvant. Alginate is an anionic polymer used for the delivery of proteins and drugs in intestinal fluids. Carrageenan forms biodegradable hydrogels, it was used for the oral administration of peptides, proteins and drugs.</td>
<td>Pectin: Wong et al., 2011</td>
</tr>
<tr>
<td>Guar gum</td>
<td></td>
<td>Guar Gum: Chaurasia et al., 2006; George and Abraham, 2007;</td>
</tr>
<tr>
<td>Acacia gum</td>
<td></td>
<td>Acacia gum: Lamprecht et al., 2007;</td>
</tr>
<tr>
<td>Starch</td>
<td></td>
<td>Starch: Sturesson and Wikingsson, 2000; Wikingsson and Sjöholm, 2002;</td>
</tr>
<tr>
<td>Alginate</td>
<td></td>
<td>Alginate: Suksamran et al., 2009; Zhang et al., 2011; Carrageenan: Briones and Sato, 2010 )</td>
</tr>
<tr>
<td>Carrageenan</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Microbial source</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthan gum</td>
<td>Xanthan gum was used for the production of hydrophilic matrix for</td>
<td>(Xhantan gum: Maiti et al., 2007; Ray et al.,</td>
</tr>
<tr>
<td>Polymers</td>
<td>Characteristics</td>
<td>References</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Dextran</td>
<td>Anionic polymer with affinity for proteins, used for gastro-resistant formulations.</td>
<td>Dextran: Martins et al., 2007; Gellan: Narkar et al., 2010; Prezotti et al., 2014</td>
</tr>
<tr>
<td>Gellan</td>
<td>Forms strong gels even at low concentrations, has mucoadhesive properties, used for stomach specific release of drugs.</td>
<td></td>
</tr>
</tbody>
</table>

**Animal origin**

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan</td>
<td>Cationic, pH sensitive polymer with mucoadhesive properties. Used for oral gene delivery, oral vaccination, delivery of proteins and drugs.</td>
<td>Chitosan: Lubben and Verhoef, 2001; Guliyeva et al., 2006; Gelatine: Huang et al., 2014; Phromsopha and Baimark, 2014</td>
</tr>
<tr>
<td>Gelatine</td>
<td>Used for production of carriers for water soluble drug and gastro resistant formulations in association with other polymers.</td>
<td></td>
</tr>
</tbody>
</table>

### 2.2.1.2.1 Chitosan

Chitosan is a proven biocompatible natural polymer; it is the N-deacetylated derivative of chitin produced from crustacean shells, fungi, and insects. It is a polycation, soluble in acidic environment, with versatile mechanical proprieties (De Vos et al., 2013). It is composed by monosaccharides linked by linear β (1→4) bonds, in particular by 2-amino-2-deoxy-β-D-glucan combined with glycosidic linkages. Chitosan has been used for several biomedical applications (Berger et al., 2004), in ocular applications (Felt et al., 1999), implants (Azab et al., 2006) and in the pharmaceutical industry as an excipient (Illum, 1998). An interesting property of this natural polymer is its pH sensitivity which comes from the presence of the amino groups making it interesting for pH sensitive formulations. Below their pKa (6.3) the
amino groups of chitosan become positively charged; leading to the swelling of the polymer. This process is called *dynamic swelling* (Krishna Rao et al., 2006; Gunasekaran et al., 2006), and depends on two principal mechanisms: a *mass transfer* in the bulk of the polymer, and an *electrostatic repulsion* (Gunasekaran et al., 2006) among the polymeric chain. The ionization of the polymer under a certain pH leads to the formation of positive charges, which produce a high concentration of ions inside the gel. This produces the migration of the counter ions inside the polymer matrix, forming a difference of osmotic pressure between the inside and the outside of the gel. The difference in osmotic pressure causes the transfer of water molecules from the surroundings into the hydrogel reducing the difference of concentration between the inside and the outside of the gel. The water intake represents the primary reason of swelling. In addition, with crosslinking the material can maintain its three-dimensional structure after swelling. The reverse process occurs if the polymer is in basic conditions, causing hydrogel shrinkage. Another mechanism proposed for the swelling at acidic pH of this polymer involves the electrostatic repulsion of the polymeric chains. After the ionization of the amino groups, an electrostatic repulsion can occur between the charged polymeric chains, causing a stretching of the crosslinked bonds and the swelling of the material (Gunasekaran et al., 2006).

Microparticles can be produced by ionic crosslinking of chitosan with polyanions such as tripolyphosphate (TPP) (Shu and Zhu, 2002), hexametaphosphate (SHMP) (Gupta and Jabrail, 2006) or sulphuric acid (Cui et al., 2008). The ionic interaction with polyphosphate anions often produces microparticles with poor mechanical strength (Shu and Zhu, 2002; Angelova and Hunkeler, 2001). The use of SHMP produces more rigid microparticles due to the high density charge of SHMP anions, which provide strong electrostatic interactions with chitosan amino groups (Gupta and Jabrail, 2006). Chitosan has also been complexed with negatively charged materials such as alginate (Y. Zhang et al., 2011) and proteins (Kurukji et al., 2014) for the encapsulation and pH triggered release of molecules. Chemical crosslinking of chitosan is obtained by reaction with aldehydes (such as glutaraldehyde), glyoxal or ethylene glycol diglycidyl ether (Bhumkar and Pokharkar, 2006). The reaction with glutaraldehyde consists of a process involving the formation of a Shiff’s base with the amino groups of chitosan and the carbonyl groups of glututaraldehyde, as shown in Figure 5 (Wang et al., 2004).
The addition of another polymer able to react with the crosslinker, can reduce the chitosan crosslinking density and increase the number of protonable amino groups available. In this work the possibility to produce an interpenetrating network PVA-Chitosan for the production of pH-sensitive microparticles, using glutaraldehyde as crosslinker for both the polymers (Wang et al., 2004) and form a PVA-grafted-Chitosan particles for the delivery of drugs (Krishna Rao et al., 2006) was evaluated.

2.2.1.2.2 Alginate

Alginate is a water soluble linear anionic polysaccharide obtained from brown algae exhibiting excellent biocompatibility and biodegradability, it has many different applications in the field of biomedicine. It is composed of β-D-mannuronopyranosyl (called M blocks) and α-L-guluronopyranosyl (called G blocks), shown in Figure 6. Homopolymeric regions (composed by only M or only G blocks) are interspaced by MG heteropolymeric regions. The ratio and distribution of M blocks and G blocks influences the pH sensitivity of the material.
and the interaction with cations because it affects the relative position of the carboxylic groups (Alvarez-Lorenzo et al., 2013).

![Chemical structure of Alginate, modified from literature (Alvarez-Lorenzo et al., 2013).](image)

**Figure 6** Chemical structure of Alginate, modified from literature (Alvarez-Lorenzo et al., 2013).

Alginate can be ionically crosslinked using divalent or polyvalent cations, it was reported that the ionic interaction with Ca\(^{2+}\) ion is preferred as it binds selectively the glucuronic acid units in a way that the Ca\(^{2+}\) ions are located into electronegative cavities (like eggs in a box) from which the name “egg-box model” was derived (Grant et al., 1973; Fundueanu et al., 1999). Crosslinking alginate with Mg\(^{2+}\) ions has also been reported, for the production of monodisperse microspheres via a microchannel emulsification device (Khalid et al., 2015). The Mg\(^{2+}\) crosslinked alginate spheres showed stability over 10 days and were used for the encapsulation of L-ascorbic acid. Other divalent cations, such as Pb\(^{2+}\), Cu\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\), and Mn\(^{2+}\), will also crosslink alginate gels, but their application is avoided because they are reported to be toxic to cells (De Vos et al., 2013). Reports mention the production of alginate microparticles by ionotropic gelation with positively charged polymers such as chitosan (Y. Zhang et al., 2011; Wang et al., 2016) and polylisine (Ferreiro et al., 2002). The chemical crosslinking of alginate was obtained with glutaraldehyde (Kulkarni et al., 2000) even though the use of less toxic crosslinkers as genipin (Chen et al., 2004) is preferred for drug delivery. Due to its chemical structure alginate offers a wide range of functionalization possibilities making the polymer versatile for the use in drug delivery (Alvarez-Lorenzo et al., 2013). Alginate has become one of the most important biomaterials for several applications in drug delivery; often in association with other biocompatible polymers. Alginate- whey microparticles (Déat-Lainé et al., 2013) and alginate-chitosan (Y. Zhang et al., 2011) microparticles were produced for the oral administration of insulin. (Rastogi et al., 2007) reported the production of Ca\(^{2+}\) crosslinked alginate microparticles for the prolonged oral release of isoniazid. Ionically crosslinked alginate (with Ca\(^{2+}\)) was also used as coating material (of gelatine microparticles) to provide protection of a drug from the acidity of the
gastric juice due to its stability in low pH solutions; and for the subsequent delivery in intestinal fluids (Annan et al., 2008). Chitosan-alginate microparticles were used for the colon targeted delivery of drugs in the treatment of the “inflammatory bowel disease” (Wang et al., 2016) as well as for protein (Chen et al., 2004) and antineoplastic (Yu et al., 2008) drug delivery. Alginate and polylysine microparticles were produced for the encapsulation and delivery of nucleotides into the intestine (Ferreiro et al., 2002), the use of alginate has been reported for the production oral vaccine delivery vehicles (Kim et al., 2002). García-González et al., (2015) produced aerogel microspheres (obtained by an emulsion-gelation process followed by a supercritical CO2-assisted drying) for the oral administration of poorly water soluble drugs.

2.2.1.2.3 Gelatine

Gelatine is a colourless, flavourless, brittle (when dried) material that comes from the partial hydrolysis of the collagen from skin, connective tissue, etc. of various animals such as fish, pigs, cattle (Tiwari et al., 2014). Gelatine is a heterogeneous mixture of amino acids, a typical structures contains many residues of glycine, proline and 4-hydroxyproline as shown in Figure 7 (Hamman, 2010). Gelatine is a material advantageous for medical and pharmaceutical uses because it is biodegradable and biocompatible (Young et al., 2005). This material has been used as excipient in the pharmaceutical industry, or as plasma expander and sealant for vascular prostheses (Young et al., 2005). Gelatine is particularly sensitive to variation in environmental temperature, and gelling and melting point are two important parameters to consider when using this material for the production of microparticles for drug delivery. Generically, mammalian gelatines are characterized by a higher gelling and melting temperatures: porcine and bovine gelatine gel in a range from 20 to 25°C, and they melt between 28-31°C. In comparison, gelling temperature for fish gelatine is between 8 to 25°C, while the melting point is 11 to 28°C. This can be due the lower imino groups content in fish gelatine compared to mammalian gelatine, which seems to reduce the propensity of intermolecular helix formations (Karim and Bhat, 2009). In some cases the gelatine is added to the formulation to improve the mechanical, or biological, properties of other polymers; for example gelatine with alginate microparticles were produced for the delivery of drugs in the intestinal fluid (Huang et al., 2014), Eudragit coated gelatine microparticles were used for the
production of a gastro-resistant formulation for the oral administration of Nifedipine (Li et al., 2009)

**Figure 7** A typical structure of gelatine (-Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro-) modified from literature (Hamman, 2010).

### 2.2.2 Polymeric microparticles for cell encapsulation

The encapsulation of a living microorganism as well as cells, for the purpose of a therapy, is a relatively recent and promising technique advantageous for a broad range of sectors, from medicine to the food industry. The technology of cell encapsulation is applied as a method to deliver therapeutics in a desired rate and for longer period by controlled, or triggered, release of an active ingredient. Unlike the encapsulation of proteins, encapsulated cells can synthetize the therapeutic product, giving a physiological concentration of a drug with time (Murua et al., 2008). Furthermore, due to the in-situ manufacture of the active ingredient the risk of a toxic release of a therapeutic is avoided in the case of unexpected breaking of the encapsulated particles (Murua et al., 2008). The immobilization of cells in particles is widely discussed in tissue engineering; the possibility to encapsulate non-human cells reduces the host’s immune system response, facilitating transplantation as an alternative to the limited donor tissues available (Murua et al., 2008). The technique of cell encapsulation is being used for the production of a “Bio artificial Pancreas” (Nedovic, V., & Willaert, 2006), immunobarrier for islet cells transplantation (Nedovic, V., & Willaert, 2006; Duvivier-Kali et al., 2001; de Vos et al., 2006), the encapsulation of stem cells (Ma et al., 2003; Wang et al., 2009). Encapsulation of living cells was also adopted for the production of chemicals such as alcohol, organic acids, steroids, antibiotics, vaccines (Yoshida et al., 2003). Encapsulated
yeast in alginate and carrageenan beads were also used as biocatalysts in organic solvents for complex reduction reactions (Green et al., 1996). There are reports (Mandwal et al., 2004) of the use of immobilized yeast in a polyacrylamide hydrogel for the production of L-phenylacetyl carbinol; an intermediate in the synthesis of L-ephedrine (used as anti-asthmatic and decongestant). A variety of materials have been tested for the encapsulation of living cells: alginate (Cheong et al., 1993; Green et al., 1996; Song et al., 2003; de Vos et al., 2006; Shintaku et al., 2007; Graff, Hussain, et al., 2008; Annan et al., 2008; Wang et al., 2009; De Vos et al., 2013) is the most common and versatile; chitosan (Raymond et al., 2004; De Vos et al., 2013); gelatine (Hyndman et al., 1993; Annan et al., 2008); cellulose (Schaffellner et al., 2005; De Vos et al., 2013); dextran (Murua et al., 2008); carrageenan (Green et al., 1996; Raymond et al., 2004; Bhatia et al., 2005); poly(lactide-co-glycolide) (PLGA) (Murua et al., 2008; De Vos et al., 2013); Poly (Ethylene Glycol) (Koh et al., 2002; De Vos et al., 2013) have all been used individually, and in blends (Bhatia et al., 2005; Murua et al., 2008).

The release of probiotic cells (bacteria or yeast) in the intestinal-colon area is reported to be beneficial for the treatment of several pathologies (Anal and Singh, 2007), but to obtain the beneficial action it is crucial that the microorganism is viable when reaching the required site i.e. able to form colonies when re-cultured on solid agar. A severe loss of cell viability is reported after administration due to the harsh environment of the stomach (i.e. low pH) and the high bile salts concentration in the intestine (Cook et al., 2012). To overcome this problem, many formulations have been tested for the encapsulation of probiotics with the purpose of protecting them against the adverse conditions of the GI tract, some example are: the production of chitosan-coated alginate microparticles for the encapsulation of *Lactobacillus gasseri* and *Bifidobacterium bifidum* (Chávarri et al., 2010) for the specific release into the intestine; the encapsulation of *Bifidobacterium adolescentis* in alginate-coated gelatine microparticles to enhance their survival during the passage through the GI tract (Annan et al., 2008); and the intestinal delivery of *Lactobacillus acidophilus* and *Bifidobacterium lactis* by encapsulation in alginate with xanthan gum, or cellulose microparticles (Albertini et al., 2010). To achieve a targeted drug release in the intestinal area, it is also possible to coat the microparticles using a coating material resistant to the stomach acidic environment and which is soluble in basic-neutral conditions i.e. Eudragit S100 (L. Zhang et al., 2011). Eudragit coated microparticles were produced for the colonic release of therapeutics (L. Zhang et al., 2011; Paharia et al., 2007) or proteins (Hori et al.,
2005), while it has not yet been applied for the coating of encapsulated cells. In this thesis the use of gelatine as an encapsulating material for yeast cell with Eudragit S100 as coating material, for the targeted release of the yeast in the intestine-colon tract, is reported. The Eudragit coating was selected due to its formation in mild conditions (no crosslinking reactions but the formation of a layer by solvent evaporation) and the mechanical strength of the formed coating layer over the delicate gelatine microparticles (obtained by thermal gelation).
2.3 MICROPARTICLES PRODUCTION METHODS

2.3.1 Conventional techniques of microparticles production

Along with the right material, the choice of the right encapsulation technique is fundamental for the success of an encapsulation process. An overview of the most common techniques of microparticle production is reported in this chapter. Conventional methods of particle production are derived from three basic techniques: spray drying, phase separation (coacervation) and solvent extraction/evaporation (Freitas et al., 2005). The method of electrospaying is also briefly discussed as it has received increasing interest for the production of particles for pharmaceutical applications (Bock et al., 2012).

2.3.1.1 Spray drying technique

The spray drying technique is based on three steps which are 1) atomization, 2) solidification of the droplets or solvent evaporation 3) particle collection (Nandiyanto and Okuyama, 2011). The first and most important step of the process, is the atomization, where a solution (or slurry), called precursor, is converted into small droplets. The choice of the right atomizer is one of the most important in order to produce droplets with the desired dimension and with a good size distribution. The most common atomization technique is “temperature assisted evaporation”, which consists of the vaporization of the solution-slurry under certain temperature, lower than the evaporation temperature of the precursor. One of the problems related to this atomization technique is the high price. To overcome the problem, on the market are available different types of atomizers, that use different driving forces to assist the atomization process, such as pressure, centrifugal, electrostatic, or ultrasonic energy. The most common atomizers are rotary (where the driving force is the centrifugal energy), a pressure nozzle (atomization by pressure energy), two-fluid nozzle (which use the kinetic energy) and ultrasonic nebulizer (atomization by ultrasonic energy). The solidification step is also called droplets-to-particles conversion. This part of the process is crucial for the removal of the solvent from the produced droplets, and their solidification into particles. The solvent can be principally evaporated by three treatments: solvent evaporation by heated carrier gas, solvent evaporation by heating in a hot furnace or reactor, solvent evaporation by solvent diffusion using a diffusion dryer. The last step consists of the collection of the solid particles produced; for that purpose principally cyclones, filter bags, or an electric field precipitator are
used. The principal problem related to this technique is the use of high temperature, and for that reason it cannot be used with highly temperature-sensitive compounds.

2.3.1.2 Solvent extraction or evaporation

Another method of particle production is the technique of solvent extraction or evaporation. This technique does not require high working temperature or phase separation inducing agents, which is an advantage for the production of particles designed for pharmaceutical uses. Although it needs a careful choice of the material that has to be used and the conditions of encapsulation (Freiberg and Zhu, 2004). The phase separation technique could principally be divided into the following steps (Freitas et al., 2005):

I) The dispersion or dissolution of the therapeutic or the drug into a solvent (often an organic solvent) that contains also the polymeric material that will form the microparticles;

II) The emulsification of this polymeric phase into a second one that is immiscible with it (often an aqueous phase containing an emulsifier);

III) Removal of the solvent from the dispersed phase through the continuous phase (e.g. solvent evaporation) which produces the transformation of the liquid droplets into solid particles;

IV) The last passage is harvesting and drying of the particles.

2.3.1.3 Coacervation or phase separation

The technique of the coacervation and phase separation is one of the first process reported as an industrial method of production of microspheres and microcapsules as well. It is defined as a partial desolvation of a homogenous polymeric solution, into two phases: the coacervate (rich of polymer) and the coacervation medium (poor of the polymer). The process requires the use of desolvation agents to produce the phase separation, this is case called simple coacervation. Complex coacervation involves the interaction (a complexaction) of polymers with opposite charge. For the production of the microspheres, the gradual introduction of the desolvating agents produces the formation the formation of thin droplets called “coacervate nuclei”, that contains a high concentration of the polymer compared to the bulk of the solution (coacervation medium). The further addition of the coacervation agents allows to the “primary droplets” to grow larger and larger, and to coalesce, forming bigger droplets. Adjusting parameters like stirrer speed, stabilizer concentration etc. it is possible to obtain
droplets with size range from about 100 µm upwards. After the formation of the droplets, with processes like solvent removal and crosslinking reaction, it is possible to obtain the corresponding microparticles (Arshady, 1990).

2.3.1.4 Electrohydrodynamic atomization

The Electrohydrodynamic atomization (EHDA) technique is also called electrospraying and is a method of production of micro-structured materials (Xie et al., 2015). The process consists in breaking up a liquid jet into fine drops by the action of an external electric field. The generated electric stress overcomes the surface tension forces producing fine drops (process of atomization). A normal EHDA setup consists of a few components: a syringe pump, a syringe with a metal needle serving as a nozzle, an electric power source and a grounded collector. Once the drops are emitted from the tip of the needle, solvent evaporation occurs increasing the solute and charge concentrations. The solute within the drops evaporates forming solid microparticulates on the collector. In some cases the EHDA setup is equipped with chamber with air/ nitrogen flow to collect the particles through a filter. The use of a closed chamber produces a slow evaporation of the solvent leading to the formation of smaller particles with a smoother surface. In the EHDA technique it is crucial to select the proper solvent to dissolve the polymer as the surface morphology and particle size is strictly dependent on the solvent physicochemical properties. For EHDA using water a controlled inert gas or vacuum environment is required. There are reports of the production of microparticles by electrospraying for pharmaceutical applications loaded with different types of drugs or proteins (Bock et al., 2012).

2.3.2 Microparticles produced by emulsification

2.3.2.1 Emulsions

Emulsions are by definition colloidal dispersions of one or more immiscible phases: the discontinuous, dispersed or inner phase is presents as drops in a continuous or external phase. Presence of surfactants is required to avoid coalescence of the two phases, showing that the emulsions are thermodynamically unstable systems. Surfactants are required to reduce the interfacial tension existing between the two immiscible phases; the work (W) required for the production of an emulsion can be expressed as (Kontogeorgis and Kiil, 2016):
where \( \gamma \) is the interfacial tension and \( \Delta A \) is the change in the surface area.

Destabilization of the emulsions can occur with three main mechanisms: creaming, flocculation or coagulation, and coalescence. Creaming or sedimentation phenomena can be caused by a density difference between the two phases, it is a separation caused by the upward or downward movement of the droplets. Flocculation is described as the aggregation of the droplets which collide due to attractive forces. Coalescence occurs when the drops merge losing their identity. Stability of the emulsions can be improved by changing the interfacial tension, providing a mechanically strong and elastic interfacial film, having a relatively small amount of dispersed phase and by narrowing the drop size distribution (Kontogeorgis and Kiil, 2016).

Generally the emulsions can be classified as water in oil (W/O) or oil in water (O/W) according to the type of liquid forming the dispersed and continuous phase. These emulsions are called single emulsions, multiple emulsions can be produced by emulsifying a single emulsion into an outer continuous phase. Multiple emulsions can be water in oil in water (W/O/W) or oil in water in oil (O/W/O). The properties of an emulsion mainly depend on the emulsion microstructure which is a function of the mean droplet size and size distribution (Barbosa-Canovas et al., 2009). In order to obtain stable emulsions an emulsifying agent is required to lower the interfacial tension. A surfactant (or surface active agent) is an amphiphilic molecule composed by a “polar” head (ionic- anionic or cationic, or polar group) and a hydrophobic part (Kontogeorgis and Kiil, 2016). This chemical structure makes the surfactant able to absorb at the air-liquid surface or at the water-oil interface reducing the surface or interfacial tension. Surfactants can be classified depending on the nature of the head as anionic, non-ionic or cationic.(Kontogeorgis and Kiil, 2016). The decrease of the surface tension (and interfacial) is observed below a certain concentration called the Critical Micelle Concentration (CMC) above which the surfactants form a new entity often spherical called a micelle (Barbosa-Canovas et al., 2009). A second classification of surfactants can be done considering the hydrophilic-lipophilic balance (HLB). The HLB is an empirical parameter first introduced by William C. Griffin (Griffin, 1949) which gives information
about the hydrophilic-lipophilic nature of a surfactant or emulsifier, it can be calculated accordingly to **Equ. 2**.

\[
HLB = 20 \times \frac{M_h}{M}
\]  

**Equ. 2**

Where \(M_h\) is the molecular weight of the hydrophilic portion of the surfactant molecule and \(M\) is the molecular weight of the whole molecule. In (Griffin, 1949) it is reported that surfactant with a HLB number between 4 and 6 is appropriate for the production of w/o emulsions while with HLB numbers in the range 8-18 it is possible to produce o/w emulsions. In this work o/w emulsions where mainly produced, the surfactant used to stabilize the water drops and their chemical structure are reported in **Figure 8**.

![Figure 8](image-url)

Span 80 (HLB= 4.3)

PGPR (HLB= 1.5- 2)

ABIL EM 90 (HLB= 5)
Figure 8 Oil soluble surfactants used in this thesis: Span 80 (sorbitane monooleate), PGPR (polyglycerol polyricinoleate) and ABIL EM 90 (modified polyether–polysiloxane).

2.3.2.2 Methods of emulsion production

Several conventional methods have been described for the production of emulsions, mechanical processes are the most frequently applied; these methods often involve the production of a primary emulsion by gentle stirring which is subsequently homogenized to obtain a further reduction of the drop size. Rotor-stator systems are applied for the continuous or discontinuous production of emulsions, the mechanical energy is the driving force for the droplet production. For the production of fine-dispersed emulsions high-pressure systems such as high-pressure homogenizers are commonly used; with this method the mechanical energy required for the drop formation is provided by a pressure difference. Emulsions are also produced by ultrasound systems in which the drop breaking occurs due to the turbulence caused by ultrasound waves. This method has been mainly applied for a lab scale production as it generates drops with a wide size distribution (Barbosa-Canovas et al., 2009). As discussed above these methods of emulsion production are based on droplet disruption, which can be potentially a disadvantage for the encapsulation of delicate compounds. Emulsions can also be produced by droplets formation from microstructured or membrane systems. The production of drops using microfluidic devices consists of injecting one liquid (the dispersed phase) through a single microchannel into a second perpendicular microchannel channel transporting the continuous phase; in a configuration called T-junction (Figure 9 a). In an alternative configuration the drops are introduced into a microchannel and subsequently break up in smaller drops while passing through a constriction (flow focusing devices) (Vladisavljevic, 2016) as shown in Figure 9 c and d. For these systems the drop generation is driven by the shear exerted by the flow of the continuous phase, the continuous phase can also flow with a cross-flow mode (i.e. T and Y junction) or co-flow mode (flow focusing systems) (Maan et al., 2015). Spontaneous drop break up systems are also available, they are characterized by two different channel depths forming a shallow structure called terrace. The dispersed phase is initially compressed in a disk-like shape on the terrace and when it reaches the continuous phase channel it spontaneously breaks into spherical drops (Maan et al., 2015). The main advantage of the microchannel devices is the production of extremely uniform sized emulsions with a CV% of around 3% (Vladisavljevic, 2016), but the main
disadvantage is the low production rate and difficult scale up nature for larger throughput (Spyropoulos et al., 2014a). Using microfluidic emulsification devices drops can be formed by several microchannels in parallel, it was suggested that it may be possible to achieve a throughput up to 100 kg of emulsion per hour (Vladisavljević et al., 2012) with a CV% up to 5%.

Figure 9 Examples of microfluidic devices (a) T- junction (Ushikubo et al., 2014), (b) Y-junction (Ushikubo et al., 2014), (c) Flow- focusing system for the production of a single emulsion (Hughes et al., 2013) and (d) a double emulsion (Hughes et al., 2013).

2.3.3 Emulsions production by Membrane Emulsification

With membrane devices the emulsion drops are generated by injection of the dispersed phase or an emulsified mixture through the pores of a membrane. The process is called Membrane Emulsification (ME) and uniform emulsions can be produced (CV% between 10- 20%) with a higher productivity compared to microfluidic devices (several tonnes per hour) (Vladisavljević et al., 2012). Nakashima and Shimizu in the late 1980, fabricated a particular glass membrane called SPG (Shirasu Porous Glass) (Nakashima and Shimizu, 1986), that was successfully used for the production of uniform emulsion of kerosene in water and water in kerosene. In the last 20 years the interest on this method increased thanks to the possibility to control effectively the droplet dimensions by varying the operating conditions of the process (Vladisavljević and Williams, 2005). The membrane emulsification technique overcomes
some of the disadvantages typical of the classical methods of emulsions production such as: unreliable scale-up, insufficient droplet uniformity, high mechanical stress due to high forces applied, and poor batch to batch reproducibility (Spyropoulos et al., 2011). The process involves the use of an applied force to press the dispersed phase through a porous membrane into the continuous phase. The droplets are formed by a direct permeation of the dispersed phase through the micropores of a membrane and a shear stress applied over the membrane surface produces the detachment of the formed droplets. Compared to the conventional method of emulsion production (Spyropoulos et al., 2011) the shear stress applied in the ME is lower allowing the use of highly sensitive ingredients. Furthermore, the ME process allows the production of emulsions with lower energy consumption ($10^4$-$10^6$ J m$^{-3}$) compared to the conventional method mentioned above ($10^6$-$10^8$ J m$^{-3}$) (Joscelyne and Trägårdh, 1999). It is possible to inject a pure dispersed phase, in this case the process is called direct ME, or a pre-mixed dispersed phase (pre-mix ME process) (Charcosset, 2009). With the conventional method of the direct ME droplets are produced in situ by pressing through the membrane pores the pure dispersed phase, into an immiscible second phase (continuous phase). The membranes used can be hydrophobic or hydrophilic depending on the type of emulsion required: a hydrophobic membrane is normally used to produce W/O emulsions, otherwise a hydrophilic membrane is used for the production of O/W emulsions. Theoretically the membrane surface (where the droplets are formed) should not be wet by the dispersed phase (Nakashima et al., 1992). The detachment of the formed droplets is assisted by a shear stress applied over the membrane surface, recirculating the continuous phase with a low-shear pump or by its agitation (Vladisavljević and Williams, 2005). In pre-mix membrane emulsification instead, a preliminary emulsified phase (rather than a pure phase) is injected through the membrane into a third liquid. The first emulsion is generally produced by conventional methods like a stirrer or a homogenizer; then it is forced through the membrane. With the pre-mix higher dispersed phase fluxes are achieved (typically above $1$ m$^3$ m$^{-2}$ h$^{-1}$) compared to the direct ME ($0.01$-$0.1$ m$^3$ m$^{-2}$ h$^{-1}$) due to the generally lower viscosity than pure dispersed phase (Lloyd et al., 2015), allowing the production of smaller droplets with the same type of membrane and phase composition (Vladisavljević and Williams, 2005).

In the process of ME several factors affect the final drops size such as the shear stress applied, the membrane type and pore size, dispersed phase flux, surfactant used, viscosity of the dispersed and continuous phase (Joscelyne and Trägårdh, 1999; Vladisavljević and
Williams, 2005; Charcosset, 2009; Nazir et al., 2010; Spyropoulos et al., 2014a). Many different membrane types are now available for ME and choice of the most appropriate depends on the type of emulsion required (e.g. W/O or O/W). The first type of the membrane developed specifically for membrane emulsification was the Shirasu porous glass (SPG), which is characterized by interconnected pores and a wide range of dimensions of the pores is available (0.05 μm – 20 μm) (Nakashima and Shimizu, 1986). The SPG membranes have also a high porosity, between 50-60% (Charcosset, 2009). The SPG membranes can also be rendered hydrophobic by chemical modifications (Kukizaki and Wada, 2008). Microsieve membranes (Holdich et al., 2012) are also often used in ME which are instead ultra-thin foils with linear pores and very low internal pore area, one of the advantages of this type of membrane is the possibility to reach high transmembrane flux with low transmembrane pressure (Wagdare et al., 2010). Most common microsieve membranes used in ME are: silicon nitride AquamarijnTM microsieves, nickel microsieves produced by using UV-LIGA process (Nazir et al., 2010), produced by Micropore Technology Ltd (Egidi et al., 2008), laser drilled alluminium and stainless steel foil (Vladisavljević and Williams, 2006). Nickel microsieve membranes can be produced with different geometry of the pores, including slotted pores (Holdich et al., 2006). Microsieve membranes are also less inclined to foul compared to the SPG membrane, which have low surface porosity and high tortuosity of the pores. Membrane pore size is a crucial parameter affecting the drop size; in literature there is reported a linear correlation between the average pore diameter of the membrane and the average droplet diameter (Vladisavljević and Williams, 2005). In ME an important role is also played by the emulsifier type, it quickly diffuse to the oil-water interface to facilitate the droplet detachment from the membrane and stabilize the emulsion reducing the interfacial tension (Vladisavljević et al., 2012). The surfactant should not reduce too much the value of interfacial tension because in that case the dispersed phase would just pass through the membrane pore without forming the droplets. The velocity in which the surfactant’s molecules reach the interface is also important: the faster the emulsifier absorbs at the interface, the smaller the resulting drops are (Vladisavljević et al., 2012). It should be avoided that the surfactant molecules are adsorbed on the membrane surface, because this could foul the membrane pores and, more likely, assist the dispersed phase to spread over the membrane (Vladisavljević, 2015). It was shown (Nakashima et al., 1993) that surfactant molecules should not have functional groups with opposite charge to that of the membrane surface to avoid membrane fouling. In order to obtain the detachment of the droplets from the
membrane surface, a shear stress is generated (in some cases obtained by the movement of the continuous phase) in a controlled manner to allow a better control over the drop size. Depending on the principle of the shear stress generation several ME techniques have been described (Spyropoulos et al., 2014a), they can be classified as stationary membrane systems (or moving continuous phase ME) as Crossflow ME (Figure 10 a); where shear is generated by the recirculation of the continuous phase across the membrane surface (Nakashima et al., 2000; Joscelyne and Trägårdh, 1999; Ho et al., 2013), the Stirred ME (Figure 10 b) (Santos et al., 2015); where the rotation of a stirrer generates the movement of the continuous phase, Pulsed ME (Figure 10 c) (Holdich et al., 2012) in which the cross-flow of the continuous phase is combined with its pulsation across the membrane surface, Pulsed back-and-forward ME (Figure 10 d) (Piacentini et al., 2014) where the continuous phase is moved back and forward tangentially to the membrane surface in a pulsed cyclic flow and nonstationary membrane devices (or moving membrane) such as Vibrating (Holdich, 2010) or Rotating (Figure 10 e and f respectively) (Vladisavljević and Williams, 2006) ME where the shear is produced by the vibration or rotation of the membrane respectively, Azimuthally Oscillating ME (Figure 10 g); where a non-stationary cylindrical membrane is rotated backward and forward (Silva et al., 2015).

Figure 10 Different ME systems classified dependently to the principle of shear generation; (a) Cross-flow ME, (b) Stirred ME, (c) Pulsed ME, (d) Pulsed back and forward ME, (e) Vibrating ME, (f) Rotating ME and (g) Azimuthally oscillating ME.
2.3.3.1 Droplet size modelling

There are several forces acting on a growing droplet at the membrane pore opening, those forces can be grouped in *detaching forces* and *retaining forces* (Spyropoulos et al., 2014b) **Figure 11**.

**Figure 11** Forces acting on a growing drop at the membrane pore opening.

The key retaining force during the drop formation is the capillary force, $F_{Ca}$ (**Equ. 4**) keeps the drop on the membrane surface and it is the effect of the dispersed phase adhesion around the edge of the pore opening. The key detaching force is the drag force ($F_D$) (**Equ. 5**) it is created by the continuous phase flowing past the droplet and parallel to the membrane surface. The other detaching forces are: $F_{stat}$ static pressure difference force which due to a pressure difference between the inside and the outside of the droplet, the dynamic lift force, $F_L$ which is the result of the asymmetric velocity profile of the continuous phase near the droplet, $F_B$ is the buoyancy force due to the density difference between the dispersed and the continuous phase (it is only important for very large drop sizes (Kosvintsev et al., 2008)), $F_I$ is the inertial force and it is associated with the mass of the fluid flowing out from the opening of the pore (Rayner et al., 2004). It was shown that $F_B$ and $F_I$ are from 6 to 9 times smaller than the drag and capillary forces therefore they can be neglected for force balance type model (Rayner et al., 2004). In this work for the droplet diameter calculation the simplest approach introduced in (Kosvintsev et al., 2005) was adopted. As the pressure inside a deforming drop will be uniform, it is possible to write a force balance between the two main forces acting on a drop growing at a single pore: the capillary force $F_{Ca}$ and the net detaching force $F_D$ (Peng and Williams, 1998; XU et al., 2005; Kosvintsev et al., 2008)
\[ F_{ca} = F_D \]  \hspace{1cm} \text{Equ. 3}

\( F_{ca} \) is calculated from Young-Laplace equation for the capillary pressure, considering \( \theta = 0 \) (fully wetting):

\[ F_{ca} = 2\gamma\pi r_p \]  \hspace{1cm} \text{Equ. 4}

where \( \gamma \) is the interfacial tension between the two phases, \( r_p \) is the pore radius. The drag force is expressed as a modified Stoke’s equation for Newtonian liquids (Keh and Chen, 2001; Kosvintsev et al., 2005);

\[ F_D = B3\pi x\tau \left( \frac{x^2}{2} - r_p^2 \right) \]  \hspace{1cm} \text{Equ. 5}

where \( B \) is a constant that was taken as equal to 3 (Kosvintsev et al., 2005) to consider the nearness of a wall (Keh and Chen, 2001), \( x \) is the formed droplet diameter and \( \tau \) is the shear stress. When the detaching force prevails on the retaining force the drop is detached from the membrane surface, solving \text{Equ. 6} for \( x \) gives the resulting drop diameter

\[ 2\gamma\pi r_p = 9\pi x\tau \left( \frac{x^2}{2} - r_p^2 \right) \]  \hspace{1cm} \text{Equ. 6}

\[ x = \frac{\sqrt{18 \tau^2 r_p^2 + 2\sqrt{81 \tau^4 r_p^4 + 4 r_p^4 \tau^2 \gamma^2}}} {3 \tau} \]  \hspace{1cm} \text{Equ. 7}

For the simple Dispersion cell system (discussed in \textit{subchapter 5.1}) the value of the shear stress \( \tau \) varies depending on the distance from the centre of the membrane, and it can be calculated from the following equations (Stillwell et al., 2007) using an approach described in (Ciofalo et al., 1996):

\[ \tau = 0.825 \eta \omega \delta \quad r < r_{\text{trans}} \]  \hspace{1cm} \text{Equ. 8}
\[ \tau = 0.825 \eta \omega r_{\text{trans}} \left( \frac{r_{\text{trans}}}{r} \right)^{0.6} \frac{1}{\delta} \quad r > r_{\text{trans}} \quad \text{Equ. 9} \]

Where \( \eta \) is the coefficient of dynamic viscosity of the continuous phase, \( \omega \) is the angular velocity. In order to determine the drop dimension, it can be assumed that the appropriate shear stress to use in the equation is the maximum value, the maximum value of shear (\( \tau_{\text{max}} \)) is reached at distance \( r_{\text{trans}} \) from the centre of the membrane;

\[ \tau_{\text{max}} = 0.825 \eta \omega r_{\text{trans}} \frac{1}{\delta} \quad \text{Equ. 10} \]

Where \( r_{\text{trans}} \) represents the transitional radius, the point in which the rotation of the continuous phase changes from a free to a forced vortex and it can be calculated using Equ. 11 (Kosvintsev et al., 2005)

\[ r_{\text{trans}} = \frac{D}{2} \frac{1.23 \left( 0.57 + 0.35 \frac{D}{T} \right) \left( \frac{b_h}{T} \right)^{0.036} n_b^{0.116}}{1000 + 1.43 Re} \quad \text{Equ. 11} \]

where \( b_h \) is the blade height, \( T \) is the cylinder width, \( D \) is the stirrer width, and \( n_b \) is the number of blades. \( Re \) represents the Reynolds Number calculated for a stirred vessel \( Re = \frac{\rho \omega D^2}{2\pi \eta} \), where \( \rho \) is the continuous phase density. The shear at the membrane surface is calculated assuming that it is equal to zero in the boundary layer over a thickness (\( \delta \)) given from the Landau-Lifshitz (Landau and Lifshitz, 1959) equation:

\[ \delta = \sqrt{\frac{\eta}{\rho \omega}} \quad \text{Equ. 12} \]

However, Equ. 7 (page 41) does not take into account the “necking” effect: the existence of a neck between the forming drop and the membrane pore. In (Dragosavac et al., 2008; Egidi et al., 2008) a modified expression of the capillary force is reported to include the effect of the “neck”.
When modelling the drop size for the Pulsed (Oscillatory) flow ME system (please refer to subchapter 3.5), the shear stress at the membrane surface was calculated on the wave equation for the shear stress (Holdich et al., 2012)

\[ \tau = v_0 \sqrt{\frac{\omega_f \mu \rho}{2}} [\sin(\omega_f t) - \cos(\omega_f t)] \]  \hspace{1cm} \text{Equ. 13}

Where \( \omega_f \) is the angular frequency calculated as

\[ \omega_f = 2\pi f \]  \hspace{1cm} \text{Equ. 14}

where \( f \) is the frequency of the oscillation while \( v_0 \) is the peak velocity both related to the angular frequency and the amplitude of the oscillation \( a \)

\[ v_0 = \omega_f a \]  \hspace{1cm} \text{Equ. 15}

The peak shear event occurs when the value of wall shear provided by \textbf{Equ. 13} is at maximum, the maximum shear is reached twice per cycle and this value is included in \textbf{Equ. 7} (page 41) for the determination of \( x \) (Holdich et al., 2012; Piacentini et al., 2013; Silva et al., 2015).

\[ \tau_{max} = \omega_f^{3/2} a (\rho \mu / 2)^{1/2} = 2a (\pi f)^{3/2} (\rho \mu)^{1/2} \]  \hspace{1cm} \text{Equ. 16}
3. EXPERIMENTAL

3.1 DISPERSION CELL SYSTEM

3.1.1 Membranes

The W/O emulsions produced using the Dispersion Cell system were obtained using flat disk membranes all supplied by Micropore Technologies Ltd (Redcar, UK). The membranes used were nickel or stainless steel membranes, either PTFE or FAS (fluorinated compounds) coated to obtain the hydrophobicity. The membranes had uniform cylindrical pores with diameters of 10, 20 and 30 µm and distance between the pores of 80 or 200 µm. For some of the experiments the pores were distributed into a narrow ring portion placed at distance $r_{trans}$ from the centre of the membrane; in this area the shear reaches its maximum value. This type of membrane is named “ring membrane”, further details are given in the experimental section (please refer to subchapters 4.1.1.2.1 and 4.1.1.2.2). A schematic representation of the whole and ringed membrane is shown in Figure 12 which also reports the diameters measured, area of the full membrane was 0.000855 m² while the area of the ringed membrane was 0.000185 m².

![Figure 12](image)

*Figure 12* Schematic representation of the whole membrane (a) and the ringed membrane (b).

The pores on the membranes are oriented in a perfectly hexagonal array with a pore in the centre of each hexagon as illustrated in Figure 13 a and b. Using Equ. 17 (Dragosavac et al., 2008) the porosity of the membrane ($\varepsilon$) was determined,
\[ \varepsilon = \frac{3A_{pore}}{A_{hexagon}} = \frac{3 \left( \frac{d_p^2 \pi}{4} \right)}{\frac{3\sqrt{3}}{2} L^2} = \frac{\pi}{2\sqrt{3}} \left( \frac{d_p}{L} \right)^2 \]  

Equ. 17

Where \( d_p \) is the pore diameter

\( L \) is the space between the pores

a schematic explanation of the membrane porosity calculation is reported in Figure 13 a.

**Figure 13** Schematic representation of the hexagonal array of the pores on a disk membrane, 
\( L \) represents the distance between the pores (Dragosavac et al., 2008) (a) and microphotograph of a 30 \( \mu \)m pore size and 200 \( \mu \)m pore spacing membrane (b).

The porosity measured for the membranes used in this work was between 0.2 and 12.8%.
3.1.2 General procedure

The process of emulsification using the Micropore Technology Ltd. Dispersion Cell can be described reporting a general working procedure. A schematic illustration of the Dispersion Cell device is provided in Figure 14. The chamber at the bottom of Dispersion Cell base is initially filled with continuous phase and the remaining air is removed using a plastic syringe. The metallic membrane is then placed in the base (above the chamber) with the shiny side up, a PTFE o-ring is placed on the top of the membrane to ensure the sealing of the system after the positioning of the glass cylinder (the ring is resistant to the organic solvent as it does not swell). The glass cylinder is tighten at the base to avoid oil leakage. The required amount of continuous phase is gently poured into the glass cylinder, a maximum volume of 100 cm$^3$ was used. Subsequently the two blades paddle stirrer is fixed on the glass cylinder right above the membrane surface. The stirrer is governed by a DC power supply INSTEK, model: PR3060 which provides the shear at the membrane surface. The speed of the stirrer was tested from 1 to 16 V providing velocities from 200 to 2600 rotations per minutes. The corresponding shear was calculated accordingly with the composition of the emulsion produced and reported in each section. The dispersed phase was injected by using a syringe pump (World Precision Instrument Inc., AL-1000, UK) which is connected to the base of the Dispersion Cell with a rubber tube and a T- junction to the tip of a glass syringe (containing the dispersed phase) and (on the other side) to the tip of a plastic syringe used to remove the air bubbles from the tube. The syringe pump provided a constant flow rate from 0.17 to 10 mL min$^{-1}$ corresponding for the standard membrane to a flux between 12 to 702 L h$^{-1}$m$^{-2}$ and for the ringed membrane to a flux between of 54 to 3250 L h$^{-1}$m$^{-2}$. To produce the emulsion the desired amount of the dispersed phase is injected into the Cell while the stirrer provides desired shear stress. Once the emulsion is produced, the stirrer is removed from the top of the glass cylinder and the emulsion is simply recovered by pouring it into a beaker.
Figure 14 Schematic illustration of the Dispersion Cell device for Membrane emulsification.

The Dispersion Cell was used to determine the optimal conditions for production of W/O emulsions using several formulations. The Dispersion Cell is particularly advantageous as a lab-scale device for the investigation of a specific formulation as it is portable, it requires low energy, and the amount of continuous phase used is quite low (maximum of 100 cm³). A summary of the principal formulations tested using the Dispersion Cell is reported in Table 3 with the range of shear stress applied and the flux, membrane type and the membranes pore size and pore spacing is also reported.

Table 3 Basic formulations of the emulsions produced with the Dispersion Cell and membrane types used. DP: dispersed phase, CP: continuous phase, DP flux: flux of the dispersed phase.

<table>
<thead>
<tr>
<th>Emulsion type</th>
<th>DP</th>
<th>CP</th>
<th>Shear stress (Pa)</th>
<th>DP flux (Lh⁻¹m⁻²)</th>
<th>Membrane type (coating material)</th>
<th>Membrane pore size/spacing (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W/O</td>
<td>15 wt.% PVA</td>
<td>2 wt.% PGPR in Miglyol</td>
<td>2-59</td>
<td>12-1250</td>
<td>Nickel standard (PTFE)/stainless steel ringed</td>
<td>10/200</td>
</tr>
<tr>
<td>W/O</td>
<td>15 wt.% PVA-1 wt.%</td>
<td>2 wt.% ABIL EM 90 in</td>
<td>35</td>
<td>210-270</td>
<td>Nickel standard</td>
<td>10, 20/200</td>
</tr>
</tbody>
</table>
3.1.3 Flat disk membrane cleaning

After the production of the emulsion the glass cylinder is unscrewed from the base and the membrane is collected for the washing. For the hydrophobic (coated) membranes the prolonged washing in water, long time of sonication and the use of strong acid and base can damage or remove the hydrophobic coating. The PTFE coating is thermally bonded and it is less resistant comparing to the FAS coating which is chemically bonded. The PTFE or FAS membrane was washed initially with running water and washing up liquid for few seconds to remove the excess of oil and polymeric phase, subsequently it was placed in a beaker and submerged with warm soapy water. The membrane was then sonicated for maximum 30 seconds and dried using the compressed air. To preserve the hydrophobicity the membranes were soaked in pure oil phase without any surfactant. The stainless steel membrane was not
coated, hence it was washed by sonication in warm soapy water for 1 minute and subsequently rinsed with pure water. Afterwards the membrane is placed in a beaker and a solution of 2 wt.% citric acid (Fisher Scientific, UK) was added. The membrane in citric acid is sonicated for 1 minute and rinsed in deionized water. After the drying the membrane was left in pure oil phase.

3.2 EMULSIONS CHARACTERIZATION

3.2.1 Droplet size analysis and uniformity determination

The sizes of freshly prepared aqueous droplets in the oil phase, as well as solid particles were determined using a Leitz Ergolux optical microscope with an attached Pulnix TM-6CN monochrome camera, or an optical/fluorescent microscope (GXML3201, GX microscope) with an attached Retiga 6000 colour camera. The scale was changed accordingly with the appropriate ratio pixels/μm determined for each magnification lens depending on the microscope type. The droplets sometimes burst when placed on a microscopic slide due to surface tension effects, so photographs of the droplets had to be taken very quickly. For each experiment, numerous photos were taken and at least 300 droplets, or particles, were measured. The measurement of the drop size was done using the Java-based image processing package ImageJ available for the online download, or using the modified Matlab script “imfindcircles” available on line (more detailed description is reported in the Appendix D.2). The conversion pixels into μm was done using measuring a microscope graticule slide and the software Microsoft paint®. The droplet size was reported as $D_{av}$, mean average droplet diameter using the following formula:

$$D_{av} = \frac{\sum_{i=1}^{k} n_{i}d_{i}}{N} \quad \text{Equ. 18}$$

Where $d_{i}$ is the $i^{th}$ diameter of the droplet,

$n_{i}$ is the number of drops in the size range,

$N$ is the total number of the droplets counted.

The uniformity of the emulsion is reported as a value of Coefficient of Variation % (CV%) calculated as follows:
The densities of both continuous and dispersed phases were measured using a specific glass density bottle (Pycnometer). The dispersed and continuous phase viscosities were measured using a Rheometer AR100-N (TA instrument, USA), with a cone-plate configuration. The cone geometries used were: a 4 cm in diameter, 1.59° geometry with a truncation of 56 µm for the more viscous solutions or a 6 cm in diameter, 0.59° geometry with a truncation of 27 µm for less viscous solutions. For the formulations containing the porcine skin gelatine (in order not to solidify) the viscosity measurements were performed at 40 °C (temperature during the emulsification process) while for all the other formulations the measurements were done at 20 °C. Most of liquids reported in Table 4 showed a Newtonian behaviour, the only exceptions were the phases composed by 3 wt.% CS, 15 wt.% PVA+ 1 wt.% CS and 15 wt.% PVA+ 3 wt.% CS. For these phases a slight reduction of the viscosity with the increase of the shear rate was measured, the values of (maximum) shear rate at which the measurements were taken are reported at the bottom of the table. The equilibrium interfacial tensions of the W/O emulsions were measured using the Du Nouy ring method (Lunkenheimer and Wantke, 1981) using White Electric Instrument Tensiometer (model DB2KS). Further information for the use of the Tensiometer are reported in the Appendix A. All the physical measurements performed on the continuous and dispersed phase used are shown in Table 4.
Table 4 Densities, viscosities and interfacial tensions measured for the main formulations used in this work. PVA: Poly (Vinyl alcohol), CS: chitosan, PG: porcine skin gelatine, FG: fish gelatine.

<table>
<thead>
<tr>
<th>Continuous phase</th>
<th>Dispersed phase</th>
<th>Equilibrium interfacial tension (mN m⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viscosity (mPa s)</td>
<td>Density (Kg m⁻³)</td>
</tr>
<tr>
<td>Miglyol</td>
<td>8.1±0.1</td>
<td>910.5±0.1</td>
</tr>
<tr>
<td>2 wt.% PGPR in Miglyol</td>
<td>11.3±2.2</td>
<td>15 wt.%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 wt.% Span 80 in Miglyol</td>
<td>8.4±1.2</td>
<td>15 wt.%</td>
</tr>
<tr>
<td>2 wt.% ABIL EM 90 in Miglyol</td>
<td></td>
<td>15 wt.%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wt.% CS</td>
<td>36.8±2.5</td>
<td>1008.4±0.5</td>
</tr>
<tr>
<td>2 wt.% CS</td>
<td>33.1±2.3</td>
<td>1014.6±0.5</td>
</tr>
<tr>
<td>3 wt.% CS</td>
<td>61.4(+)</td>
<td>1022.1±0.4</td>
</tr>
<tr>
<td>15 wt.% PVA+ 1 wt.% CS</td>
<td>496.1(++)</td>
<td>1045.2±0.1</td>
</tr>
<tr>
<td>5 wt.% PVA+ 1 wt.% CS</td>
<td>217.0±5.3</td>
<td>1015.5±0.1</td>
</tr>
<tr>
<td></td>
<td>15 wt.% PVA + 3 wt.% CS</td>
<td>15,100.0(***)</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>Gelatine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 wt.% FG</td>
<td></td>
<td>59.6±0.79</td>
</tr>
<tr>
<td><strong>2 wt.% Span 80 in kerosene</strong></td>
<td>1.9±0.1</td>
<td>808.3±1.0</td>
</tr>
<tr>
<td>5 wt.% PG + 2 wt.% CS</td>
<td>4.5±0.1</td>
<td>1010.82±0.6</td>
</tr>
<tr>
<td>5 wt.% PG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(*) Shear rate = 1,300 s⁻¹
(**) Shear rate = 200 s⁻¹
(***) Shear rate = 2.6 s⁻¹
3.2.3 Contact angle measurements

Contact angle measurements were done using pure deionized water or a solution of 15 wt.% of PVA on the surface of a nickel standard membrane made hydrophobic by a pre- treatment using PTFE (poly(tetrafluoroethylene)), and untreated stainless steel membrane. The measurements and photos were obtained using a Drop Shape Analyzer, DSA 100, Krüss, Germany. The results are reported in Table 5, and images of the drop sitting on the membrane surface are shown in Figure 15.

Table 5 Contact angle measurements (dispersed phase on the membrane surface)

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Dispersed phase</th>
<th>Theta(average) (deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophobic nickel</td>
<td>Pure water</td>
<td>122.2 ± 0.1</td>
</tr>
<tr>
<td>Hydrophobic nickel</td>
<td>15 wt.% PVA</td>
<td>102.7 ± 0.1</td>
</tr>
<tr>
<td>Stainless steel</td>
<td>Pure water</td>
<td>60.1 ± 0.7</td>
</tr>
<tr>
<td>Stainless steel</td>
<td>15 wt.% PVA</td>
<td>40.8 ± 0.4</td>
</tr>
</tbody>
</table>

Figure 15 15 wt.% PVA drop on (a) the hydrophobic surface of the nickel membrane and (b) surface of the stainless steel membrane

3.3 PARTICLES PRODUCTION

3.3.1 Formulations and experimental procedure

3.3.1.1 Poly (vinyl) alcohol emulsion in Miglyol

Poly (Vinyl Alcohol) (PVA) droplets were obtained using the Dispersion Cell system. The continuous (oil) phase was obtained by mixing Miglyol® 840, propylene glycol...
dicaprylate/dicaprate (SASOL, Germany) with an oil soluble surfactant. According to the manufacture’s description (‘Miglyol® produc brochure’, 2016), Miglyol is suitable for pharmaceutical preparations (oral, parenteral, topical and rectal formulations) as carrier and solvent. Three emulsifiers were tested: 2 wt.% PGPR, polyglycerol polyricinoleate, (ABITEC, USA), 2 wt.% ABIL EM 90, modified polyether-polysiloxane (EVONIC industrials, Germany), and 2 wt% Span 80, sorbitan monooleate (Sigma Aldrich, UK). The dissolution of ABIL EM 90 and Span 80 in Miglyol was obtained mixing the solutions at room temperature using a magnetic stirrer and clear solutions were produced. For the preparation of the 2 wt.% PGPR in Miglyol phase heating to 50°C (Surh et al., 2007) of the oil was required to dissolve the emulsifier. The water phase in the W/O emulsions contained pure PVA in water (MW 13.000-23.000 g mol⁻¹, degree of hydrolysis 87-88% Sigma Aldrich, UK) in different concentrations, PVA solution was prepared by dissolving the predetermined amounts of PVA in warm water containing HCl (Fisher Scientific, UK) (required for the PVA crosslinking reaction) in a final concentration of 0.1 M (unless differently specified). PVA was dissolved by heating up the solution up to a temperature of 70-80 °C and stirred for at least 2h. Prior to the use the PVA solution was cooled to room temperature. The prepared PVA solution can be stored in a sealed bottle for a maximum of 1 week. Where appropriate, the inner polymer phase contained 3000 ppm of Blue Dextran (BD) (MW= 2,000 kDa) or with 20000 ppm of CuSO₄×5H₂O (MW= 249.685 g mol⁻¹) corresponding to 5000 ppm of Cu²⁺ (both supplied by Fisher Scientific, UK). The BD or copper sulphate was directly added to the PVA solution and the solutions were gently mixed in order to complete the dissolution of the compounds.

3.3.1.1 PVA microparticles solidification

To crosslink PVA microparticles glutaraldehyde (GA) was used. Solution of 50 wt.% GA in water was used (Sigma Aldrich, UK) and was diluted to reach the appropriate concentrations. Production of the PVA particles was performed following a “two-step” procedure. A primary W/O emulsion was produced using the Dispersion Cell with nickel or stainless steel membrane both with 10 µm pore diameter and 200 µm pore spacing. Previous work by Pan et. al 2012 (Pan et al., 2012) showed that the contact angle of the hydrophilic membrane can be changed so the membranes were pre-soaked in Miglyol (pure continuous phase) for at least 30 min to increase the hydrophobicity of the membrane surface. The initial volume of continuous oil phase in the cell was 100 cm³ and the experiments were run until the dispersed
phase concentration reached 10 vol.%. Once the desired amount of polymeric phase had passed through the membrane, both the pump and the agitator were switched off, the droplets were collected and analysed. In a second step a secondary emulsion was prepared in a separate beaker using a homogenizer (IKA® T 10 ULTRA-TURRAX®, Germany) operating at 30,000 RPM for 3 min (giving the droplet sized between 5-20 μm). The secondary emulsion was made of the GA diluted in water up to the required concentration and the same continuous phase of the primary emulsion. The volume ratio of GA solution and continuous phase in the secondary emulsion was 1:1. The volume ratio GA: polymer (PVA) was 1:1. The secondary emulsion was gently poured into the primary emulsion under continuous stirring using a magnetic stirrer. The reaction time for all samples was 90 min at room temperature. By homogenizing the crosslinker with the continuous phase at very high shear it was possible to obtain very small droplets of GA. In this way the surface area of GA droplets was increased promoting the GA diffusion through the continuous phase consequently reacting with the aqueous polymeric droplets. Using this method it was possible to avoid the “extraction step” reported by other authors (Thanoo, 1993; Wang et al., 2005) which consists of the extraction of GA into organic solvents to make it miscible with the oil phase of the primary emulsion. After the solidification, the stirring was stopped and particles were left to settle at the bottom of the beaker for few minutes; the oil was gently removed and the remaining solid particles were washed in toluene which dissolved the remaining oil. The toluene was left to evaporate and the particles were collected, freeze dried (AdVantage 2.0 BenchTop Freeze Dryer) and stored in air tight containers until further analysis. A summary of the “two-step” procedure for the production of the solid particles is shown in Figure 16.
Figure 16 Scheme of the “two-step” PVA microparticles production: (a) the first step - production of the primary emulsion using the Dispersion Cell (DC), (b) the second step – production of solid microparticles using secondary emulsion which contained the crosslinker.

3.3.1.1.2 Swelling of PVA microparticles

The swelling study was performed on the PVA particles crosslinked with different concentrations of GA (1-50 vol.%). 0.35 g of PVA particles were placed in 20 ml of release media made of phosphate buffer saline solution (pH= 7.3) (Sigma Aldrich, UK). The prepared samples were placed in a shaking water bath at 37°C. The microparticles were left to swell for 24h (no further change in particle size was observed after this time) the excess of water was removed and the diameter of swollen particles was measured. For the determination of the $D_{av}$ of the dried microparticles, a microparticles sample was initially washed with acetone and dried at room temperature; afterwards the sample was freeze-dried to ensure that no water remained inside the particles.
3.3.1.1.3 In vitro release

For the in vitro release microparticles were produced using the polymeric solutions containing BD or Cu²⁺. For the release of BD 1 g of the loaded microparticles was added to 10 mL of a PBS solution (pH= 7.4), unless differently specified the release medium contained a small amount of 2 wt.% SDS (sodium dodecyl sulphate) added to the suspension to avoid the particle agglomeration. The samples were placed into a shaking bath at 37°C and the release was checked at regular time intervals up to 24h. Presence of BD in the release medium was detected using the UV-Vis spectrophotometer (Lambda 35 Perkin Elmer, UK) performing a scan of the wavelengths from 200 to 700. Peak of absorbance for the BD was at 620 nm. Calibrations of the UV-Vis spectrophotometer for BD are reported in Appendix B. Presence of residual GA was also checked at 280 and 235 nm (Gillett and Gull, 1972). For the release of Cu²⁺ 0.35g of the loaded microparticles was placed in 20 ml buffer solutions with pH values of 3 or 7. The buffer solutions were made of acidified water (using HCl (Fisher Scientific, UK)) or pure deionized water in order to minimize the interference of other ions during the Cu²⁺ detection. At predetermined time intervals 5 ml of the release media was removed and replaced with 5 ml of fresh media to maintain the volume or the release media. The amount of Cu²⁺ released was assayed using an Atomic Absorption Spectrophotometer (AAS) (Spectra AA-200 Varian, UK operating at wavelength of 244.2 nm); The amount of Cu²⁺ was calculated from the corresponding calibration curve, made for each release media used (for further details please see Appendix C) Samples in triplicate were averaged for each experiment. The release profile of Cu²⁺ was reported as “Cumulative release percentage” which was calculated as follows:

\[
CR = \frac{\sum_{i=1}^{m} C_i V_i + V_o C_a}{m} \times 100 \quad (%) \quad \text{Equ. 20}
\]

where \( V_o \) is the volume of the release media (=0.02 L),

\( C_a \) is the concentration of the compound determined at a specific time interval (mg L⁻¹),

\( V_i \) is the volume of the replaced media (= 0.005 L),

\( C_i \) represents the concentration of the encapsulated compound in the previous sample (mg L⁻¹),

Appendix B.
\( m \) is the mass of the encapsulated compound in the sample (mg).

**3.3.1.2 Pure chitosan or PVA blended with chitosan emulsions in Miglyol**

For the preparation of pH sensitive formulations the chitosan polymer (CS) (MW 50,000-190,000 g mol\(^{-1}\) Sigma Aldrich, UK) was used with or without the addition of PVA for the production of the water phase of the W/O emulsions. CS is slightly soluble in solutions of acetic acid with a pH below 6. The solubility of CS depends on the polymer charge: the acidic environment protonates the amino groups making the polymer water soluble, on the other hand, if the pH is increased the polymer loses the charge and becomes essentially hydrophobic (Pillai et al., 2009). CS was dissolved in warm (50-60°C) 6 vol.% of CH\(_3\)COOH. For the production of the polymeric blends, the solution of CS in the required concentration is mixed with the PVA solution prepared as described previously when producing pure PVA particles (chapter 5.3.1.1). Once the two polymers were dissolved separately, the solutions were mixed together according to the required polymer ratio and stirred constantly for at least 2h. In the blends final acid concentrations was 3 vol.% of CH\(_3\)COOH (needed for CS) and 0.05 M of HCl (needed for the PVA crosslinking). Where appropriate the polymeric phase contained 13500 ppm CuCl\(_2\times2\)H\(_2\)O (MW= 170.48 g mol\(^{-1}\) supplied by Fisher Scientific, UK) as copper source. As model drug (anionic) 3000 ppm of sodium salicylate (SS) (MW=160.11g mol\(^{-1}\) Sigma Aldrich, UK) were used. For the production of the W/O emulsions Miglyol was used as oil and 2 wt.% PGPR or 2 wt.% ABIL EM 90 were tested as emulsifiers. The membranes used for the production of the emulsions in this section were nickel or stainless steel membranes with 10 or 20 µm pore size and 200 µm pore spacing. The 20 µm membrane was mainly used due to high viscosity of the dispersed phase.

**3.3.1.2.1 CS or PVA-CS microparticles solidification**

The solidification of the polymeric CS or PVA-CS drops of the primary emulsion was performed using the “two-step” procedure described Figure 16 the reaction time was 90 minutes for all the samples and the reaction temperature for these formulations was raised to 75 °C. Washing of CS or PVA-CS microparticles was done using isohexane; the obtained particles were subsequently freeze-dried and stored.
3.3.1.2 Swelling of CS or PVA-CS particles microparticles and in vitro release

The release of Cu$^{2+}$ from the CS or PVA-CS particles was assayed following the same procedure described for the pure PVA microparticles (see section 5.3.1.1.2), while SS concentration in the sample was analysed using the UV-Vis spectrophotometer operating at wavelength of 300 nm. The instrument was previously calibrated for the sodium salicylate (SS) detection using standard solution at predetermined SS concentrations (calibrations for the UV-Vis spectrophotometer are reported in Appendix B). The release of SS with time and depending on the pH of the buffering solution is reported as “Cumulative release %” calculated using Equ. 20 (page 58).

3.3.1.3 Pure gelatine (G) or gelatine-chitosan (G-CS) microparticles

The continuous (oil) phase (O) of the W/O emulsion was mainly 2 wt.% Span 80 (Sigma Aldrich, UK) in low odour kerosene (Sigma Aldrich, UK). Also as a continuous phase Miglyol containing ABIL EM 90, PGPR or Span 80 was also tested. The dispersed (water) phase (W) was composed of pure gelatine (G) (porcine skin, gel strength 300, Type A (Sigma Aldrich, UK)) dissolved in deionized water or gelatine (G) was blended with chitosan (CS) (MW 50,000–190,000 g mol$^{-1}$ Sigma Aldrich, UK). 30 µm standard nickel membranes with 80 or 200 µm pore spacing were used for the emulsion produced. Chitosan and gelatine solutions were prepared separately and mixed together afterwards; CS was dissolved in warm (50-60ºC) 2 wt.% CH$_3$COOH in water. Gelatine solution was prepared by dissolving gelatine in warm water (50-60ºC). For the preparation of the blend, the two polymeric solutions were mixed together in appropriate ratio and stirred for at least 2h, at 40ºC to avoid the gelatine solidification. For the production of the W/O emulsions the Dispersion Cell was placed in a water bath operating at 40ºC to avoid the solidification of the gelatine presents in the formulations.

3.3.1.3.1 Re-hydration and encapsulation of baker’s yeast cells (Saccharomyces Cerevisiae)

Where appropriate, the dispersed phase contained Baker’s yeast cells (Saccharomyces cerevisiae) purchased from a local supermarket (sachets of “Fast Action Dried Bread Yeast”, Sainsbury, UK). The yeast cells were selected for the encapsulation due to its reported probiotic activity (Graff, Chaumeil, et al., 2008), furthermore it is easy to track the cell viability by checking glucose metabolism with time. The median size of the yeast cells was 4
μm, determined using ImageJ. An initial yeast suspension was prepared by adding 3.5 g of the dried yeast powder to 200 mL of ultrapure water (Pure Milli-Q water) or a Ringer’s solution (Sigma Aldrich, UK). The suspension was stirred for at least 10 minutes and centrifuged at 1200 RPM for 3 minutes. The supernatant was discarded and the washing procedure was repeated 3 times in order to make sure that all the salts, emulsifiers and other additives presents in the sachet are removed. The final volume of water removed was 175 mL. Considering the yeast cell as having a spherical shape with an average diameter of 4 μm and a dry mass density of 1.33 g mL⁻¹, the calculated maximum concentration of yeast in the suspension was 3.14 x 10⁹ cells mL⁻¹. The initial cell suspension was subsequently diluted for use by factors of 10 and 100, giving cell concentrations of 3.14 x 10⁷ and 3.14 x 10⁸ cells mL⁻¹. A manual cell counting using a hemocytometer was also performed to check the cell density. For the stock suspension higher value than 3.18 x 10⁹ cells mL⁻¹ was measured, thus the lower cell density calculated from the initial weighed yeast was considered more reliable for cell density characterization. It is worth of pointing out that the yeast cell density used is in accordance, or higher than, the generally accepted required dose of probiotics having health benefits (10⁶- 10⁷ cells mL⁻¹ (Burgain et al., 2011)).

### 3.3.1.3.2 Solidification of gelatine microparticles containing baker's yeast cells (Saccharomyces cerevisiae)

To obtain solid microparticles, the liquid drops in emulsion were solidified using either a thermal gelation (placing the emulsion in ice), or ionic crosslinking using Sodium hexamethaphosphate SHMP (Fisher Scientific, UK). Sodium sulphate and Sodium tripolyphosphate (purchased from Sigma Aldrich, UK) were also evaluated as ionic crosslinkers. For the dispersed phase composed by the blend of G:CS in a ratio of 5:2 (G:CS (5:2)) the emulsion was initially cooled with ice for 30 minutes to set the gelatine, subsequently 10 mL of a 10 wt.% SHMP solution was gently dripped into the emulsion to crosslink the chitosan. The reaction occurred under continuous stirring for 3h. When pure gelatine (5 wt.%) was used as dispersed phase, solidification was obtained by thermal gelation: the beaker containing the emulsion is placed in an ice bath, under continuous stirring for 4h to avoid the drops coagulation. In both cases the obtained solid particles were washed using 2 wt.% Tween 20 polyethylene glycol sorbitan monolaurate (Sigma Aldrich, UK) to remove any remaining kerosene phase.
3.3.1.3.3 Gastro resistant coating for gelatine microparticles

Polymers Eudragit S100 (Evonik Industries, Germany) was used for the production of gastrointestinal resistant coatings on the microparticles: Coating was performed on gelatine microparticles using the oil in oil (O<sub>1</sub>/O<sub>2</sub>) solvent evaporation method previously described by L. Zhang et al. (2011) (the method of the solvent evaporation was also evaluated). The polymeric coating solution (O<sub>1</sub>) was prepared using 2 wt.% Eudragit S100 dissolved in a solution of ethanol:acetone (Fisher Scientific, UK) at a volume ratio of 4:1. To prevent microparticles aggregation during the subsequent coating process, 20 mg of zinc stearate (Sigma Aldrich, UK) was added to the organic phase which acted as an anti-sticking agent. The zinc stearate was not completely soluble in the coating solution therefore; to minimize the zinc stearate particle diameter the coating mixture was homogenized using a homogenizer (IKA® T 10 ULTRA- TURRAX®, Germany) at maximum speed (30,000 RPM) for 3 min.

3 mL of gelatine microparticles suspension was added to 10 mL to the coating solution (O<sub>1</sub>) and stirred for 2 minutes providing a uniform dispersion of the particles. Coating solution (O<sub>1</sub>) containing the microparticles was gently poured into 50 mL of 2 wt.% Span 80 in Paraffin oil (Sigma Aldrich, UK) (O<sub>2</sub>). The O<sub>1</sub>/O<sub>2</sub> emulsion was stirred using the Dispersion Cell impeller at 5V (corresponding to 800 RPM) for 4h. The process of solvent evaporation was checked every hour; samples of the O<sub>1</sub>/O<sub>2</sub> emulsion were withdrawn and examined under the optical microscope. Reduction of the O<sub>1</sub> droplet size was observed until complete solvent evaporation and Eudragit polymer deposition on the gelatine microparticles occurred. The coated microparticles were washed using isohexane and dried at room temperature overnight.

3.3.1.3.4 Gelatine microparticles loaded with yeast - in vitro dissolution study

Simulation of the gastrointestinal transit conditions was obtained varying the pH of the dissolution medium over time: the acidic stomach fluid was reproduced using a pH 1.2 for 2h, afterwards a pH 7 (or pH 8) medium was used to mimic the intestinal-colon area for a maximum period of time of 3h (or up to complete particle dissolution) (Paharia et al., 2007). The dissolution medium was prepared from a phosphate buffer saline (PBS, Sigma Aldrich, UK) solution (pH 7), adjusting the pH with HCl, or NaOH (Sigma Aldrich, UK). An initial amount of 15 mg of dried particles was added to 1 mL of dissolution medium at pH 1.2 and shaken in an orbital incubator (Sartorius Certomat BS-1, Sartorius AG, Germany) at 37ºC for 2h. After 2h in an acidic environment, the microparticles were observed under the microscope.
microscope and subsequently collected from the acidic buffer and re-suspended in 1 mL of pH 7 buffer for maximum 3h with shaking. Again the microparticles were recovered, observed and re-suspended in pH 8 buffer in order to mimic the environmental conditions during the passage from the intestine to the colon (tests in- vitro). Microphotographs of the microparticles were taken at regular time intervals, until the dissolution of the particles and complete cell release.

3.3.1.3.5 Viability of the yeast cells released from gelatine microparticles

The viability of the released cells released from the microparticles was tested using two methods: 1. Consumption of D(+) glucose D(+) -Glucose anhydrous, (Fisher Scientific, UK) by yeast cells was determined with time

2. Fluorescent probes LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells, Molecular Probes, Invitrogen (California, US).

Once released from the microparticles, the yeast cells were centrifuged using an Eppendorf centrifuge at 1000 RPM for 2 minutes and re-suspended in a 1 mL of a 6 mM Glucose solution. The glucose solution with the yeast cells was subsequently placed in an orbital incubator at 37ºC and rotation speed of 150 RPM. At predetermined time intervals the cell suspension was centrifuged and 50 µl of the glucose solution was withdrawn and glucose concentration was determined using a glucose analyser (model G5, Analox Instruments Ltd., UK). The glucose consumed with time was calculated as % of the initial glucose present in the solution according to the following equation:

\[
\% \text{ Glucose consumed} = \frac{(\text{Glu}_i - \text{Glu}_n) \times 100}{\text{Glu}_i}
\]

Where \(\text{Glu}_i\) is the concentration of the initial glucose solution (6 mM),

\(\text{Glu}_n\) is the amount of glucose measured in the sample at different time intervals.

Fluorescent probes were also used to determine the fraction of live/dead cells. Component A of the LIVE/DEAD ® Viability/Cytotoxicity Kit (Calcein AM) is retained in living cells giving a bright green fluorescence if excited using a fluorescein optical filter (485 ± 10 nm). In dead cells component B (Ethidium homodimer-1) enters the cells with a damaged membrane and binds to the nucleic acid giving a bright red fluorescence when excited under
a typical rhodamine optical filter (530 ± 12.5 nm). The samples were prepared by addition of the two probes to the yeast suspension and incubation at 37°C for 40 minutes, prior to the observation with the epifluorescence microscope Nikon Ti Eclipse.

3.3.1.4 Alginate microparticles

Alginate (Sodium Alginate, MW= 216.121 g mol⁻¹, Sigma Aldrich UK) drops were obtained using the Dispersion Cell system with a nickel hydrophobic membrane having 30 µm pore size and 200 µm pore spacing. The continuous phase (O) of the W/O emulsion was made of 2 wt.% Span 80 in kerosene. The Alginate polymer was dissolved in warm (60- 70 °C) deionized water under vigorous stirring for a maximum of 2h. Where appropriate, the dispersed phase contained Baker’s yeast cells (Saccharomyces cerevisiae) rehydrated following the procedure described in section. 5.3.1.3.1. The predetermined amount of yeast cell dispersion was added to the alginate solution and the polymeric phase was agitated up to the formation of a homogenous phase. The amount of cells encapsulated was between 3x 10⁷ and 1.88x 10⁸ cells mL⁻¹ calculated according to the procedure described in section 5.3.1.3.1. The dispersed phase was injected using the Dispersion Cell and the final concentration of the dispersed phase in the emulsion was 10%. Once produced the emulsion was subsequently transferred in a beaker and stirred.

3.3.1.4.1 Solidification of alginate microparticles (crosslinking procedure)

Two positively charged ionic crosslinkers were tested for the solidification of the alginate drops with or without cell: MgSO₄, magnesium sulphate (Sigma Aldrich, UK) or CaCl₂ calcium chloride (Sigma Aldrich, UK). Different concentrations of both crosslinkers were used.

Two ways of solidification were tired: crosslinker addition directly to W/O emulsion and addition of W/O emulsion to the saturated crosslinker solution. Unless differently specified, the crosslinker solution (water solution) was directly added to the emulsion under continuous stirring for a maximum of 24h. Once solid, microparticles were recovered by filtration using a vacuum pump and washed using a mixture of acetone: water in a volume ratio of 1:2. Once washed the microparticles were left to dry at room temperature. Dried microparticles were recovered and stored for further analysis.
In a second approach the alginate drop solidification is obtained pouring the W/O emulsion produced using the Dispersion Cell into a saturated solution of CaCl₂ constantly agitated using a magnetic stirrer. The solidification reaction is stopped after 1h and the microparticles were collected and washed as reported above.

3.3.1.4.2 Viability of the yeast cells released from alginate microparticles

Survival of the yeast to the encapsulation process was assayed adding 10 mg of the alginate microparticles containing the yeast in 1 mL of a 6 mM glucose solution, the samples were placed in the orbital incubator at 37°C with a set rotation speed of 150 RPM. At regular time intervals sampling of the glucose solution was done using a micropipette and 10 µl was analysed using the glucose analyser.

3.4 PARTICLES CHARACTERISATION

3.4.1 Scanning Electron Microscopy (SEM) analysis with Energy Dispersive X-ray Spectrometer (EDX)

The solid produced microparticles and membranes structure were observed by Scanning Electron Microscopy using the Tabletop microscope TM3030, (Hitachi, Japan) operated at 15 kV for pure imaging. The samples were previously prepared applying an ultra-thin layer of electrically- conducting material (Gold/Palladium- Au/Pd) by sputter coating. For the observation of the internal structure of the microparticles the samples were mounted in a resin and cut into slices using a Base Sledge Microtome (Leitz, Weitezer, UK). Microparticles elemental analysis was done using the SEM coupled with the Energy Dispersive X-ray Spectrometer (EDX) for multiple elemental analysis (i.e. point/area analysis, line scan and mapping).

3.5 FORMULATION OPTIMIZATION FOR THE SCALE UP AND USE OF THE PULSED (OSCILLATORY) FLOW SYSTEM

3.5.1 Disk membranes used in the Dispersion Cell (preliminary investigation)

For the continuous production of W/O emulsions the performance of a PTFE and FAS coated membranes were preliminary investigated using the Dispersion Cell. Surfaces of both tested nickel membranes where transformed to hydrophobic by coating the surface either by thermally bonded PTFE or using a chemical reaction to apply the FAS coating. Membranes
used in the Dispersion Cell had 30 µm pore size and 200 µm pore spacing. The membrane performance was evaluated considering the production of several emulsions and the required soaking time. For these tests the continuous phase was composed by 2 wt.% Span 80 in kerosene and as dispersed phase was 5 wt.% gelatine containing yeast cells. The use of the gelatine required the heating therefore the equipment (Dispersion Cell) was placed in the water bath.

Where appropriate pig gelatine was substituted with fish gelatine. Fish gelatine compared to the pig gelatine remains liquid at 11 to 28 °C (Karim and Bhat, 2009) allowing operation at room temperature. Where appropriate Miglyol oil (‘Miglyol® produc brochure’, 2016) was used as a replacement of kerosene (‘Kerosene safety data sheet’, 2016) as it is not toxic nor flammable.

3.5.2 Tubular membranes used in the pulsed flow system with experimental procedure

For the production of the W/O emulsions using the Pulsed system 20 wt.% of FG in water was used as dispersed phase. The continuous phase was made of 2 wt.% ABIL EM 90 in Miglyol. The interfacial tension measured for this system is shown in Table 4 (page 51). The system was run at room temperature. The membrane used was a tubular membrane which was previously FAS coated with 20 µm pore size and 80 µm pore spacing, photograph of the membrane used is reported in Figure 17 a. The membrane had a working length of 60 mm with an internal diameter of 14 mm. The dispersed phase was injected in the system using a syringe pump (World Precision Instrument Inc., AL-1000, UK) (red pump shown in Figure 17) with a flow rate of between 5 to 30 mL min⁻¹ corresponding to a transmembrane flux between 103 to 620 L h⁻¹m⁻². The continuous phase was injected within the system using a peristaltic pump (Watson-Marlow Sci-Q 300, UK) (green pump reported in Figure 17) that can be operated up to a maximum flow rate of 2000 mL min⁻¹. Once the whole system was assembled (with the membranes placed in the membrane module) it was filled up with the continuous phase, the pulsation was activated and the system is run for at least 30 minutes without injecting the dispersed phase. At this stage the recirculation of the oil phase is done with the peristaltic pump set at the maximum rate. This procedure is required to remove all the air entrapped within the system which could cause the formation of foam and coalesce of the drops during the emulsification process. The pulsation is generated by the oscillation of a bellow, designed for the use in a diaphragm pump, which is filled with the continuous phase.
The bellow is placed in the metallic case as shown in Figure 17 g. The oscillation of the bellow was regulated by an accelerometer connected to a National Instrument Analogue to Digital converter (NI cDAQ-9174) interfaced with a software (LabView). From the software is possible to obtain information about the frequency and the amplitude of the oscillation, as reported in (Piacentini et al., 2013) the frequency is determined by the direction of the travel while the amplitude is determined from the acceleration data. An example of the LabView software interface during a running experiment is shown in Figure 18, from the “control” panel showed in Figure 18 a it is possible to select the required value of frequency (e.g. 20 Hz) and the target value of peak to peak displacement expressed in mm. It is possible to set manually the voltage from the “voltage generator” window or by clicking on the button “Feedback on” the voltage is automatically adjusted to reach the target value of displacement set. From the widow showed in Figure 18 b the actual value of frequency and displacement detected are reported. Shape of the oscillation is reported from the software and it was in regular waveform (i.e. sine wave) (Holdich et al., 2012).
Figure 17 Photographs of the tubular membrane (a) the membrane module (b) and the assembled Pulsed (Oscillatory) device (c) together with the peristaltic pump for the circulation of the continuous phase (d) and the syringe pump for the injection of the dispersed phase (e), with (g) is pointed the metallic case where the bellow is placed, (f) is the beaker where the emulsion is collected.
Figure 18 Interface of the LabView executive program with (a) the control panel and (b) the detected values of frequency and peak to peak displacement.

For the production of the W/O emulsions using the Pulsed system the frequency used were in a range between 10 to 30 Hz while the displacement ranged between 0.5 and 3.5 mm. The Pulsed system was previously used for the production of O/W emulsions (Holdich et al., 2012; Piacentini et al., 2013) using sunflower oil as dispersed phase and 2 wt.% Tween 20 in water containing polymers as continuous phase.

In this work the system was adjusted for the production of W/O emulsions; the high viscosity of the dispersed and continuous phase of the formulation tested in this set of experiments could cause the vibration of the membrane. Breaking of the membrane was experienced operating with high fluxes and high values of shear stress. The use of more resistant membranes might be considered as an option for the optimization of the Pulsed (Oscillatory) Flow system for the production of highly concentrated and uniform W/O emulsions.
3.5.3 Tubular membrane cleaning procedure

After the production of the required amount of emulsion the pulsed system is dismantled and the membrane module is opened, the membrane was removed from the membrane module and the excess of oil and water phase is initially wiped away. The membrane is subsequently rinsed with tap water and washing up liquid, then is placed in a glass cylinder and warm soapy water is added up to complete submersion of the membrane. Washing is done using a sonicating bath for a maximum of 3 minutes, the membrane is then dried using the compressed air and it is left in the oil phase to increase the hydrophobicity.
4. RESULTS

4.1 PVA MICROPARTICLES FOR DRUG DELIVERY

4.1.1 W/O emulsion production

4.1.1.1 Influence of the emulsifier type

Droplets stability in W/O emulsions is not greatly affected by the hydrophobic membrane used to produce the emulsion, but it is by the type of the emulsifier used to stabilise the droplets within the emulsion. To investigate the influence of the oil soluble emulsifier on the drop size and stability the Dispersion Cell was used. The dispersed phase (W) was pure PVA (15 wt.%) dissolved in water containing 0.1 M HCl. Oil used for preparation of the continuous phase (O) was Miglyol and the emulsifier was changed in order to achieve the stability of the emulsion and avoid phenomena of coalescence and/or breaking of the drops. The dispersed phase flux used in tests was 113 L h\(^{-1}\)m\(^{-2}\), while the applied shear stress was 35 Pa. Three oil soluble (non-ionic) emulsifiers were tested: Span 80, ABIL EM 90 and PGPR all in a concentration of 2 wt.%. This surfactant concentration was selected as it gave stable emulsions, in accordance with data reported in literature (Kukizaki, 2009). Once the emulsions were produced, samples were taken and observed under the microscope. Microphotographs were taken and analysed to determine the average droplet size (D\(_{av}\)) and the coefficient of variation (CV%). From Figure 19 it is possible to see that using Span 80 the size of the drops produced is on average 86 µm, while for drops stabilised using ABIL EM 90 and PGPR have a similar average diameter of 30 µm. Furthermore, Span 80 produced emulsions with a high CV% (30 %) while the lowest CV% (18%) was obtained using PGPR as emulsifier in the continuous phase. Using the emulsifier ABIL EM 90 a CV% of 23% was obtained. Hence, Span 80 produced emulsions with larger and less uniform drops. This effect is confirmed by observation the microphotographs reported in Figure 20. Figure 20 a shows the emulsion prepared using as emulsifier 2 wt.% Span 80 characterized by a turbid background and big polymeric drops. Figure 20 b and c are respectively the emulsions obtained using ABIL EM 90 and PGPR as emulsifier, which displayed a similar D\(_{av}\) and CV%. Slightly less uniform emulsions were produced using ABIL EM 90 compared to the ones produced using PGPR; but in both cases the emulsions were stable and no coalescence was observed. In Table 4 (page 51) are reported the values of interfacial tensions measured for the different system tested. Compared to pure Miglyol all the tested emulsifiers were able
to reduce the interfacial tension, in some cases a drastic reduction of the interfacial tension could produce a flow of the dispersed phase through the membrane pores; without forming the drops at the membrane surface (Kim et al., 2014). The use of Span 80 reduced the interfacial tension from 6.6 mN m\(^{-1}\) (for the system composed by pure Miglyol and 15 wt.% PVA) down to 1.6 mN m\(^{-1}\) which could be too low for the right formation of the drops in this system. PGPR produced a slight higher interfacial tension if compared to the ABIL EM 90, as a consequence the resulting emulsions were more stable and more uniform. The molecule of Span 80 (\(M_w= 428.61 \text{ g mol}^{-1}\)) is slightly smaller than the ABIL EM 90 (\(M_w= 522.89 \text{ g mol}^{-1}\)) and PGPR (\(M_w= 520.704 \text{ g mol}^{-1}\)), hence the Span 80 molecules diffuse faster to the interface producing a higher reduction of the interfacial tension.

Additionally, in (Taylor, 2011) it is reported cohesive forces at the interface are much stronger between the PGPR molecules than Span 80 molecules. Although both the emulsifiers have good surface properties, the extended structure of the ricinoleate chain (of PGPR) and the orientation of the molecules might produce a stronger interface than the oleate chain (of Span 80).

![Figure 19](image)

**Figure 19** Influence of the emulsifier type on average droplet diameter and droplet size distribution. Dispersed phase composition: 15 wt.% PVA in water, dispersed phase
flux = 113 l h⁻¹m⁻², shear stress applied = 35 Pa. Error bars represent the standard deviation of the measurements.

![Microphotographs of emulsions produced using different surfactants in continuous phase](image)

Figure 20 Microphotographs of emulsions produced using different surfactants in continuous phase (a) 2 wt.% Span 80 in Miglyol, (b) 2 wt.% ABIL EM 90 in Miglyol, (c) 2 wt.% PGPR in Miglyol. Dispersed phase 15 wt.% PVA in water.

Based on these observations, PGPR and ABIL EM 90 were selected as more appropriate for the stabilization of the PVA drops into Miglyol.

4.1.1.2 Effect of process operating parameters

4.1.1.2.1 Influence of the shear stress and dispersed phase flux on droplet size and size distribution

Using a standard nickel membrane with pore size 10 µm and pore spacing 200 µm, W/O emulsions were produced. The composition of the dispersed phase was 15 wt.% PVA in water containing 0.1 M HCl, the continuous phase was made of 2 wt.% PGPR in Miglyol. The dispersed phase flux was increased from 12 to 270 L h⁻¹m⁻², while the shear stress was
varied from 22 to 59 Pa. Figure 21 reports the average droplet size as a function of the flux and shear stress; it can be seen that the increase of the dispersed phase flux produced on average an increase of the mean droplet size. For instance, at a constant shear of 22 Pa, a flux of 12 L h⁻¹ m⁻² produced drops of 40 µm; increasing the flux to 112 and 270 L h⁻¹ m⁻² droplet size 49 and 64 µm were produced respectively. This effect was already reported in previous work (Egidi et al., 2008; Dragosavac et al., 2008): assuming that the drop detachment time is constant, the increase of the dispersed phase flux produces an increase of the amount of dispersed phase flowing in the drop during the detaching stage generating larger drops. Same set of experiments was repeated applying a shear of 35, 43 and 59 Pa. It is interesting to notice that for 35 and 43 Pa the effect of the increase of the dispersed phase flux is still evident; while for the emulsions produced at higher shear (59 Pa) did not show a significant difference in size increasing the dispersed phase flux. The combination 59 Pa and 12 L h⁻¹ m⁻² gave droplet sized 36 µm; the increase of the flux to 112 L h⁻¹ m⁻² produced droplet sized 38 µm, while for the highest flux used (270 L h⁻¹ m⁻²) 43 µm drops were obtained. Reduction of the Dₐᵥ is obtained increasing the shear stress applied; for instance, at constant value of 112 L h⁻¹ m⁻² as dispersed phase flux; increasing the shear stress from 22 to 59 Pa produced a decrease of the Dₐᵥ from 50 µm to 38 µm. Effect of the shear stress influence was more evident for the emulsions produced at 270 L h⁻¹ m⁻² where the shear variation from 22 to 59 Pa gave a reduction from 64 to 44 µm of the average droplet size.
Figure 21 Variation of the mean droplet size ($D_{av}$) and coefficient of variation (CV%) depending on the dispersed phase flux and shear stress applied using a full nickel standard membrane with 10 µm pore size and 200 µm pore spacing (dispersed phase: 15 wt.% PVA in water + HCl 0.1 M, continuous phase: 2 wt.% PGPR in Miglyol). Error bars represent the standard deviation of the measurements.

From Figure 21 it is possible to see that the less uniform emulsions were obtained at the lowest dispersed phase flux used, independently from the shear stress applied. The extreme case of very low dispersed phase flux and very high rotation speed (high shear stress) produced the emulsion with the lowest uniformity; corresponding to a CV% of 38%. A flux of 12 L h$^{-1}$m$^{-2}$ was considered insufficient for uniform emulsion production independently from the shear stress applied, while the use of a too high shear could produce a breaking up of the formed drops into smaller ones producing an increase of the CV% (Vladisavljević and Williams, 2005; Stillwell et al., 2007; M. Dragosavac et al., 2012).

As a general rule for the formation of uniform drops in membrane emulsification a uniform shear field is required (Stillwell et al., 2007). For the Dispersion Cell system; the paddle-blade stirrer produces a varying radial shear field; which reaches its maximum value at the transitional radius ($r_{trans}$) where the vortex changes from free to forced (Kosvintsev et al.,...
Hence, more uniform drops are generated by a narrow annular region on the disk membrane surface (Stillwell et al., 2007). To check this a ring membrane was used which had active pores only in the area of the maximal shear stress. Emulsions were produced keeping constant the composition of dispersed and continuous phase while varying the rotation speed of the paddle (hence the shear stress applied) and the dispersed phase flux. The stainless steel membrane was characterized by a mean pore diameter of 10 µm and 200 µm as pores spacing. For this set of experiment the dispersed phase flux was gradually increased from 50 to 1250 L h⁻¹m⁻² and the shear was varied from 22 to 59 Pa. Figure 22 shows the Dₐv and CV% measured for the produced emulsions, including (where applicable) the values of the standard deviation of the measurements as error bars. Increasing the transmembrane flux, larger drops were produced for all values of shear stress tested, and it was possible to produce droplet between 28 and 53 µm. It is interesting to notice that a little difference exists between the sizes of the resulting drops produced setting the shear at 22 or 35 Pa. For the highest value of shear applied (59 Pa) the increase of the drop size depending on the transmembrane flux was less pronounced. As comparison, a similar trend is reported in previous paper (M. Dragosavac et al., 2012) for the production of W/O emulsions using a membrane with 20 µm as pore diameter and 200 µm pores spacing. The increase of the flux up to 3200 L h⁻¹m⁻² produced a small change in the drop size for high rotation speed of the paddle-blade stirrer. As expected the increasing shear stress produced smaller drops, for example at a constant transmembrane flux of 1250 L h⁻¹m⁻² 53 µm drops were generated applying a shear of 22 Pa, this value gradually decreased to 39 µm applying a shear of 59 Pa. Figure 22 shows that uniform droplets were obtained for almost all the fluxes and shear stress applied, with CV% lower than 30%. As previously observed, the use of very high shear such as 59 Pa produced in some cases a CV% of 27-35%; as breaking up of the drops is likely to occur (Vladislavljević and Williams, 2005; M. Dragosavac et al., 2012). When comparing Figure 21 and Figure 22 it can be seen that the use of the ring membrane reduced the CV% which can be contributed to the formation of the drops only in the region of the maximal shear.
Figure 22 Variation of the mean droplet size (Dₘ) and coefficient of variation (CV%) depending on the dispersed phase flux and shear applied using stainless steel ringed membrane with 10 µm pore size and 200 µm pore spacing (dispersed phase: 15 wt.% PVA in water + HCl 0.1 M, continuous phase: 2 wt.% PGPR in Miglyol). Error bars represent the standard deviation of the measurements.

4.1.1.2.2 Effect of the membrane type: comparison between nickel and stainless steel membrane

Influence of the membrane type on emulsion droplet size and uniformity was investigated comparing the nickel membrane with the stainless steel membrane; both membranes had 10 µm pore size and 200 µm pore diameter. Figure 23 reports the comparison of the results obtained using the nickel membrane and stainless steel membrane. Average droplet size and size distribution are reported as a function of the shear and flux. Dispersed phase used was 15 wt.% PVA in water; the continuous phase was composed by 2 wt.% PGPR in Miglyol. Using a dispersed phase injection rate between 10 to 230 mL h⁻¹, the transmembrane flux obtained for the nickel membrane was between 12 to 270 L h⁻¹m⁻². Applying a shear stress from 22 to 59 Pa droplet sized between 36 to 64 µm were obtained. With the stainless steel membrane
the dispersed phase injection rate applied was between 10 to 50 mL h⁻¹ producing a flux through the membrane between 54 to 270 L h⁻¹m⁻². At comparable values of shear stress (22-59 Pa) and dispersed phase flux generated using the nickel membrane, a slight reduction of the droplet size was observed for the stainless steel membrane; the mean droplet diameter was between 28 to 40 µm. Difference of the average droplet size and size uniformity between the two membrane tested could also be a consequence of the difference in the hydrophobicity of the membranes surface. The surface of the nickel metal membrane is hydrophobised by a thermally bonded PTFE coating on the metal surface (Morelli et al., 2017), while no further surface modification were made on the stainless steel membrane. Measurement of contact angles were made for water and 15 wt.% PVA on hydrophobic nickel and stainless steel membranes and the results are reported in Table 5, page 53. In addition the photographs of the droplets sitting on different membrane surfaces are reported within Figure 15, page 53. According to the contact angle measurements the PVA droplet wets the stainless steel membrane far more than the nickel membrane (Figure 15 a and b) giving a contact angle of 60°. The stainless steel is more hydrophilic than the nickel and, therefore, one would assume that wetting of the membrane surface by the dispersed phase is more likely to happen for the stainless steel membrane. Hence, the smaller drops, and better size distribution, coming from the stainless membrane, cannot be attributed to the membrane surface wetting properties—nickel is more hydrophobic and should wet better with the oil phase. An SEM analysis of the membranes surface as well as the membranes pore cross-section was performed. A clean nickel membrane and stainless steel membrane were sliced and observed using the SEM. In Figure 24 is possible to see that the nickel membrane shows a conical surface above the pores (lines within SEM image, Figure 24 a–c); which could be a consequence of the coating treatment with PTFE. On the other hand, the pores of the stainless membrane show a cylindrical internal shape and sharp openings without conical configuration (Figure 24 d–f). During the formation of the drop, in the conical portion of the nickel membrane’s pores the droplets can grow before being detached by the action of the shear applied. Thus the smaller and more uniform drops are more likely to be (also) a consequence of the sharp pore opening; i.e. flat membrane surface. In addition having a polymeric phase highly acidic (PVA 15% + HCl 0.1 M) the use of the stainless steel as membrane material represents a further advantage as it is more resistant to acidic pH than nickel.
Figure 23 Influence of the dispersed phase flux and shear stress on average droplet size and size distribution. Comparison between the results obtained using the 10 µm nickel membrane (Nm) shown with the full signs and 10 µm stainless steel membrane (SSm) shown with the hollow signs. Dispersed phase: 15 wt.% PVA in water, continuous phase: 2 wt.% PGPR in Miglyol. Error bars represent the standard deviation of the measurements.
Figure 24 SEM analysis of hydrophobic 10 µm nickel membrane: (a) pore cross-section. (b) Pore top view. (c) Membrane side view. Red lines mark the pore edges. Stainless steel 10 µm membrane: (d) Pore cross-section. (e) Pore top view. (f) Top view of the membrane surface.
4.1.1.2.3 Model

The shear-capillary model was used for the theoretical determination of the droplet size at a given value of the shear stress applied. This model has been validated in previous works (Kosvintsev et al., 2005; Stillwell et al., 2007; Dragosavac et al., 2008; M. Dragosavac et al., 2012), it was reported that the model slightly underestimate the real drop size obtained (Stillwell et al., 2007). It has to be taken into account that the shear-capillary model does not consider the contribution of the dispersed phase flux as having an influence on the final drop size. Hence, it represents the size of the smallest drops that can be produced at a given set of operating parameters (Dragosavac et al., 2008). It is expected to be in very good agreement for emulsions obtained with a very low dispersed phase flux (Dragosavac et al., 2008; M. Dragosavac et al., 2012). The experimental values of droplet diameter were compared to the theoretical ones determined using the Equ. 7, page 41. Figure 25 shows the average droplet size of emulsions produced using from 12 to 270 L h\(^{-1}\) m\(^{-2}\), data from the nickel and stainless steel membrane were both included. The curve shows the theoretical values of the \(D_{\text{av}}\) calculated using Equ. 7 (page 41) at different shear stress. For all the experiments 15 wt.% in water was used as dispersed phase and 2 wt.% PGPR in Miglyol as continuous phase. It is noticeable that for low flux (12 L h\(^{-1}\) m\(^{-2}\)) the experimental values are closer to the theoretical ones. An increase of the flux produces a gradually higher divergence between the experimental droplet size and the model predicted ones; as it was observed in previous works (Dragosavac et al., 2008; M. Dragosavac et al., 2012; Hanga and Holdich, 2014). It is reported that two main effects are responsible of this divergence (M. Dragosavac et al., 2012). First, during the drop formation it was observed that a neck is formed which holds the drop connected to the pore opening (Van der Graaf et al., 2005); during this time the drop grows as an additional amount of dispersed phase flows inside (Van Der Graaf et al., 2006; M. Dragosavac et al., 2012). Consequently, the resulting drops are larger in size than the ones predicted by the shear-capillary model. The second reason of divergence is the reduced number of surfactant molecules covering the drop surface during the formation. In the model, the equilibrium interfacial tension is taken into account; which is lower than the dynamic interfacial tension. The interfacial tension increases due to the insufficient surfactant molecules coverage of the drop surface, consequently the formed drops are larger (M. Dragosavac et al., 2012).
The influence of the dispersed phase flux on the drop formation and detachment has been previously investigated by Schröder and Schubert (1999). According to Schröder and Schubert the droplets formed on the membrane surface are influenced by the number of the active pores on the membrane surface. The number of active pores within the model has to be estimated which is an additional fitting parameter which needs to be taken into consideration (Holdich, 2010).

Figure 25 Comparison between the theoretical droplet size calculated using the model (Equ. 7, page 41) (line) and the average droplet diameter obtained under different conditions of shear and transmembrane flux. Nm – nickel membrane, SS – stainless steel membrane (dispersed phase: 15 wt.% PVA; continuous phase: 2 wt.% PGPR).

4.1.1.3 Influence of PVA concentration

Viscosity of the dispersed and continuous phase have an effect on average droplet size and uniformity of the emulsions. The increase of the continuous phase viscosity is reported to have an influence on the diffusivity of the surfactant molecules; according to Z. Wang et al. (Wang et al., 2000) increase of the continuous phase viscosity leads to a decrease of the diffusion coefficient of the emulsifier. As a consequence the rate of reduction of the interfacial tension decreases as well producing emulsions with larger drops. The increase of the dispersed phase viscosity produces a reduction of the dispersed phase flux; according to the Darcy’s law (Joscelyne and Trägårdh, 1999; Charcosset, 2009; Kukizaki, 2009).
Kukizaki’s work (Kukizaki, 2009) is shown that for a system composed by mixtures of decane and paraffin used as dispersed phase and 3 wt.% SDS in water was used as continuous phase, the increase of the dispersed phase viscosity produced a reduction of the average droplet size. This effect is a consequence of the reduction of the flow of dispersed phase in the drop during the formation. The values of measured interfacial tension is reported to be higher increasing the viscosity of the dispersed phase, which should theoretically produce larger drops. Reduction of mean drop size as a consequence of the more viscous dispersed phases used is also reported by I. Kobayashi et al’s work (2005) In this section a study of the effect of the amount of polymer used for the preparation of the dispersed phase is reported. Increasing concentrations of PVA in water were used as dispersed phase to produce emulsions using as continuous phase 2 wt.% ABIL EM 90 in Miglyol. Table 4 (page 51) shows that increasing the amount of PVA the viscosity of the polymeric phase is increased from 24 mPa s to 2550 mPa s. At the same time, an increase of the interfacial tension is measured as the dispersed phase viscosity becomes higher (from 1.6 to 3.5 mN m⁻¹) (Table 4, page 51). The membrane used in this set of experiments was a stainless steel membrane with 10 µm pore size and 200 µm pores spacing. Dispersed phase injection rate was constantly set at 80 mL h⁻¹; giving a dispersed phase flux of 434 L h⁻¹m⁻². The shear stress applied was 35 Pa. In Figure 26 is reported the average drop size and uniformity of emulsions obtained using from 10 to 25 wt.% of PVA solutions. It is possible to see that for all the PVA concentrations used, drops of 39- 42 µm were obtained, measured coefficients of variation were in the range of 16- 21%. In this case no difference in terms of size and size uniformity of the drops was observed increasing the viscosity of the dispersed phase. The interfacial tensions measured (using the Du Nouy ring method) and reported in Table 4 (page 51) are corresponding to the interfacial tension for the systems at the equilibrium (equilibrium interfacial tension). It is possible that the actual interfacial tension existing during the drop formation (dynamic interfacial tension) is higher and constant for the systems analyzed; giving the same drop size. On the other hand, it is possible that the effect of the increasing viscosity (which should lead to a reduction of the $D_{av}$) could be counterbalanced by the effect of the increasing equilibrium interfacial tension (which should produce larger drops).
Figure 26 Influence of the PVA concentration in the dispersed phase average droplet size and size distribution. Continuous phase: 2 wt.% ABIL EM 90 in Miglyol, membrane used: stainless steel membrane with 10 µm pore diameter, 200 µm pore spacing. Dispersed phase flux= 434 L h$^{-1}$m$^{-2}$, shear stress= 35 Pa.

4.1.2 Droplets solidification

Aim of this section is the production of uniform spherical PVA microparticles for the encapsulation and sustained release of active molecules by oral administration. According to Rathbone et al. (2002) the average particle size of the microparticles used for oral drug delivery is between 20 and 120 µm (Rathbone et al., 2002). PVA microparticles have already being produced using emulsification methods; where the drops formation was achieved by mechanical stirring (Thanoo, 1993; Bachtsi and Kiparissides, 1995; Shah et al., 1997; Varshosaz and Koopaie, 2002). Dispersion Cell was used in order to have a fine control over PVA droplets which were then converted to solid uniform microparticles after suitable crosslinking. Solidification of the liquid PVA drops produced with the membrane to solid microparticles was achieved by chemical reaction with the GA (crosslinking). Various authors have reported (Shah et al., 1997; Thanoo et al., 1993; Mandal, 2000; Varshosaz and Koopaie, 2002) that the release pattern of a drug from the microparticles is strongly dependent on the extent of the crosslinking reaction; the release rate is reported to be inversely proportional to the crosslinking density which is directly proportional to the concentration of GA used. Effect of the crosslinking reaction was investigated as well as the swelling properties of the PVA microparticles obtained and release of model molecules is reported.
4.1.2.1 PVA crosslinking

The chemical crosslinking reaction of PVA with GA is a condensation polymerization which removes two water molecules per each GA molecule used. The reaction is catalysed by the presence of a strong acid, and it consists in the formation of acetal bridges between the pendant hydroxyl groups of PVA (Hassan and Peppas, 2000). Hence, the process of droplet solidification into solid microparticles produces a shrinkage and reduction of the $D_{av}$. In Figure 27 is reported the effect of the crosslinking reaction on the $D_{av}$ of the liquid drops during the solidification step. The starting emulsions were produced using as dispersed phase a solution of 15 wt.% PVA + HCl 0.1 M, as continuous phase Miglyol containing 2 wt.% PGPR as surfactant. The membrane used was a nickel membrane with 10 μm pore size and 200 μm pores spacing. The dispersed phase flux was 270 L h$^{-1}$m$^{-2}$ for all the experiments. Droplets between 244 to 53 μm were produced varying the shear stress applied from 2 to 35 Pa, solidification of the drops was achieved using 50 vol.% GA in water in a volume ratio 1:1 with the volume of dispersed phase injected. To check that the solidification of the drops occurred, a small sample was withdrawn and washed with acetone. Acetone dissolves the oil and removes the excess water, thus establishing if the dispersion is of droplets, or solidified particles which do not dissolve. Using 50 vol.% GA in water in a volume ratio 1:1 with the volume of dispersed phase injected microparticles were formed after 90 min. Solid microparticles were re-suspended in water and observed under the optical microscope. Size of the solid un-swollen microparticles was between 160 to 35 μm. On average crosslinking using 50 vol.% GA in water in a volume ratio 1:1 reduced initial droplet diameter approximately 30%. Largest shrinkage was observed for the largest particles - starting form 244 μm drops microparticles of 160 μm were obtained. At comparable GA concentration used for the reaction; larger drops are characterized by a lower degree of crosslinking. It is possible that during the washing step the acetone removes a higher amount of water from the larger microparticles.
Mean diameter of liquid droplet (dark blue) and of the solid microparticles obtained chemical crosslinking (light blue) at different values of shear stress used to produce the initial emulsion. Membrane used: nickel with 10 µm pore size and 200 µm pore spacing, dispersed phase used: 15 wt.% PVA in water + HCl 0.1 M, continuous phase: 2 wt.% PGPR in Miglyol. Dispersed phase flux= 270 L h⁻¹m⁻².

4.1.2.1.1 Influence of the crosslinker concentration

Influence of the crosslinker concentration required to produce solid microparticles was investigated adding to the primary emulsion increasing concentrations of GA from 1 to 50 vol.% in a volume ratio with the dispersed phase injected of 1:1. The reaction was conducted for a maximum of 90 min for all the samples under continuous agitation. Once solid microparticles were obtained, the stirring was stopped to allow the particles to settle at the bottom of the beaker. The remaining oil was removed by simply pouring it away from the beaker, remaining particles were washed with acetone and re-suspended in a solution of 2 wt.% SDS in water allowing the complete separation (where possible) of the particles for the observation under the optical microscope. The whole process of washing and re-suspension in water was fast to avoid the microparticles drying at this stage; which could partially affect the final shape and size of the microparticles. Figure 28 presents microphotographs of the produced microparticles using different GA concentrations, the starting emulsion is also reported for comparison. It is possible to see that for 50 to 10 vol.% of GA the size of the particles is the same for all the samples. 5 vol.% GA microparticles show a slightly larger diameter and the shape of some of them is not perfectly spherical. 2 and 1 vol.% GA still
produced solid microparticles; from the observation under the optical microscope they appeared to have a soft consistency and they did not separate by resuspension in the surfactant solution. The size difference observed for less crosslinked samples (1-5 vol.% GA) can be explained considering that a higher amount of water is retained by these particles. SEM analysis of the 50 and 5 vol.% crosslinked microparticles was performed and the images are presented in Figure 29. It is possible to see a difference of the microparticles surface depending on the amount of crosslinker used for the solidification: less crosslinked microparticles showed a corrugated surface which is less visible for higher crosslinked microparticles. The samples need to be completely dried for the observation with the SEM, hence the particles were freeze dried to remove all water present. As the less crosslinked particles are characterized by a more elastic polymeric network, during the drying stage the crosslinked polymeric segments collapse forming these creases. Internal structure of the produced microparticles for 5 and 50 vol.% of GA was examined by slicing the dried particles. The cross-section was observed on the SEM, internally the particles were solid and homogeneous as shown in Figure 30. It is believed that the GA can easily diffuse from the droplets of the secondary emulsion into the polymeric droplets of the primary emulsion. Having in mind that the droplets size is quite small GA molecules have quite short pathway for diffusion. According to this analysis the reaction of GA is not limited to the surface of the drops and solid spheres are produced.
Figure 28 Influence of the GA concentration on microparticles production; comparison with the initial emulsion produced. Operating conditions of production: as dispersed phase 15 wt.% PVA in water, continuous phase: 2 wt.% PGPR in Miglyol, shear stress: 35 Pa, dispersed phase flux: 434 L h⁻¹m⁻², membrane used: stainless steel with 10 µm pore diameter, 200 µm pore size. Solid microparticles washed and re-suspended in 2 wt.% SDS in water.

Figure 29 SEM images of the microparticles surface once dried, crosslinked using (a) 50 vol.% and (b) 5 vol.% of GA.

Figure 30 SEM cross-section of PVA microparticles produced using (a) 50 vol.% and (b) 5 vol. % GA for the crosslinking reaction.

4.1.2.1.2 Influence of crosslinker amount on microparticles swelling properties

PVA crosslinked network is reported as having the capability to sorb water and swell stretching the interconnected polymeric chains, the extent of this stretching is directly related to the degree of crosslinking. Furthermore, the capability of the crosslinked PVA
microparticles to incorporate water and swell is correlated to the release profile of an encapsulated substance within the particles (Varshosaz and Koopaie, 2002). PVA microparticles produced with different amount of GA showed a different swelling degree. The starting emulsion was prepared using the same operating parameters for all the experiments: the dispersed phase was 15 wt.% of PVA in water, the continuous phase was 2 wt.% PGPR in Miglyol. The membrane used was the stainless steel membrane with 10 µm pore size and 200 µm pores spacing. The dispersed phase flux was 434 L h⁻¹m⁻² and shear stress of 35 Pa. Using these operating parameters, droplets sized 45 µm were obtained with a CV of the 20 %. GA in different concentrations (from 5 to 50 vol.%) was added to the emulsion in a volume ratio with the dispersed phase of 1:1. The reaction for all the experiments was stopped after a maximum of 90 min. Once solid microparticles were washed with acetone and left at room temperature for few seconds to dry. To remove completely the water the microparticles were freeze-dried. The particles were than observed under the microscope and microphotographs were taken. Pictures of the dried microparticles were analysed and the average microparticles diameter determined. In order to study the swelling properties of the differently crosslinked microparticles they were allowed to swell completely in water at 37 °C for 24h under continuous agitation using a shaking water bath. Again, samples of the swollen microparticles were taken and analysed under the optical microscope. Figure 31 shows mean particles diameter (in the dried and swollen state) as a function of the amount of GA used for the solidification. It is possible to see that for GA concentration higher than 10 vol.% the difference of Dₐᵥ between the dried and swollen state is minimal. At these concentration of GA the swelling of the microparticles was limited. On the contrary, microparticles crosslinked with less than 5 vol.% GA showed on average a size in the dried state of 30 ± 2 µm approximatively, while after the swelling the particles size was of 47 ±2 µm on average. For a higher GA amount the number of linkages between the polymeric chains is higher, this reduces the ability of the polymeric network to sorb water and swell. Furthermore, the reduction of the swelling can be attributed to the decrease of the free hydroxyl groups of PVA hence a reduced hydrophilicity of the material for high crosslinking densities (Shah et al., 1997). Microphotographs of the swollen particles are shown in Figure 32, it is visible that microparticles crosslinked with 10, 25 and 50 vol.% of GA have the same diameter. Particles crosslinked with 2 and 5 vol.% GA incorporated the water and were able to swell and the spherical shape was not compromised by the swelling. For the particles crosslinked using 1 vol.% GA, the swelling produced a loss of the spherical shape for some
of them, showing the softness of the material as well as the stickiness as the particles aggregated and did not separate even after 24h under continuous shake in the presence of the surfactant (2 wt.% SDS in water).

Figure 31 Average microparticles size crosslinked with different amount of GA in the dried (black squares) and swollen state (blue diamonds). Starting emulsion conditions of production: dispersed phase: 15 wt.% PVA + HCl 0.1 M, continuous phase: 2 wt.% PGPR in Miglyol, dispersed phase flux= 434 L h⁻¹m⁻², shear stress= 35 Pa. Volume ratio polymeric solution: GA solution= 1:1, time of reaction= 90 minutes.
The crosslinking reaction of PVA with GA requires the presence of a strong acid as catalyst (as previously discussed in chapter 3.1.1.1). Hydrochloric acid was added to the PVA solution before the addition of the crosslinker. In order to determine the minimum amount of HCl required for the solidification, different concentrations of acid were added to the polymeric solution. Starting from a concentration of 0.1 M of HCl as reported in other papers
(Mansur et al., 2008) the amount of acid was gradually reduced up to 0.0125 M in the polymeric solution. The acidified solution of PVA was subsequently emulsified in the 2 wt.% of PGPR in Miglyol using an homogenizer. Final concentration of the dispersed phase in the emulsion was 10 %. Crosslinking of the PVA drops was obtained adding 50 vol.% of GA solution to the emulsion in a volume ratio PVA solution: GA solution 1:1. The reaction was stopped after 90 min for all the experiments. A sample of the resulting microparticles was taken, washed quickly with acetone to remove the remaining oil and re-suspended in water. The sample was subsequently observed under the microscope and pictures were taken. Figure 33 reports the microphotographs obtained using a decreasing amount of HCl. It was observed that for concentrations of 0.1 and 0.05 M of HCl solid microparticles are be formed. For further decrease of the amount of acid at comparable conditions of reaction the PVA drops are not solidified by the action of the GA: by addition of the acetone the spherical shape is no longer maintained and clusters of polymer are visible. The amount of acid is directly affecting the crosslinking reaction and the formation of solid microparticles resistant enough to be dehydrated and stored. The role of the acid in the mechanism of crosslinking reaction is the protonation of the carbonyl oxygen of the GA to make the carbonyl carbon more electrophile; it also allows the loss of H₂O as leaving group (McMurry, 2000). Hence, presence and amount of acid is essential for the reaction to happen. It was seen that at these conditions the minimum amount of acid required is 0.05 M of HCl. However, to ensure the amount of HCl always in excess; 0.1 M was used for the further experiments.
Figure 33 Microphotographs of the resulting microparticles prepared using different amount of strong acid (HCl) required for the crosslinking reaction. Dispersed phase used: 15 wt.% PVA in water with appropriate HCl concentration, continuous phase: 2 wt.% PGPR in Miglyol. Emulsions prepared using the homogenizer.

4.1.3 Encapsulation of model compounds and release

4.1.3.1 Blue Dextran (BD) Encapsulation

PVA microparticles containing BD as water soluble model drug were produced. The operating conditions used for the microparticles production are summarized in the Table 6. The appropriate amount of BD was directly dissolved in the polymeric solution made of 15 wt.% of PVA and 0.1M of HCl to obtain a final concentration of 3000 ppm. The liquid drops containing the sugar were subsequently solidified by reaction with GA. 1 g of the obtained microparticles was added to the release medium (PBS solution pH= 7.4), samples at regular time intervals were taken and analysed. Sample A and sample B reported in the table below were crosslinked using respectively 25 and 50 vol.%, furthermore, the produced particles were different in size (sample A= 52 µm; sample B= 26 µm), providing a different pathway length for the Dextran diffusion. The release was checked at different time intervals by withdrawing few mL of the release medium and measuring the concentration of BD using a spectrophotometer, up to a time of 24h. Figure 34 shows the release of BD in 24h. The release was analysed using the UV-Vis spectrophotometer and a scan at several wavelengths
(λ= 200-700 nm) was performed for both samples. A peak at λ= 620 nm was expected, but (as shown in the Figure 34) it was not found any peak of absorbance at that wavelength for both the samples A and B. The sugar was not released from the two samples produced as no traces of it were found in the spectrum. This result can be explained considering that the BD can be itself crosslinked as polymer containing pendant groups able to react with GA in a crosslinking reaction (Brøndsted et al., 1998); the size of the sugar molecule (M_w~ 2,000 kDa) could also prevent the diffusion through the polymer matrix of the crosslinked PVA particles produced. Peaks around 220 nm were found and attributed to impurities. According to Gillett and Gull, 1972; the pure monomeric GA absorbs at 280 nm and GA’s impurities (as its polymer) has an absorbance peak at 235 nm, no peaks were found after 24h in those regions of the spectrum. The particles were left in the release medium up to 5 days and no peaks were present. It was assumed that no unreacted GA is released from the produced particles.

Table 6 Operating conditions for the production of microparticles containing BD as model molecule

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Continuous Phase</th>
<th>Polymeric phase composition</th>
<th>Crosslinker concentration (wt.%)</th>
<th>Dispersed phase flux (L h⁻¹ m⁻²)</th>
<th>Shear (Pa)</th>
<th>Particles size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Miglyol + ABIL 2% w/w</td>
<td>PVA 15% w/w - BD 3000ppm</td>
<td>50</td>
<td>270</td>
<td>35</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>B</td>
<td>Miglyol + ABIL 2% w/w</td>
<td>PVA 15% w/w - BD 3000ppm</td>
<td>25</td>
<td>110</td>
<td>35</td>
<td>26 ± 2</td>
</tr>
</tbody>
</table>
**Figure 34** Spectrum of the release medium analysed using the UV-VIS spectrophotometer within a range of wavelengths between 200 and 700 nm; release time = 24h.
4.1.3.2 Copper (Cu²⁺) encapsulation

15 wt.% PVA crosslinked microparticles were used for encapsulation of copper ions. Different amounts of GA were tested for crosslinking of the particles containing Cu²⁺ in order to study the effect of the crosslinking reaction on the release profile. For this set of experiments 30 µm particles were used. Copper sulphate pentahydrate (CuSO₄ 5H₂O) was directly added to the dispersed phase of the emulsion prior to the crosslinking reaction. Copper sulphate was selected because of the small dimensions of the molecule (CuSO₄ 5H₂O, Mw= 249.68 Da) and possibility to detect small changes of concentration by using the Atomic Absorption Spectrophotometer. Previous papers have used Cu²⁺ as an analogue drug molecule (Imbrogno et al., 2015). The copper sulphate added to the dispersed phase of the emulsion (composed by 15 wt.% PVA and 0.1 M of HCl) could react with the hydrochloric acid producing copper chloride and sulphuric acid. Copper chloride is reported to be soluble in acetone; for this reason the washing of the particles for this set of experiments was done using toluene instead of acetone in order to prevent loss of the encapsulated Cu²⁺. Microparticles were subsequently freeze- dried. The behaviour of the particles was studied in acidic conditions (pH = 3) to mimic the gastric fluid, and neutral conditions (pH = 7) to mimic the intestinal tract. Normally, the gastric emptying time is about 3- 4h (Paradkar, 2008), after a normal meal. Thus, the release was assayed up to 3h, required for possible drug absorption at stomach level. Figure 35 shows the release of Cu²⁺ from 15 vol.% PVA particles as a function of the time depending on the amount of GA used. The release of Cu²⁺ decreases with the increase of the crosslinker concentration; for example the particles prepared with 25 vol.% of GA released less than 1% of Cu²⁺ after 3h, while the particles prepared with the 1 vol.% of GA released up to 70% of Cu²⁺ in 3h. The results obtained are in accordance with the literature (Thanoo et al., 1993; Bachti and Kiparissides, 1995; Shah et al., 1997; Varshosaz and Koopaie, 2002) as the increase of the crosslinking density produced a reduction of the release rate. High initial release of Cu²⁺ was observed from the PVA particles, giving the so called “burst release” (Huang and Brazel, 2001). “Burst release” of Cu²⁺ in the early minutes, could be attributed to a diffusion of Cu²⁺ from the particles surface. It is interesting to notice that no release from PVA particles was observed if GA concentration for crosslinking was 10 vol.% or above. High crosslinking density (GA concentrations higher that 10 vol.%) prevented the swelling of the PVA hydrogels, as a consequence the amount of Cu²⁺ to diffuse out was limited. For the 2 vol.% and 5 vol.% GA
samples, after the reaching the “peak” concentration in the early minutes, the release reached a constant value. The release pattern obtained from 1 vol.% GA microparticles was a sustained release, as after the initial “burst” the detected concentration gradually increased over the 3h.

Figure 35 Release with time of copper ion from microparticles of 15 wt.% PVA crosslinked with different amount of GA. The size of the dried microparticles used was approximately 30 µm ± 2 µm and CV% = 20 ± 3%. Error bars represent the standard deviation of the measurements.

Figure 36 represents cumulative release of Cu²⁺ after 3h depending on the pH of the release medium. 15 wt.% PVA microparticles were crosslinked with 10 vol.% of GA. Even though the resulting concentration of Cu²⁺ is higher at pH 7, a maximum of 0.7% of Cu²⁺ was released in 3h; showing that the microparticles crosslinked with 10 vol.% of GA do not show any significant Cu²⁺ release no matter the pH. In J. Varshosaz and N. Koopaie’s work (2002) even for lower concentration of GA (1.25 vol.%), the crosslinked PVA hydrogel did not respond to variation of the pH. Same result is reported by Yang et al. (2008), swelling behaviour of pure PVA hydrogels is reported to be independent to pH variations.
Figure 36 Cu$^{2+}$ release pH dependent from 15 wt.% microparticles crosslinked with 10 vol.% GA after 3h. The size of the dried microparticles used was approximately 30 µm ± 2 µm and CV% = 20 ± 3%. Error bars represent the standard deviation of the measurements.

Based on these experiments, the produced PVA microparticles can be used to achieve a sustained release of a model compound after the oral administration. However, the release will not be specific for a section of the gastrointestinal tract. With the aim to have a targeted release into the stomach, a pH sensitive polymer was added to the formulation. In the next chapter the use of chitosan as material for the production of microparticles sensitive to the acidic environment will be evaluated.
4.2 PVA-CHITOSAN MICROPARTICLES FOR DRUG DELIVERY

4.2.1 W/O emulsion production

The use of chitosan as pH sensitive material for the production of polymeric blends with PVA or on its own was investigated for the manufacturing of microparticles for the release in acidic environment. Concentrations of chitosan from 1 to 3 wt.% were tested with or without PVA in the formulations. In the following sections a preliminary investigation of the emulsion composition and influence of the operating parameters is presented.

4.2.1.1 Influence of the emulsifier

Different blends of PVA and chitosan were used as a dispersed phase of the W/O emulsions production. Investigation of the appropriate emulsifier type was done using the Dispersion Cell device. For this set of experiments two blends were used as dispersed phase: the PVA 5 wt.%-CS 1 wt.% and PVA 15 wt.%-CS 1 wt.% in water. Compared to pure PVA; viscosity of the blend PVA 5 wt.%-CS 1 wt.% was 2 times higher while the blend PVA 15 wt.%-CS 1 wt.% was 5 times more viscous than pure 15 wt.% PVA solution, as reported in Table 4 (page 51). The membrane used was full nickel membrane with 20 µm pore diameter and 200 µm pores spacing. The dispersed phase flux was set to 270 L h⁻¹ m⁻² and the shear stress was set to 35 Pa. 2 wt.% PGPR and ABIL EM 90 in Miglyol were used to produce the emulsions. Figure 37 reports the effect of the composition of the continuous phase on the average drop size and size distribution of the emulsions prepared using the two polymeric blends as a dispersed phase. It is shown that the use of PGPR as oil soluble emulsifier produced the same size of drops for both the dispersed phases used (Dav= 45 µm). Using ABIL EM 90 droplet sized between 36 and 40 µm were obtained. At comparable conditions of production, slightly larger drops were obtained for the more viscous dispersed phase (PVA 15 wt.%-CS 1 wt.%). Un-uniform emulsions were produced using PGPR: the CV % for both the polymeric blends was higher than 34%. Better uniformity was achieved using ABIL EM 90, a CV% below 30 % was obtained. In Figure 38 microphotographs of the produced emulsions are presented; it is possible to see that for the dispersed phase made of PVA 5 wt.%-CS 1 wt.% the use for PGPR as emulsifier produced a polydisperse emulsion (Figure 38 a), while using ABIL EM 90 (Figure 38 b) an improvement of the size uniformity was achieved. From Figure 38 c is possible to see that with a dispersed phase PVA 15 wt.%-CS 1 wt.% using PGPR as surfactant a very unstable emulsion is produced. The value of 34% as CV% measured is not
reliable as from the observation under the microscope it was seen that the formation of bigger drops occur rapidly after the production of the emulsion due to the instability and coalesce of the drops. Using ABIL EM 90 for the production of the PVA 15 wt.%-CS 1 wt.% polymeric drops (Figure 38 d) the stability improved and uniform drops were obtained. It was seen that the use of PGPR is not appropriate for the formation of the drops using these blends of polymers. It is hypothesized that after the emulsification process the phenomenon of coalesce is due to interactions of CS with the stabilizer (PGPR). The hydrophilic part of PGPR contains polyricinoleic acid, characterized by the presence of groups – COOH. An interaction between carboxylic groups and the CS amino groups in an acidic environment can occur, destabilizing the emulsifier and interfering with its action. Thus, a slight increase of the interfacial tension was measured for the system 2 wt.% PGPR in Miglyol oil and PVA 15 wt.%-CS 1 wt.% (Table 4, page 51).

Figure 37 Influence of the emulsifier type on average droplet size ($D_{av}$) and distribution (CV%). Dispersed phase flux= 270 L h$^{-1}$m$^{-2}$, shear stress= 35 Pa. Membrane type was a nickel membrane with 20 µm pore size and 200 µm pores spacing. Error bars represent the standard deviation of the measurements.
4.2.1.2 Influence of the process operating parameters

Figure 39 reported the average droplet size and size uniformity of the emulsions obtained using three compositions of the dispersed phase. The emulsions were produced using a nickel standard membrane with 20 µm pore size and 200 µm pores spacing, or a stainless steel membrane with 10 µm pore size and 200 µm pores spacing. The shear stress applied was for all samples 35 Pa. Using as dispersed phase the blend PVA 5 wt.%- CS 1 wt.%; the dispersed phase injection rate was varied from 3 to 5 mL min⁻¹, giving a flux from 210 to 350 L h⁻¹ m⁻² for the nickel standard membrane. As expected, the increase of the dispersed phase flux applied produced an increase of the average drop size from 36 to 50 µm. For the dispersed phase made of PVA 15 wt.%- CS 1 wt.%; using a flux of 210- 270 L h⁻¹ m⁻² droplets sized ~
40 µm were produced. As comparison the resulting emulsion produced using the stainless steel membrane is shown, for a dispersed phase injection rate of 0.35 mL min\(^{-1}\) a flux of 114 L h\(^{-1}\)m\(^{-2}\) is generated, producing droplets sized on average 32 µm. Lower value of CV% was obtained using the stainless steel membrane (CV% = 23%) or the nickel membrane at lower dispersed phase flux (CV% = 20%).

**Figure 39** Influence of the dispersed phase flux on average droplet size and size uniformity. Blends of PVA and CS were used as dispersed phase, continuous phase used was 2 wt.% ABIL EM 90 in Miglyol. Membrane types used: nickel membrane (Nm) 20 µm pore size; 200 µm pores spacing, stainless steel membrane (SSm) 10 µm pore size; 200 µm pores spacing. Shear stress = 35 Pa. Error bars represent the standard deviation of the measurements.

For the dispersed phase PVA 15 wt.\% - CS 3 wt.\% a CV % higher than 40 % was measured, due to the high viscosity of this dispersed phase (~ 15000 mPa s) the production of this emulsion was challenging as this polymeric blend was difficult to inject through the membrane pores even using low fluxes and the 20 µm membrane. Damage (bending) of the membrane was experienced. It was not possible to measure the interfacial tension for the system 15 wt.\% - CS 3 wt.\% as dispersed phase and 2 wt. % ABIL EM 90 in Miglyol as
continuous phase using the ring method, the viscosity of the dispersed phase was too high and unreliable values were registered.

For the production of pure chitosan microparticles, several concentrations of the polymer were used for the preparation of the dispersed phase of the primary emulsion. 1, 2 and 3 wt. % of chitosan in water solutions were prepared adding 3 vol.% of CH3COOH to dissolve completely the polymer, as continuous phase was used 2 wt. % ABIL EM 90 in Miglyol. In Figure 40 are presented the resulting D_av and CV% of emulsions produced using a nickel membrane with 20 µm pore diameter and 200 µm pores spacing or; for the dispersed phase made of 3 wt.% of chitosan, a stainless steel membrane with 10 µm pore diameter and 200 µm pores spacing. The shear stress applied was 59 Pa for all the samples while the flux was varied (270- 434 L h⁻¹m⁻²). For a dispersed phase flux of 270 L h⁻¹m⁻² the increase of the chitosan concentration into the dispersed phase produced an increase of the average droplet size from 32 to 53 µm. From Table 4 (page 51) is it possible to see that the viscosity of the 1 and 2 wt.% chitosan solution did not change significantly, while 3 wt.% chitosan in water was two times more viscous, nevertheless, increasing the amount of chitosan into the dispersed phase an increase of the measured value of the equilibrium interfacial tensions was measured, which is in accordance with the larger size obtained. Drops sized 56 µm on average were obtained using the 3 wt.% chitosan solution as dispersed phase injected through the stainless steel membrane with smaller pores than the nickel membrane; but using a higher dispersed phase flux. The uniformity of the emulsions produced was not as good as for the blends PVA- CS (CV%= 20- 23%) or pure PVA drops (CV% below 20%), as the lowest CV% measured was 23- 26%. The emulsions resulted to be stable and no obvious phenomenon of coalescence was observed. From Figure 39 and Figure 40 is noticeable that the use of the stainless steel membrane for both the formulations (PVA and chitosan blends or pure chitosan solutions) improved the uniformity of the droplet size. The material of the membranes is playing an important role in this effect: even though; as previously reported, the PTFE- coated membrane resulted to be more hydrophobic than the stainless steel (chapter 4.1.1.2.2), the PTFE hydrophobic coating of the nickel membrane is thermally bonded on the membrane surface (Morelli et al., 2017) and the acidic nature of the dispersed phase injected could potentially remove it. It was hypnotized that the nickel membrane becomes less hydrophobic with multiple uses producing also less uniform drops.
Figure 40: Droplet size and size distribution of the emulsion as a function of chitosan concentration in the dispersed phase. Shear stress= 59 Pa. Membranes: SSm: stainless steel membrane, Nm: nickel membrane. Error bars represent the standard deviation of the measurements.

4.2.2 Particle solidification using glutaraldehyde (GA)

Chitosan and PVA can be chemically crosslinked using the GA. The reaction of chitosan with GA involves the amino groups of chitosan and the carbonyl carbon of GA. In this reaction (as well as the reaction of PVA with GA) the acid acts as catalyst. Due to the presence of the amino and carboxyl groups in the repeating unit of chitosan, the polymer is soluble in diluted acidic solution; hence the polymeric blends were acidified with 3 vol.% of CH₃COOH and a 0.05 M of HCl. Quantity of the acids used was not changed, the study of the crosslinking reaction was performed varying the concentration of GA into the secondary emulsion from 5 to 25 vol.%. The volume ratio between the GA and amount of dispersed phase added in the primary emulsion was 1:1 for all the samples. The polymeric blends as well as the chitosan solution used for the preparation of the primary emulsion are reported in Table 7 including a summary of the concentrations of GA tested. It was determined a time of approximately 90 min for the formation of the solid particles. At this stage acetone was used to wash the particles which were subsequently re-suspended in pure water for the observation under the
optical microscope. Acetone is used to determine the hardness of the microparticles since it removes the remaining oil of the emulsion and the water - if the microparticles were solidified they would not disintegrate when washed with acetone. Figure 41 shows the microparticles obtained by solidification of the PVA 15 wt.%- CS 1 wt. % drops using different concentrations of GA (5-25%). As it can be seen 10 vol.% was the limiting GA concentration to obtain solid microparticles. Particles crosslinked using 5 vol.% GA (Figure 41 a), lost completely their shape and agglomerations is visible. Using 10 vol.% GA (Figure 41 b) solid microparticles are formed, resistant to acetone washing, however a structure similar to a “deflated” sphere is shown. Figure 41 c reports particles crosslinked using 25 vol.% GA sample Resulting particles were kept their spherical shape hence they were more rigid than the 10 vol.% GA crosslinked sample.

Table 7 Conditions of microparticles production for the polymeric blends tested and pure chitosan.

<table>
<thead>
<tr>
<th>Continuous phase</th>
<th>Polymeric phase composition</th>
<th>Crosslinker concentrations tested (vol.%)</th>
<th>Volume ratio (mL polymeric phase: mL GA solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 wt.% ABIL EM 90 in Miglyol</td>
<td>PVA 15 wt.%- CS 1 wt.%</td>
<td>5-10-25</td>
<td>1:1</td>
</tr>
<tr>
<td>2 wt.% ABIL EM 90 in Miglyol</td>
<td>PVA 5 wt.%- CS 1 wt.%</td>
<td>10-25</td>
<td>1:1</td>
</tr>
<tr>
<td>2 wt.% ABIL EM 90 in Miglyol</td>
<td>PVA 15 wt.%- CS 3 wt.%</td>
<td>10</td>
<td>1:1</td>
</tr>
<tr>
<td>2 wt.% ABIL EM 90 in Miglyol</td>
<td>CS 1 wt.%</td>
<td>5-10-25</td>
<td>1:1</td>
</tr>
<tr>
<td>2 wt.% ABIL EM 90 in Miglyol</td>
<td>CS 2 wt.%</td>
<td>5-10</td>
<td>1:1</td>
</tr>
<tr>
<td>2 wt.% ABIL EM 90 in Miglyol</td>
<td>CS 3 wt.%</td>
<td>10</td>
<td>1:1</td>
</tr>
</tbody>
</table>
Figure 41 Effect of the GA concentration on the process of particles formation. Dispersed phase: PVA 15 wt.% - CS 1 wt. %. Continuous phase: 2 wt.% ABIL EM 90 in Miglyol. Dispersed phase flux= 210 L h⁻¹m⁻², shear stress= 35 Pa. GA concentration used for particles crosslinking: (a) 5 vol.%, (b) 10 vol.%, (c) 25 vol.%.

Figure 42 reports the microphotographs of the produced chitosan microparticles crosslinked with 5 and 10 vol.% of GA. It can be seen that for 1wt.% of chitosan (Figure 42 a and b) the crosslinking reaction did not produce solid microparticles resistant to the acetone treatment, independently from the amount of GA used for the solidification. 1 wt.% of chitosan was insufficient to obtain particles from the liquid drop. A similar result is observed by increasing the chitosan concentration to 2 wt.% (Figure 42 c) and using 5 vol.% GA for the crosslinking reaction; visually the particles formed are extremely soft and transparent, also they agglomerate forming clusters. 10 vol.% GA and 2 wt.% of chitosan produced solid particles shown in Figure 42 d which were separated and resistant. 3 wt.% chitosan drops were directly crosslinked using 10 vol.% of GA, in Figure 42 e is possible to see that solid and separated microparticles were obtained, resistant enough to be dried and stored for further
analysis. 10 vol.% of GA was enough for the formation of all microparticles: PVA- CS blends and the 2- 3 wt.% chitosan solutions, hence it was the concentration selected.

Figure 42 Effect of the GA concentration on the process of particles formation: (a) 1 wt.% CS particles crosslinked using 5 vol.% GA, (b) 1 wt.% CS particles crosslinked using 10 vol.% GA, (c) 2 wt.% CS particles crosslinked using 5 vol.% GA, (d) 2 wt.% CS particles crosslinked using 10 vol.% GA, (c) 3 wt.% CS particles crosslinked using 10 vol.% GA. Continuous phase: 2 wt.% ABIL EM 90 in Miglyol. Dispersed phase flux= 270 L h⁻¹m⁻², shear stress= 59 Pa.
4.2.3 Encapsulation and release of copper ions

4.2.3.1 Optimization of the Cu\(^{2+}\) source and microparticles washing procedure

For the analysis of the release profile of drug from the obtained particles, Cu\(^{2+}\) was encapsulated as model compound. It was observed that the dissolution of CuSO\(_4\) directly into the dispersed phase of the emulsion made of blends of PVA and chitosan in different ratio or pure chitosan produced a precipitation of the chitosan. It was hypothesized that the presence of the acidic environment of the dispersed phase could produce in a certain quantity sulphuric acid from the reaction of the copper sulphate with the HCl present in the blends PVA- CS or with the acetic acid present in the pure chitosan solutions. It is reported in literature (Kumbar et al., 2002) that chitosan can be ionically crosslinked using sulphuric acid as the anion SO\(_4^{2-}\) can react with the positively charged amino groups of the chitosan forming ionic bonds. Hence the presence of the anion SO\(_4^{2-}\) in the polymeric dispersed phase prevented the dissolution of chitosan. For this reason the source of Cu\(^{2+}\) was changed from copper sulphate to copper chloride for the formulations containing chitosan. To obtain a final concentration of 5,000 ppm of Cu\(^{2+}\) in the dispersed phase, a polymeric phase containing 13,500 ppm CuCl\(_2\) \(2\text{H}_2\text{O}\) (MW = 170.48 g mol\(^{-1}\)) was made. Polymeric microparticles were so produced using for all the compositions of the dispersed phase 10 vol.% of GA for the crosslinking reaction in order to use for all the samples the lowest concentration required to make solid microparticles. The washing procedure was carefully changed accordingly to the solubility of the CuCl\(_2\) in acetone (refer to section 4.1.3.2). Initially toluene was used to remove Miglyol from the microparticles; it is shown in Figure 43 that using toluene in the washing step of the microparticles was not appropriate. Microparticles were softened (Figure 43 a). Toluene was incompatible with the chemical composition of these microparticles, and was substituted with isohexane (Figure 43 b) which was effective to eliminate Miglyol but did not damage the microparticles.
Figure 43 Miglyol washing. (a) toluene, (b) isohexane. Dispersed phase: PVA 15 wt.%- CS 1 wt.%. Continuous phase: 2 wt.% ABIL EM 90 in Miglyol. GA concentration: 10 vol.%.

4.2.3.2 Cu$^{2+}$ detection and time dependent release

PVA 15 wt.%- CS 1 wt.% microparticles and CS 3 wt.% microparticles were freeze-dried and subsequently observed using the SEM Hitachi desktop; EXD analysis of the elements present in the polymeric particles was done to check if Cu$^{2+}$ was successfully encapsulated. An elemental mapping was tested as a quick method to determine encapsulation. Obtained images show presence of the elements using different colours. Figure 44 reports elemental maps for the sample PVA 15 wt.%- CS 1 wt.% crosslinked using 10 vol.% of GA.
Figure 44  EDX analysis (elemental mapping) of the PVA 15 wt.%- CS 1 wt.% sample crosslinked with 10 vol.% GA containing CuCl₂ as model compound encapsulated.

As expected, it is possible to see that the carbon is the element present in the largest amount; followed by the oxygen. Those two elements can be attributed to the chemical nature of the polymer and cross linker used; while presence of Cu²⁺ (shown with the yellow colour in Figure 44) cannot be attributed to any the chemical composition of the two polymer used for the particles or the crosslinker. The presence of the encapsulated Cu²⁺ is also confirmed by the presence of chlorine (shown in purple in Figure 44). Even though the elemental mapping is a qualitative analysis, the intensity and the brightness of the colours in the obtained images have a correlation with the amount of the element present. In this case, is possible to see that the purple colour appears to be more intense than the yellow colour, which is in accordance with the chemistry of the material encapsulated as chlorine concentration is double compared to the Cu²⁺ concentration. Elemental mapping was also performed on 3 wt.% chitosan particles, and results presented in Figure 45.
In this case a section of the particle was analysed. From the only SEM image is possible to see that internally the CS particles appear to have a porous structure. In Figure 45 detected carbon is shown with the red colour and again it represents the element present in the largest amount. Oxygen is shown in green, carbon and oxygen are the main constituents of the polymeric particles hence the related colours are higher in intensity and delimiting the microparticles area. Cu\(^{2+}\) and chlorine were also detected, the yellow colour referring to the presence of Cu\(^{2+}\) resulted to be spread in the whole sample rather than being limited to the particles area. This effect could probably be the consequence of reduced quantity of Cu\(^{2+}\) present, the intensity of the colour is not enough to delimit the particles threshold. In purple the chlorine is showing the edges of the microparticles and as well as for the PVA 15 wt.%-CS 1 wt.% sample the intensity is higher than the one deriving from the presence of Cu\(^{2+}\).

4.2.3.2.1 Atomic Absorption Spectrophotometry

Release of the encapsulated Cu\(^{2+}\) was assayed with time in acidic conditions (pH= 3), the Cu\(^{2+}\) released in the medium was detected using the AAS (Atomic Absorption Spectrophotometer) as done for the previous samples produced in this work. As previously reported in section 4.1.3.2 it is generically accepted that the gastric emptying time is about 3-4h (Paradkar, 2008), after a normal meal. For this reason the release from the particles was
tested within a maximum of 3h which is the limiting time for a drug to be absorbed at stomach level. **Figure 46** shows the release from particles made by either pure CS or blending of PVA with CS in different ratios, all the samples were crosslinked using 10 vol.% of GA. For comparison the release of Cu$^{2+}$ from pure PVA microparticles is also reported. It is visible that Cu$^{2+}$ was gradually released over time reaching up to a maximum of the 80% in 3h depending on the polymeric blend. Faster release is obtained from the polymeric blends of PVA 15 wt.%- CS 1 wt.% and PVA 5 wt.%- CS 1 wt.% which gave a similar release profile. Particles produced using 3 wt.% CS showed a very low release of Cu$^{2+}$, only 5 wt.% of the encapsulated Cu$^{2+}$ was released in 3h. This effect is due to the higher crosslinking density: at comparable amount of crosslinker used (10 vol.% of GA) the GA reacts with a lower concentration of polymer (3 wt.% of CS). The sample made by the combination of 15 wt.% PVA and 3 wt.% of CS, gave intermediate release. Such behaviour could be attributed to the high viscosity of this polymeric phase (**Table 4**, page 51), which as a result produces more dense particles, less able to swell and to release the Cu$^{2+}$. As comparison the release of Cu$^{2+}$ from pure PVA microparticles is also reported in **Figure 46**, it resulted to be extremely low (near to 0 %) even after 3h, in these conditions.

**Figure 46** Release of Cu$^{2+}$ ions time dependent in acidic environment for microparticles made of either the polymeric blends of PVA and chitosan or pure chitosan. All the samples were crosslinked using 10 vol.% GA. The size of the dried microparticles used was approximately 30 µm ± 2 µm and CV% = 20 ± 3%. For comparison the
results obtained from pure PVA particles crosslinked with 10 vol.% of GA is also reported. Error bars represent the standard deviation of the measurements.

4.2.3.3 Release of Cu²⁺- pH dependent

To investigate pH sensitivity of the prepared formulations, the Cu²⁺ from the microparticles was tested at pH= 3 and pH= 7, and the results are reported in Figure 47. The release time was 3 h. Visually, the formulations containing both polymers, released more Cu²⁺ at acidic pH. More evident is the Cu²⁺ concentration difference at the two pHs for microparticles made of PVA 15 wt.%- CS 1 wt.% and PVA 5 wt.%- CS 1 wt.%: the release in acidic environment was approximately 82%, while about 1% of Cu²⁺ was released from the same microparticles in 3h in neutral conditions (pH= 7). Particles made with only CS (crosslinked with 10 vol.% of GA) did not show a significant release of Cu²⁺ at any pH. It is evident that even though CS is a pH sensitive polymer, pure chitosan crosslinked matrix shows a reduced pH sensitivity as the most of the amino groups present in chitosan react with the crosslinker in the formation of the chemical bridges (Berger et al., 2004). Presence of a second polymer in the formulation able to react with GA is required to produce pH sensitive particles, since it can reduce the crosslinking density of the CS and increase the number of free amino groups (Berger et al., 2004). It was also observed that the viscosity of the polymeric blend does have an effect on the release trend; if it is too high, the density of the deriving polymeric network increases as well causing a lower diffusion rate of substances and molecules encapsulated in the particles; similar observation is reported in the work by T. Uhlich et al. (1996). None of the prepared microparticles showed a significant release in neutral conditions.
Figure 47 pH dependent Cu\(^{2+}\) Release of microparticles made of either the polymeric blends of PVA and chitosan or pure chitosan. All the samples were crosslinked using 10 vol.% GA. The size of the dried microparticles used was approximately 30 µm ± 2 µm and CV\%= 20 ± 3%. Error bars represent the standard deviation of the measurements.

4.2.4 Release of sodium salicylate

To study the behaviour of a drug molecule, sodium salicylate (SS) was encapsulated within the PVA- CS particles crosslinked with 10 vol.% of GA. The sample that gave the highest release of Cu\(^{2+}\) was chosen (PVA15 wt.%- CS1 wt.%) for these tests. The release was tested at pH =3 and 7. As shown in Figure 48, the release of SS with time in acidic environment is reported. It is possible to see that the microparticles released the SS slowly reaching only 23% of the drug in 3h. SS is an anionic drug (Feng et al., 2009) (MW= 160.11 g mol\(^{-1}\)) and it was hypothesized that in an acidic environment the ionic amino groups of CS can delay the diffusion of the negatively charged molecule of SS; which can interact ionically with the charged group of CS. Comparing the release of Cu\(^{2+}\) with the SS, the delay of the SS release can be explained considering the drug-polymer ionic interaction (Puttipipatkhachorn et al., 2001) and the MW of the SS molecule which is much higher than the Cu\(^{2+}\) ion. On the other hand, Cu\(^{2+}\) is a positively charged ion, which can be repelled from the CS polymeric matrix, facilitating release.
Figure 48 Sodium salicylate cumulative release time dependent from microparticles of PVA 15 wt.%- CS 1 wt.% crosslinked with 10 vol.% GA. The size of the dried microparticles used was approximately 30 µm ± 2 µm and CV% = 20 ± 3%. Error bars represent the standard deviation of the measurements.

Figure 49 reports the release of SS depending of the pH of the release media; acidic (pH= 3) and neutral (pH= 7) solutions were used. It is shown that a little difference of the SS concentrations is measured in the two release media. A small amount of SS was released at pH=7 from the PVA-CS particles after 3h; approximately 13%, while the release in acidic environment was slightly higher reaching up to 23%. However, this behaviour was expected since CS is not ionized at pH= 7 and the ionic interaction between the polymer and the SS do not occur. SS is released in a small quantity at pH 7 probably from the surface of the particles. Type and charge of the molecule encapsulated in the CS based microparticles play a fundamental role in the release profile that can be obtained. Amount of crosslinker must be also considered in order to achieve the desired release rate of the molecule with time.
Figure 49 PH dependent release of sodium salicylate from microparticles made of PVA 15 wt.%- CS 1 wt.% crosslinked with 10 vol.% GA. The size of the dried microparticles used was approximately 30 µm ± 2 µm and CV%= 20 ± 3%. Error bars represent the standard deviation of the measurements.

Using the technique of ME uniform microparticles were successfully produced, modifying the chemistry of the formulation it was possible to achieve a pH sensitive release of the encapsulated materials. The technology of ME has been already applied for the production of microparticles for the encapsulation of ions, molecules or enzymes (Imbrogno et al., 2015; Piacentini, Yan, et al., 2017; Piacentini, Dragosavac, et al., 2017). While, to-date, Song et al. (Song et al., 2003) is the only work reported for the encapsulation of cells using an SPG membrane. In that work there were no details of cell density used. ME is not an obvious choice for cell encapsulation: the microfilters used would be expected to filter out the suspended cells, rather than allow the passage of the cells from one side of the membrane to the other. In the following chapter the encapsulation of yeast into particles produced using the ME technique with the micro-sieve type metal membranes is investigated. The micro-sieve metal membranes are appropriate for cell encapsulation, as conditions that may cause membrane blockage, or cell filtration, can be avoided. A study of the most appropriate formulation to achieve a release of the cells into the intestinal-colon area is also reported.
4.3 GELATINE -CHITOSAN MICROPARTICLES FOR CELL ENCAPSULATION AND COLONIC RELEASE

4.3.1 W/O emulsion– with and without yeast cells

4.3.1.1 Effect of the emulsifier on drop size and emulsion stability

Preliminary investigation of the emulsifier type using Miglyol as oil phase for the preparation of the W/O emulsions was performed. Using standard nickel membrane with 30 µm as pore size and 80 µm pores spacing, a dispersed phase made of 5 wt.% gelatine blended with 2 wt.% chitosan (G:CS (5:2)) with 2 vol.% CH₃COOH was injected into the Dispersion Cell. Dispersed phase injection rate used was between 5 and 20 mL min⁻¹ corresponding to a flux from 350 to 1400 L h⁻¹m⁻². Resulting average drop size and size distribution is reported in Figure 50, for all the experiments reported in the graph the rotation speed of the stirrer was set on 1500 rpm. It is possible to see that, independently from the emulsifier type used, the measured value of CV% was higher than 30%. The lowest value CV% for this set of experiments (CV%= 33%) was obtained using 2 wt.% of ABIL EM 90 in combination with an intermediate value of dispersed phase flux (700 L h⁻¹m⁻²). The increase of the dispersed phase flux produced an increase of the average drop size (except for the very high flux used: 1400 L h⁻¹m⁻²); but corresponding CV% measured was high. The variation of the dispersed phase flux did not produce a consistent improvement of the drop size uniformity. Increasing the concentration of ABIL EM 90 to 4 wt.% polydispersed emulsions were produced with a CV% higher than 50%. The use 2 wt.% of PGPR in Miglyol was not successful for the production of uniform drops at any flux used. Figure 51 reports some example microphotographs of the emulsions obtained changing the emulsifier type, ABIL EM 90 (Figure 51 a) and PGPR (Figure 51 b) gave drops with a wide range of size but no breaking of the drops was observed. 2 wt.% Span 80 was also tested as oil soluble emulsifier in Miglyol, Figure 51 c shows the obtained emulsion. It is visible that the drops are extremely unstable and they collapse when touching each other or the glass slide. Presence of small dots in the background can be attributed to the partial dissolution of the Span 80 in the oil phase, which could affect the stabilization of the drops. Variation of the emulsifier type and flux applied did not give satisfactory results in terms of stabilization of the drops and drop size uniformity. As a consequence, the use of a different oil type was considered for the subsequent tests.
Figure 50 Effect of the emulsifier on average drop size and uniformity at different fluxes. Oil used Miglyol. Standard nickel membrane with 30 µm pores and 80 µm pore spacing. Stirrer rotation speed 1500 rpm.

Figure 51 Effect of the emulsifier on average drop size and size uniformity. Standard nickel membrane with 30 µm pores and 80 µm pore spacing. Flux= 700 L h⁻¹ m⁻², stirrer rotation speed 1500 rpm. Dispersed phase: G:CS (5:2). Continuous phase Miglyol with: (a) 2 wt.% ABIL EM 90. (b) 2 wt.% PGPR. (c) 2 wt.% Span 80.

4.3.1.2 Effect of the oil type used as continuous phase

Figure 52 shows the emulsions produced using Miglyol or kerosene as oil phase. The dispersed phase used was G:CS (5:2). The membrane used for all the experiments in this
section was a nickel standard membrane with 30 µm pore size and 80 µm pores spacing. Rotation speed of the stirrer was set to 1500 rpm. Most uniform emulsions produced with Miglyol were obtained adding to the oil 2 wt.% of ABIL EM 90 as surfactant and 700 L h⁻¹ m⁻² as dispersed phase flux (please refer to section 4.3.1.1). Using the same operating conditions, Miglyol oil was substituted with kerosene. 2 wt.% ABIL EM 90 was used as emulsifier. It is possible to see that on average larger drops were produced using kerosene with a CV% higher than 50%. The resulting emulsion was unstable and polydispersed as probably coalescence of the drops occurs. 2 wt.% Span 80 in kerosene was tested; the operating conditions were the same as reported above, from Figure 52 it is visible that the $D_{av}$ resulted to be similar to the $D_{av}$ obtained with the Miglyol oil but with a significant improvement of the size uniformity (CV% = 21%). Kerosene, with Span 80 as emulsifier, resulted to be more effective for the production of uniform sized emulsions with this composition of dispersed phase.

![Figure 52](image)

**Figure 52** Effect of the oil type used for the preparation of the continuous phase on average drop size and size uniformity for different values of dispersed phase flux. Dispersed phase: G:CS (5:2), rotation speed of the stirrer 1500 rpm. Membrane used was a nickel standard membrane with 30 µm as pore diameter and 80 µm pores spacing.
4.3.1.3 **Effect of the operating parameters on drop size and uniformity**

4.3.1.3.1 **Operating temperature**

Effect of the temperature used during the emulsification process is shown in Figure 53, the emulsions were prepared at room temperature or submerging the whole Dispersion Cell within a water bath set to a constant temperature of the 40°C. The membrane used for this test was a nickel standard membrane with 30 µm as pore diameter and 200 µm pores spacing. Continuous phase used was 2 wt.% Span 80 in kerosene and the dispersed phase was G:CS (5:2) with 2 vol.% CH₃COOH. The gelatine present in the dispersed phase is liquid for temperatures higher than 25°C (Karim and Bhat, 2009), as soon as the solution cools down the gelatine starts to coagulate which is inconvenient during the emulsification process as the viscosity of the dispersed phase gradually changes. Initially the emulsification process was carried out straight after the heating of the dispersed phase, but having the Dispersion Cell at room temperature (room T). In a second set of experiments, to prevent the possible coagulation of the dispersed phase, a water bath was used. The dispersed phase flux used was 350 L h⁻¹m⁻². In Figure 53 is possible to see that for comparable values of shear applied; the drop size of the emulsions produced at room T was similar to the emulsions prepared at T= 40°C. The uniformity was comparable with CVs% lower than 30% for all the prepared samples. However, in order to avoid viscosity changes due to the fluctuation of the environmental temperature, the use of the water bath was preferred for the production of emulsions containing pig gelatine.
Effect of the temperature of operation on the $D_{av}$ and CV% varying the shear stress. Dispersed phase flux = 350 L h$^{-1}$m$^{-2}$. Membrane used was a nickel standard membrane with 30 µm as pore diameter and 200 µm pores spacing.

4.3.1.3.2 Membrane pore spacing

Effect of the shear stress on the average drop size and size distribution of emulsions produced using as dispersed phase G:CS (5:2) and continuous phase 2 wt.% Span 80 in kerosene, is shown in Figure 54 compares the results obtained using two nickel membranes having 30 µm pore size and 80 or 200 µm pores spacing respectively. The size of the drops decreased as the shear stress applied is increased in accordance with the data reported in literature (Kosvintsev et al., 2005; Egidi et al., 2008; Dragosavac et al., 2008; M. M. Dragosavac et al., 2012; Holdich, 2010). It was observed that using the same membrane type (nickel standard membrane with 30 µm pore size and 200 µm pores spacing) the increasing of dispersed phase flux from 490 to 700 L h$^{-1}$m$^{-2}$ did not produce a significant change in the drop size for all the shear stress tested. It is also interesting to notice that at comparable values of shear stress applied and dispersed phase flux, the $D_{av}$ and CV% were independent from the distance between the pores of the membrane.
Figure 54 Influence of the shear stress applied on the average drop size and distribution at different values of dispersed phase flux. Membrane types used were: a nickel standard membrane with 30 µm as pore diameter and 80 µm pores spacing (Nm 30/80 µm) or 200 µm pores spacing (Nm 30/200 µm).

The effect of the membrane pores spacing on the drop size and size uniformity has already been investigated in the presence of a shear field and different values of dispersed phase flux (Egidi et al., 2008). For membrane with a regular pore array, if the diameter of a drop formed at the pore becomes bigger than the distance between the membrane pores, the shape of the drops is deviated from spherical to spheroidal for high dispersed phase flux. Hence, increasing the dispersed phase flux, the drop sizes increase up to a point where each drop formed is influenced by the presence of the drops formed at adjacent pores. This produces a faster detachment of the drops from the membrane resulting in smaller and more uniform drops. This additional force acting on a forming drop at the pore opening is called push-off and it is a detaching force. In Egidi et al.’s work (Egidi et al., 2008) is reported that using a 200 µm pore spaced membrane (with 20 µm pore diameter), no evidence of the existence of the push-off force was found. The increase of the dispersed phase flux produced an increase of the average drop size and the increase of the shear stress led to a reduction of the $D_{av}$. While, using an 80 µm pore spaced membrane, the trend observed was slightly different: for
high dispersed phase flux (higher than 1000 L h\(^{-1}\)m\(^{-2}\)) the further increase of the flux did not produce larger drops. For high shear stresses applied, highly uniform emulsions were obtained when the effect of the push-off force was predominant hence for high injection rates. For low shear stresses applied the increasing of the flux produced smaller drops. In the present work, the size of the produced drops was (in some cases) larger than the space between the pores in the case of the 80 µm pore spaced membrane. Using a flux of 700 L h\(^{-1}\)m\(^{-2}\) drops from 43 to 130 µm were produced with a 30 µm pore sized and 80 µm pore spaced membrane; but no evidence of the action of the push-off force was observed. At these operating conditions the two membranes resulted to be interchangeable. It was experienced that the membrane with 30 µm pore size and 80 µm pore spacing was less resistant to mechanical forces than the 200 µm pore spacing membrane and more susceptible to the bending. Hence, the 200 µm membrane was preferred for the subsequent set of experiments.

4.3.1.3.3 Effect of transmembrane flux and shear stress

Variation of average drop diameter and uniformity with dispersed phase flux and shear stress was investigated using as dispersed phase G:CS (5:2) or 5 wt.% of pure gelatine in water. The continuous phase for all the experiments was 2 wt.% Span 80 in kerosene. The membrane used was a nickel standard with 30 µm pore size and 200 µm pore spacing. Emulsions were produced with, or without, addition of yeast cells to the dispersed phase and the results are shown in Figure 55. Initially a dispersed phase flux of 350 L h\(^{-1}\)m\(^{-2}\) was used and the stirrer rotation speed was changed from 200 to 1500 rpm generating a shear stress from 1 to 17 Pa. Consequently, the \(D_{av}\) decreased with increasing shear stress for all the samples. This trend is in accordance with data reported previously (Dragosavac et al., 2008; M. Dragosavac et al., 2012; Morelli et al., 2016). Modifying the shear stress applied it was possible to produce drops sized between 60 µm to 340 µm using the 30 µm membrane. The corresponding CV% was in a range between 17 to 24%. The same set of experiments were repeated adding yeast cells in the dispersed phase; for comparable values of shear stress and dispersed phase flux the mean drop size and uniformity did not change significantly suggesting that the presence of the cells did not influence the emulsion characteristics. Furthermore, it is possible to see that the use of pure gelatine instead of a dispersed phase containing G:CS (5:2) did not produce a variation of the mean drop size and the CV% obtained was 23-25% using 350 L h\(^{-1}\)m\(^{-2}\). On the other hand, reduction of the flux to 70 L h\(^{-1}\)m\(^{-2}\) produced less uniform emulsions. The dotted line within Figure 55 reports the theoretical values of drop size calculated using
the shear-capillary model (Kosvintsev et al., 2005); as demonstrated in another work (Dragosavac et al., 2008) the shear stress is not uniform on the membrane surface, the maximum shear reachable is at radial distance from the centre of the membrane and the maximum shear is normally used in Equ. 7 (page 41) for the determination of the theoretical values of \( D_{av} \). In Figure 55 the results obtained using a dispersed phase flux of 70 L h\(^{-1}\)m\(^{-2}\) were much closer to the theoretical values calculated using the model. The shear-capillary model does not include the dispersed phase flux as a parameter affecting the drop size, it is the theoretical ‘smallest’ drop size formed at zero flux rate, therefore reducing the transmembrane flux the divergence between real and theoretical drop size decreases. The literature does contain models that add a volume to the drops caused by the injection volume flow rate \( (J) \) and the drop formation time \( (t) \):

\[
V = V_0 + tJ \tag{Equ. 22}
\]

where \( V_0 \) is the volume of the drop predicted by a force balance, e.g. Equ. 7 (page 41), and \( tJ \) is the extra volume of the drop caused by operating at a high injection rate and the finite time required for droplet formation (Suárez et al., 2013). However, in order to apply Equ. 22 the drop formation time has to be assessed and this will depend on the fraction of pores that are actively generating drops, which is rarely a known value. Thus, for the purpose of illustrating the key parameters influencing the drop formation in the system studied here the simple force balance model, as represented by Equ. 7 (page 41), is presented.
Figure 55 Effect of dispersed phase flux and shear stress on size distribution and drop uniformity. The hollow square marks represent experiments performed using a dispersed phase of G:CS (5:2) with and without yeast respectively. Hollow circle and triangle marks represent experiments performed using a dispersed phase of G 5 wt.% with and without yeast respectively. Error bars represent the standard deviation of the measurements.
**Figure 56** Effect of the shear stress applied on the average drop size of emulsions prepared using 350 L h^{-1}m^{-2} as dispersed phase flux; with and without yeast encapsulated.
within the dispersed phase. Continuous phase used was 2 wt.% Span 80 in kerosene. Membrane used was a nickel standard membrane with 30 µm pore size and 200 µm pore spacing.

Figure 56 shows images of emulsions produced using the same dispersed phase flux (350 L h⁻¹m⁻²) and varying the shear stress. The dispersed phase was G:CS (5:2) with and without the yeast cells; the continuous phase was 2 wt.% Span 80 in kerosene. The average drop size reduced gradually with increasing shear stress from 1 to 17 Pa. Visually, the size and size uniformity were the same at comparable values of shear stress for emulsions with or without the yeast encapsulated in the drops. The yeast solution added to the dispersed phase was prepared using only pure water, the obtained emulsions were stable: no bursting of the drops was observed.

4.3.1.3.4 Effect of the salts present in the dispersed phase

To encapsulate the yeast, a certain amount of the yeast suspension was added directly to the dispersed phase prior to the emulsification process. The preparation of the yeast suspension was described in section 3.3.1.3.1. For the re-hydration and washing of the cells a Ringer’s solution was initially used taking into consideration that cells placed in hypotonic solutions (such as pure water) will be affected by the low solutes concentration of the environment (Kent, 2000). The Ringer’s solution contains several salts as sodium chloride, potassium chloride, calcium chloride and sodium bicarbonate, which makes it isotonic with the body fluids of animals. Hence, in a first attempt, 1 vol.% of the yeast solution prepared using the Ringer’s solution (to avoid possible cell damage) was added to a dispersed phase made of G:CS (5:2) dissolved into buffer acetate (pH= 4.5) (CH₃COONa with CH₃COOH) in order to keep the pH constant. Formulation of the dispersed phase and specifically the amount of salts present has an effect of the final emulsion stabilization. In a second experiment the dispersed phase was prepared minimizing the amount of salts present: the yeast solution was now prepared using only pure water for the washing and it was added to the dispersed phase in a concentration of 1 vol.%. The dispersed phase was made of G:CS (5:2) in pure CH₃COOH (2 vol.%). Figure 57 are presented the resulting emulsions obtained, all the samples were prepared using a 30 µm pore size and 80 µm pore spaced nickel standard membrane, a shear stress of 17 Pa and a dispersed phase flux of 490 L h⁻¹m⁻². It is visible in Figure 57 a that the emulsion obtained was highly unstable, characterized by collapse of the drops and
coalescence, while reducing the amount of salts present in the dispersed phase, the stability of the emulsion was greatly improved, as visible in Figure 57 b. However, this aspect might be optimized for the encapsulation of a different or more susceptible type of cell using the technique of Membrane Emulsification.

![Figure 57](image)

**Figure 57** Effect of the salts present in the dispersed phase: (a) emulsion produced using a dispersed phase containing G:CS (5:2) dissolved in buffer acetate and 1 vol.% yeast cell solution washed using the Ringer's solution, (b) emulsion produced using as dispersed phase G:CS (5:2) dissolved in 2 vol.% CH₃COOH and with 1 vol.% yeast cell solution washed in pure water. Shear stress= 17 Pa, dispersed phase flux= 490 L h⁻¹m⁻². Membrane used: 30 µm pore size and 80 µm pore spaced nickel standard.

4.3.1.3.5 Effect of the cells presence in dispersed phase

Size and uniformity of emulsion drops was investigated as a function of the amount of yeast cells added to the dispersed phase. The initial yeast dispersion (3.14×10⁹ cells mL⁻¹) was prepared using dried yeast powder re-suspended in pure water. For the preparation of the dispersed phase, a calculated volume of the initial yeast dispersion was added to the polymeric dispersed phase. The dispersed phase was stirred for few minutes at 40°C until uniform dispersion of the cells within the phase was reached. The dispersed phase containing the cells was subsequently injected into the emulsification apparatus through a 30 µm pore sized membrane with 200 µm pore spacing. The amount of yeast dispersion injected was gradually increased up to a final concentration of 3.14×10⁸ cells mL⁻¹. During the emulsion production, the operating parameters were: dispersed phase flux 350 L h⁻¹m⁻², shear stress 17 Pa. **Figure 58** shows the mean drop size as a function of the yeast concentration: it is possible
to see that the amount of cells did not affect the size of the emulsion produced. Using these operating parameters, drops sized ~66 μm were obtained, a good uniformity was achieved with a CV between 19% and 23% as reported in Figure 58. Cell concentration of $3.14 \times 10^8$ cells mL$^{-1}$ was the maximum used in order to avoid membrane damage as formation of cell agglomerates was observed for higher yeast concentration. The agglomerates could potentially block the membrane. Images of the produced emulsions containing yeast cells are shown in Figure 59; the microphotographs were taken using a magnification of 20X and 40X objective lenses to show the cells trapped in the liquid drops. Uniform distribution of the cells within the drops was achieved; the cells did not agglomerate; shape and colour were normal. The emulsions showed a similar size of the drops and up to a concentration of $3.14 \times 10^8$ cells mL$^{-1}$ no drops breaking or coalescence was observed, showing that the emulsions characteristics are not affected by the amount of cell encapsulated. Although the number of cells per drop was high (especially for a concentration of $3.14 \times 10^8$ cells mL$^{-1}$), no yeast cells were found in the continuous phase, suggesting that the cells were successfully trapped within the drops.
Figure 58 Average drop size and CV at different cell concentrations. Dispersed phase: G5 wt.%. Continuous phase: 2 wt.% Span 80 in kerosene. Transmembrane flux= 350 L h\(^{-1}\)\(\text{m}^2\), shear stress= 17 Pa. Membrane used was a nickel standard membrane with 30 µm pore size and 200 µm pore spacing. Error bars represent the standard deviation of the measurements.
Figure 59 Microphotographs of emulsions produced increasing the concentration of cells in the dispersed phase; $3.14 \times 10^7$ cells mL$^{-1}$ (a), $9.42 \times 10^7$ cells mL$^{-1}$ (b), $1.88 \times 10^8$ cells mL$^{-1}$ (c), $3.14 \times 10^8$ cells mL$^{-1}$ (d). Dispersed phase: G 5 wt.$\%$, Continuous phase: 2 wt.$\%$ Span 80 in kerosene. Dispersed phase flux= 350 L h$^{-1}$m$^{-2}$, shear stress= 17 Pa. Membrane used was a nickel standard membrane with 30 µm pore size and 200 µm pore spacing.

The passage of the cells through the membrane, i.e. no filtering effect, was checked by injecting the cell suspension with the highest cell density used ($14 \times 10^8$ cells mL$^{-1}$) through a membrane with 30 µm pore size and 200 µm pore spacing. For this test the yeast suspension in aqueous phase was injected through the membrane in the absence of any organic phase. After 10 mL of the cell suspension passed through the membrane, i.e. the same volume used in the ME, the pump was switched off and the suspension passed through the membrane was recovered and observed under the microscope. To check no cells were filtered, and remained below the membrane, the membrane was turned upside down and the cell suspension injected again. This is termed ‘back-flushed’ in Figure 60, which shows: a the cell suspension before the injection through the membrane; b the cell suspension injected through the membrane; and c the cell suspension using the membrane turned upside down and back-flushed. It is
possible to observe that the number of cells for the three images does not appreciably change and it is reasonable to deduce that they have the same cell concentration. Similar tests were done at the lower cell concentrations used and identical results were found: no membrane fouling observed. Hence, the non-tortuous pore channel membrane used did not appear to retain any of the yeast cells at the cell densities used in this work, and, therefore, the encapsulation efficiency of the yeast cells will be 100%.

Figure 60 Microphotographs of the cell suspension with the highest cell density (3.14 x 10^8 cells mL^{-1}) used as dispersed phase; (a) starting cell suspension, (b) filtered cell suspension and (c) back flushed cell suspension.

4.3.2 Microparticles formation: thermal gelation and ionic crosslinking

4.3.2.1 Pure gelatine drops solidification by thermal gelation

Formation of pure gelatine microparticles was obtained by thermal gelation; the starting emulsion was prepared using the Dispersion Cell. The dispersed phase was made of gelatine (in a concentration from 1 to 5 wt.% ) containing 9.42 x 10^7 cells mL^{-1} of yeast. 2 wt.% Span 80 in kerosene was used as the emulsion continuous phase. Once produced the emulsion was
cooled down using an ice bath. Samples of the particles were taken with time and observed under the microscope: the gelation was considered complete when particles were effectively containing the cells without leakage. Increasing the amount of gelatine in the dispersed phase the gelation time was reduced from 5h (for the 1 wt.% gelatine) to 3h (for the 5 wt.% gelatine). Figure 61 shows the microphotographs of the produced samples; it is visible that the 1 wt.% gelatine microparticles were partially visible and not completely formed; some of the encapsulated cells were released in the surrounding medium as probably the amount of gelatine was insufficient for the formation of solid microparticles. Distinct particles were visible for the 2 wt.% and 5 wt.% gelatine samples, the formed particles contained the yeast without leakage. However, it was not possible to dry completely the pure gelatine particles, as they were not hard enough to keep the spherical shape. Therefore the gelatine particles were washed in 2 wt.% Tween 20 in water, centrifuged and stored (still wet) in the fridge to prevent the gelatine from melting.

![Figure 61](image)

**Figure 61** Gelatine microparticles formed by thermal gelation of the liquid drops in ice using increasing concentrations of gelatine in the dispersed phase.
4.3.2.2 Ionic crosslinking of chitosan

For the production of ionically crosslinked G: CS microparticles a preliminary investigation of the crosslinking of pure chitosan was done. 2 wt.% of chitosan dissolved in a 2 vol.% CH₃COOH solution was used as dispersed phase, as continuous phase 2 wt.% Span 80 in kerosene was used. The emulsion concentration was 10% for all the samples. All the emulsions in this set of experiments were produced using the homogenizer for no longer than 1 minute to avoid the formation of very small drops difficult to observe. Crosslinking was tested using the sulphate ion adding a solution containing 5 or 20 wt.% of sodium sulphate with or without 1N of NaOH directly to the emulsion. Sodium tripolyphosphate (STPP) was also tested as ionic crosslinker in a concentration from 1 to 10 wt.% All the samples were left to react at least for 3h; images of the resulting particles are presented in Figure 62. It is visible that using sodium sulphate the drops were not solidified. The addition of acetone to the sample produced a collapse of the polymeric drops on the glass slide. It was observed that the sodium tripolyphosphate in a concentration of 1 wt.% did not produce solid microparticles as well, but increasing the crosslinker concentration slightly harder particles were obtained. Formation of aggregates is visible for 5 and 10 wt.% of tripolyphosphate. With the same method of investigation, sodium hexametaphosphate (SHMP) was used as ionic crosslinker in a concentration of 10 and 20 wt.% in water, the result is shown in Figure 63. The obtained particles were solid and resistant to the drying process with acetone, but formation of agglomerates was observed for the 10 wt.% SHMP. The reaction occurred within 4h for all the samples. Increasing the temperature of reaction to 50°C did not produce a significant improvement; the obtained chitosan particles were melted and attached on each other.
Figure 62 2 wt.% CS crosslinked with sodium sulphate and sodium tripolyphosphate (STPP) in different concentrations.
Figure 63 2 wt.% CS microparticles produced using increasing concentrations of sodium hexametaphosphate (SHMP) as ionic crosslinker and increasing the temperature of reaction. The microphotographs were taken using magnifications of 10X (photograph of the 10 wt.% SHMP system) and 20X objective lenses (photographs of the 20 wt.% SHMP and 20 wt.% SHMP, T=50°C).

4.3.2.3 Gelatine- CS microparticles formation

4.3.2.3.1 Gelatine- CS microparticles production by thermal gelation

G:CS (5:2) drops containing a concentration of yeast of $9.42 \times 10^7$ cells mL$^{-1}$ were produced using the Dispersion Cell. To solidify the liquid drops, thermal gelation in ice was used. The beaker containing the emulsion was put in an ice bath for 4h. Afterwards the sample was washed using 2 wt.% Tween 20 in water and concentrated using the centrifuge. In Figure 64 is shown the starting emulsion and the product of the crosslinking procedure. It is visible that after 4h in ice no particles were formed and all the yeast present in the liquid drops was released in the surrounding medium. The process of solidification by only thermal gelation is not appropriate for this formulation.
Considering the previous observations, a combination of thermal gelation of the gelatine and ionic crosslinking of chitosan was tested as solidification process to obtain solid G:CS microparticles. The starting emulsion was produced using the Dispersion Cell; the dispersed phase of the emulsion was made of G:CS (5:2) containing $9.42 \times 10^7$ cells mL$^{-1}$ of yeast. As continuous phase 2 wt.% of Span 80 in kerosene was used. The final emulsion was 10% concentrated. Once obtained the drops; the emulsion was placed in the ice bath for 30 min. Subsequently, the SHMP was added. In Figure 65 are reported the microphotographs of the emulsion during the reaction process taken at different times. In this test 2 mL of a 10 wt.% SHMP solution was added to the emulsion after the cooling step. The starting emulsion is reported for comparison, it is visible that after 1h the drops are still liquid and removing the water the drops collapsed. After 3h of reaction the microparticles are visible but not solid enough to be dried. In 4h the reaction is complete, solid particles are formed and no aggregation occurred. A second test was done increasing the amount of the 10 wt.% SHMP solution to 10 mL and the whole procedure of solidification was repeated. Figure 66 shows the microphotographs taken during the crosslinking reaction; visually, after 1h of reaction the shape of the particles is already visible even though the particles are not completely solid. After 2h some of the drops were solidified into microparticles with a spherical shape; however the reaction was considered complete after 3h as all the microparticles were solid and the process of drying did not damage them. For this composition of the dispersed phase the use of a combination of thermal gelation and ionic crosslinking was considered the most appropriate for the production of solid microparticles which can be dried and stored. The

**Figure 64** Gelatine: CS (5:2) solidification by thermal gelation (4h in ice).

### 4.3.2.3.2 Gelatine- CS microparticles production by thermal gelation in combination with ionic crosslinking
The optimal amount of SHMP was 10 mL (in a volume ratio with the dispersed phase injected of 1:1) in a concentration of 10 wt.%. Lower amount of SHMP leads to a slower process of solidification and particle agglomeration.

**Figure 65** Gelatine: CS (5:2) microparticles crosslinked in ice for 1, 3 and 4 h with 2 mL of 10 wt. % SHMP.
4.3.3 Eudragit S100 coating of the gelatine- CS microparticles

To achieve a release of the yeast specifically in the intestinal- colon area, the obtained microparticles were coated using a material resistant to the acidic stomach environment but which is soluble in basic-neutral conditions: Eudragit S100.

4.3.3.1 Eudragit S100 solubility

A preliminary study was done for the determination of the solubility of the Eudragit polymer in different solvents. Eudragit S100 is an anionic colpolymer of methacrylic acid and methilmethacrylate (‘EUDRAGIT® product brochure’, 2016). It is reported to be soluble in methanol, ethanol, isopropyl alcohol and 1M of NaOH giving clear to cloudy solutions; while is practically insoluble in water. Several concentrations of Eudragit S100 (from 2 to 10 wt.%) were used for the preparation of suspensions using different types of solvents. In Table 8 summarises performed tests. 5 wt.% of Eudragit was dissolved in 1M NaOH or in 3 and 10
vol.% of water in acetone. It formed cloudy solutions by stirring; by heating up clear solutions were produced.

Table 8 Summary of the solvents used for the dissolution of Eudragit S100 in different concentrations.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Eudragit S100 wt.%</th>
<th>Soluble</th>
<th>Insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH 1M</td>
<td>5</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>3 vol.% acetone in water</td>
<td>5</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>10 vol.% acetone in water</td>
<td>5</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>Ethanol: Acetone (4:1)</td>
<td>2</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>Ethanol: Acetone (2:1)</td>
<td>5-10</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>5</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>Ringer’s solution pH 7</td>
<td>2</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>PBS+ HCl pH 7</td>
<td>2</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>PBS pH 7.4</td>
<td>2</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>PBS + NaOH pH 8</td>
<td>2</td>
<td>√</td>
<td></td>
</tr>
</tbody>
</table>

Mixtures of ethanol and acetone in different ratios were tested and formation of stable Eudragit suspensions were obtained up to a concentration of 10 wt.%.

PBS and PBS with NaOH or HCl solutions were tested to mimic the gastrointestinal fluids during the microparticles dissolution tests. Eudragit in a concentration of 2 wt.% was added to the PBS solutions having different pHs; the produced suspensions are shown in Figure 67 a. It is possible to see that all the samples were milky suspensions but they were stable as no sedimentation was observed up to two days. At these conditions the variation of pH (from 7 to 8) did not visibly affect the dissolution of the Eudragit. Eudragit was found to be insoluble in water (forming an insoluble film on the water surface) and in the Ringer’s solution; forming an insoluble precipitate at the bottom of the beaker. In Figure 67 b the suspension obtained adding Eudragit to the Ringer’s solution is shown, as comparison the suspension made of Eudragit it PBS+ HCl is also reported. With the arrow is indicated precipitate of Eudragit formed at the bottom of the beaker. It is also visible that a certain amount of Eudragit is floating on the liquid surface. From these observations it was hypothesized that solvents containing the hydroxyl or carboxyl groups or able to generate hydroxide ions are more likely to dissolve the Eudragit S100 compared to pure water or the Ringer’s solution.
Choice of the appropriate solvent for the coating process and dissolution of the coated particles was based on these data. PBS solutions at different pH were used for the release studies.

**Figure 67** Dissolution test for Eudragit S100 in different solvents; (a) PBS solutions at different pHs, (b) comparison of the Eudragit suspensions in PBS with HCl and the Ringer’s solution.

### 4.3.3.2 Eudragit S100 coating process

#### 4.3.3.2.1 Coating by solvent evaporation

Microparticles made of G:CS (5:2) without cells or 5 wt.% of pure gelatine microparticles containing the cells were coated using 5 or 10 wt.% of Eudragit S100 dissolved in a mixture of acetone and ethanol or either pure acetone. **Table 9** reports the summary of all the coating tests performed using as the solvent evaporation method.
Table 9 Summary of the coating operating conditions tested using the process of solvent evaporation.

<table>
<thead>
<tr>
<th>Microparticles type</th>
<th>Coating solution</th>
<th>Washing solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>G:CS (5:2)</td>
<td>5 wt.% Eudragit S100 dissolved in acetone: ethanol (2:1)</td>
<td>Acetone: water (3:1)/ microparticles re-suspended in water</td>
</tr>
<tr>
<td>G:CS (5:2)</td>
<td>10 wt.% Eudragit S100 dissolved in acetone: ethanol (2:1)</td>
<td>Acetone: water (3:1)/ microparticles re-suspended in water</td>
</tr>
<tr>
<td>G 5 with yeast</td>
<td>10 wt.% Eudragit S100 in acetone</td>
<td>Acetone/ microparticles re-suspended in water</td>
</tr>
<tr>
<td>G 5 with yeast</td>
<td>1st cycle: 5 wt.% Eudragit S100 dissolved in acetone: ethanol (2:1)</td>
<td>Acetone: water (3:1)/ microparticles re-suspended in water</td>
</tr>
<tr>
<td></td>
<td>2nd cycle: 10 wt.% Eudragit S100 dissolved in acetone: ethanol (2:1)</td>
<td></td>
</tr>
</tbody>
</table>

0.1 g of G:CS microparticles were added to 10 mL of the coating solution (Table 9), the particles suspension was left to evaporate under continuous stirring. Once all the solvent was completely evaporated the washing solution was directly added to the beaker containing the particles and stirred again for few minutes. The microparticles were filtered and re-suspended in water. Figure 68 shows the coated microparticles using 5 or 10 wt.% of Eudragit respectively, for comparison the uncoated microparticles are also shown. The images refer to microparticles re-suspended in water after the coating and washing process. The material visible in the background of the Figure 68 a is diatomaceous earth added to keep the microparticles separated. Compared to the smooth surface of the uncoated microparticles, the coated ones (Figure 68 b and e) showed a roughened surface due to the deposition of the Eudragit polymer. In the background the presence of clusters of Eudragit polymer is visible. However, it was hypothesized that the coating layer is not uniformly distributed on the microparticles surface: it was expected a reduction of the microparticles size as the presence of the coating layer should have reduced the permeability of the microparticles to the water and subsequently the swelling; while the size of the coated microparticles did not change from the uncoated ones. As reported in Table 9, the G5 microparticles containing yeast were also coated using the method of the solvent evaporation. The gelatine microparticles were not dried, the wet microparticles were directly added to the
coating solution. In the first attempt the coating solution was made of 10 wt.% Eudragit S100 in acetone. The obtained microparticles were washed and dried at room temperature; subsequently they were observed under the optical microscope showing that the coating layer was not present as the microparticles collapsed. A second attempt consisted in coating the microparticles in two cycles: the first coating cycle was done using the same technique as described above; subsequently the obtained particles (in water) were coated again using a higher concentration of the Eudragit. Pictures of the obtained particles are shown in Figure 69. After the first coating cycle a discontinuous layer surrounding the particle is visible; after the second coating cycle (using a higher amount of Eudragit) the microparticles size is visibly reduced but the microparticles were “trapped” in a film of Eudragit and it was not possible to recover them. The process of coating the microparticles using the solvent evaporation method was not efficient for the production of a uniform coating layer on the microparticles surface. The increase of the Eudragit concentration in the coating solution formed a layer of polymer in the beaker which trapped the microparticles.
**Figure 68** G: CS (5:2) microparticles (a) without coating re-suspended in water (b) coated using as coating solution 5 wt. % and (c) 10 wt.% Eudragit S100 dissolved in acetone: ethanol (2:1).

**Figure 69** G5 microparticles coated using a solution of Eudragit S100 in acetone: ethanol (2:1) in two cycles.
4.3.3.2.2 Coating by oil in oil solvent evaporation

The method of oil in oil solvent evaporation was tested for the formation of a uniform coating layer on the microparticles surface. The method was described previously in other work (L. Zhang et al., 2011). The coating reaction occurred in 4h, the reaction was monitored by observation of the particles in the O₁/O₂ emulsion (for the O₁/O₂ emulsion composition please refer to Chapter 3.3.1.3.3, page 61) during the formation of the Eudragit shell. Figure 70 a shows an uncoated gelatine particle suspended in water as comparison, an image of the early stage of the reaction (t= 0 minutes) is shown in Figure 70 b: a liquid shell surrounding the gelatine drop surface composed by the organic phase containing the coating material dissolved is visible. It is possible to see in the background of the image the presence of larger drops of coating solution (O1). With time, evaporation of the solvent of the coating solution is visible, the Eudragit polymer gradually formed a solid layer on the particle surface and in the background a reduction of the coating solution drop size is noticeable (Figure 70 c). The reaction was stopped and considered complete once the O₁ drops were almost invisible leaving the solid Eudragit polymer. The particles were washed and dried at room temperature, giving a white powder. A small sample of coated particles was re-suspended in water and observed under a microscope. Figure 70 d shows one of the coated particles re-suspended in water. Compared to the un-coated microparticles, the coated ones are smaller in size: the mean size of un-coated particles (in water) was 100± 2 µm, and water wet coated microparticles were 70± 2 µm in diameter. The water is extracted from the particles by the O₁ phase during the process of coating producing shrinkage. When re-suspended in water the coating layer prevents water absorption, thus swelling is reduced, proving that using this method the coating layer formed is continuous and the water cannot permeate. Furthermore, the uncoated gelatine microparticles were too delicate for the drying process, so the addition of a coating layer was also advantageous for the final formulation as the coated particles were dried and the storage for longer time was possible.

Figure 71 shows the coated microparticles re-suspended in water after being dried at room temperature, in Figure 71 a the process of coating was done adding diatomaceous earth to the particles solution which was subsequently mixed with the coating solution. It is visible that the coating process led to the formation of aggregates of particles which were impossible to separate. The addition of zinc stearate as anti-sticking agent directly to the coating solution produced separated microparticles; in Figure 71 b is visible that the microparticles were
coated separately and the final dried product was a free flowing powder. However, presence of polymer residuals are visible in the background, the only washing procedure was insufficient for the complete removal of the debris.

**Figure 70** (a) Uncoated microparticle in water (b) O₁/O₂ emulsion during the coating process after 0 minutes and (c) 4h, (d) microparticle re-suspended in water after washing in hexane and drying at room temperature.

**Figure 71** Coated G5 microparticles using the oil in oil solvent evaporation method (a) without zinc stearate and (b) with the zinc stearate into the coating solution.

4.3.3.3 Un-coated and coated particles: dissolution pH and temperature dependence release of the yeast cells

In-vitro release studies were performed on the particles to test the yeast release with time at different pH of the medium. All the experiments were carried out at 37°C. Three formulations were used for this test and compared. **Figure 72** shows images of the particles at different pH and time intervals. The yeast encapsulated particles made of G:CS (5:2) did not show any cell release; this formulation was extremely resistant to the acidic environment (pH 1.2) and to neutral (pH 7) conditions even after 5 days. The crosslinked matrix did not dissolve as shown in **Figure 72 a**. The trapped yeast cells were immobilized within the polymeric matrix and the formulation failed to release the cells. Behaviour of the G:CS formulation at the tested pH
can be explained by the formation of a strong ionically crosslinked polymeric matrix. A complex coacervation occurs between proteins and the polyanion (Wang et al., 2014) preventing the dissolution of the two polymers in the release medium. As the G:CS did not dissolve within the required timeframe and pH, the coating was not performed for this particles and no further analysis were conducted on this formulation. Figure 72 b shows the gelatine microparticles that were initially placed at pH 1.2, to mimic the stomach conditions. Unlike the previous formulation, the pure gelatine particles released very rapidly; they did not resist the acidic environment and released their contents after 5 minutes. The test was also conducted at neutral pH (pH 7), in this case the gelatine particles dissolved slower, after 15 minutes the yeast particles were swollen and almost invisible, but the encapsulated yeast was still retained inside the polymer. After a time of 30 minutes, the gelatine particles were completely dissolved and the content released in the surrounding medium. The pure gelatine microparticles did not meet the requirements for a gastro-resistant formulation: they did not protect the yeast cells from the acidic environment of the stomach for a period of at least 2h (Paharia et al., 2007). Therefore, the Eudragit S100 coating was added to the gelatine particles to preserve the gelatine polymer during the residence at pH 1.2; allowing dissolution of the particles only in a neutral, or basic, environment (pH between 7 and 8) (‘EUDRAGIT® product brochure’, 2016) typical of the intestinal-colonic area. Eudragit coated microparticles were tested and the results are shown in Figure 72 c. In the images, background debris of the coating material is present. At pH 1.2, after 2h, the microparticles still encapsulate the yeast without alteration of their shape, or swelling. The coated microparticles were removed from the acidic medium and transferred to a neutral environment at pH 7; no change was visible after 30 minutes, but a significant modification is noticeable after 2h: the coating gradually dissolved, for some particles a visible breaking of the Eudragit shell exposing the gelatine internal part is noticeable. At this stage, however, the cells were not released from the internal gelatine matrix. After 3h the microparticles were completely dissolved and the structure was no longer visible, the yeast cells were released and they are visible as small spots on the background of the image. In a basic environment the release was faster: it is possible to see that after 15 minutes at pH 8 the external coating was almost dissolved, but the particle shape is still visible. In 30 minutes the microparticles released completely the content, some residual part of the particles is still visible. Although the yeast cells were already released, the complete dissolution of the residual polymer was observed after 1h. The Eudragit S100 methacrylic polymer is insoluble in acids, protecting
the microparticles from dissolution in the stomach environment. However, the formed Eudragit S100 layer on the microparticles dissolves at pH above 7, leaving the gelatine material exposed, and simple melting of the gelatine at 37°C produced the release of yeast cells in this medium.
Figure 72 Microparticles dissolution test with time at different pH; (a) G:CS (5:2) microparticles encapsulating yeast, (b) Un-coated G5 microparticles encapsulating yeast, (c) Eudragit S100 coated- G5 microparticles containing yeast.

4.3.3.4 Yeast cells viability test

4.3.3.4.1 The glucose consumption test

Yeast cells released from Eudragit S100 coated gelatine microparticles (Figure 72 c) were collected and transferred into a 6 mM glucose solution. The glucose concentration was measured at regular time intervals to check for yeast cell viability. Figure 73 shows the reduction in glucose concentration measured with time, in terms of glucose consumed. The test was repeated for three samples and standard deviation of the three measurements is reported in the graph as error bars. All the glucose measurements were conducted using the glucometer, the standard deviation was high for some of the data as the instrument resulted to be extremely sensitive to little variations of the environment and solutions temperatures as well as the amount of solution injected in the reaction chamber (10 µl). However, for this type of test the accuracy of the measurements was considered sufficient as the purpose of the experiment consisted in checking if a reduction of glucose concentration occurred. In Figure 73, the “% glucose consumed” refers to the amount of glucose consumed by the yeast as a percentage of the initial glucose concentration; calculated using the formula Equ.21 (shown in section 3.3.1.3.5).

\[
\% \text{Glucose consumed} = \frac{(\text{Glu}_i - \text{Glu}_n) \times 100}{\text{Glu}_i} \tag{Equ.21}
\]

After 24h the measured concentration of glucose was about 0.4 mM; hence the yeast were able to metabolize up to the 90% of the glucose in one day. The measurements were conducted up to 5 days but no further reduction of the glucose amount was measured. It was hypothesized that the consumption does not reach the 100% because of the cell death due to the absence of nutrients or the increasing amount of ethanol as by-product of the cell metabolism which is toxic for the yeast. The data from Figure 73 shows that the encapsulation process did not damage the cells and that they were successfully protected from an acidic environment (pH 1.2), equivalent to the conditions of the stomach, and after
release when the conditions are no longer acidic the cells are viable and their metabolic activity is preserved.

Figure 73 Released yeast glucose consumption with time as percentage of the initial amount of glucose consumed. Error bars represent the standard deviation of the measurements.

4.3.3.4.2 Viability test using fluorescent probes

Two fluorescent probes for cell dyeing were used to distinguish living cells from dead cells. Using an epifluorescence microscope the yeast cells encapsulated in the Eudragit coated particles (Figure 72 c) and the yeast released from the particles (Figure 72 c) were observed. In Figure 74 a microparticles encapsulating the yeast is shown. From the image it is possible to see yeast cells coloured in green and a small number of cells coloured in red. The number of living cells is higher than the dead cells; however, the result may not be reliable as the cells are still held within a gelatine matrix. The polymeric material could interfere with the absorption of the fluorescent dyes and the results might be unreliable. For this reason the yeast cells released, as described above, were also dyed and checked by fluorescence: Figure 74 b shows these results. The cells were treated with the fluorescent dyes right after being released from the particles. According to this analysis, the living cells are far greater in number compared to dead ones, showing that the encapsulation process was successful, the cells survived a period of time (2h) in acidic conditions and were successfully released (and viable) on returning the particles to a neutral aqueous environment. These conditions mimic
what would be expected to occur during the passage of the encapsulated particles through the human gastric system. Thus successfully delivering living cells to the colon.

Figure 74 Fluorescent microphotographs of (a) yeast cell still entrapped into the particles and (b) the released cell from the particles. Green-yellow coloured cell are living, red-orange coloured cell are dead.

Possibility to immobilize the cells into particles (without having the release) is also an interesting field of research and it finds applications in medicine or the food and beverage industry (Yoshida et al., 2003; Murua et al., 2008). In the next chapter the use alginate as polymeric material for the production of porous microparticles (De Vos et al., 2013) is described for the immobilization of the yeast.
4.4 ALGINATE MICROPARTICLES FOR YEAST ENCAPSULATION

4.4.1 W/O emulsion production

4.4.1.1 Effect of the operating parameters of the emulsification process

Drop size variation and emulsion uniformity depending on the shear stress applied was investigated using as dispersed phase a solution of 2 wt.% of alginate dissolved in water with or without addition of yeast. The use of higher alginate concentrations was inconvenient due to the increase of the dispersed phase viscosity, which could damage the membrane during the injection. Lower concentrations would be insufficient to solidify the drops into particles. Continuous phase of the emulsions produced was 2 wt.% Span 80 in kerosene. The membrane used was a hydrophobic nickel standard membrane with 30 µm pore diameter and 200 µm pore size. The dispersed phase injection rate used for all the samples was 5 mL min\(^{-1}\) corresponding to a flux of 350 L h\(^{-1}\)m\(^{-2}\). The rotation speed of the paddle was increased from 200 to 1500 rpm generating a shear stress from 1 to 17 Pa. Figure 75 reports the values of \(D_{av}\) and CV% obtained varying the shear. Error bars are reported showing the standard deviation of the measurements. Effect of the shear was evaluated on emulsions without yeast, it is visible that the increase of the shear led to a gradual reduction of the drop size from 270 to 84 µm. For all the samples produced the CV% was below 24%, hence uniform drops were obtained at these operating conditions. For comparison, the graph shows the average drop size and CV% of emulsions obtained adding the yeast to the dispersed phase in a final concentration of 9.42x 10\(^7\) cells mL\(^{-1}\) using 17 Pa as applied shear. At comparable value of shear and dispersed phase flux (350 L h\(^{-1}\)m\(^{-2}\)) the resulting drops were had the same size and the uniformity was consistent as well. This data suggested that the addition of the yeast did not have an effect on the emulsion properties. In Figure 76 are reported the microphotographs of the emulsions produced increasing the shear stress. As discussed above, smaller drops were produced by the action of the shear. Visually the emulsions obtained had a clear background showing that the emulsifier is properly dissolved in the continuous phase and it resulted to be appropriate for this formulation. No coalescence or drop bursting was observed for these emulsions.
Figure 75 Influence of the shear stress applied on emulsion drop size and size distribution, comparison between emulsions without yeast and with yeast encapsulated. Dispersed phase flux was 350 L h$^{-1}$m$^{-2}$. Membrane used was a hydrophobic nickel membrane with 30 µm and 200 µm pore spacing. Error bars represent the standard deviation of the measurements.
4.4.1.2 Influence of yeast cell content on droplet size

The influence of the amount of cells encapsulated was examined adding the yeast to the dispersed phase of the emulsions; the appropriate amount of yeast was added to the 2 wt.% alginate in water and mixed up to complete dissolution. The amount of cells in the dispersed phase was increased from $3.14 \times 10^7$ to $1.88 \times 10^8$ cells mL$^{-1}$. Emulsions were produced using as continuous phase 2 wt.% Span 80 in kerosene. The dispersed phase flux used was 350 L h$^{-1}$ m$^{-2}$ and the shear stress applied was 11 Pa; these operating conditions were the same for all the experiments performed. Resulting average drop size and size distribution are shown in Figure 77; it is possible to see that up to a concentration of $9.42 \times 10^7$ cells mL$^{-1}$ the size of the drops did not vary significantly and for all the samples the CV% was below 21%. Further increase of the yeast cells in dispersed phase (above $1.88 \times 10^8$ cells mL$^{-1}$) produced on average larger drops sized 140 µm and the CV% increased accordingly. In Figure 78 are
reported the images of the emulsions produced using a dispersed phase containing $9.42 \times 10^7$ cells mL$^{-1}$ and $1.88 \times 10^8$ cells mL$^{-1}$ and the difference of the droplet size can be observed. For the highest cell density sample ($1.88 \times 10^8$ cells mL$^{-1}$) presence of smaller and larger drops simultaneously is visible. For this sample the stability was not as good as for the less concentrated ones and phenomenon of coalescence was observed. The yeast solution was prepared using a dried yeast powder (Sainsbury's Fast Action Dried Yeast). The powder contained beside yeast (94%) calcium sulphate, sorbitan monostearate, ascorbic acid, alpha-amylase, salt and wheat starch. Presence of additives, emulsifiers and salts in the dispersed phase deriving from the initial yeast preparation might destabilize the resulting emulsions producing polydispersed samples (bursting of the drops occurs due to the osmotic pressure). The yeast was washed several times to try and eliminate additional ingredients but it is reasonable to consider that the process of washing was less effective when higher amount of yeast powder was used.

**Figure 77** Influence of the amount of yeast encapsulated on emulsion drop size and uniformity. Operating parameters of the emulsification process were: flux= 350 L
and shear stress applied= 11 Pa. Error bars represent the standard deviation of the measurements.

Figure 78 Microphotographs of emulsions containing (a) 9.42x 10^7 cells mL^-1 and (b) 1.88x 10^8 cells mL^-1 in the dispersed phase.

4.4.2 Alginate drops solidification by ionic crosslinking

4.4.2.1 Magnesium sulphate as ionic crosslinker

The solidification of the liquid alginate drops into particles was obtained by ionic crosslinking. The positively charged magnesium ion was tested as ionic crosslinker, solutions of anhydrous magnesium sulphate (MgSO₄) in different concentrations were used. A 2 wt.% (or 10 wt.%) MgSO₄ solution was made adding the required amount of the salt in water and slight heating to facilitate the complete dissolution. The solution was cooled at room temperature. The starting emulsion was prepared injecting 10 mL of a 2 wt.% alginate solution as dispersed phase into the emulsification apparatus. Continuous phase was 2 wt.% Span 80 in kerosene. The dispersed phase flux was 350 L h^{-1}m^{-2}, the shear stress applied was 11 Pa. Afterwards, 5 mL of the crosslinking solution (2 wt.% of MgSO₄) was directly added to the emulsion and stirred continuously using the Dispersion Cell stirrer set at 1200 rpm. The reaction was run up to 24h. Figure 79 reports the microphotographs of the resulting particles; the starting emulsion is also reported for comparison (Figure 79 a). The Figure 79 b refers to the particles obtained after 24h of reaction; it is visible that solidification of the drops occurred but formation of aggregates was obtained. Rotational speed was too high and could cause droplet break-up. Rotation speed of the stirrer during the crosslinking reaction was reduced to 500 rpm. Figure 79 c shows the obtained particles after 24h of reaction; in this case no clusters were formed and the particles had a spherical shape. However, they resulted
to be extremely soft and “jelly-like”; hence the particles were not strong enough to go through the process of centrifugation or filtration. A different approach was then tested: to facilitate the crosslinker reaching the alginate drops of the emulsion; a secondary emulsion was produced by mixing 5 mL of 10 wt.% MgSO₄ solution with 25 mL of 2 wt.% Span 80 in kerosene. The secondary emulsion was produced by homogenization for few seconds, afterwards it was added to the primary emulsion produced with the Dispersion Cell. The reaction was conducted for 24h and solidification of the particles was checked using the acetone procedure. From Figure 79 d it is visible that particles were formed but they resulted to be fragile and they did not resist to the washing procedure; thus it was not possible to recover the microparticles.

Figure 79 Ionic crosslinking using MgSO₄ as crosslinker; (a) starting emulsion, (b) crosslinking using 2 wt.% MgSO₄; rotation speed= 1200 rpm, (c) crosslinking using 2 wt.% MgSO₄; rotation speed= 500 rpm, (d) crosslinking using 10 wt.% MgSO₄. (in a secondary emulsion), rotation speed= 500 rpm. Time of reaction= 24h. Magnification used was 10X and 20X objective lenses.
**4.4.2.2 Calcium chloride as ionic crosslinker; effect of the crosslinking process on the yeast glucose consumption**

The use of calcium chloride as ionic crosslinker was evaluated. The starting emulsion was obtained using the Dispersion Cell; the dispersed phase was composed by 2 wt.% alginate in water (containing $9.42 \times 10^7$ cells mL$^{-1}$) which was injected into a continuous phase made of 2 wt.% of Span 80 in kerosene. A first approach consisted in adding directly to the starting emulsion 100 mL of a solution on CaCl$_2$ with a concentration of 40 wt.% The reaction was stopped after 24h; solid microparticles were obtained. The microparticles were recovered by filtration under vacuum using as washing solution a mixture of acetone and water in a volume ratio of 1:2. The use of a small amount of acetone produced a further hardening of the particles which are shown in **Figure 80 a**. Visually the obtained particles did not have a perfect spherical shape but it was possible to dry them obtaining a powder. In a different approach a saturated solution of CaCl$_2$ (CaCl$_2$ solubility in water= 7.45 g mL$^{-1}$ at 20°C) was used for the crosslinking. While stirring continuously the W/O emulsion was added slowly into 100 mL of CaCl$_2$ saturated solution. The mixture was stirred for 1h. The resulting microparticles are reported in **Figure 80 b**. The microparticles were solid and resistant to the drying process; however, shape of particles was elongated. This form is a consequence of the rapid solidification due to the ionic crosslinking with the calcium ion, the liquid alginate drops start to solidify as soon as they touch the calcium solution but the reaction does not occur instantaneously, forming a “tail” while they sink. The ionic crosslinking using the calcium ion resulted to be more effective for the production of hard microparticles resistant to the washing and drying process. Although, the spherical shape is lost during the solidification of the drops into solid microparticles.
4.4.2.2.1 Crosslinking time

Influence of the crosslinking time was evaluated using a very low concentration of crosslinker (5 wt.% CaCl₂ solution) to solidify the alginate drops. Ideally, for higher crosslinker concentrations (as 40 wt.% of the first attempt) the reaction occurs faster; hence the reaction time required for the 5 wt.% solution to solidify the drops was considered the maximum time. 100 mL of the crosslinking solution was added directly to the starting emulsion produced with the Dispersion Cell; the reaction was monitored with time showing that solid particles are formed after 1h. For comparison pictures of the particles were taken after 24h of reaction. The samples produced at these operating conditions were named sample 1. Figure 81 reports respectively the images of samples 1.1 crosslinked in 1h, and sample 1.2 crosslinked in 24h. As shown in the images; the particles are formed and solid after 1h of reaction although the crosslinking using 5 wt.% of CaCl₂ produced a loss of the spherical shape and formation of debris. Increasing the time of reaction up to 24h produced particles with an irregular shape as well and particles aggregates were formed. Hence, the reaction was considered complete after 1h as the particles obtained were resistant to the drying process with acetone, subsequently they were washed with a mixture of acetone: water in a volume ratio of 1:2 and dried at room temperature.
Figure 81 Microparticles crosslinked using 5 wt.% of CaCl$_2$ (a) sample 1.1; picture taken after 1h, (b) sample 1.2; picture taken after 24h. The microparticles shown are re-suspended in water.

To check the influence of crosslinking time on the cell viability, the two samples produced (sample 1.1 and 1.2) were re-suspended in a 6 mM glucose solution and the glucose concentration reduction with time was checked. Figure 82 shows the percentage of glucose consumed by the encapsulated yeast in sample 1.1 and 1.2 with time up to 150h. From the graph it is possible to see that the % of glucose consumed by sample 1.1 and sample 1.2 was similar. A slight reduction of the consumption rate was observed for sample 1.2 however, the glucose metabolism reached the 90% within 50h, showing that yeast viability was not affected by the longer time of reaction. It is reasonable to consider that 1h is a sufficient time to crosslink the drops forming solid alginate particles.
Figure 82 Percentage of glucose consumed with time depending on the time of reaction. Sample 1.1 (full circle signs) was produced in 1h of reaction, sample 1.2 (star signs) was obtained in 24h of reaction using a 100 mL of 5 wt.% CaCl₂ solution.

4.4.2.2 Washing procedure

Influence of the washing procedure on the microparticles formation and yeast viability was examined. For this set of experiment the microparticles were produced by adding 100 mL of a 40 wt.% CaCl₂ solution directly to the starting emulsions obtained using the membrane. The mixture was stirred for 1h with the Dispersion Cell stirrer set at 500 rpm. Once solid the microparticles obtained were washed with two different procedures. Sample 2.1 was washed with 2 wt.% Tween 20 in water and subsequently centrifuged at 800 rpm (3 times) and 1000 rpm (1 time). Sample 2.2 was filtered under vacuum and washed using a mixture of acetone: water (1:2). The obtained particles were dried at room temperature. A small amount of sample 2.1 and 2.2 was re-suspended in water and observed under the optical microscope. Figure 83 reports the images of the microparticles obtained with these two washing methods. It is possible to notice that the microparticles of sample 2.1 were almost transparent showing a soft nature, furthermore using the Tween 20 solution it was more difficult to remove completely the remaining oil: Several cycles of washing and centrifuging were necessary to clean the particles from the oil phase producing loss of the sample.

Figure 83 Microparticles produced using a 40 wt.% CaCl₂ solution and washed with (a) a 2 wt.% Tween 20 in water solution (sample 2.1) and (b) a acetone: water (1:2) solution (sample 2.2). The microparticles shown are re-suspended in water.

The use of the acetone: water solution was more effective in removing the kerosene furthermore the process was less time consuming. Since it was possible to filter the particles
and dry them directly from the filter paper, the loss of sample was reduced. Acetone is an organic solvent that could potentially damage the yeast, the analysis of the glucose consumption was performed on sample 2.1 and sample 2.2 to check cell survival. Figure 84 reports the glucose consumed by the encapsulated yeast in sample 2.1 and 2.2. The percentage of glucose consumed by sample 2.1 reached the maximum in 125h. Sample 2.2 showed a slower consumption rate as in 125h only the 30 % of the glucose present in the solution was metabolized. A glucose consumption of the 85% was reached after 175h. This data shows that the yeast cells are viable and able to metabolize the glucose present in the solution surrounding the particles. However, it was hypothesized that the washing using acetone (in a small amount) produces a further hardening of the particles, thus the diffusion of the glucose through the alginate membrane requires longer time; increasing also the time of glucose consumption.

Figure 84 Percentage of glucose consumed with time depending on the washing procedure used. Sample 2.1 (hollow triangle signs) was washed using 2 wt.% Tween 20 in water, sample 2.2 (full squares signs) was washed using a mixture of acetone: water (1:2). All the samples were crosslinked with a 40 wt.% CaCl₂ solution. Crosslinking time was 1h.

4.4.2.2.3 Glucose consumption depending on the amount of crosslinker used

The glucose consumption with time was evaluated depending on the amount of crosslinker used for the microparticles production. 2 wt.% alginate microparticles containing $9.42 \times 10^7$ cells mL$^{-1}$ were produced using respectively 5- 25- 40 wt.% of CaCl₂ or a saturated solution.
of CaCl$_2$. The starting emulsions for all the samples were prepared using 2 wt.% Span 80 in kerosene as continuous phase, operating conditions of emulsions production were: dispersed phase flux= 350 L h$^{-1}$m$^{-2}$, shear stress= 11 Pa. The drops were solidified adding 100 mL of the CaCl$_2$ solution with the appropriate concentration directly to the emulsion. The reaction was conducted under continuous stirring using the stirrer with a rotation speed of 500 rpm. The reaction was stopped for all the samples after 1h. The obtained particles were washed using a solution of acetone: water (1:2) and dried at room temperature overnight. Another sample was produced by gently pouring the starting emulsion in 100 mL of a CaCl$_2$ saturated solution. The particles were washed after 1h and dried with the same procedure described above. 10 mg of particles were added to a 6 mM solution of glucose and the glucose consumption checked with time. **Figure 85** reports the data gathered. It is noticeable that the glucose consumption is slower for high crosslinked microparticles, while is 0% for the samples prepared using the saturated solution. For the 5-25 and 40 wt.% CaCl$_2$ crosslinked particles the consumption reached a maximum of 85-100% of the initial glucose present in the solution. This suggests that the encapsulated yeast is viable and the difference in the velocity at which the glucose is consumed can be attributed to the crosslinking density. High crosslinked alginate particles are characterized by more interconnected polymeric chains giving rise to a slower diffusion of the glucose through. The sample made with a saturated solution does not produce a reduction of the glucose up to 150h, which can be explained by the death of the yeast encapsulated or the formation of a too dense polymeric network which prevented the glucose permeation.
Figure 85 Glucose consumption as a function of the microparticles crosslinking density. The samples were crosslinked with a concentration from 5 wt.% to 40 wt.% of CaCl₂ or a saturated solution. All the samples were washed using an acetone: water (5:2) solution.

According to the data gathered, ME is suitable for the production of uniform microparticles for living cell encapsulation. In the previous chapters, the samples were produced using the Dispersion Cell device for a lab-scale investigation of the emulsification process and formulations. Possibility to scale-up the process of ME for the production of W/O emulsions (crucial for the subsequent microparticles production) is investigated in the next chapter. The Pulsed (Oscillatory) Flow system selected for the scale-up was previously used for the production of O/W emulsions with hydrophilic membranes (Holdich et al., 2012; Piacentini et al., 2013). Hydrophobic membranes are required for the production if W/O emulsions, hence in preliminary investigation, effect of the membrane coating type is also reported.
4.5 MEMBRANE COATING

4.5.1 Effect of the membrane coating on the emulsion drop size and uniformity

The importance of membrane wettability was demonstrated by Nakashima et al., (1992). They showed that membrane wetting by the dispersed phase should be avoided for successful production of monodispersed emulsions; therefore, in the production of W/O emulsions, the membrane should be thoroughly wetted by the continuous oil phase, in order to minimize the spreading of the dispersed phase on the membrane. Hydrophilic membranes can be converted into hydrophobic membranes by coating the membrane surface with different hydrophobic materials (Yamazaki et al., 2002; Vladisavljević and Williams, 2005) and this thesis have investigated the use of PTFE (polytetrafluoroethylene) and FAS (Fluoroalkyl Silane) coating. For testing of two membrane coatings the dispersed phase flux was 350 L h⁻¹ m⁻², and the shear stress was 17 Pa. The dispersed phase was composed by 5 wt.% gelatine in water, with a yeast concentration of 3.14 x 10⁷ cells mL⁻¹. The continuous phase was made of 2 wt.% Span 80 in kerosene. The emulsions were produced at 35-40 °C. The results obtained using the two membranes (new PTFE and FAS coated) are compared in Figure 86. The PTFE coated membrane had a thermally bonded PTFE coating on the metal surface, which made recoating the membrane (to maintain its hydrophobicity with multiple use) troublesome. In addition PTFE coated membrane requires a delicate washing procedure to avoid membrane coating damage: warm soapy water and an ultrasonic bath for a maximum of 30 s was used for the washing. To overcome this disadvantage, a FAS coated membrane (obtained from Micropore) was also tested for the production of the W/O emulsions. The FAS is chemically bonded to the membrane surface (Jeong et al., 2001), it is more resistant to the washing and recoating of the same membrane is possible. Figure 86 a.1 shows the surface of PTFE coated membrane while Figure 86 b.1 the surface of the FAS coated membrane. Both used membranes had 30 µm pore diameter, 200 µm pore spacing (30/200 µm). The emulsion produced with the PTFE coated membrane was characterized by an average droplet size of 62 µm with a CV% of 18%. For the emulsion produced with the FAS coating the average droplet size measured was of 82 µm while the calculated CV% was 22%. The drops produced using the FAS coated membranes were noticeably larger in size than the ones produced using the PTFE coated membrane, suggesting that the PTFE coated membrane has a greater degree of hydrophobicity than the FAS coated membrane, when new. However, FAS coating may be
more practical for membranes intended for multiple uses due to the higher resistance and possibility to recoat the same membrane.

**Figure 86** Dispersed phase flux = 350 L h\(^{-1}\)m\(^{-2}\), and the shear stress = 17 Pa. The Dispersed phase was 3.14 x 10\(^7\) cells mL\(^{-1}\) dispersed in 5 wt.% gelatine. The continuous phase was 2 wt.% Span 80 in kerosene. (a) Emulsion produced using (a.1) standard PTFE coated nickel membrane 30/200 µm, (b) emulsion produced using (b.1) standard FAS coated nickel membrane 30/200 µm.

**4.5.2 PTFE and FAS coated membrane for multiple uses**

Performance of a standard PTFE coated and standard FAS coated membrane were evaluated for multiple emulsions production. The membranes had 30 µm pore diameter and 200 µm pore spacing. The dispersed phase of the emulsions produced was made of G:CS (5:2) containing 3.14x 10\(^7\) cells mL\(^{-1}\) or 9.42x 10\(^7\) cells mL\(^{-1}\). The continuous phase was 2 wt.% Span 80 in kerosene. Operating parameters of the process for all the samples were: 350 L h\(^{-1}\) m\(^{-2}\) as dispersed phase flux and 17 Pa as shear stress. The Dispersion Cell was submerged in a water bath with a constant temperature 35- 40°C during the production of the emulsions. **Figure 87** reports the microphotographs of emulsions obtained using a new PTFE or FAS
coated membrane (Figure 87 a- PTFE and c- FAS) and PTFE or FAS coated membrane (Figure 87 b- PTFE and d- FAS) after they have been used 4 times. After the use; the membrane (PTFE or FAS coated) was washed according to the procedure described in section 3.1.3. After the washing, the membrane was dried and soaked overnight in pure kerosene without any surfactant. From Figure 87 a- PTFE it is visible that the emulsion obtained with the new PTFE coated membrane was uniform (CV%= 18%) while a reduction of the uniformity is visible after multiple uses: the fourth emulsion produced using the same membrane had a CV%= 35% (Figure 87 b- PTFE). Uniform drops were produced with a new FAS coated membrane with a measured CV% of 22% (Figure 87 c- FAS), a CV% of 24% was measured for the fourth emulsion produced using the same FAS coated membrane (Figure 87 d- FAS). It is noticeable that a new PTFE coated membrane gives monosized emulsions as this type of coating resulted to be more hydrophobic than the FAS, however with the multiple uses the PTFE coating is partially removed producing less uniform drops. The FAS coating showed to be more effective for production of multiple W/O emulsions with a good uniformity. The membranes can be easily recoated with FAS coating in order to regenerate the hydrophobicity when required.
4.5.2.1 Influence of the soaking time

Prior to the emulsification process the membrane is soaked for at least 30 minutes in the oil used as continuous phase without presence of any emulsifier to improve the hydrophobicity of the membrane. The effect of the soaking time on the uniformity of the emulsions obtained was investigated using both PTFE and FAS coated membranes with 30 µm pore size and 200 µm pore spacing. Figure 88 shows the emulsions obtained using the PTFE coated membrane soaked for 30 minutes or overnight, for the emulsions production a shear stress from 7 to 17 Pa was used. The dispersed phase flux was 350 L h⁻¹ m⁻². The dispersed phase was 5 wt.% gelatine containing 9.42x 10⁷ cells mL⁻¹, as continuous phase 2 wt.% Span 80 in kerosene was used. The temperature of emulsions production was 35- 40 °C.
Figure 88 Microphotographs of emulsions produced at different values of shear stress using a nickel standard PTFE coated membrane with 30 µm pore size and 200 µm pore spacing soaked for 30 minutes or overnight in kerosene. The dispersed phase flux used was 350 L h⁻¹ m⁻².

It is visible that the uniformity of the emulsions obtained using a membrane soaked into kerosene for longer time was improved. Using a membrane soaked for 30 minutes emulsions with a CV% of 24%–33% and 27% were produced applying respectively a shear stress of 7-11 and 17 Pa. At comparable values of shear stress applied; the obtained CV% was lowered
to 23%- 17% and 15% respectively using the same membrane but soaked overnight into the oil. This data shows that the PTFE coated membrane is subject to a reduction of the hydrophobicity for shorter soaking times. A longer time of soaking means also an increase of the overall time of emulsions and subsequently microparticles production. Same investigation was done using a FAS coated membrane. The shear stress applied was 17 Pa, the dispersed phase flux used was 350 L h⁻¹ m⁻². The system temperature was kept at 35- 40 °C. As previously discussed the FAS coated membrane gave larger and less uniform drops compared to the PTFE membrane under the same operating conditions. In Figure 89 are reported the images of the emulsions produced using the FAS coated membrane, using the membrane soaked 30 minutes a CV% of 20% was obtained. Increasing the soaking time by leaving the membrane overnight in kerosene produced a negligible increase in CV%.

![Figure 89 Emulsions produced using a standard nickel FAS coated membrane with 30 µm pore size and 200 µm pore spacing soaked for 30 minutes or overnight in kerosene. Shear stress applied was 17 Pa and dispersed phase flux was 350 L h⁻¹ m⁻².](image)

Figure 90 summarizes the obtained values of $D_{av}$ and CV% of the emulsions produced in this section using the PTFE or FAS coated membrane showing the effect of the soaking time. Using a PTFE membrane soaked for 30 minutes presence larger drops was observed comparing to the emulsions produced using the overnight soaked PTFE membrane. The CV% obtained using the 30 minutes soaked PTFE membrane was higher than the CV% of the emulsions produced with the overnight soaked PTFE membrane (probably as a consequence of the spreading of the drops at the membrane surface). The $D_{av}$ of the emulsions produced with the FAS coated membrane did not change depending on the time of soaking and the obtained CV% was similar for both samples. Visually, the FAS coated membrane gave reproducible results independently from the soaking time. Error bars are
reported showing the standard deviation of the measurements. According to this observation the use of the FAS coated membrane could potentially reduce the time of emulsions production; 30 minutes resulted to be a sufficient soaking time to obtained uniform emulsions.

![Figure 90](image)

**Figure 90** Influence of the membrane soaking time on $D_{av}$ and CV% of the emulsions produced using a nickel PTFE or FAS coated membrane with 30 µm pore size and 200 µm pore spacing. Dispersed phase flux = 350 L h$^{-1}$m$^{-2}$. Error bars represent the standard deviation of the measurements.
4.6 SCALE UP OF W/O EMULSIONS PRODUCTION BY MEMBRANE EMULSIFICATION

4.6.1 W/O emulsions production, Dispersion Cell preliminary tests

For the W/O emulsion production scale up using the Pulsed (Oscillatory) Flow system, the optimization of the continuous phase composition was required. Miglyol is less flammable, less toxic and less volatile than the previously used kerosene. Furthermore the use of more viscous oil reduces the risk of leakages and loss of oil through the metallic connectors during the emulsification process. The use of Miglyol oil for the production of the W/O emulsions was tested using the Dispersion Cell and the results compared with ones obtained using kerosene as continuous phase. The dispersed phase was made of 10 wt.% fish gelatine in water. The emulsions were produced using a standard nickel FAS coated membrane with 30 µm pore size and 200 µm pore spacing. The dispersed phase flux was 350 and 490 L h⁻¹ m⁻². Figure 91 reports the 

\[D_{av}\] and CV% of emulsions produced using 2 wt.% Span 80 in kerosene or 2 wt.% ABIL EM 90 in Miglyol. Using Span 80 in kerosene; at comparable values of shear stress (11 Pa) the use of a higher flux produced more uniform drops: at 490 L h⁻¹ m⁻² the CV% obtained was 29%. Resulting size of the drops produced in Miglyol at 22 Pa was in accordance with the size of the drops produced in kerosene (applying a shear between 11 to 27 Pa); Miglyol can be successfully used in this formulation as replacement for the kerosene. Comparable CVs% were obtained using kerosene or Miglyol for the preparation of the continuous phase.

ABIL EM 90, PGPR and Span 80 were tested in Miglyol as oil soluble emulsifiers; Figure 92 shows the emulsions obtained varying the emulsifier type. For this test the dispersed phase used was 20 wt.% fish gelatine in water, the shear stress applied was 22 Pa and the flux was 350 L h⁻¹ m⁻². It is visible that both ABIL EM 90 and PGPR were able to stabilize the polymeric drops in Miglyol while the use of Span 80 produced extremely unstable emulsions causing bursting of the drops on the glass slide and at the bottom of the beaker. The PGPR as emulsifier was not preferred as the dissolution in Miglyol required the heating of the oil and longer mixing time compared to the ABIL EM 90; hence for comparable emulsifying properties the surfactant which is dissolved more easily in the oil was selected.
Figure 91  Influence of the continuous phase composition on emulsion droplet size and size uniformity. The membrane used was a standard nickel FAS coated membrane with 30 µm pore size and 200 µm pore spacing. Dispersed phase flux used was 350 L h⁻¹ m⁻².

Figure 92  Microphotographs of emulsions produced using as continuous phase (a) 2 wt.% ABIL EM 90 in Miglyol, (b) 2 wt.% PGPR in Miglyol and (c) 2 wt.% Span 80 in Miglyol. Membrane used was a nickel standard FAS coated membrane with 30 µm
pore size and 200 µm pore spacing. Dispersed phase flux used was 350 L h⁻¹ m⁻², shear stress applied 22 Pa.

Influence of the dispersed phase viscosity was investigated using as continuous phase 2 wt.% ABIL EM 90 in Miglyol and increasing concentrations of fish gelatine in the dispersed phase. The emulsions were produced using a nickel standard FAS coated membrane with 30 µm pore size and 200 µm pore spacing and the results are reported in Figure 93.

Figure 93 Effect of the amount of gelatine in the dispersed phase on the emulsion $D_{av}$ and CV%. Membrane type used was nickel standard FAS coated membrane with 30 µm pore size and 200 µm pore spacing. Continuous phase used: 2 wt.% ABIL EM 90 in Miglyol. Dispersed phase flux used was 350 L h⁻¹ m⁻², shear stress applied 22 Pa.

The dispersed phase was varied from 5 wt.% to 30 wt.% of fish gelatine in water. The viscosity measured for the 5- 10- 20- 30 wt.% fish gelatine solutions were 4.0±0.1- 12.0±0.5- 59.6±0.79- 380.5±0.8 mPa s⁻¹ respectively (the viscosity measurements were taken at a constant temperature of 40°C to avoid solidification of the gelatine, the phases showed a
Newtonian behaviour). The operating conditions of emulsions production were: 350 L h\(^{-1}\)m\(^{-2}\) as dispersed phase flux and 1200 rpm as rotation speed of the stirrer corresponding to 22 Pa. It is visible from **Figure 93** that increasing the amount of gelatine in the dispersed phase produced a reduction of the CV\% obtained. The emulsions obtained using 5 wt.% of fish gelatine as dispersed phase had a \(D_{\text{av}}\) of 70 µm and a CV\% of 40\%. Microphotographs of the emulsions are presented in **Figure 94**, it is visible that the 5 wt.% fish gelatine sample is characterized by the presence of smaller drops which suggests that at this shear the low viscosity of the dispersed phase could cause breaking of the drops. Increasing the viscosity of the dispersed phase it is noticeable a slight reduction of the emulsion drop size for fish gelatine concentrations of 10, 20 and 30 wt.%. It is believed that the increasing viscosity of the dispersed phase could lead to a reduction of the dispersed phase flux, producing a reduction of the drop size. This trend was observed in previous work (Kukizaki, 2009). The CV\% gradually reduced up to 23\% for the sample prepared using 30 wt.% fish gelatine. However, 20 wt.% of fish gelatine was selected for the subsequent experiments because the viscosity of the resulting solution was not too high and a reasonable uniformity of the drops was achieved.
4.6.2 Pulsed (Oscillatory) Flow system for W/O emulsion production scale up

4.6.2.1 O/W emulsions production; preliminary tests

The Pulsed (Oscillatory) Flow system was initially tested for the production of O/W emulsions in order to investigate the influence of the operating parameters on the mean drop size and size distribution. The composition of the O/W emulsion was 2 wt.% Tween 20 in water as continuous phase and sunflower oil as dispersed phase. For the tests the “generator voltage” was set on 1 V while the frequency was varied from 1 to 15 Hz. The membrane used was a hydrophilic tubular membrane with 20 µm pore size and 80 µm pore spacing. The system was initially filled with the continuous phase using a peristaltic pump and subsequently the pulsation was activated using the LabView executive software. The system was run for at least 30 minutes to ensure that all the air was removed. For the first set of experiments the continuous phase flow rate was 80 mL min\(^{-1}\) and the dispersed phase flow...
rate was 20 mL min$^{-1}$ (CP/DP flow rate$= 80/20$ mL min$^{-1}$). The resulting average drop size and uniformity of the emulsions obtained are shown in Figure 95, it is visible that increasing the frequency of the oscillation a reduction of the drop size is obtained and the trend is in accordance with the data reported in literature (Holdich et al., 2012). The measured CV% was above 30% using 1 and 3 Hz but it decreased up to 23% increasing the frequency from 5 to 15 Hz. It was observed that for frequencies below 5 Hz the signal detected by the accelerometer was unstable and the shape of the oscillation was not in a regular waveform (i.e. sine wave), for low frequencies the amplitude detected was varying with time. In a second set of experiments the injection rates of the continuous and dispersed phase were changed: the CP flow rate was lowered to 70 mL min$^{-1}$ and the DP flow rate was increased to 30 mL min$^{-1}$ (CP/DP injection rate$= 70/30$ mL min$^{-1}$). The frequency used was the one that gave the lowest CV% with the previous combination of injection rates. The average drop size was similar to the $D_{av}$ obtained using a CP/DP injection rate of 80/20 mL min$^{-1}$. The uniformity of the emulsions produced with a CP/DP flow rate of 70/30 mL min$^{-1}$ was equivalent to the one obtained with a CP/DP flow rate$= 80/20$ at comparable voltage and frequency of the pulsation.

![Graph showing influence of frequency on drop size and CV%](image)

**Figure 95** Influence of the frequency on the $D_{av}$ and CV%, the membrane used was a tubular hydrophilic membrane with 20 µm pore size and 80 µm pore spacing. The voltage
was 1 V for all the experiments. Continuous phase was 2 wt.% Tween 20 in water, dispersed phase was sunflower oil.

4.6.2.2 W/O emulsions production

For the production of W/O emulsions in a continuous rate the use of the Pulsed (Oscillatory) Flow system was tested, the influence of the shear stress generated by the pulsation was investigated on the $D_{av}$ and uniformity of the emulsions. For these tests the continuous phase was made of 2 wt.% ABIL EM 90 in Miglyol and the dispersed phase was 20 wt.% of fish gelatine in water. The membrane used for the production of the O/W emulsions showed the previous section (subchapter 4.6.2.1) was made hydrophobic by FAS coating. The continuous phase flow rate was initially set at 80 mL min$^{-1}$ while the dispersed phase flow rate was 20 mL min$^{-1}$ corresponding to a dispersed phase flux of 413 L h$^{-1}$m$^{-2}$. The continuous phase was pulsed along the membrane module using frequencies between 10 to 30 Hz and peak to peak displacement values from 0.5 to 3.5 mm. The shear stress generated at these operating conditions was between 0.4 to 14.5 Pa. The resulting drop size obtained with the variation of the shear are reported in Figure 96, the line reports the theoretical $D_{av}$ calculated using the model (Equ. 7, page 41). The experimental drop size measured was in accordance with the $D_{av}$ determined using the model for equivalent values of shear stress applied. From Figure 96 however is visible that the emulsions produced were characterized by values of CV% above 30 for almost all the experiments. It was observed the formation of oil drops within the water drops of the W/O emulsions obtained. Initially it was hypothesized that the dispersed phase flow could be insufficient to avoid the continuous phase permeation through the membrane during the emulsification process when the pulsation is activated. Hence, in a second set of experiments the continuous phase flow was decreased to 60 mL min$^{-1}$ and the dispersed phase flow was increased to 30 mL min$^{-1}$ generating a dispersed phase flux of 620 L h$^{-1}$m$^{-2}$. Using frequencies between 10 and 25 Hz in combination with peak to peak displacement values of 1.5 or 2.5 mm (i.e. amplitudes of 0.75 or 1.75 mm) the shear stress generated was between 3.3 to 7.8 Pa. The $D_{av}$ of emulsions produced at these operating conditions are reported in Figure 96 for comparison with the previous results; visually the experimental $D_{av}$ are in accordance with the model. The size of the drops produced with a shear of 7.8 Pa is smaller than the predicted $D_{av}$ for this value of shear; this could be a consequence of drops breaking due to the extreme conditions of production. For this set of experiments the resulting uniformity was improved as the CV% obtained was in a range of 25-30%. Error bars report
the standard deviation of the measurements done on three samples. Adjustment of the CP/ DP flow rate improved the uniformity but formation of inner oil drops was still observed. Other combinations of CP/ DP flow rate were tested (90/ 10, 60/ 20, 60/ 25 mL min\(^{-1}\)) giving extremely polydisperse emulsions hence the data were not included in the graph. It was hypothesized that this phenomenon is due to the membrane vibration during the process of emulsification giving a pre-mixing of the two phases, as a consequence of the high viscosity of the emulsions produced.

![Figure 96](image)

**Figure 96** Influence of the shear stress on emulsions drop size and size distribution varying the continuous phase injection rate and the dispersed phase flux. Membrane used was a FAS coated with 20 µm pore size and 80 µm pore spacing. DP: 20 wt.% fish gelatine in water, CP: 2 wt.% ABIL EM 90 in Miglyol. Error bars represent the standard deviation of the measurements.

### 4.6.2.3 Comparison of emulsions produced using the Dispersion Cell system or the Pulsed (Oscillatory) Flow system

The results obtained using the Pulsed system were compared to the \(D_{av}\) and CV% of emulsions produced using the Dispersion Cell system. The way in which the shear is generated is different for the two systems; for the Dispersion Cell the shear is generated by
the rotation of the stirrer on the top of a flat disk membrane; while for the Pulsed system the shear is generated by the pulsation of the continuous phase within the tubular membrane mounted in the membrane module. Consequently, the maximum shear stress is calculated using different equations (please refer to section 2.3.3.1); however, the theoretical $D_{av}$ depending on the shear stress applied was calculated using the shear-capillary model. Figure 97 shows the average drop size vs the shear stress obtained using the Dispersion Cell system for the production of 15 wt.% PVA drops in a continuous phase made of 2 wt.% PGPR in Miglyol. The emulsions were produced using a hydrophobic PTFE coated (disk) membrane with 10 µm pore size and 200 µm pore spacing. The dispersed phase flux for this set of experiments was 12 L h$^{-1}$m$^{-2}$ and the shear stress applied was between 2 and 17 Pa. For comparison is reported the resulting average drop diameter of emulsions produced with the Pulsed system using a dispersed phase made of 20 wt.% fish gelatine in water and 2 wt.% ABIL EM 90 in Miglyol as continuous phase. These emulsions were produced applying a shear stress between 3.3 and 7.8 Pa, 60 mL min$^{-1}$ was used as continuous phase flow and 620 L h$^{-1}$m$^{-2}$ as dispersed phase flux. The membrane was a hydrophobic FAS coated (cylindrical) membrane with pore sized 20 µm and 80 µm pore spacing. The models reported in the graph with the line and the “dash-dot” line were calculated taking into account the pore size of the membranes. These two formulations were selected for the comparison has they had similar interfacial tensions, as reported in Table 4 (page 51): 2.2 mN m$^{-1}$ was measured for the system 15 wt.% PVA in Miglyol with 2 wt.% PGPR and 2.5 mN m$^{-1}$ for the system 20 wt.% fish gelatine in Miglyol with 2 wt.% ABIL EM 90. From Figure 97 it is visible that the size of the drops is in accordance with the predicted $D_{av}$ within a range of shear of 2-5.6 Pa while a slight divergence is noticeable for shear above 6 Pa. However, the data illustrates that the $D_{av}$ of emulsions produced with either the Dispersion Cell or the Pulsed Flow system can be predicted using the shear-capillary model and that the tests performed using the Dispersion Cell can be reproduced on a large scale using the Pulsed system.
Figure 97 Average droplet size and size distribution as a function of the applied shear stress. Comparison between the Dispersion Cell and the Pulsed (Oscillatory) system using membranes with 10 or 20 µm pore size. Error bars represent the standard deviation of the measurements.

4.6.2.4 Continuous production of W/O emulsions using the Pulsed (Oscillatory) Flow system

Scaling up the process of W/O emulsions production using the Pulsed system presents the main advantage of the continuous production of emulsions with a high dispersed to continuous phase ratio (up to the 30%) within a single pass. The possibility to produce emulsions in a continuous rate was tested running the system for 3h. The continuous phase was circulated within the system using the peristaltic pump for 1h, afterwards the dispersed phase was injected. After the production of the first emulsion (named Sample 1) the injection of the dispersed phase was stopped while the continuous phase was re-circulated an additional hour without opening the membrane module. After 1 hour time, the dispersed phase was pumped within the system and a second emulsion was produced (Sample 2) keeping constant all the operating parameters. The whole process was repeated a third time and the emulsion collected was Sample 3. The continuous phase was injected with a flow rate of 60 mL min⁻¹ and the dispersed phase flux was 620 L h⁻¹ m⁻² (i.e. 30 mL min⁻¹). The shear stress was 3.3 Pa generated with a frequency of 20 Hz and a peak to peak displacement of 1.5 mm. The resulting drop size and uniformity of the emulsions produced within 3h in total are shown in Figure 98. It is visible that measured $D_{av}$ was approximately 100 µm for all the
emulsions produced, Sample 1 showed a slight lower CV% (26 %) compared to Sample 2 and 3 produced after 2 and 3h respectively. However, reproducible results were obtained running the system for 3h in continuous without washing the membrane which is advantageous if a high quantity of emulsion is required, furthermore the high concentration of dispersed phase in the emulsion is extremely advantageous as a higher amount of particles can be obtained with a lower consumption of continuous phase.

Figure 98  $D_{av}$ and CV% of emulsions produced running the Pulsed (Oscillatory) system continuously for 1h time. Continuous phase injection rate= 60 mL min$^{-1}$, dispersed phase flux= 620 L h$^{-1}$m$^{-2}$. Shear stress applied= 3.3 Pa.
4.7 RESULTS SUMMARY

The Membrane Emulsification technique was successfully applied for the production of microparticles for the encapsulation of model molecules or probiotic cells and their potential oral delivery. Size of the microparticles obtained was above 28 μm in accordance with the size of particles commonly used for the oral administration (20-120 μm) (Rathbone et al., 2002). The formulations were modified accordingly with the final use of the microparticles, while the size and size distribution were controlled optimizing the process of emulsions production.

The Dispersion Cell device with flat disk membranes having cylindrical pores was used for the generation of the polymeric drops in a controlled manner. Varying the operating parameters of the process such as the shear stress applied at the membrane surface, dispersed phase flux and membrane type it was possible to manufacture uniform emulsions (under the best working conditions CV%=16%) with drops sized from 28 to 340 μm. The Dispersion Cell system resulted to be extremely useful for the preliminary investigation of the emulsion composition and best operating conditions for drop generation with the desired size.

A post-emulsification treatment was adopted to convert the liquid polymeric drops into solid microparticles. The solidification procedure was varied accordingly with the polymeric material selected and the final application. Using PVA and blends of PVA with CS as the emulsion dispersed phase, the solidification of the drops was obtained using glutaraldehyde. It was seen that the extent of the crosslinking reaction had a direct effect of the swelling properties of the material and, as a consequence, on the release rate of the encapsulated model compound. The use of a cationic polymer in the formulation (i.e. chitosan) in association with a second polymer able to react with the GA (i.e. PVA), produced microparticles able to release the model molecule when the environmental pH is acidic.

Alternative formulations were developed for the encapsulation of living cells, the cell passage through the membrane pores during the process of the initial emulsion production was tested showing that selecting a membrane with the appropriate pore size (in this case a 30 μm membrane was used) no filtration of the cell occurs. It was demonstrated that the emulsions properties (D_{av} and CV%) are not affected by the presence of the cells in the dispersed phase. The solidification of the liquid drops containing cells was obtained in mild conditions: by thermal gelation of the gelatine or with a combination of thermal gelation and ionic
crosslinking for the blends gelatine-CS. The release of the cells in neutral-basic conditions (to mimic the intestinal-colon tract) was obtained by coating the particles with the gastro-resistant polymer Eudragit S100.

Immobilization of yeast into particles was obtained using alginate as encapsulating material. Alginate drops were solidified by ionic crosslinking and it affected the porosity of the resulting material. Regardless the material used it was demonstrated that the yeast survived the process of encapsulation and it was shown that Membrane Emulsification is a suitable technique for the production of drops and particles containing cells with the required diameter and with a high uniformity.

Hence, the industrial application of the technique of Membrane Emulsification is object of great interest. As the Dispersion Cell device was conceived for a lab-scale investigation, alternative devices have been developed for the process scale up. In this work the use of the Pulsed (Oscillatory) Flow system for the production of W/O emulsions was tested. Polymeric drops of fish gelatine were successfully generated in Miglyol oil containing a surfactant and a high concentration of the dispersed phase in the emulsion was obtained (~30%) in a single pass.

For the continuous production of W/O emulsions an alternative coating for the production of hydrophobic membranes was tested, performances of a FAS (fluorinated compounds) coated membrane were compared with the classical PTFE coated membrane. The chemically bonded FAS coating resulted to be less hydrophobic than the thermally bonded PTFE producing larger drops at comparable conditions of production, but it resulted to be more appropriate for multiple uses, furthermore using a FAS coated membrane was possible to reduce the membrane soaking time required to obtain uniform emulsions.

A simple shear-capillary model was adopted for the prediction of the drop size for both the Dispersion Cell and the Pulsed systems. It was shown that the experimental results were in accordance with the predicted ones using the model but a divergence between experimental and theoretical size is visible when increasing the dispersed phase flux during the drop production.
5. CONCLUSIONS AND FUTURE WORK

Dispersion Cell system and formulations tested

Poly (Vinyl) Alcohol (PVA) microparticles were obtained starting from W/O emulsions produced using the Dispersion Cell system. Droplets with a narrow size distribution (CV% = 16% under the best operating conditions) were generated using hydrophobic membranes (nickel and stainless steel) having 10 μm pore size and 200 μm pore spacing. Controlled mean drop diameter from 28 to 288 μm were obtained applying a shear stress from 2 to 59 Pa and using 12- 1250 L h⁻¹m⁻² as dispersed phase flux. 15 wt.% PVA in water was used as dispersed phase for most of the emulsions produced and using 2 wt.% ABIL EM 90 or 2 wt.% PGPR in Miglyol stable emulsions were obtained. The stainless steel membrane (pre-soaked in the oil continuous phase) produced smaller and more uniform droplets compared to a PTFE coated nickel membrane. Contact angle measurements confirmed the hydrophobicity of the nickel membrane therefore the pore surface (conical for the nickel and flat for the stainless steel membrane) had a greater influence on the size and uniformity possibly with the drop forming on an area greater than the pore itself for drops produced using the nickel membrane. Solidification of the PVA drops was obtained by a chemical crosslinking reaction using glutaraldehyde (GA); the amount of GA used was varied from 1 to 50 vol.% . Using 50 vol.% of GA a reduction of approximately the 30% of the size was obtained passing from the liquid drops to the solid microparticles. 1 vol.% was the lowest GA concentration which produced solid microparticles. The crosslinking degree directly affected the capability of the PVA microparticles to incorporate water and swell: it was shown that concentrations of GA from 50 to 10 vol.% did not produce a visible microparticles swelling while for concentrations of GA below 5 vol.% the size of the solid microparticles increased significantly after 24h in water. This behaviour to swell is directly correlated with the ability of the particles to release an encapsulated compound. Blue Dextran (BD) and Cu²⁺ were used as model molecules for the encapsulation. No release of BD was detected up to 5 days, while 70% of the encapsulated Cu²⁺ was released from the PVA particles prepared with 1 vol.% of GA copper within 3h. The release profile of Cu²⁺ from the PVA particles was characterized by an initial “burst release” probably due to the release of Cu²⁺ from the surface of the particles. However, no difference in the release of Cu²⁺ was seen from the PVA microparticles at different pH (3 and 7) suggesting that, at these conditions, PVA does not have pH sensitivity.
For the production of pH sensitive microparticles PVA and chitosan were selected as polymeric materials. Uniform emulsions were initially obtained using as dispersed phase mixtures of PVA and chitosan (CS) in different ratios or pure chitosan and 2 wt.% ABIL EM 90 in Miglyol as continuous phase. Membranes used were a stainless steel ringed or a nickel standard membrane with 10 or 20 μm pore size respectively and 200 μm pore spacing. Average drop diameters obtained were between 32 to 56 μm applying a shear stress from 35 to 59 Pa and a dispersed phase flux between 114 to 434 L h⁻¹ m⁻². Lowest values of CV% obtained were 20 and 23%. Crosslinking of the drops was performed using GA and it was seen that 10 vol.% was the lowest crosslinker concentration required to obtain solid microparticles for all the formulations tested in this section. Copper and sodium salicylate (SS) were encapsulated in separate samples and the release was tested with time at pH=3 and 7. Successful encapsulation of Cu²⁺ in the microparticles was confirmed by SEM analysis with Energy Dispersive X-ray Spectrometer (EDX) and using the release tests. Blending chitosan and PVA it was possible to have a release of Cu²⁺ up to 80% in 3h under acidic conditions. No significant release (less than 3%) was observed at neutral conditions. Pure CS microparticles did not show any pH sensitivity and the release of Cu²⁺ did not exceed 5% within 3h. Due to the higher MW and the ionic interaction with the polymeric matrix the release of SS was almost 4 times lower compared to Cu²⁺ after 3h in acidic environment and around 10% in neutral environment.

Microparticles for cell encapsulation and pH depended release were obtained blending Gelatine with Chitosan and applying a polymeric coating on the microparticles surface. The Dispersion Cell was used for the production of the W/O emulsions with hydrophobic membranes of 30 μm pore diameter and 80 or 200 μm pore spacing. The aqueous dispersed phase composed of a mixture of gelatine and chitosan, or just gelatine with yeast cells, used as an example of encapsulating a living organism. The oil continuous phase used was 2 wt.% SPAN 80 in kerosene. The dispersed phase flux was varied from 70 to 700 L h⁻¹ m⁻² and the maximum shear stress at the membrane surface was between 1–27 Pa. Using these operating parameters uniform (CV below 25% for most of the samples) emulsions were obtained with drops sized between 34 and 340 μm. Tests with and without cells showed that the emulsion drop size, and uniformity, was not affected by the addition of the cells in the disperse phase. Tests were performed up to a maximum yeast cell concentration in the encapsulated in the drops of 3.14×10⁸ cells mL⁻¹. The yeast cell encapsulation efficiency was 100%, checked by
ascertaining that the non-tortuous pore channel membrane used did not filter any yeast cells when operating under the flow rate and by observation of no cells occurring in the organic continuous phase. The process of solid microparticles formation consisted of a thermal gelation and/or ionic crosslinking using SHMP (sodium hexametaphosphate). Eudragit S100 coating was performed on gelatine microparticles encapsulating cells using the oil in oil solvent evaporation method. The dissolution of the yeast loaded particles was checked at different time intervals in acidic (pH 1.2), neutral (pH 7) and slightly basic (pH 8) environments to mimic the transit conditions through the gastro-intestinal tract. The Eudragit coated particles did survive the acidic environment for 2h without dissolving, or releasing, the yeast cells. After surviving acidic conditions, dissolution of the particles occurred at pH 7 within 3h, and within 1h at pH 8, with subsequent yeast release. A targeted pH dependent release of cells in simulated intestine-colon conditions was achieved. The cell viability after the release was demonstrated by the ability of the yeast to metabolize up to 90% of glucose added to the growth medium in 24h. A confirmatory test was performed using a live/dead cell staining with two fluorescent probes which showed that living yeast cells predominated. Yeast cells were chosen as a proof of concept showing that ME is a promising method for cell encapsulation; the process can be applied to a variety of micro-organisms according to the cell type and specific requirements.

Immobilization of yeast cell within particles was obtained using alginate as encapsulating material. Emulsions made of 2 wt.% of alginate as dispersed phase and 2 wt.% SPAN 80 in kerosene as continuous phase were initially produced using 350 L h⁻¹ m⁻² as dispersed phase flux and shear stress from 1 to 17 Pa. Using a 30 μm pore sized and 200 μm pore spacing nickel membrane, drops with average diameter between 84 to 270 μm were produced and the CV% obtained did not exceed 24%. The presence of the yeast inside the drops did not affect the emulsion drop size and size distribution, only for the highest concentration tested (1.88x 10⁸ cells mL⁻¹) a slight increase of the CV% was observed. Magnesium ion was tested for the ionic crosslinking of alginate but the use of calcium ion resulted to be more appropriate for the formation of solid alginate microparticles. Solidification of the alginate drops was obtained by addition to the emulsion of a CaCl₂ solution (in different concentrations) or by pouring the emulsion within a saturated solution of CaCl₂. A solution of acetone: water (1:2) was used for the washing and further harvesting of the particles. It was shown that the yeast survived the process of particles production and washing procedure analysing the glucose
consumed by the yeast (still encapsulated in the particles) with time. It was seen that the glucose consumption rate was influenced by the amount of crosslinker used for the microparticles production: increasing the CaCl₂ concentration from 5 to 40 wt.% a reduction of the glucose consumption with time was observed, for particles produced with a saturated solution of CaCl₂ the amount of glucose consumed was approximately 0% up to 150h. This data suggested that the crosslinking density has a direct effect on the alginate porosity and a highly crosslinked polymer matrix delays the glucose diffusion.

**Membrane performance for W/O production**

The performance of hydrophobic membranes coated with PTFE and FAS (fluorinated compounds) was compared showing that the PTFE coated membrane gave smaller drops than the FAS coated membrane. From this result it was concluded that the PTFE coated membrane is characterized by a higher degree of hydrophobicity, but the coating method makes it less favourable for repeated use after cleaning. FAS coated membranes are promising for repeated production of W/O emulsions: it was seen that the FAS coating is more resistant and it does not require a long soaking time (30 minute in pure oil phase was sufficient to obtain uniform emulsions), furthermore a re-coating of the same membrane is possible if required. Hence, the use of the FAS coated membranes for a continuous production of W/O emulsions resulted to be more appropriate.

**Pulsed (Oscillatory) Flow system for process scale up**

Crucial for the scale up of the microparticles production process is the manufacturing of the emulsions with a high concentration of dispersed phase, the Pulsed (Oscillatory) Flow system was tested for the production of W/O emulsions on a large scale. The Pulsed system was successfully used to generate drops of 20 wt.% fish gelatine using 2 wt.% ABIL EM 90 in Miglyol as continuous phase. Operating parameters used were: 80 mL min⁻¹ as CP flow rate in combination with 413 L h⁻¹ m⁻² as DP flux or 60 mL min⁻¹ as CP flow rate in combination with 620 L h⁻¹ m⁻² as DP flux. The shear stress applied was between 0.4 to 14.5 Pa. Drops between 40 and 280 μm were produced, however, the lowest value of CV% obtained was 25%. Emulsions concentrated up to the 30% were produced. The experimental results obtained with the Pulsed systems were in accordance with the results obtained using the Dispersion Cell showing that it is possible to optimize the emulsion composition and operating parameters using the lab-scale device and replicate the experiments using the
Pulsed system. However, more uniform emulsions were produced using the Dispersion Cell. Breaking of the tubular membrane was experienced showing that a vibration of the membrane occurs during the emulsion production probably due to the high viscosity of the phases. Nevertheless, due the great potential of the Pulsed system as method of scale up (possibility to produce emulsions with a high concentration of dispersed phase), the working conditions should be optimized in order to achieve higher emulsions uniformity. Possibility to produce emulsions in continuous manner was tested running the system for 3h, the emulsions produced within this time had the same $D_{av}$ and CV% showing the reproducibility of the results.
5.1 RECOMMENDATIONS AND FUTURE WORKS

Dispersion Cell system

The Dispersion Cell was extremely useful for the investigation of the formulations in this work, it is easy to dismantle and to clean but, most importantly, it is possible to generate the emulsions using a small amount of dispersed and continuous phase (lowest volume used was 50 mL). The use of Dispersion Cell is highly recommended for the preliminary tests when a new emulsion composition for Membrane Emulsification is tried and for the determination of the best operating conditions of production.

PVA microparticles

Possibility to obtain a sustained release from the PVA microparticles was shown as a function of the crosslinker used, an additional study of the influence of the microparticles size on the release rate is suggested as further parameter to consider when designing a microcarrier for the sustained release of a drug using this formulation. The use of an alternative crosslinker as the glyoxal (smaller aldehyde compared to the GA) might be tested to vary the crosslinking density and influence on the release profile might be investigated.

PVA-CS and CS microparticles

It was shown that the use of SS as model drug produced a delay in the release of compared to the release of copper, the higher molecular weight of SS is one of the reasons but also the charge of the molecule (SS is negatively charged) might be responsible of the slower release obtained. It is recommended when designing a drug delivery system to take into consideration the chemistry of the molecule to encapsulate; in this case the use of a cationic polymer as encapsulating material could partially retain the negatively charged drug molecule encapsulated. Influence of the charge of molecule encapsulated on the release rate using this formulation should be investigated. The use a positively charged model molecule with a molecular weight similar to SS could be used for the study of the release with time and depending on the pH.

CS blended gelatine or Eudragit S100 coated gelatine microparticles

The Eudragit S100 coated gelatine microparticles containing the yeast were dried at room temperature and stored in glass bottles; in some cases, some of the produced samples were
used after 1 week of storage. However, the released yeast was vital and able to consume glucose. For the potential commercialization of this formulation as carrier of probiotic cells it is advised to determine for how long it is possible to store the dried microparticles before being used. Dried samples of the microparticles containing cells could be stored for several months and vitality of the yeast evaluated as a function of the storage time.

**Alginate microparticles**

Influence of the crosslinking reaction on the yeast glucose metabolism with time was shown, it was hypothesized that this effect is due to the formation of a more dense polymeric network which delays the diffusion of glucose through the matrix of the microparticles. A further investigation could be done on the material porosity: a BET (Brunauer-Emmett-Teller) surface area characterization on microparticles samples solidified using increasing amount of crosslinker is suggested. Thus, it will be possible to correlate the yeast glucose metabolism time with the crosslinking density of the alginate particles. Alternatively, the use of other ions (e.g. $\text{Sr}^{2+}$ or $\text{Ba}^{2+}$) for the formation of the alginate gel might be considered and a study on the porosity (and permeability to the nutrients) of the final material depending on the cation used for the ionic crosslinking is suggested. Incorporation of other polymers to the alginate represents also a popular direction in the probiotic encapsulation research field; chitosan could produce hydrogels by ionic gelation with alginate, which would provide an additional protection of the probiotic cells to the acidic stomach environment.

Possibility to immobilize mammalian cells is also highly recommended as a future work, in this field the production of modified alginate hydrogels containing cell adhesion ligands onto the surface would be extremely interesting for the production of microparticles for mammalian cells adhesion.

**Scale up using the Pulsed (Oscillatory) system**

Advantages of the Pulsed system for the process scale up for the W/O emulsions production were discussed, however some issues were also reported associated with some operating settings. The uniformity of produced emulsions was not optimal, a premix of the dispersed phase with the continuous oil phase was observed probably due to the vibration of the membrane, oil drops were found inside the water drops of the dispersed phase for all the samples produced. Furthermore, membrane breaking occurred. It is recommended to improve the mechanical strength of the membrane (variation of the membrane material or membrane
length) for the production of W/O emulsions using this system and further investigation of
the process operating parameter is required to reduce the CV% of the emulsions produced
and reduce the premix.
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APPENDIX

A. Tensiometer

All the interfacial tension measurements reported in this work were obtained with a White Electric Instrument Tensiometer (model DB2KS) using the Du Nouy ring method. The instrument is equipped with a precision lift mechanism to vertically move a glass beaker where the sample liquid/s are poured. The measurement of the surface tension is performed immersing a platinum ring (Du Nouy ring with defined geometrical dimensions) into the liquid. The ring hanging from the balance hook is then carefully pulled up, by lowering the sample beaker, through the surface of liquid. For the measurement of the interfacial tension the platinum ring is placed inside the dense liquid (water-polymeric solution) and pulled out to the light liquid (oil phase), until the detachment of the dense phase by lowering the sample cup. Tare of the instrument is required for the correct measurement hence for the determination of the surface tension the tare is done when the ring is outside the liquid; while for the interfacial tension the tare is done when the ring is submerged in the upper liquid. Wetting of the ring with the bottom liquid (i.e. the polymeric solution) is required prior to the immersion in the phases. The gravimetric assembly is recording the torque while pulling through the surface or the interface. The surface tension or interfacial tension is the maximum force needed to detach the ring from the liquid surface or from the bottom liquid to the upper liquid (A.Ullah, 2012). The instrument displays the determined value of interfacial tension in mN m\(^{-1}\).

![Figure 99 Schematic representation of the interfacial tension measurement using the Du Nouy ring method, the image is taken from a copyright free web page](http://www.mastrad.com/tsd1.htm).

It is extremely important that the ring and the glass beaker are completely clean and free from impurities which could interfere with the correct measurement. A cleaning procedure is
followed and repeated for each measurement for each phase. Prior to each use the ring and the glass beaker are washed with acetone, water, aqua regia and again rinsed in water in this order; afterwards the ring is cleaned using the flame.

B. UV-VIS SPECTROPHOTOMETER

The UV- Vis Spectrophotometer (Lambda 35 Perkin Elmer, UK) was used for the analysis of the Blue Dextran (BD) and Sodium Salicylate (SS) concentration in water solutions. The spectrophotometer is equipped with different types of light sources required for the different spectral regions. Tungsten and deuterium lamps are used for visible (400-700 nm) and the ultraviolet (200-400 nm) regions, respectively. Absorbance measurements can be performed at a specific wavelength (that can be manually set by the operator) or it is possible to perform a scan of the spectrum from 200 to 700 nm. For all the samples an initial spectra scan was done in order to determine the wavelength of the peak of absorbance. The results were compared with the data reported in literature; for BD the peak of absorbance is expected as 620 nm while for the SS at 300 nm. Calibrations were done accordingly with the expected working range of concentrations. For the BD the standard solutions measured had a concentration between 5 and 500 ppm (calibration reported in Figure 100) and for the detection of the SS standard solutions were prepared with a concentration between 1 to 100 ppm. The standard solutions of SS were prepared in PBS or acidified water with HCl; a slight divergence of the calibration curves was found as shown in Figure 101.
C. ATOMIC ABSORPTION SPECTROPHOTOMETER

The determination of the Cu$^{2+}$ ion concentration into water solutions was determined using the Atomic Absorption Spectrophotometer (Spectra AA-200 Varian, UK) interfaced with the “SpectrAA worksheet Oriented AA Software”. For Cu$^{2+}$ ion detection the flame type selected was acetylene/ air and the lamp current was set on 4 mA; these operating settings were recommended from the user’s guide provided. PROMT mode was selected as mode of operation. For the instrument calibration standard solutions were made dissolving a
predetermined amount of copper in pure Milli-Q water, PBS buffer solution or acidified water with HCl accordingly with the composition of the release medium used. The standard solutions prepared were in a concentration rage of between 0.05 to 1000 ppm of Cu\textsuperscript{2+}, the maximum standard concentration was selected considering the maximum concentration that should be measured. From the “calibration tab” the linear (Lambert-Beer) calibration algorithm was selected. The wavelength has to be set depending on the ion type to analyse and the expected concentration working range as reported in the manufacturer guide. Initially the wavelength used was 234.8 nm recommended for the detection of Cu\textsuperscript{2+} concentrations from 0.03 to 10 ppm. The standards measured had a concentration between 0.05 to 3 ppm. The corresponding values of absorbance obtained are reported in Figure 102. Each standard solution was measured 3 times and standard deviation of the values is reported as error bars. No difference of absorbance was found between the three different solvents used for the Cu\textsuperscript{2+} dissolution showing that the presence of other ions in the solution does not affect the Cu\textsuperscript{2+} detection. In the user’s guide is reported that no interferences have been recorded for Cu\textsuperscript{2+} in the acetylene/air flame. Calibration using higher Cu\textsuperscript{2+} concentrations (from 10 to 1000 ppm) was performed using the recommended wavelength of 244.2 nm that is reported to be appropriate for a working range between 10 to 2000 ppm. The standard solutions were prepared using acidified water with HCl. Figure 103 shows the detected value of absorbance as a function of the Cu\textsuperscript{2+} concentration. The instrument calibration was done twice; before starting the samples analysis (Calibration 1 in Figure 103) and after 20 samples measured (Calibration 2 in Figure 103); it is visible that the calibration curves did not vary significantly. To avoid signal variation due to thermal or humidity changes, the samples produced during the release studies were all analysed in the same assay (for the storage the samples were frozen), the wavelength of 244.2 nm (used in Figure 103) resulted to be most reliable for the concentrations obtained.
Figure 102 Calibration for Spectra AA using Cu²⁺ dissolved in different release medium in a concentration between 0.05 and 3 ppm.

Figure 103 Calibration for Spectra AA using Cu²⁺ dissolved in different release medium in a concentration.
D. IMAGE ANALYSIS

D.1 Image J

Size of the produced drops and microparticles was determined by a direct analysis of the images acquired using an optical microscope Leitz Ergolux with attached Pulnix TM-6CN camera or an optical/fluorescent microscope (GXML3201, GX microscope) with an attached Retiga 6000 colour camera. The liquid drops of the emulsions had the tendency to burst on the microscope glass slide due to surface tension phenomena. Therefore, the microphotographs were taken quickly and analysed using a Java-based image processing software (free download available from internet [https://imagej.nih.gov/ij/download.html](https://imagej.nih.gov/ij/download.html)). The size of semi-solid and solid microparticles in the dried or wet state were analysed using the same method. For each sample at least 300 drops or particles were measured to obtain a representative estimation of the size.

![Figure 104](image)

**Figure 104** Image analysis using ImageJ; (a) microphotograph of the microscope graticule slide for calibration (b) microphotograph of a W/O emulsion (c) image conversion into a binary image (d) numbered drops outlined ready for the measurement.
Prior to the image analysis the scale has to be set (i.e. conversion of pixels into microns): the length in pixels of 100 µm was measured on the microphotograph of a microscope graticule slide reported in Figure 104 a, the measurement is done using the software Microsoft Paint®. The image to analyse was imported to the software ImageJ (Figure 104 b) and converted into a 8- bit image. Brightness and contrast of the image were modified and threshold adjusted in order to obtain a clear black and white image of the circles representing the drops; subsequently the borders of connected drops were separated using the watershed function together with manual corrections if required (Figure 104 c). Drops touching the edges of the microphotograph were neglected from the counting using the function “exclude on edges”. The Feret’s diameter and standard deviation were set as measurements to be displayed and the outlines of the circles considered into the counting were shown (Figure 104 d). The measurements performed were then imported into an excel file where the average drop size and the value of Coefficient of Variation % is calculated.

D.2 Matlab script imfindcircles

A more powerful tool for drops and particles size analysis was adopted using the programming language Matlab® developed by MathWorks®. Microphotographs of emulsions and microparticles were analyzed using a modified version of the “circles detector” script “imfindcircles” available from the MathWorks online documentation (https://it.mathworks.com/help/images/ref/imfindcircles.html). The script is run within a folder containing the images to analyse and it automatically detects circular objects or circles within the images. Before call imfindcircles on an image, a conversion to a grey scale image is required using rgb2gray, with the method imadjust the intensity values (i.e. saturation and contrast) were modified varying the numbers in brackets to obtain an image with clearly visible circles, the converted image is shown in Figure 105 b.

```matlab
imgrey = rgb2gray(currentimage);
imadjusted = imadjust(imgrey,[0.3 0.4],[[]);
imageBW = im2bw(imadjusted);
```

In the second step of the analysis imfindcircles is run on imageBW, the command needs a range of radius (expressed in pixels) to search for circles which is added within the square brackets. Imfindcircles has an internal detection threshold which determines the sensitivity of the algorithm. The parameter “Sensitivity” (which is a number between 0 and 1) is used to modify the internal threshold; increasing the “Sensitivity” value lower the
threshold and leads to detect a higher number of circles but also reduces the accuracy of the detection.

```
[centers, radii] = imfindcircles(imageBW,[10 200],
'ObjectPolarity','dark', 'Sensitivity',0.85,'Method','Phasecode');
```

With the reported below function `viscircles` the detected circles are drawn on the image and it is possible to check that most of the drops were detected as visible in Figure 105 c. The diameter is then calculated from the detected radius and the measurements are saved as an excel file generated in the original folder. The values of diameter are manually converted into µm accordingly with the scale previously measured as described in Appendix D.1.

```
h = viscircles(centers,radii);
radius = [radius; radii];
diameter= radius*2;
```

Figure 105 Image analysis using Matlab® (a) microphotograph of a W/O emulsion (b) image converted in a grey-scale image using the scripts `rgb2gray` and `imadjust` (c) circles measured by the software running `imfindcircles`. 
LIST OF PUBLISHED WORK


CONFERENCE PROCEEDINGS

- “Crosslinked Poly (Vinyl Alcohol) microparticles for controlled drugs release produced by Membrane Emulsification”. Proceeding presented at XXII International Conference on Bioencapsulation (2014); Bratislava, Slovakia, Printed proceeding

- “Poly Vinyl alcohol and Chitosan microparticles for controlled drug delivery produced with the Membrane Emulsification Technique”. Proceeding presented at Particulate Systems Analysis Conference (2014); Manchester, UK, Proceeding from a library database

- “PH sensitive chitosan-based microparticles produced by Membrane Emulsification”. Proceeding presented at XXIII International Conference on Bioencapsulation (2015); Delft, The Netherlands, Printed proceeding


- "Microparticles for yeast encapsulation produced by Membrane Emulsification". Proceeding presented at XXIV International Conference on Bioencapsulation (2016), Lisbon, Portugal, Printed proceeding
CONFERENCE PARTICIPATION

2014
• Health and Wellbeing conference; Loughborough University, UK (oral presentation and poster)
• Research Challenges in Focus 2014; Loughborough University UK (oral presentation and poster)
• XXII International Conference on Bioencapsulation 2014; Bratislava, Slovakia (oral presentation)
• Particulate Systems Analysis Conference 2014; Manchester, UK (“short talk” oral presentation)

2015
• 7th Training School on Bioencapsulation, February 2015; Strasbourg, France (poster)
• XXIII International Conference on Bioencapsulation, September 2015; Delft, The Netherlands (poster)
• Conference of 5th UK-China and 13th UK Particle Technology Forum, July 2015, Leeds, UK. (Oral presentation)

2016
• 8th Training School on Microencapsulation, June 2016, Cork, Ireland. (Technical demonstration)
• Innovations in Encapsulation 2016 Conference, June 2016, Edinburgh, UK. (Poster)
• XXIV International Conference on Bioencapsulation, September 2016, Lisbon, Portugal. (Oral presentation)

AWARDS
- Prize for research impact at the Health and Wellbeing Conference 2014, Loughborough University, UK.
- Best short talk prize at Research Challenges in Focus 2014, Loughborough University, UK
- Best Poster running up, Innovations in Encapsulation Conference 2016, Edinburgh, UK